PASTEURELLA MULTOCIDA-TOXIN INDUCED ATROPHIC RHINITIS IN PIGLETS

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PASTEURELLA MULTOCIDA-TOXIN INDUCED ATROPHIC RHINITIS IN PIGLETS

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Abstract

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Progressive atrophic rhinitis (AR) is a complex of disease symptoms caused by infection with toxigenic *Pasteurella multocida*. Environmental and animal factors contribute to the severity of the disease. Their impact and relationship with severity of disease are inadequately understood and remain to be quantified in their effects. In this thesis, two areas of interest in atrophic rhinitis have been studied. A challenge model with *Pasteurella multocida* derived toxin (Pm-T) to mimic the disease was developed. Next, the impact of some aspects of climatic environment and the relationship with the severity of AR on health and metabolism of piglets were studied. Furthermore, investigations on the role of the immune system in atrophic rhinitis have been conducted with emphasis on mechanisms underlying the apparent lack of conventional (classic) immune responses to Pm-T. The Pm-T challenge resulted mainly in a lower food intake with concomitant lower weight gain, and in a reduced heat production caused by decreased activity of the pigs. Immunological features of Pm-T suggest T cell involvement in the pathogenesis of AR. Though the immune responses during AR remain far from understood, it is hypothesized that AR has autoimmune like features, with Pm-T triggering T cells to initiate destruction of nasal bony tissue.

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STELLINGEN

1 De in de praktijk genoemde verbeteringen van het stalklimaat ter controle van de ernst van een AR-uitbraak, worden waarschijnlijk grotendeels veroorzaakt door verminderde kolonisatie mogelijkheden van AR pathogene *Pasteurella multocida* op de neusslijmvliezen.
W.J. Smith. CEC report (1983): 151-162; R.F.W. Goodwin. Vet.Rec. 123 (1988): 566-568; Dit proefschrift.

2 De multi-factoriële aetiologie van AR in het varken kan, onafhankelijk van het infectieuze agens, met het ontwikkelde Pm-T challenge model bestudeerd worden.
Dit proefschrift.

3 De opmerking van McCaw dat: "AR acts as the 'canary in the coal mine' for production environments and management practices that do not meet the needs of the pig. Turbinate atrophy and nasal deviation are easily seen by owners, whereas increased severity of pneumonia and poor pig performance are often not recognized." negeert de gevolgen van subklinische AR.
M.B. McCaw. *The Compendium* 16 (Dec 1994): 1615-1618.

4 Koude en tocht hebben geen invloed op de ernst van met Pm-T geinduceerde AR symptomen.
Dit proefschrift.

5 Een challenge met Pm-T leidt niet tot een detecteerbare humorale immuunrespons. Het gebruik van serologische diagnostiek, het vaststellen van antilichamen tegen Pm-T, als enkelvoudig bewijs dat het dier in contact geweest is met Pm-T, lijkt dan ook voor de praktijk ongeschikt.
Dit proefschrift.

6 Door zijn gedrag aan te passen kan een varken, binnen zekere fysiologische grenzen, de gevolgen van (met Pm-T induceerde) subklinische AR beperken.
Dit proefschrift.

7 Ondanks dat er vaccins zijn die in de praktijk het 'probleem' AR binnen de perken houden, behoeven de mechanismen waardoor de specifieke neusafbraak bij AR ontstaat verdere opheldering.

8 Variatie in reaktie op Pm-T tussen biggen onder gelijke condities lijkt eerder regel dan uitzondering; dit suggereert de bijdrage van een genetische component aan het ziektebeeld.

9 De negatieve publieke opinie over dier-experimenteel onderzoek blijkt om te slaan, indien - zoals in het geval AR - het klinisch en subklinisch beeld zichtbaar (te maken) is (kromme neuzen; conchae atrofie).

10 De grootste tragiek van wetenschappelijk onderzoek is door budgettaire perikelen een intrigerende hypothese niet te kunnen toetsen.

11 Als 'proefkonijn' participeren in een (koffie)proef leidt tot koffieleut en koffiedik kijken.

12 Niets is zo moeilijk als het zetten van de laatste punt .

P.M. van Diemen
Pasteurella multocida-toxin induced atrophic rhinitis in piglets
Wageningen, 24 april 1995

VOORWOORD

Voor U ligt het resultaat van 4 jaar rondsnuffelen in varkensneuzen; een proefschrift met een 'Huisvesting en Klimaat'- en een 'Immunologie'-poot. Het onderzoeksproject is uitgevoerd bij de Vakgroep Veehouderij (Gezondheidsleer en Reproduktie) op 'Zodiac' en op de proefaccomodatie 'de Haar' van de Landbouwuniversiteit te Wageningen.

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The Pig

In England once, there lived a big, and wonderfully clever pig
to everybody it was plain that piggy had a massive brain
he worked out sums inside his head, there was no book he had not read
he knew what made an airplane fly, he knew how engines worked and why ..

He knew all this, but in the end, one question drove him round the bend
he simply couldn't puzzle out, what life was really all about
what was the reason for his birth, why was he placed upon this earth
his giant brain went round and round, alas, no answer could be found ..

Till suddenly one wondrous night, more in a flash, he saw the light
he jumped up like a belly dancer, and yelled, by gum, I've got the answer

They want my bacon slice by slice, to sell at a tremendous price
they want my tender juicy chops, to put in all the butcher shops
they want my pork to make a roast, and that's the part that cost the most
they want my sausages in strings, they want my chitterlings

The butcher shop, the carving knife, that is the reason for my life !

(Uit: Dirty Beasts, Martin Butler)

CONTENTS

ABBREVIATIONS

AR	atrophic rhinitis
Pm$^+$	toxin-producing *Pasteurella multocida* strains
Pm-T	*Pasteurella multocida* derived toxin
Bb	*Bordetella bronchiseptica*
VCA	ventral conchae atrophy
DCA	dorsal conchae atrophy
cBS	change in brachygnathia superior
TPR	turbinate perimeter ratio
TAR	turbinate area ratio
DL	Dutch Landrace pigs
GY	Groot York (Large White) pigs
AHS	Animal Health Services
UCT	upper critical temperature
LCT	lower critical temperature
ELISA	enzyme-linked immunosorbent assay
LST	lymphocyte stimulation test
SI	stimulation index
PBL	peripheral blood leucocytes
APC	antigen-presenting cell
MHC	major histocompatibility complex
TCR	T cell receptor
App	*Actinobacillus pleuropneumoniae*
KLH	keyhole limpet haemocyanin
TT	tetanus toxoid
OA	ovalbumin

Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

In today's pig production, upper respiratory tract infections, such as *Pasteurella multocida* which leads to atrophic rhinitis (AR), are common and insidious diseases of swine. They are often considered causes of decreased rate of gain, inefficient feed conversion, and increased time to market, although these parameters do not absolutely correlate with the severity of lesions.

The severity of observed clinical, pathological or anatomical deformations specific in AR, might be attributable to a number of factors including virulence of the microbial agents, differences in immune status, condition, age-related and possibly genetic susceptibility of the pig (De Jong, 1985; Rutter, 1985). Aerial conditions, management factors and hygiene are involved in the epidemiology of the disease (Robertson *et al.*, 1990). Therefore, AR is considered to be a disease with a multifactorial etiology. Although above mentioned factors are reported to be of importance, the impact of most of them and their relation with severity of AR are as yet inadequately understood and remain to be quantified in their effects.

Environmental factors play an important role in the health and production of livestock. Incidence and severity of disease can be related to fluctuating ambient temperature and sometimes increased air velocity (Verhagen *et al.*, 1987; Kreukniet *et al.*, 1990). The thermoregulatory demand of an animal related to a climatic condition affects partitioning of energy (metabolism) within an animal, and possibly the reaction of that animal to an invading pathogen. Coldness and draught will increase maintenance requirement (heat production) so that less energy is available for body weight gain (Verstegen *et al.*, 1987). Also, when an animal experiences disease, the maintenance requirement will be increased at the cost of efficiency of production (Verstegen *et al.*, 1987). This increase is, among others, due to an increased energy demand of the immune system and fever (Baracos *et al.*, 1987). Results with regard to effect of atrophic rhinitis on productivity mention no or little reduction in daily gain up to a 15% reduction in growth rate (Rutter, 1985). Whereas information on the effects of AR on food intake and partitioning of energy (heat production, efficiency and digestibility) is lacking.

It has been demonstrated that a single environmental stimulus, e.g. cold air, effectively reduced resistance to disease-causing organisms in pigs, like *Actinobacillus pleuropneumoniae* (*App*) (Verhagen, 1987), and transmissible gastroenteritis (TGE) virus (Shimizu *et al.*, 1978). Climatic stress can alter humoral as well as cell mediated immunity (Shimizu *et al.*, 1978; Kreukniet *et al.*, 1990; Scheepens, 1991). Good environmental management may reduce morbidity and mortality rate of a disease. This,

however, requires knowledge of the pathogenic process(es) leading to disease, and more specifically of the (immune) responses of animals to invading pathogens.

It is acknowledged that toxin producing strains of *Pasteurella multocida* (Pm^+) cause the pathogenic processes of atrophic rhinitis, leading to age-related irreversible destruction and reabsorption of nasal bony tissues in pigs (De Jong and Nielsen, 1990; Foged *et al.*, 1992). Sera from severely affected pigs in field outbreaks lack toxin-neutralising antibodies. Also, experimental infections with intranasally applied toxigenic *Pm* or Pm-T generally lead to sporadic and low humoral immune responses to Pm-T as estimated by biological and immunological tests (Rutter, 1988; Foged *et al.*, 1992). This suggests that Pm-T does not initiate a (protective) humoral response. On the other hand, little is known about the role of the cellular immune response in AR. The pathogenic effects caused by Pm^+ and Pm-T are known, but the mechanisms underlying pathogenesis of atrophic rhinitis, the mode of action, need to be clarified.

Considering the above mentioned problem areas, the objectives of the present research were, first, to develop a challenge model to mimic atrophic rhinitis, which would allow investigations on AR (Pm-T) in a broad field of interest (e.g. breed differences, productivity, behaviour, immune responses). And second, to study the effects of AR (Pm-T) on immune parameters, energy metabolism (heat production), and performance of weaned piglets, by means of this model. Moreover, pathogenesis ultimately leading to specific, irreversible nose bone destruction was explored.

In Chapter 2, a review is given of literature concerning state of knowledge on atrophic rhinitis. The economic relevance, the multiple etiology of AR and the control strategy in the Netherlands are briefly described. Environmental and animal factors which are thought to contribute to the severity of the disease symptoms are discussed and effects of *Pasteurella multocida* toxin are summarized. Gaps in knowledge are pointed out.

In the third Chapter, development of the challenge model to induce subclinical AR in pigs is described. With this model, the impact of environmental temperature and the relationship with the severity of AR on health and metabolism of piglets were studied in climatically controlled respiration chambers.

Chapter 4.1 describes the effects of induced moderate atrophic rhinitis on energy metabolism and performance of piglets under two different climatic conditions. The effects on level and changes in heat production and activity under the same conditions are described in Chapter 4.2. Visa versa, the effects of exposure to adverse climatic conditions on severity of atrophic rhinitis-like symptoms are studied (Chapter 4.1).

In Chapter 5, the role of the immune system in AR – particularly the immunological aspects of *Pasteurella multocida* toxin – is studied. First, specific immune responses to Pm-T and Pm-T induced conchae atrophy were compared with AR immunity, initiated with a vaccine. Serum antibody titres and *in vitro* lymphoproliferation to *Pasteurella multocida*-derived toxin and toxoid were studied (Chapter 5.1). In the successive Chapter 5.2, the effects of intranasally administered *Pasteurella multocida* toxin on the cellular and (T cell dependent) humoral immune responses of piglets were studied by means of an antigen cocktail containing Keyhole Limpet Haemocyanin (KLH), Ovalbumin (OA) and Tetanus Toxoid (TT). In Chapter 5.3, two pilot studies are depicted. Through immunosuppressant treatment and through returning Pm-T-stimulated T cells to the pig, an attempt was made to establish T cell involvement in the pathogenic process of AR.

In the General Discussion (Chapter 6), the major findings of the previous Chapters are discussed. Though as yet not understood, several features of Pm-T indicate the involvement of the immune system in the pathogenesis of AR. Literature in support of experiments that have led to the hypothesis that 'AR is a disease with autoimmune-like features, with Pm-T triggering T cells to destroy nose tissue' is incorporated. Subsequently, consequences and implications of the proposed concept are outlined and discussed.

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Chapter 2

ABOUT ATROPHIC RHINITIS IN PIGLETS - A REVIEW

P.M. van Diemen

ABOUT ATROPHIC RHINITIS IN PIGLETS - A REVIEW

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Abstract

The present state of knowledge on atrophic rhinitis is presented. Environmental and animal factors which are thought to attribute to the severity of the disease symptoms are discussed. The economic relevance, the multiple etiology of AR and the control strategy in the Netherlands are briefly described. The toxigenic effects of *Pasteurella multocida* toxin are summarized and attention is given to the role of the immune system in atrophic rhinitis. Lines for future research are suggested.

Key words: Atrophic rhinitis, *Pasteurella multocida* toxin, climatic environment, immunity, piglets

INTRODUCTION

'Was ist die Schnuffelkrankheit der Schweine?'. With this question, in 1829, Franque asked attention for a disease in pigs which he called '*Schnuffelkrankheit*'. He described this disease as gradually arising, beginning with inflammation of the nasal mucous membrane, followed by deformation of the muzzle. At this point, affected animals breathed audible, might have bleeding noses, and stopped growing or lost weight, thus causing considerable economic losses. He suggested that the disease was transmitted from parents to offspring and affected preferably short nosed pigs. He stressed that its character and cause should be studied as soon as possible.

Since then, much research has been conducted on the infectious origin and much has been learned about the etiology of '*Schnuffelkrankheit*' or atrophic rhinitis (AR). Infectious atrophic rhinitis is a world wide spread disease of the upper respiratory tract of pigs (Pedersen and Nielsen, 1983). Pathologically, AR is characterized by a chronic rhinitis resulting in interference with remodelling and deformation of the bony structures underlying the nasal mucosa of a young, rapidly growing pig (Done, 1983). Anatomically it is characterized by deformity of snout and by tooth malapposition and possibly growth retardation (Done, 1983). It is acknowledged that toxin producing strains of *Pasteurella multocida* cause these progressive and generally irreversible turbinate lesions. The

severity of conchal damage is highly variable both within and between affected farms (de Jong, 1985; Rutter, 1985). At the 10th International Pig Veterinary Society Congress in Rio de Janeiro, Brazil, 1988, the definition of progressive atrophic rhinitis (AR) as a disease caused by infection with toxigenic *Pasteurella multocida* (Pm^+), was accepted (de Jong and Nielsen, 1990). The clinical diagnosis of progressive AR is confirmed by the detection of Pm^+. Thus herds harbouring Pm^+, even though only slight or subclinical disease is present, can be identified.

In addition to the above mentioned infectious cause of atrophic rhinitis, environmental and animal factors are said to contribute to the severity of the disease symptoms. An infection with Pm^+ can be present for some time before clinical disease symptoms occur.

This review briefly describes the economic relevance, the multiple etiology of AR and the combating strategy in the Netherlands. Furthermore, the toxigenic effects of *Pasteurella multocida* toxin are summarized and its possible influence on bone metabolism is discussed. Moreover, attention is given to the role of the immune system in atrophic rhinitis, and known effects of climatic environmental factors on health and performance of pigs are outlined.

ECONOMIC RELEVANCE OF ATROPHIC RHINITIS

The direct and indirect costs of disease are an important economic factor in today's intensive pig industry. Atrophic rhinitis undoubtedly causes economic losses through retarded growth rates, medication costs, extra labour and inability to sell (breeding-)stock, although these parameters do not absolutely correlate with the severity of lesions. However, exact figures for economic significance are difficult to obtain and depend on the extent of observation and recording. The severity of the disease in pigs and concomitant productivity losses on commercial units are highly variable. Results with regard to effects of AR on productivity losses vary from no or little reduction in daily gain to a 10-15% reduction in growth rate (Rutter, 1985). De Jong (1985) stated that especially severely affected animals showed a (5-20%) reduction in growth. However, no unequivocal definition of AR or of an AR-affected herd was used. Thus, other disorders of the nose may blur the estimation of economic significance of AR. Various factors, including other microorganisms, may also cause rhinitis as opposed to atrophic rhinitis (Rutter, 1985). The relation between growth performance and AR can only be elucidated if the latter is clearly defined.

Growth retardation can be caused by a lower food intake, a lower availability of nutrients and/or by an increased maintenance requirement (heat production) of exposed

(affected) animals (Verstegen *et al.*, 1987). Smith (1983) stated that pigs with AR may convert food to meat as good as their contemporaries, but because of a depressed food intake, their rate of daily gain is also depressed. If a quantitative relation between degree of turbinate lesions and individual food intake or maintenance requirement could be demonstrated, this would be of tremendous help to estimate the economic impact of AR.

Also factors affecting the estimation of economic significance of AR may have a different impact in different areas or herds. These are, among others, incidence of clinically affected pigs, inspection/combatting policy, intensity of treatments and vaccinations (Nielsen, 1983). Indirect economic losses, due to subclinical disease levels e.g. growth retardation, can occur too. When the conchal bones are damaged, part of the filtering system of the upper respiratory tract is damaged. Finally, lungs become more susceptible to secondary infections, like *App*-infections or (enzootic) pneumonia due to e.g. mycoplasmata. In most affected pig herds, also other problems, like diarrhoea, are present (de Jong, 1983).

COMBATING STRATEGIES IN THE NETHERLANDS

Most disease combating strategies for pigs in The Netherlands are focussed on the top breeders. By making the breeding farms free of diseases, the production flow, through to multipliers and fatteners will be low(er) risk.

Since 1980, atrophic rhinitis is combated under the auspices of the Regional Animal Health Services (AHS). After at least 6 months of checkups on the presence of clinical symptoms of various diseases, among which AR, a breeding farm could receive a 'Health Certificate' (de Jong, 1985). Animal health certification is part of a national animal health care programme to improve the health status of the cattle, poultry and swine production sector (Anonymous, 1987). After establishing the role of the toxigenic *Pasteurella multocida* (*Pm*$^+$) in AR in the mid-eighties, a check for the presence of *Pm*$^+$ was included in the monitoring programme and '*Pm*$^+$-free' Certificates are currently issued. Bacteriological checks are made routinely by taking samples of the nose mucosa (nose swabs) of piglets between the 4 and 10 weeks of age and of pigs of 3 to 6 months old. Additional sampling of the tonsils might be useful in minimizing the proportion of false negatives (increasing the sensitivity), especially when the number of *Pm*$^+$ bacteria may be relatively low in the carriers (de Jong *et al.*, 1994).

In Table 1, the number of farms with and applicants for the '*Pm*$^+$-free' Certificate through the monitoring programme of AHS in The Netherlands are given. Since 1989, nearly all breeding herds participate in the monitoring system. Due to this programme,

the prevalence of infection in breeding herds harbouring the toxigenic Pm is less than 1% at present (Voets *et al.*, 1994).

Table 1 - Survey[a] of the Dutch Animal Health Services, Monitoring programme '*Pm*$^+$-free'. Number of breeders and applicants.

	1988	1989	1990	1991	1992	1993
'*Pm*$^+$-free' certificate	97	440	423	493	487	596
(infected)	(5)	(4)	(3)	(1)	(3)	(2)
'applicant' for certificate	–	198	190	224	300	233
(infected)		(4)	(5)	(2)	(3)	(–)
Total '*Pm*$^+$-free + applicant'	97	638	613	717	787	829
Total 'Health Certificate'	1420	1398	1369	1375	1342	1115*
% '*Pm*$^+$-free + applicant'	7	45	45	53	58	74

*decrease mainly caused by business termination of small herds and rearing herds.

Since 1992, breeding herds without an AR history, but which vaccinate against AR, can obtain a '*Pm*$^+$-checked' Certificate[a]. This monitoring programme is similar to the '*Pm*$^+$-free' programme on the understanding that herds vaccinate. By taking nose and tonsil swab samples of piglets between 4 and 10 weeks of age, however, *Pm*$^+$-positive herds might be missed because of maternal protection. The sampling of older animals (eg sows and 3 to 6 month old pigs) and additional sampling of the tonsils of sows meant for culling might be useful in minimizing the number of false negative herds (de Jong and Braamskamp, 1994; de Jong *et al.*, 1994).

On multiplier and fattening farms, nevertheless, atrophic rhinitis is still a persistent problem (Voets *et al.*, 1994). By means of vaccinating pregnant sows with a vaccine consisting of *B bronchiseptica*, and toxigenic *P multocida* and/or Pm-toxoid, disease symptoms and concomittant economical losses can be controlled (Voets *et al.*, 1994). Preliminary research to the *Pm*$^+$-status of offspring born to herds in which sows are vaccinated over 3 years, indicated, however, that one third of the fattening pigs (4-6 months old) harbours the toxigenic *P multocida* (Smelt, 1989). The findings of Wallgren *et al.* (1994) suggested that pigs aged 6-12 months should be target animals when screening for presence of *Pm*$^+$ on herd level, at least in herds vaccinated against AR. Thus, the infectious cause did not resolve by longterm vaccinations. Moreover, this means that when the vaccination regime is not well executed, a clinical outbreak still may follow.

[a]Landelijk overzicht PM+ regelingen 1992, 1993. Stichting Gezondheidsdienst voor Dieren and personal communication MF de Jong, AHS East Netherland, Deventer, The Netherlands.

MULTIPLE ETIOLOGY

The occurrence of AR as a serious (economic) problem was in several countries (e.g. France, Ireland, The Netherlands) associated with the rapid increase of the number of animals per herd in the seventies (de Jong, 1983; Kobisch and Madec, 1983; O'Connor, 1983). Several reports state that atrophic rhinitis is more severe under conditions of bad ventilation and poor management. In particular high stocking densities and continuous throughput in farrowing houses and weaner accomodation have been identified as important contributing factors (de Jong, 1983; Smith, 1983). Mass or number of (dust) particles present as inspirable aerosols, and the presence of large numbers of viable bacteria may compromise the local defence mechanism of the upper respiratory tract in the pig and facilitate colonization by Pm^+ (Robertson *et al.*, 1990). It is recognized that Pm^+ causes more severe turbinate lesions in pigs of which the nasal mucosa is irritated mechanically (dry air, ammonia, dust e.g.) or by infectious agents like *Bordetella bronchiseptica* (Pedersen and Elling, 1984; de Jong and Akkermans, 1986; Rutter, 1988; Chanter, 1990). After aerial environmental and managerial defects, which contribute to the spread of AR, were identified, the prevalence decreased by implementing combating strategies, and through improvements of housing, ventilation and management (O'Connor, 1983; Schöss, 1983; Robertson *et al.*, 1990; de Jong, 1992).

The multiple etiology of atrophic rhinitis may have caused part of the diversity encountered in the assessment of economic loss per fattened pig or herd. For instance, Smith (1983) discussed that nutrition may have an indirect effect on the severity of AR. Piglets with acute rhinitis may have a poor appetite because of loss of taste and sense of smell (Smith, 1983). Feeding strategy (wet feeding, *'ad lib'* feeding) may be able to reduce sustained growth damage. *'Ad libitum'* feeding appeared to reduce the growth damage by 50% compared with a restricted feeding strategy (Paridaans *et al.*, 1981). The relation between individual rate of food intake, feeding strategy and severity of AR deserves further investigation in order to diminish economic losses in case of disease outbreaks.

Next to virulence of the microbial agents, the severity of observed clinical, pathological or anatomical deformations might be attributable to a number of more general noninfectious (husbandry) factors (Smith, 1983). The factors include differences in immune status (poor maternal immunity), poor condition, nutritional deficiencies, and an age-related and possibly genetically linked predisposition of the pig to AR, since breed and individual differences do occur (Smith, 1983; Martineau *et al.*, 1988; de Jong, 1992). Experimental work revealed that aerial conditions, management factors and hygiene are involved in the epidemiology of the disease (Smith, 1983; Robertson *et al.*, 1990). The

noninfectious factors may enhance the severity of any infectious disease, including AR. Although above mentioned factors are reported to be of importance, the impact of most of them and their relationships with severity of atrophic rhinitis are as yet inadequately understood and remain to be quantified in their effects. One of the possible approaches regard multivariate observational-analytic epidemiological studies (Martin *et al.*, 1987). Another approach is to study AR by means of experimental induction of the disease under field-like conditions.

PASTEURELLA MULTOCIDA TOXIN AND BONE METABOLISM

The mode of action of the Pm-T on the cartilaginous and osseous tissue of the nose is not known. Other osseous tissues, e.g., long bones and costochondreal junctions, do not appear to be significantly affected by the toxin (Rutter, 1988). In order to explain the apparently specific resorption of nasal turbinates in AR, the natural development of these structures and of other bones has been compared (Martineau-Doizé and Martineau, 1986; Dominick and Rimler, 1988; Martineau-Doizé *et al.*, 1990). The natural turnover of bone in the ventral turbinates resulted in a complete renewal within the first two weeks of the piglets life (Martineau *et al.*, 1982). The bones in which the natural remodelling process was most active, were the bones most affected by AR. A marked consistency between the age-dependent susceptibility to AR and the growth rate of bones of the snout was observed (Martineau-Doizé and Martineau, 1986).

In 3-week old gnotobiotic piglets intranasally inoculated Pm-T stimulated osteoclastic osteolysis and inhibited osteogenesis in turbinates by causing degeneration and death of osteoblasts (Dominick and Rimler, 1988). This suggested that the toxin upsets the balance between bone formation and resorption in favour of a net resorption (Chanter, 1990). The amount of toxin current, however, is dependent on the number of *Pm*-germs grown on the mucosals and on the level of toxin produced by the *Pm*-strain. Both growth and toxin production level fluctuate strongly. Therefore, the bacterial products (e.g. Pm-T) in the nasal mucosa might act directly on osteoclast precursors (Dominick and Rimler, 1988; Martineau-Doizé *et al.*, 1990). But the influence can also be indirect via other cells or through mediators produced by immune cells (Martineau-Doizé *et al.*, 1990; Pedersen and Elling, 1984).

On the other hand, Pm-T can increase numbers of pre-osteoclasts and osteoclasts in mouse fetal long bones *in vitro* (Kimman *et al.*, 1987), and can increase resorption activity and numbers of osteoclasts in rat long bones *in vivo* (Martineau-Doizé *et al.*, 1993). These results, combined with the observation that Pm-T is able to induce osteoclast-like cell differentiation from mouse bone marrow precursor cells (Martineau-

Doizé and Jutras, 1994) shows that Pm-T can act systemically in rodents. The specific preference for the nasal turbinates might be explained by the structure and the high natural turnover of the bony tissue.

Differences in susceptibility of the nasal bone tissue and receptors on cells may explain differences found between breeds, lines (Martineau *et al.*, 1988) or individuals, in the same fashion as breeds can differ in immune responses (Meeker *et al.*, 1987; Joling *et al.*, 1993). The age-related susceptibility as mentioned before in connection with the mechanism(s) by which nose damage develops in pigs needs further research.

EFFECTS OF *PASTEURELLA MULTOCIDA* TOXIN

Several studies have shown that the *Pasteurella multocida* derived toxin (Pm-T) is merely responsible for the pathogenic processes of AR (de Jong and Akkermans, 1986; Foged *et al.*, 1987; Rutter, 1988; Chanter, 1990). The Pm-T initiates nose damage whether applied intranasally, intramuscularly, intraperitoneally or parenterally (Martineau *et al.*, 1982; de Jong and Akkermans, 1986; Dominick and Rimler, 1986; Frymus *et al.*, 1986; Foged *et al.*, 1987; Chanter, 1990).

By biochemical and immunological methods Pm-T, a single protein, has been isolated from toxigenic *Pasteurella multocida*. During the logarithmic phase of growth it can be detected inside the bacterial cell (Nakai *et al.*, 1985; Rutter, 1988). Towards the end of growth it is released into the medium (Rutter, 1988). The toxigenic effects are the same as those of a crude extract of Pm^+ (Foged, 1992). These effects include a resorbing effect (Dominick and Rimler, 1988) on turbinate bones upon intranasal (de Jong *et al.*, 1980; Dominick and Rimler, 1986) or parenteral application (de Jong, 1983; Rutter and Mackenzie, 1984; Frymus *et al.*, 1986; Williams *et al.*, 1990); a lethal and a dermonecrotic effect in many animal species including pigs (Rimler and Brogden, 1986; Cheville *et al.*, 1988), mice (Nakai *et al.*, 1984; Rimler and Brogden, 1986; Foged *et al.*, 1987), rats (Foged *et al.*, 1987; Cheville *et al.*, 1988), rabbits (Rimler and Brogden, 1986), goats (Baalsrud, 1987), guinea pigs (Foged *et al.*, 1987) and turkeys (Rimler and Brogden, 1986); a cytopathic effect in certain cell lines *in vitro* (Pennings and Storm, 1984); and a mitogenic effect in some cultured fibroblasts (Williams *et al.*, 1986; Lax *et al.*, 1990; Rozengurt *et al.*, 1990). Also Pm-T promotes differentiation of osteoclast-like cells from proliferating mouse bone marrow cells (Martineau-Doizé and Jutras, 1994). Experiments based on neutralisation of the biological effects of Pm-T by a single monoclonal antibody (Mab), indicate that one active site on Pm-T is responsible for all these toxic activities (Foged, 1992). The signalling pathways leading to mitogenesis may be triggered to some extent by intracellular stimulation of protein phosphorylation (Rozengurt *et al.*, 1990).

Treatment by heat, formaldehyde, or proteases lead to detoxification of Pm-T as assayed in biological tests (Foged, 1992).

The characterisation and the genetic basis of the *Pasteurella multocida* toxin has been extensively reviewed by Foged (1992). Thus effects caused by Pm-T are known, but how these effects are caused needs to be clarified.

IMMUNE RESPONSES TO ATROPHIC RHINITIS (Pm-T)

Severely affected pigs in field outbreaks of AR lack toxin neutralising antibodies in their sera or show a very late and hardly detectable humoral response. Also experimental infections with toxigenic Pm or intranasally applied Pm-T generally lead to sporadic and low humoral immune responses to Pm-T as estimated by biological and immunological tests (Frymus *et al.*, 1986; Foged *et al.*, 1987; Rutter, 1988). Clinical symptoms do not relate to a detectable humoral anti Pm-T immune response (Frymus *et al.*, 1986; Nagy *et al.*, 1986; Bording Jensen and Riising, 1988). This suggests that Pm-T does not initiate a (protecting) humoral response. Whether antibodies conferred protection to subsequent infection with Pm^+ is unknown.

Why such an immune response can not be measured is not clear. One possibility is that the Pm-T acts directly and locally, without involving the systemic immune system. Another possibility is that the immune response against Pm-T is undetectably low or insufficient. Both humoral and cellular immune responses to Pm-T need to be studied in more detail.

The consequences of the lack of anti-toxin antibodies are diverse. First, antibodies should be protective against the pathogenic processes of AR. Second, sero-epidemiological studies can not be performed, only visual examination of turbinate atrophy in snout sections from slaughtered pigs can be used. Third, effects of factors on specific immune responsiveness (antibodies) can not be measured. And fourth, the lack of antibodies can point at another 'type' of immune response.

The Pm-T molecule is immunogenic, i.e. it has the ability to elicit an immune response. Several researchers (de Jong and Akkermans, 1986; Frymus *et al.*, 1986; Nagy *et al.*, 1986; Foged, 1988; Bording *et al.*, 1990) showed that sera from animals immunised with sublethal doses of crude or purified Pm-T contained neutralising antibodies against Pm-T. Thus the common lack of anti Pm-T antibodies after *in situ* infection, is not directly attributable to its immunogenic properties. Done (1971) thought it possible that genetically susceptible pigs develop antibodies to their own conchal tissues. The bacteria might trigger the destruction of the conchae of some pigs. This process continues in the absence of bacteria, getting autonomous. Done (cited by Smith,

1983) detected large amounts of immunoglobulin in the conchal mucous membranes of AR cases, where bacteriological culturing results for AR pathogens were negative. Smith (1983) mentioned that a complex relationship between the immune system and the persistence of infection seemed present in some strains of pigs.

Several (immuno)-pathological questions of how Pm-T initiates atrophic rhinitis need to be answered. First, where and how does the Pm-T enter the nose tissues? Secondly, which cells or receptors are involved? Interference of Pm-T with mucous tissue will induce an immune response probably different from that with a systemic antigen. And thirdly, does the Pm-T enter as such or is there something like a hapten carrier effect?

Pm-T was reported to be an extremely potent mitogen for fibroblasts, cell-lines *in vitro* (Frymus *et al.*, 1986; Rozengurt *et al.*, 1990) and for several cell types of different mammals (Rozengurt *et al.*, 1990; Williams *et al.*, 1990). What the effect is of Pm-T *in vivo* on different tissues of the nose, for instance on mesenchymal cells which are progenitors of osteoblasts, is unknown.

CLIMATIC ENVIRONMENT

Animals need to maintain a steady state in their internal environment irrespective of their external surroundings (Curtis, 1983). Through thermoregulatory mechanisms animals actively regulate heat loss and heat production to preserve a constant body temperature. Environmental temperatures above the upper critical temperature (UCT) will cause hyperthermia. At that point heat loss to the surrounding environment is lower than heat production, thus causing body temperature to increase. Temperatures below the lower critical temperature (LCT) will cause hypothermia when maximum heat production is reached (Mount, 1979). The zone where no extra heat loss or production occurs is the zone of thermoneutrality (Figure 1). LCT depends on factors like body weight, group size, level of nutrition, age and numerous others (Verhagen, 1987). For instance pigs can reduce heat loss to the environment by huddling. Regulation of food intake may also contribute to maintain homeothermy, extra intake can meet the increased energy demand for maintenance requirement (heat production).

Ambient temperature and increased air velocity are components of the climatic environment. Several studies described the effects of these components on health and performance of weaners and young fattening pigs (Close and Mount, 1978; Verhagen, 1987; Scheepens, 1991). In general, exposure to an adverse climatic condition increases the maintenance requirement of the animals (Verstegen *et al.*, 1987). This increase (heat production) is at the cost of energy deposited in body weight gain (Verhagen, 1987).

Especially increased air velocity resulted in reduced weight gain, due to a rise in the convective heat loss (Mount et *al.*, 1980).

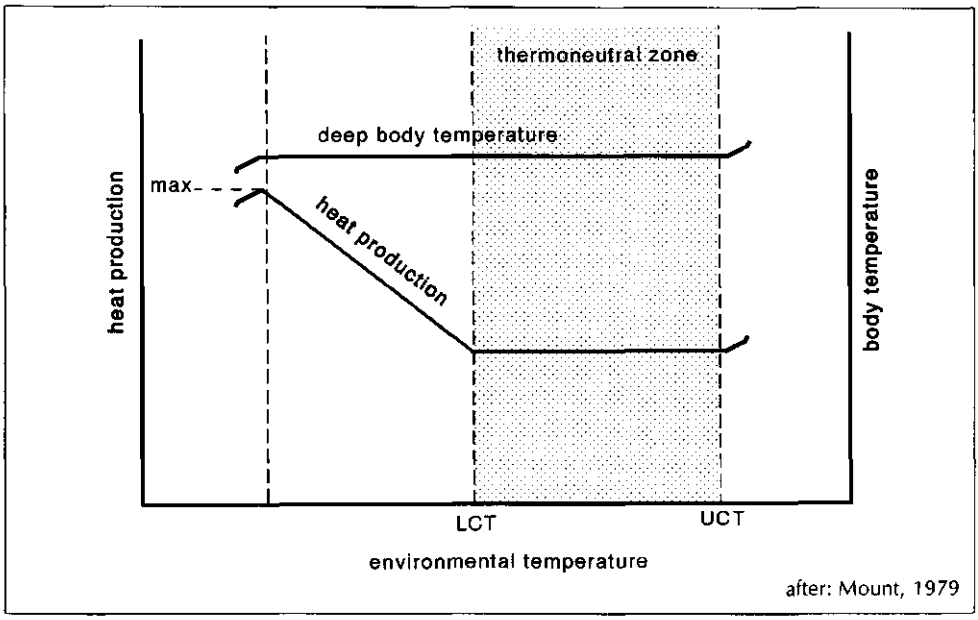

Figure 1 - Relation between heat production, body temperature and environmental temperature. UCT: upper critical temperature; LCT: lower critical temperature.

The thermoregulatory demand of an animal related to a climatic condition can affect the reaction of pigs to a pathogen. It has been demonstrated that a single environmental stimulus, e.g. cold air, effectively reduced resistance to disease-causing organisms in pigs, like *Actinobacillus pleuropneumoniae* (*App*) (Verhagen, 1987), transmissible gastroenteritis (TGE) virus (Shimizu et *al.*, 1978) or killed Aujeszky's disease vaccine virus (Noyes *et al.*, 1988). The immune response might be altered by climatic conditions too. Effects of climatic stress on immune function showed that both *in vivo* and *in vitro* responses of the pigs' immune system can be affected (Verhagen, 1987; Kreukniet *et al.*, 1990). In general, exposure to cold will increase serum antibody levels to invading antigens (e.g. *App*, Verhagen, 1987; TGE virus, Shimizu *et al.*, 1978; sheep red blood cells, Blecha and Kelley, 1981). The cell mediated immune response will be enhanced after exposure to draught (Scheepens, 1991) or exposure to cold (Kelley, 1985).

Verhagen (1987) indicated that increase in humoral immunity induced by adverse climatic conditions could be caused by the following mechanism. If the local resistance of pigs is lowered, a prolonged contact with and, in case of multiplication, a higher

exposure level to the pathogen can be the result. This hypothesis was supported by an impaired bactericidal activity of alveolar macrophages to *Escherichia coli*-infection in pigs kept at low ambient temperature (Curtis *et al.*, 1976).

In the respiratory tract there is a direct contact between the animal and its environment. During inspiration, air travels along branching passages where it is warmed and filtered on its way to the lungs. The conchal bone structures are part of this filtering system, and, owing to their large specific surface, prone to impairment by environmental conditions or colonization by microorganisms. Knowledge, however, of climatic environmental effects on the progression of atrophic rhinitis is lacking. Adverse conditions may seize upon the conchal mucous membrane causing an enhanced colonization of *Pm*$^+$ and/or passage of the Pm-T through this membrane. In both concepts more toxin might reach the underlying bony tissues resulting in more severe disease symptoms. Another possibility is that the change in immunity level (antibody mediated) caused by exposure to cold might contribute to pathological lesions. This was speculated by Kelley *et al.* (1982) in infectious diseases of cattle, referring to certain hypersensitivity states. Cold stress-induced changes in immune events depend on the type of immune response, the nature of the environmental stressor and the length of exposure. The effects of environmental stressors on immune events, and certainly on infectious diseases are complex and poorly understood (Kelley *et al.*, 1982). The physiological mechanism(s) by which adverse environmental stimuli increase the susceptibility of animals to disease is unknown.

CONCLUSION

Atrophic rhinitis is a disease of which the severity is largely determined by its multiple etiology. By means of the health monitoring programme with '*Pm*$^+$-free' Certification, dominating noncontagious factors, and vaccinating pregnant sows, sustained economic damage caused by atrophic rhinitis can be controlled. Thus, for the intensive pig industry, AR is not considered to be a major problem anymore. Strategy of the European Community, however, is to reduce the use of vaccines to control contagious diseases. Therefore, quantification of endogenous and exogenous factors in AR and knowledge about immunity to AR are indispensable. For instance, the reason why only young pigs are sensitive to Pm-T to induce nasal breakdown, in contrast to adult pigs is unknown. Identification of immune responses to AR (Pm-T) might help to develop better diagnostic values and therapeutic approaches for disease intervention.

A great deal of research into atrophic rhinitis from several angles of incidence has been conducted. But results of these studies are hard to compare because various and not

always reproducible challenge routes and agents were used to induce AR experimentally. Often the challenge system was aimed at clinical disease to study effect of medication or vaccination. To study effects of environmental and animal factors on AR, an explicit definition and a reproducible challenge model for experimental induction of atrophic rhinitis is required. Such a model would allow investigations on AR (Pm-T) in a broad field of interest to be conducted.

Preferably the induced disease symptoms should be moderate (subclinical), so that factors related to the mucosal system of the turbinates can be judged and animal welfare is not largely impaired. The model should approach infection route and disease symptoms of field infections, because then obtained results can be 'easily' translated to farm level. Then, the impact of immunological, genetic, production (metabolic) and environmental (social and climatic) factors, and their interactions on the severity of the disease might be quantified. Moreover, the mode of action, the mechanisms involved and the target receptors of the Pm-T can be studied in more detail.

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Chapter 3

INTRANASAL ADMINISTRATION OF *PASTEURELLA MULTOCIDA* TOXIN IN A CHALLENGE-EXPOSURE MODEL USED TO INDUCE SUBCLINICAL SIGNS OF ATROPHIC RHINITIS IN PIGS

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INTRANASAL ADMINISTRATION OF *PASTEURELLA MULTOCIDA* TOXIN IN A CHALLENGE-EXPOSURE MODEL USED TO INDUCE SUBCLINICAL SIGNS OF ATROPHIC RHINITIS IN PIGS

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Abstract

A challenge-exposure model was developed for dose-dependent induction of subclinical (moderate) atrophic rhinitis (AR) in conventionally raised Dutch Landrace and Large White pigs, about 4 weeks old. Under favorable climatic and housing conditions, pigs were intranasally challenge-exposed with *Pasteurella multocida*-derived toxin (Pm-T) 3 days after pretreatment by inoculation with 1% acetic acid. Pigs were challenge-exposed with 1 of the following Pm-T doses: 0 (control), 5, 13, 20 or 40 μg of Pm-T/ml of phosphate-buffered saline solution (PBS), 0.5 ml/nostril/d on 3 consecutive days. Five weeks after challenge exposure, subclinical (moderate) AR status was defined as intermediate conchae atrophy (grade 2 for ventral conchae on a 0 to 4 scale and grade 1 or 2 for dorsal conchae on a 0 to 3 scale, respectively) and perceptible difference in change in brachygnathia superior (cBS) between control and challenge-exposed pigs between the beginning and end of the study. All Pm-T-exposed pigs had nasal damage that was dose-dependent. The higher Pm-T doses resulted in higher ventral conchae atrophy and dorsal conchae atrophy scores. The cBS increased with applied Pm-T dose, resulting in significant ($P < 0.05$) differences between controls (3.88 mm) and the 13-, 20-, and 40-μg Pm-T-treated groups (7.77, 6.58 and 7.98 mm, respectively). In response to the applied dose, weight gain per week for Pm-T-exposed pigs was lower than that of controls after week 3 ($P < 0.01$). Difference from controls was 32, 54, 52, and 96 g/d/pig for 5-, 13-, 20-, and 40-μg Pm-T-treated groups respectively, in the last 2 weeks. For Dutch Landrace and Large White pigs, intranasally administered Pm-T mimicked the pathogenic effect of *in vivo* infection with toxigenic Pm strains. The optimal model to induce subclinical AR appeared to be 13 μg of Pm-T/ml (0.5 ml/nostril/d) on 3 consecutive days. Our model should enable studies of exogenous and endogenous factors involved in development of AR, independent of the colonizing ability of the Pm strain used.

Key words: Challenge-exposure model, Atrophic rhinitis, *Pasteurella multocida*-toxin, Piglets

INTRODUCTION

Infective progressive atrophic rhinitis (AR) is a disease of the proximal respiratory tract that may affect young pigs. The disease is clinically diagnosed by deformities of the snout and atrophy of the nasal conchae. Irreversible and complete disappearance of the

turbinates may be observed within 2 to 3 weeks after infection with pathogenic *Pasteurella multocida* (Pm^+) strains.

Pasteurella multocida-derived toxin (Pm-T) may be one of the agents causing the pathogenic processes of AR, that lead to irreversible destruction and reabsorption of nasal bony tissues (Foged *et al.*, 1987; Rutter, 1988; Chanter, 1990; de Jong, 1991). However, the mode of action and target receptors of the toxin are unknown. In natural infections via the mucosa of the respiratory system, the toxin appears to be a poor immunogen (Rutter, 1988).

Severity of the disease in pigs and concomitant productivity losses in commercial units are highly variable (Rutter, 1985). Experimental work indicated that aerial conditions and management factors are involved in the epidemiology of the disease (Robertson *et al.*, 1990), but relation between severity of disease and immunologic, genetic, metabolic and environmental factors are, as yet, inadequately understood.

Most challenge-exposure models aim at clinical status, and are used for toxigenic *P multocida* research, to establish pathogenic effects or to evaluate vaccines. Data available on experimental induction of AR are derived from young specific-pathogen-free (SPF) or gnotobiotic pigs challenge-exposed with Pm^+ or bacteria-free supernatants. The dose of Pm-T used is stated in micrograms per milliliter (μg/ml), mouse lethal dose (MLD_{50}) or guinea pig skin test (GPST) units, whereas various routes of challenge exposure with or without pretreatment were applied (Martineau *et al.*, 1982; Dominick and Rimler, 1986; de Jong and Akkermans, 1986; Frymus *et al.*, 1986; Nakai *et al.*, 1986; Foged *et al.*, 1987; Chanter, 1990). All exposed pigs developed progressive and irreversible turbinate lesions to a certain degree on the basis of one of the grading systems at various stages after challenge exposure. According to de Jong and Akkermans (1986), SPF pigs up to 16 weeks old had clear ventral conchae lesions with septum deviation 4 weeks after intranasal inoculation with pathogenic Pm^+.

To study effects of environmental and animal factors on AR and unravel some of the mechanisms involved, a standard challenge-exposure model for experimental induction of AR is required. Such model should approach infection route and clinical signs of field infection in conventionally raised pigs. But severity of signs of disease induced should be moderate (subclinical), so that factors related to the mucosal system of the turbinates, that may have a positive or negative effect on these signs can be studied. When the turbinates have disappeared completely, these effects are difficult to investigate.

For standardization of the model, Pm-T administration should be preferred over Pm^+ inoculation, because colonization of Pm^+ in the nasal mucosa is highly variable and dependent on irritation or damage of the nasal mucosa (Petersen and Elling, 1984; Chanter, 1990; de Jong, 1991). *Bordetella bronchiseptica* infection is ubiquitous in most

commercial pig units and induction of mucosal lesions is dependent on maternal immunity to *B bronchiseptica*. After pretreatment with diluted acetic acid, all pigs will at least have a certain degree of mucosal irritation. This will contribute to standardization of a model with *Pm*$^+$ cultures, as well as with Pm-T challenge exposure. The study reported here was designed to develop a challenge-exposure model - dose-dependent induction of subclinical (moderate) AR in conventionally raised pigs by intranasal administration of Pm-T.

MATERIALS AND METHODS

Pigs

Thirty Dutch Landrace (DL) and 30 Large White (GY) pigs were obtained from commercial farms with a *Pm*$^+$-free status (De Jong, 1985) issued by the Animal Health Service in The Netherlands. For practical reasons weaned pigs, about 4 weeks old, were studied. Pigs were assigned ad random to experimental groups by balancing body weight between groups. Littermates were distributed equally over experimental groups. At arrival, nasal swab specimens were obtained from all pigs for culture *B bronchiseptica* and toxigenic *P multocida*.

Housing and feeding

The study was carried out in 2 large identical climate-controlled chambers at the Agricultural University (Verstegen *et al.*, 1987). In each of these chambers, 2 pens, 9 m^2, were available. In each chamber, 1 breed group was housed, 15 pigs/pen. Thermoneutral environmental temperature for young pigs (25 C) was chosen. Relative humidity was maintained at 65 to 70%. Pigs were fed commercial pelleted food (16.7 kJ gross energy/g, 17% crude protein) ad libitum, using self-feeders, and had free access to water.

Experimental design

The experiment had a 2 x 5-factorial arrangement of treatments: 2 breeds and 5 Pm-T dose groups. After 4 days' acclimatization to the chambers, all pigs were intranasally administered 1%-acetic acid (0.5 ml/nostril) diluted in phosphate-buffered saline solution (PBSs). Three days later, initial challenge exposure with purified Pm-T[b] was performed. Pigs received either 0 (control), 5, 13, 20, or 40 μg of Pm-T/ml of PBSs, 0.5 ml/nostril/d on 3 consecutive days for Pm-T dose (TD) group TD_0, TD_5, TD_{13}, TD_{20}, and TD_{40},

[b]Pm-strain 5/05097-1, type D (batch 2-2-89, 123 μg/ml). Kindly provided by Dr E Rijke and Dr PK Storm, Intervet International BV, Boxmeer, The Netherlands.

respectively. Spraying was used instead of droplet application, because the latter caused vigorous sneezing and, hence, loss of toxin. Six pigs per breed per dose were studied. Two treatment groups and half of the control pigs of a breed were housed in a pen. The experiment lasted for 5 weeks after initial challenge exposure (Day 0).

All pigs were weighed once a week. Blood samples were collected before treatment and at weekly intervals during the study. Antibody produced *in vivo* and specific for Pm-T was determined by ELISA. Serial dilutions of serum were applied to Pm-T-coated wells of a microtitration-plate. After incubation for 1 hour at 37 C, the amount of Pm-T antibodies bound to the toxin was determined by incubation with a peroxidase-conjugated goat anti-swine IgG[c] and a tetramethylbenzidine substrate. Color formation was stopped after 10 minutes. All absorbances were expressed relative to absorbance of a standard positive-control serum obtained from a vaccinated pig.

Response characteristics

Brachygnathia superior (BS) was measured in millimeters at the start (BS_s) and at the end (BS_e) of the study. Because BS is a breed-associated characteristic in herds without the disease (Rutter, 1985), the change in BS between start and end of the experiment was used in calculations ($cBS = BS_s - BS_e$). Progression of AR was defined after necropsy by grade of conchae atrophy after cross-section of the snout 5 weeks after challenge exposure. The snout was sectioned between the first and second premolar teeth (Figure 1). The method of grading described by De Jong (1985) was used; ventral conchae atrophy (VCA) was graded from 0 (no lesions) to 4 (total atrophy) and dorsal conchae atrophy (DCA) was graded from 0 to 3. That author defined grade 2 for either VCA or DCA as moderate atrophy. Mean score for both nostrils was used in calculations.

Because DCA develops later than VCA in pigs affected with AR (De Jong, 1985), subclinical (moderate) AR status in this study, was defined as intermediate conchae atrophy (grade 2 for VCA and 1 or 2 for DCA) and perceptible difference in change in brachygnathia superior (cBS) between control and challenge-exposed pigs, 5 weeks after challenge-exposure.

Statistical analysis

Traits were statistically analyzed for effect of breed (B), Pm-T dose (TD) and their interaction (B × TD) using two-way ANOVA (SAS, 1985). Effects of litter, sex, and bacterial infection were not included in the model because results of preliminary analyses did not indicate effects on the traits.

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[c]GASw/IgG_{H+L}, Kpl, Gaithersburg, MD, USA

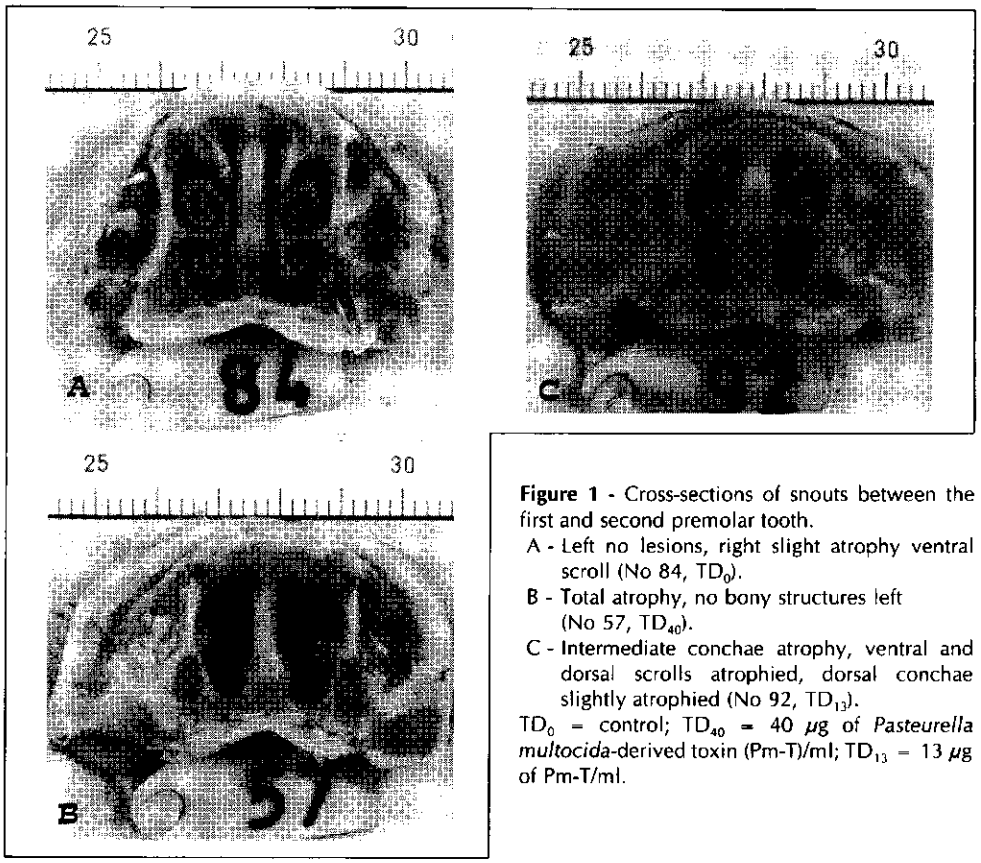

Figure 1 - Cross-sections of snouts between the first and second premolar tooth.
A - Left no lesions, right slight atrophy ventral scroll (No 84, TD_0).
B - Total atrophy, no bony structures left (No 57, TD_{40}).
C - Intermediate conchae atrophy, ventral and dorsal scrolls atrophied, dorsal conchae slightly atrophied (No 92, TD_{13}).
TD_0 = control; TD_{40} = 40 μg of *Pasteurella multocida*-derived toxin (Pm-T)/ml; TD_{13} = 13 μg of Pm-T/ml.

Except for BS_s and initial body weight (BW_i), which were affected by litter, and for BW, which was affected by sex. Also for these traits, litter and sex were not included in the model, because littermates were equally distributed over experimental groups.

Weekly measurements, observations on weight gain, and antibody formation were dependent, and were, therefore, an animal effect. Thus, a time variable (weeks) was added to the aforementioned model for analyzing effects on these traits. Effects of B, TD, and their interaction were tested against animal effect, and weeks and potential interaction terms were tested against the overall error term.

Pairwise comparisons were performed between experimental and control groups, using least-square mean differences at the overall 0.05 level of significance. Mutual relations between Pm-T dose and response characteristics and among the response characteristics were orthogonally fitted by polynomial regression (Snedecor and Cochran, 1980).

RESULTS

General

At arrival, neither *B bronchiseptica* nor toxigenic *P multocida* were detected in the GY pigs. Of 30 DL pigs, 13 were *B bronchiseptica* culture-positive and 6 of 30 were culture-positive for a non-toxigenic *P multocida* (Pm^-). At the end of the study, 6 GY and 18 DL pigs were culture-positive for *B bronchiseptica* and 10 DL pigs were culture-positive for Pm^-.

One GY pig allotted to the control group (TD_0) died of complicated umbilical inflammation a week after arrival, and 1 DL pig allotted to dose group TD_{20} died without a clear cause 5 days after arrival. None of the pigs treated with Pm-T developed detectable serum titer of Pm-T antibodies.

Body weight

At 4 weeks of age, mean initial body weight (BW_i) of GY pigs was 1.5 kg higher, compared with that of DL pigs ($P < 0.001$). The BW_i was similar between dose groups, and interaction between breed and dose groups was not detected (Table 1). Body weight gain over the experimental period (BWG_{exp}) was found to be breed-dependent ($P < 0.001$), resulting in a 6.5-kg difference in BW in favor of GY pigs. Significant effects of applied Pm-T dose or of interaction between breed and dose were not found (Table 1), but compared with controls, tendency toward lower BWG_{exp} with dose was seen.

Table 1 - Comparison of variables by dose group. Least-square means and significance level of initial body weight (BW_i), brachygnathia superior (BS_s), body weight gain over the experimental period (BWG_{exp}), ventral and dorsal conchae atrophy scores (VCA, DCA) and difference between BS_s and BS_e (cBS), using two-way ANOVA*

	Dose Pm-T (μg/ml)						Significance level		
Variable	0	5	13	20	40	SEM	B	TD	B×TD
n	11	12	12	11	12	-	-	-	-
BW_i (kg)	7.69	7.69	7.70	7.62	7.70	0.371	<0.001	0.99	0.99
BS_s (mm)	1.43	1.19	0.77	1.21	1.31	0.265	<0.001	0.48	0.56
BWG_{exp} (kg)	18.3	17.8	17.6	18.2	17.4	0.884	<0.001	0.95	0.45
cBS (mm)	3.88^a	5.69^{ab}	7.77^{bc}	6.58^{bc}	7.98^c	0.787	0.22	0.004	0.68
VCA	0.93^a	1.54^{ab}	1.88^b	2.12^{bc}	2.83^c	0.202	0.012	<0.001	0.96
DCA	0.26^a	0.58^a	0.75^a	0.88^a	1.79^b	0.235	<0.001	<0.001	0.63

* Model used $Y_{ij} = \mu + B_i + TD_j + (B \times TD)_{ij} + e$; where Y = variable, B = breed, TD = dose of *Pasteurella multocida*-derived toxin, (B×TD) = interaction between breed and Pm-T dose.
[a,b]Different superscripts indicate pairwise significant ($P < 0.05$) difference.

Mean weight gain per week (BWG_w) was significantly affected by breed and the interactions, breed×week and dose×week ($P < 0.05$). With regard to the applied dose, after week 3, weight gain per week of the Pm-T-exposed pigs was lower, compared with controls (Figure 2). Over the last 2 weeks, difference from controls in weight gain was 32, 54, 52, and 96 g/d/pig for TD_5, TD_{13}, TD_{20}, and TD_{40}, respectively.

Response characteristics

The DL pigs have a longer upper jaw than do GY pigs ($P < 0.001$), expressed in higher BS_s (1.86 vs 0.50 mm). Neither significant difference among dose groups nor significant interaction between breed and dose groups was found for this initial characteristic (Table 1). The change in BS between start and end of the study (cBS) was only dose-associated; interaction between breed and dose group was not observed. The cBS (Figure 3) increased with applied dose of Pm-T ($P < 0.05$), and this relation was linear (Table 2). When control pigs were eliminated from analyses, significant relation was not found.

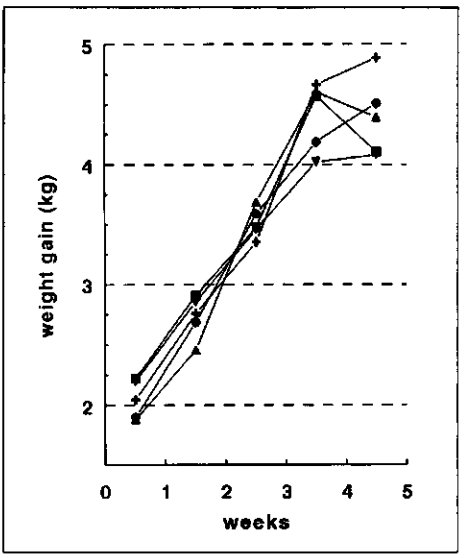

Figure 2 - Least-square mean body weight gain by dose group. -+- TD_0 = control; -▲- TD_5 = 5 μg of Pm-T/ml; -●- TD_{13} = 13 μg of Pm-T/ml; -■- TD_{20} = 20 μg of Pm-T/ml; -▼- TD_{40} = 40 μg of Pm-T/ml.

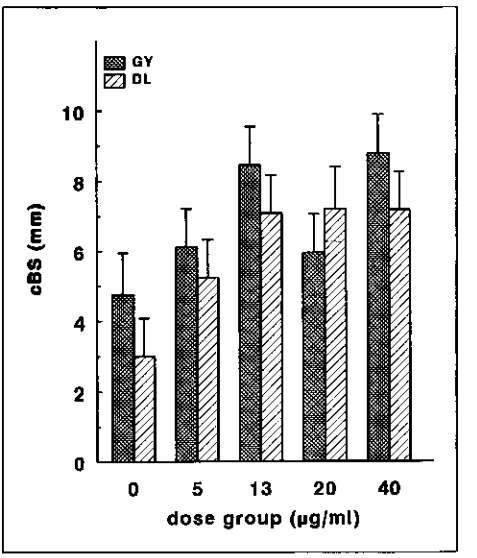

Figure 3 - Mean (± SEM) change in brachygnathia superior (cBS = BS_s - BS_e) for Large White (GY) and Dutch Landrace (DL) pigs in each Pm-T dose group.

In pairwise comparison, cBS was significantly ($P < 0.05$) different between control group TD_0 and groups TD_{13}, TD_{20}, and TD_{40}. Explanation was not found why the GY pigs of the TD_{20} group had divergently lower cBS. Without this group in the analyses, the relation between cBS and dose became a second-degree polynomial ($r^2 = 0.27$).

Conchae atrophy developed in all Pm-T-exposed pigs. One pig of each breed had maximal score (VCA 4 and DCA 3); both belonged to the TD_{40} group. Presence of *B bronchiseptica* or Pm^- infection in DL pigs did not affect their VCA or DCA score. Conchae of DL pigs were significantly ($P < 0.01$) more affected than those of GY pigs (VCA, 2.09 vs 1.62; and DCA, 1.24 vs 0.47). Progression of AR signs (VCA and DCA) caused by intranasally administered Pm-T was dose-dependent (Table 1), the grade of atrophy increased linearly with the dose of Pm-T applied (Table 2). Interaction between breed and dose groups was not found. In pairwise comparison, ventral conchae in pigs of the higher dose groups were significantly ($P < 0.05$) more affected than those of control pigs (TD_0). Dorsal conchae of pigs in group TD_{40} were significantly ($P < 0.05$) more atrophied than those of pigs of the other groups.

Table 2 - Relation between response characteristics (VCA, DCA, cBS), applied Pm-T dose, and body weight gain over the experiment (BWG_{exp}) or over the last 2 weeks (BWG_{w4}, BWG_{w5}).

Relation Y to X	With controls				Without controls			
	Intercept	$ß_1$	$ß_2$	r^2	Intercept	$ß_1$	$ß_2$	r^2
VCA to dose	1.194	0.043	NS	0.44	1.373	0.037	NS	0.35
DCA to dose	0.294	0.036	NS	0.26	0.310	0.035	NS	0.20
cBS to dose	5.065	0.084	NS	0.16	-	-	-	-
DCA to VCA	-0.187	0.152	0.177	0.68	-1.175	1.043	NS	0.68
VCA to cBS	1.179	0.108	NS	0.12	1.719	0.052	NS	0.03
DCA to cBS	0.283	0.091	NS	0.07	0.528	0.067	NS	0.03
BWG_{exp} to VCA	3.058	-0.070	NS	0.07	3.251	-0.069	NS	0.08
BWG_{exp} to DCA	2.297	-0.084	NS	0.09	2.586	-0.093	NS	0.10
BWG_{w4} to VCA	5.028	-0.338	NS	0.14	-	-	-	-
BWG_{w5} to VCA	5.067	-0.364	NS	0.11	-	-	-	-

Model used $Y = \text{Intercept} + ß_1 \cdot X + ß_2 \cdot X^2$, where X and Y = variables, $ß_{1,2}$ = regression coefficients. NS = not significant.

Mutual relations between dose and response characteristics and among the response characteristics also were determined (Table 2). The DCA and VCA were highly related to each other ($r^2 = 0.68$); DCA develops in association with the higher VCA grades. When devoid of controls, the linear relation with dose remained for VCA and DCA, but relation was not found between cBS and dose. The lower weight gain during week 4 and

week 5 (BWG_{w4} and BWG_{w5}) in Pm-T-exposed pigs was linearly related with severity of VCA. Pigs with severe conchae atrophy had less growth, compared with pigs with low conchae atrophy scores.

DISCUSSION

In this study, all Pm-T-exposed pigs had nasal damage that was dose-dependent. The GY pigs had significantly lower VCA and DCA scores than did DL pigs (including controls), whereas DL pigs had less change in brachygnathia superior (except those in group TD_{20}). Rutter (1985) reviewed conflicting findings in the relation between facial conformity and severity of AR. Others cited, may have been referring to various syndromes, because the infectious nature of AR was not known during their research. Presence of *B bronchiseptica* infection in half the DL pigs was not found to interfere with applied Pm-T doses, but might be the cause of some of the breed difference in VCA and DCA, because it has been reported that infection with *B bronchiseptica* could result in mild clinical manifestations (de Jong and Akkermans, 1986). Differences in susceptibility and sensitivity of the nasal bone tissue and receptors on cells may be explained by differences between breeds or lines (Martineau *et al.*, 1988) in similar manner as breeds can differ in immune responses (Meeker *et al.*, 1987).

Notwithstanding a difference in degree differences in response characteristics were not found in the way the breeds reacted to the applied Pm-T dose. The same challenge dose can be used for both breeds. Dosage of 13 μg of Pm-T/ml administered intranasally on 3 consecutive days met the requirements of a standard challenge-exposure model for experimental induction of moderate AR. At dosage of 13 μg/ml, both breeds had intermediate grade of conchae atrophy (VCA, 1.75 and 2.0; and DCA, 0.33 and 1.16 for GY and DL pigs, respectively) and clear change in BS (cBS, 8.46 and 7.08 for GY and DL, respectively, Figure 3), all significantly different from values in controls. In the long-nosed DL pigs, the applied 0.5-ml/nostril Pm-T solution may have spread over the longer conchae, rather than reaching the skull bones, whereas in GY pigs, the Pm-T might have reached the skull bones more easily, thus resulting in indefinite relation between cBS on the one side and VCA and DCA on the other.

Serum titer of antibodies against intranasal administered Pm-T were not detectable, which is in accordance with the poor immunogenicity associated with natural infections (Rutter, 1988). The reason why anti-toxin response is not detectable needs further research, because intramuscular administration of Pm-T causes strong antibody response, which is protective against experimental challenge exposure (Rutter, 1985; de Jong and Akkermans, 1986; Frymus *et al.*, 1986).

Our results indicate that this experimental challenge-exposure model mimics the pathogenic effect of *in vivo* infection with toxin-producing Pm strains, and response characteristics are dose-dependent. These findings correspond to results of other research workers (de Jong and Akkermans, 1986; Frymus *et al.*, 1986; Foged *et al.*, 1987), although in this study conventionally raised 4-week-old pigs and not young SPF or gnotobiotic pigs, were studied. The snouts were scored later after challenge exposure than was reported in most experiments.

Toward the end of the study, weight gain per week for Pm-T-exposed pigs was lower than that for control pigs. Controls (TD_0) had the best (18.3 kg) and pigs of group TD_{40} had the lowest BWG_{exp} (17.4 kg). This trait seemed to be dependent on severity of nasal damage. With time, this relation might have become more pronounced. Weight gain during the last 2 weeks (BWG_{w4} and BWG_{w5}) had a negative linear relation with VCA. Growth of pigs with severe conchae atrophy was more (negatively) affected than that of pigs with slight conchae atrophy. This relation and the variable productivity losses cited in literature (Rutter, 1985) need further research. They might be caused by changed efficiency in metabolism or by lower food intake of affected pigs.

Our model should enable studies of exogenous and endogenous factors involved in development of AR, independent of the colonizing ability of the Pm strain used. However, it remains to be established whether (sub)clinical signs attributable to administration of Pm-T are based on a mechanism similar to that attributable to *in vivo* infection with Pm^+ strains.

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Chapter 4

CLIMATIC ENVIRONMENT

Chapter 4.1

EFFECTS OF ATROPHIC RHINITIS AND CLIMATIC ENVIRONMENT ON THE PERFORMANCE AND ENERGY METABOLISM OF PIGS

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EFFECTS OF ATROPHIC RHINITIS AND CLIMATIC ENVIRONMENT ON THE PERFORMANCE AND ENERGY METABOLISM OF PIGS

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Abstract

Effects of subclinical atrophic rhinitis and exposure to adverse climatic environment on partitioning of energy (metabolism) and performance in pigs under field-like conditions were determined. Eight groups of 30 five-week old pigs each, were assigned to a 2 × 2 factorial arrangement of treatments: to 0 or 13 μg/ml of Pm-T challenge-exposure, and to a good or adverse climatic environment. Climatic treatment lasted 5 weeks. Pigs were fattened till 100 kg live weight. All Pm-T exposed piglets had nasal damage, which was not affected by climatic treatment. Growth retardation caused by Pm-T administration was suggested to be mainly the outcome of a lower food intake. Changes in metabolizability and maintenance requirements were not found. Growth retardation due to adverse climatic environment was related to a lower food intake as well as an increased maintenance requirement. Pm-T treated and control pigs from the good environment differed 3 days in reaching 100 kg body weight, while for those groups from the adverse environment this difference was 8 days.

Key words: Atrophic Rhinitis, *Pasteurella multocida*-toxin, Climatic Environment, Energy Metabolism, Performance, Piglets

INTRODUCTION

Environmental factors can play an important role in the health and production of livestock. Fluctuating ambient temperature and sometimes increased air velocity are important components of the indoor climatic environment. Incidence and severity of disease can be related to these components (Verhagen, 1987; Kreukniet *et al.*, 1990). This is particularly true for respiratory diseases in pigs (Verhagen, 1987; Elbers, 1991). In the respiratory tract, a direct contact between the animal and its environment exists. During inspiration, air travels along branching passages where it is warmed and filtered on its way to the lungs. The nasal turbinate bones (conchae) are part of this filtering system. And, therefore, prone to be affected by environmental conditions. When the conchae are

damaged or absent, the lungs are more susceptible to secondary infections like enzootic pneumonia or *Actinobacillus pleuropneumoniae*-infection (de Jong, 1983; Martineau *et al.*,1988). Therefore it is important that conchae function.

Toxin producing strains of *Pasteurella multocida* (Pm^+) cause progressive and irreversible lesions in the conchae (Foged *et al.*, 1987; Chanter, 1990). This disease, atrophic rhinitis (AR) is considered to be a disease with a multifactorial etiology. Aerial conditions, management factors and hygiene are involved in the epizootiology of AR (Smith, 1983; Goodwin, 1988; Robertson *et al.*, 1990). The severity of AR-symptoms is highly variable between animals both within and between affected farms. When an animal experiences disease, its maintenance requirement will increase due to an activated immune system and to fever, the efficiency of production will dwindle (Verhagen, 1987). Results reported with regard to effects of AR on productivity losses vary from no or little reduction in daily gain to a 10-15% reduction in growth rate (de Jong, 1985; Rutter, 1985). Growth of pigs with more severe conchae atrophy was more (negatively) affected than that of pigs with no or slight conchae atrophy (van Diemen *et al.*, 1994).
Reduction in growth can be the outcome of a lower food intake or a changed partitioning of energy (metabolism) in affected pigs. Coldness and draught increase maintenance requirement (heat production) so that less energy is available for body weight gain (Verhagen, 1987).

The aim of the present study was to assess the impact of exposure to adverse climatic conditions on the progression of AR symptoms, and the effects of atrophic rhinitis and climatic environment on partitioning of energy and performance of pigs under field-like conditions.

MATERIAL AND METHODS

Plan of study

Eight groups of pigs were studied in a 2 × 2 factorial arrangement of treatments: two levels of Pm-T challenge-exposure, and 2 different climatic conditions. Each group consisted of 30 five-week-old Large White (GY) piglets. The experimental period was composed of an exposure period of 5 weeks in a climatically-controlled respiration chamber and a fattening period in a conventional barn. The first day of the exposure period was defined Day 0. Each exposure period started after a preliminary period of 5-7 days. Pigs originated from 4 farms with a 'Pm^+-free' certificate of the Animal Health Service in The Netherlands (de Jong, 1985). On Day 0, the piglets (gilts and boars) were 39 ± 5 days old, and weighed 9.1 ± 2.3 kg.

Exposure period

Housing and feeding - Two by two, groups were brought to the experimental facilities. Upon arrival, these 60 piglets were randomly allocated to two groups and placed in a large, open-circuit, indirect climatically-controlled respiration chamber (Verstegen *et al.*, 1987). Within each chamber, two pens of 9 m^2 each were available (15 pigs per pen). At arrival and until Day 0, ambient temperature in chambers was 25°C, relative humidity was maintained at 65 to 70 % and air velocity was below 0.2 m/s. Lights were on from 0700 to 1900.

Piglets were fed a pelleted weaner diet *ad libitum* by self-feeders and had free access to water. Food contained 16,7 Kj of gross energy (GE) per gram and 17% crude protein. Both group-housing and *ad libitum* feeding are common in piggeries.

Experimental routine - In 4 groups, AR-like symptoms were induced with the Pm-T challenge-exposure model described by van Diemen *et al.* (1994). This model was aimed to induce moderate (subclinical) disease-symptoms, 5 weeks post challenge. Thus enabling studies on factors which may have a positive or negative effect on the disease symptoms. In short, all animals were pre-treated with an 1% acetic acid solution in water, 0.5 ml in each nostril. Three days after this pre-treatment, pigs were challenged intranasally with Pm-T (Pm-strain 5/05097-1 type D) on 3 subsequent days. The applied daily challenge-dose was 0.5 ml of a 13 μg Pm-T/ml Phosphor Buffered Saline solution (PBS) in each nostril. The other 4 groups were treated similarly with 0 μg Pm-T/ml PBS. The challenge-exposure treatment started on Day 0.

The climatic treatment as applied in this study was aimed at simulating sudden and intermittent changes in unfavourable climatic conditions. Two different climatic treatments were applied; a good environment and an adverse environment. At the good environment, the ambient temperature was thermoneutral (25°C) and air velocity was below 0.2 m/s. The adverse environment consisted of an ambient temperature below thermoneutrality (15°C) combined with draught periods. Four draught-periods were applied, one at daytime: 1300 to 1500, and 3 during the night: 2100 to 2300, 0100 to 0300 and 0500 to 0700. During each draught-period air velocity within the chamber was increased to 0.6 m/s and temperature of the air stream was lowered by 3 degrees compared with the ambient temperature in the chamber. The air stream was applied intermittently (4 minutes on and 4 minutes off) similar to Verhagen (1987). Between draught-periods, the air velocity was below 0.2 m/s. Exposure to the adverse conditions started on Day 0 and lasted throughout the exposure period.

Measurements - All animals were checked twice (start and end of exposure period) through nasal swab-samples for the presence of AR-causing toxigenic *Pasteurella multocida* and AR-predisposing factor *Bordetella bronchiseptica*. Bloodsamples were drawn on Day 0 (before treatments) and on collection days (d 7, 14, 21, 28, 35) for the detection of Pm-T specific *in vivo* antibodies. The change in brachygnathia superior (cBS) over the 35-d exposure period was measured in mm in all animals as disease characteristic (van Diemen *et al.*, 1994).

The exposure period was divided into five balance periods of one week each. Individual body weight (BW) was measured on Day 0, and at the end of each balance period (collection days: d 7, 14, 21, 28, 35). Daily food intake per pen was determined at 0800. Energy and nitrogen balances per group were measured during each balance period. Faeces with urine production was measured quantitatively per balance period per group and sampled for energy and nitrogen analysis. Gross energy (GE, $kJ \cdot kg^{-.75} \cdot d^{-1}$) values were determined by adiabatic bomb calorimetry and nitrogen content by the Kjeldahl method. Intake of metabolizable energy (ME, $kJ \cdot kg^{-.75} \cdot d^{-1}$) and ME:GE ratio were determined from the GE intake and loss of energy through faeces with urine. Heat production (HP, $kJ \cdot kg^{-.75} \cdot d^{-1}$) was measured by determining the gaseous exchange of oxygen and carbon dioxide as described by Verstegen *et al.* (1987). These exchanges were used to calculate heat production according to the formula of Brouwer (1965). On the collection days, HP was not measured. Retained energy (RE, $kJ \cdot kg^{-.75} \cdot d^{-1}$) and energy retention as protein (ER_p, $kJ \cdot kg^{-.75} \cdot d^{-1}$) and fat ($ER_f$, $kJ \cdot kg^{-.75} \cdot d^{-1}$) were calculated as described by del Barrio et *al.* (1993). The amount of metabolizable energy available for production (ME_p, $kJ \cdot kg^{-.75} \cdot d^{-1}$) was calculated as:

$$ME_p = \frac{1}{k_p} x ER_p + \frac{1}{k_f} x ER_f \qquad [1]$$

where k_p = efficiency of utilization of ME for protein was assumed to be 0.54 (ARC, 1981); and k_f = efficiency of utilization of ME for fat was assumed to be 0.74 (ARC, 1981). Energy requirement for maintenance (ME_m, $kJ \cdot kg^{-.75} \cdot d^{-1}$) was then estimated by subtracting ME_p from total ME.

At the end of the exposure period (Day 35) six piglets per group were necropsied (stunned and bled) to observe AR characteristics. Progression of AR was defined by the grade of ventral and dorsal conchae atrophy (VCA and DCA, respectively) after cross-sectioning the snout between the first and second premolar tooth. The method of grading described by de Jong (1985) was used; VCA was graded from 0 (no lesions) to 4 (total atrophy) and DCA from 0 (no lesions) to 3 (total atrophy). The average of both nostrils was used in calculations.

Fattening period

The remaining 24 pigs per group were transferred to a conventional barn. They were housed in 2.75 × 2.20 m pens, 3 to 4 pigs per pen. About 30% of the floor was covered with slats. Ambient temperature was kept above 15°C. Pigs had free access to food and water. The first four weeks pigs were fed a grower diet containing 17 kJ GE per gram and 17% crude protein. Thereafter they were fed a finisher diet containing 20 kJ GE per gram and 16.5% crude protein. Individual BW was measured once a week. A pig was slaughtered (stunned and bled), and AR characteristics were defined, when the 100 kg body weight (D_{100kg}) was reached. Foodintake (FI) was measured weekly per pen. For analyzing weight gain and FI, the end of the fattening period was defined as the week the first pig reached 100 kg live weight (week 15).

Statistical analysis

The individually measured values of BW_0, BW_{35}, VCA, DCA, cBS, and D_{100kg} were averaged per group, separately for exposure and fattening period. These means were analyzed for effect of Pm-T challenge-exposure, climatic treatment and their interaction using a two-way analysis of variance (SAS, 1989).

The effects of Pm-T challenge, climatic treatment, time (week), and their interactions on the energy balance traits (GE, ME, ME:GE, HP, RE, ER_p, ER_f, and ME_m) were tested by means of a F-test using Equation [2], with data of traits within groups taken as repeated measurements:

$$Y_{ijkl} = \mu + PmT_i + CT_j + (PmT \times CT)_{ij} + e_{1,ijk} + period_l + interactions + e_{2,ijkl} \qquad [2]$$

where Y_{ijkl} = trait at challenge-exposure i, climatic treatment j, group k, and balance period l; μ = overall mean; PmT_i = the effect of challenge-exposure i (i = 1,2); CT = the effect of climatic treatment j (j = 1,2); $e_{1,ijk}$ = error term 1, which represents the random effect of group k within challenge-exposure i and climatic treatment j (k = 1,2); $week_l$ = the effect of balance period l (l = 1,..,5); and $e_{2,ijkl}$ = error term 2. The effects of Pm-T challenge-exposure, climatic treatment and their interaction were tested against error term 1. The effect of period and potential interactions were tested against error term 2.

Data on WG and FI were averaged per group and per week. They were analyzed separately for the exposure period (week 1-5) and fattening period (week 7-15) by Equation [2]. Sexe of animals and/or bacterial infection on were not included in the model because preliminary analyses showed no such effects nor of interactions with and treatments on averaged traits in either the exposure period or fattening period.

RESULTS

Exposure period

General - In all groups *Bordetella bronchiseptica* and non-toxigenic *Pasteurella multocida* were detected in about 50% of the piglets. These bacterial infections did not affect the performance traits or the disease symptoms. Seven piglets (4 controls and 3 Pm-T treated) were discarded from the study: 4 pigs because of lameness and tailbiting, and 3 pigs died of causes not related to AR (coli-diarrhoea, enteritis, *Streptococcus*-infection). None of the piglets experimentally treated with Pm-T developed detectable serum levels of antibodies against Pm-T.

AR-characteristics - All necropsied Pm-T treated pigs had developed nasal damage significantly different from their non Pm-T treated contemporaries ($P < 0.05$). Ventral and dorsal conchae atrophy scores were solely affected by Pm-T treatment (Table 1). Mean VCA scores were 1.96 and 3.29 for control and Pm-T challenged pigs, respectively. Mean DCA score was 0.29 for control and 1.69 for Pm-T challenged pigs. Brachygnathia superior (cBS) tended to change more in Pm-T challenged than in controls piglets ($P < 0.051$), 6.41 vs 1.40 mm, respectively (Table 1). Effects of climatic treatment and of interaction between treatments on the AR-characteristics were not found.

Table 1 - Least Square Means (SEM) and significance level of body weight on Day 0 (BW_0) and Day 35 (BW_{35}), ventral and dorsal conchae atrophy (VCA, DCA), change in Brachygnathia superior (BS), weight gain (WG), and food intake (FI) of pigs within a Pm-T challenge treatment and climatic treatment (CT) combination in the exposure period (week 1-5).

	Pm-T treatment							
	0 µg/ml		13 µg/ml			P-value[1]		
climatic treatment	good	adverse	good	adverse	SEM	PmT	CT	PmT×CT
BW_0 (kg)	8.95	8.99	9.25	9.24	0.57	0.65	0.96	0.97
BW_{35} (kg)	25.88	23.26	25.73	22.46	1.38	0.75	0.10	0.83
VCA	2.04[a]	1.88[a]	3.25[b]	3.33[b]	0.27	0.008	0.884	0.666
DCA	0.21[a]	0.38[a]	1.83[b]	1.54[b]	0.41	0.027	0.886	0.604
cBS (mm)	1.70	1.09	8.16	4.66	1.82	0.051	0.321	0.470
WG (g/d/pig)	486	402	471	384	26	0.545	0.030	0.959
FI (g/d/pig)	762	684	731	656	54	0.613	0.232	0.988

[1]In these three separate columns the effect of Pm-T treatment (PmT), climatic treatment (CT) and their interaction (PmT×CT) on the depicted traits are given (level of significance $P < 0.05$).
[a,b]different letters within a row indicate a significant difference $P < 0.05$.

Body weight - At Day 0, mean initial body weight (BW_0) of the piglets was similar for all treatment groups (Table 1). Mean weight gain (WG) over the exposure period was affected by climatic environment ($P < 0.030$). No effect of Pm-T challenge or of interaction between treatments on WG was found (Table 1). Pigs kept under adverse conditions had a reduced WG of 85 g/d compared with pigs kept under good conditions. Effect of Pm-T treatment on WG did not change with time (Figure 1). Difference in WG between the two climatic treatments was significant from week 3 onwards (Figure 2).

Food intake - In general, Pm-T treated animals ate less (30 g/d) than their contemporaries though not significantly (Table 1). In time, the difference in FI between the control and Pm-T treated animals increased (Figure 1). In week 4 and 5 this difference tended towards significance ($P < 0.1$). Pigs kept in the adverse environment had a lower FI (70 g/d) than pigs in the good environment. During the exposure period, the difference in FI between the good and adverse environment increased with time ($P < 0.0008$) (Figure 2).

Metabolism characteristics - The effects of Pm-T treatment and climatic treatment on the energy metabolism characteristics are given in table 2. The metabolizability of the dietary energy (ME:GE) was not affected by the applied Pm-T treatment. The maintenance requirement (ME_m) as well as the heat production (HP) were (although not significantly) reduced in the Pm-T treated pigs compared with the not Pm-T treated controls.

Table 2 - Effect of Pm-T treatment and climatic treatment (CT) on gross energy (GE), metabolizable energy (ME), ME:GE ratio, heat production (HP), retained energy (RE), energy retained as protein (ER_p) and fat (ER_f) and maintenance requirement (ME_m) of pigs during exposure period.

	Pm-T treatment					P-value[1]		
	0 µg/ml		13 µg/ml					
climatic treatment	good	adverse	good	adverse	SEM	PmT	CT	PmT×CT
GE ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	1599	1513	1520	1462	57	0.316	0.273	0.817
ME ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	1304	1203	1250	1163	36	0.263	0.060	0.848
ME:GE	0.813	0.795	0.822	0.796	0.01	0.719	0.119	0.768
HP ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	751	754	721	733	18	0.226	0.712	0.807
RE ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	552[a]	448	528	430[b]	19	0.310	0.006	0.891
ER_p ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	244	216	235	207	7	0.308	0.020	0.993
ER_f ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	309	233	294	223	19	0.523	0.017	0.894
ME_m ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	435	488[a]	417[b]	478	11	0.272	0.006	0.749

[1]In these three separate columns the effect of Pm-T treatment (PmT), climatic treatment (CT) and their interaction (PmT×CT) on the depicted traits are given (level of significance $P < 0.05$).
[a,b]different letters within a trait indicate a significant difference $P < 0.05$.

Table 3 - Effect of time (balance weeks) on gross energy (GE), metabolizable energy (ME), ME:GE ratio, heat production (HP), retained energy (RE), maintenance requirement (ME_m), and energy retained as protein (ER_p) and fat (ER_f) of young pigs.

week	1	2	3	4	5	SEM	P-value[1]
GE ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	1266^a	1493^b	1621^b	1618^b	1619^b	30	0.0001
ME ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	1000^a	1209^b	1313^b	1311^b	1316^b	26	0.0001
ME:GE	0.790^a	0.810^b	0.810^b	0.810^b	0.812^b	0.005	0.0207
HP ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	667^a	727^b	762^{bc}	774^{cd}	768^{cd}	9	0.0001
RE ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	334^a	482^b	550^b	537^b	548^b	19	0.0001
ER_p ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	171^a	224^b	245^b	244^b	244^b	6	0.0001
ER_f ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	163^a	258^b	306^b	293^b	304^b	13	0.0001
ME_m ($kJ \cdot kg^{-.75} \cdot d^{-1}$)	464	446	447	463	453	6	0.1116

[1]Interactions of Pm-T challenge, climatic treatment and their interaction with time parameter (week) not significant.
[a,b]different letters within a trait indicate significant differences ($P < 0.05$)

Climatic treatment (CT) increased the maintenance requirement (ME_m)and decreased the retained energy (RE) and the energy retained in protein (ER_p) and fat (ER_f) significantly ($P < 0.05$). The intake of metabolizable energy (ME) was lowered by CT ($P < 0.06$). The effect of CT was most clear in week 5. Interaction between Pm-T and climatic treatments were not found.

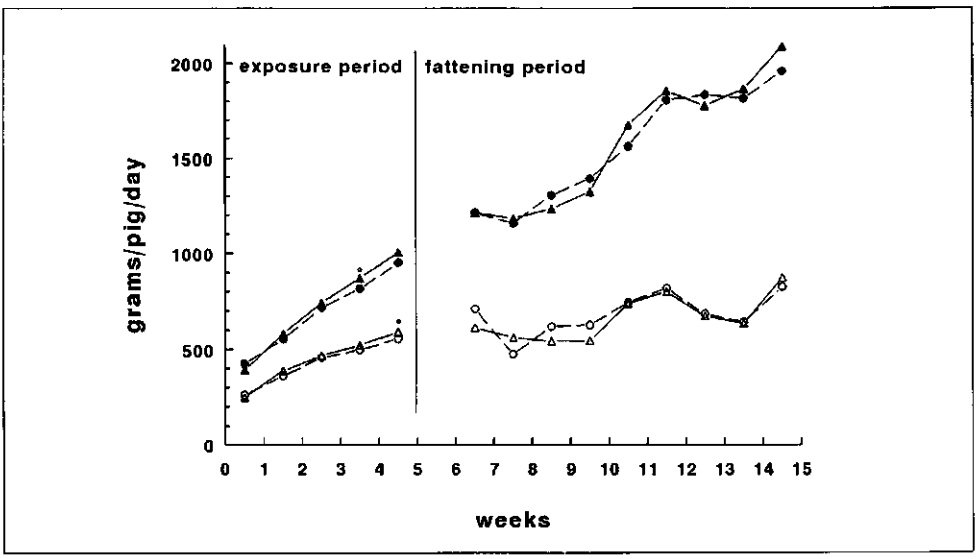

Figure 1 - Mean weight gain (WG) and food intake (FI) of pigs as affected by Pm-T treatment (0 μg/ml and 13 μg/ml) during the exposure period (week 1-5) and the fattening period (week 7-15).
—▵— WG 13 μg/ml; —○— WG 0 μg/ml; —▲— FI 13 μg/ml; —●— FI 0 μg/ml; ° $P < 0.1$.

All characteristics except ME_m increased with time (Table 3). No interaction between the time parameter (balance periods) and treatments occurred. Only heat production (HP) tended to be affected by the interaction of the climatic environment with the time parameter ($P < 0.067$). The first two weeks, the pigs in the adverse environment showed a higher HP, and the last two weeks, the pigs in the good environment showed a higher HP. In week 2 and 5 these differences were significant ($P < 0.001$). Influences of atrophic rhinitis in relation to climatic environment on level and changes in heat production and activity of pigs both between and within days will be described elsewhere (van Diemen *et al.*, 1995)

Fattening period

General - A total of 170 out of 185 pigs which entered the fattening period reached 100 kg live weight. Fourteen pigs were culled during the fattening period (5 Pm-T treated, 9 control pigs). One control pig was a runt pig and therefore discarded from analyses.

AR characteristics - At the end of the fattening period, nasal damage of Pm-T treated pigs differed from controls (Table 4). Mean VCA scores were 2.80 for challenged and 1.80 for control pigs. Mean DCA scores were 0.94 and 0.10 for Pm-T challenged and control pigs, respectively.

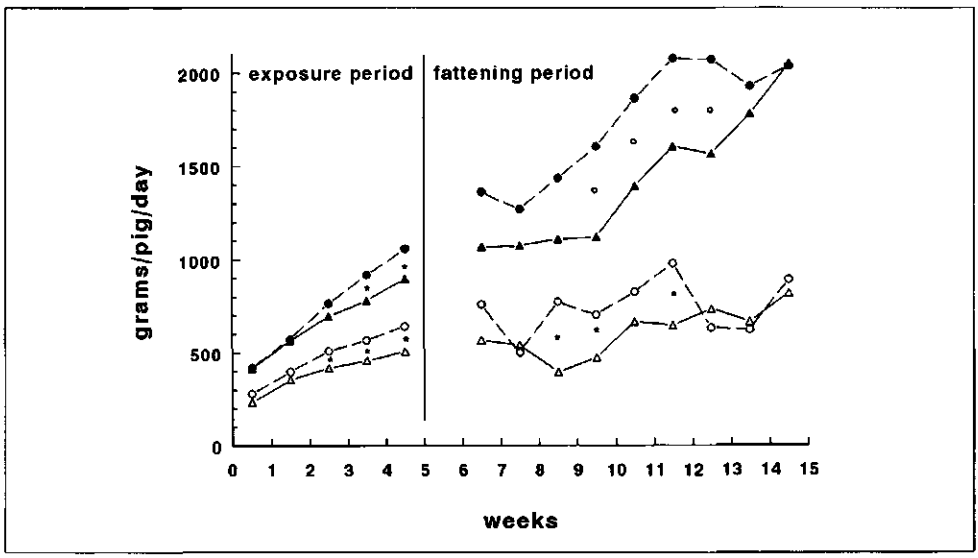

Figure 2 - Mean weight gain (WG) and food intake (FI) of pigs as affected by climatic treatment ('good' and 'adverse' environment) during the exposure period (week 1-5) and the fattening period (week 7-15). —▵— WG adverse; —○— WG Pm-T good; —▲— FI adverse; —●— FI good; * $P < 0.05$; ° $P < 0.1$.

Interaction between date of determination and severity of AR-characteristics was significant for DCA ($P < 0.0049$) and tended towards significance for VCA ($P < 0.1$). Pairwise comparisons showed that for Pm-T challenged pigs the nose damage was less severe at the end of the fattening period than at the end of the exposure period ($P < 0.05$) (Table 4). In the not Pm-T treated (control) animals no differences were found.

Table 4 - Least Square Means (SEM) and significance level of ventral and dorsal conchae atrophy (VCA, DCA), weight gain (WG), food intake (FI), and days to 100 kg live weight (D_{100kg}) of pigs per treatment combination (Pm-T, CT) in the fattening period (week 7-15).

	Pm-T treatment							
	0 µg/ml		13 µg/ml			P-value[1]		
climatic treatment	good	adverse	good	adverse	SEM	PmT	CT	PmT×CT
VCA	1.82[a]	1.79[a]	2.69[b]	2.92[b]	0.15	0.003	0.534	0.438
DCA	0.13[a]	0.06[a]	0.91[b]	0.94[b]	0.21	0.017	0.928	0.823
WG (g/d/pig)	741[a]	592[b]	743[a]	628[b]	42	0.661	0.027	0.690
FI (g/d/pig)	1748	1423	1724	1409	130	0.741	0.004	0.934
D_{100kg}	137[a]	144[b]	140[ab]	152[c]	0.99	0.006	0.001	0.089

[1]In these three separate columns the effect of Pm-T treatment (PmT), climatic treatment (CT) and their interaction (PmT×CT) on the depicted traits are given (level of significance $P < 0.05$).
[a,b]different letters within a row indicate a significant difference $P < 0.05$.

Weight gain - Effect of Pm-T treatment and of interaction between Pm-T treatment and climatic treatment on WG were not observed (Figure 1, Table 4). Climatic treatment affected WG ($P < 0.027$). Mean WG was 742 and 611 g/d/pig, respectively for pigs from good and adverse environment. Until week 12, pigs from the good environment had a higher WG than the pigs from the adverse conditions (Figure 2). After week 12 WG was comparable.

Over treatments, D_{100kg} ranged between 105 and 208 days. The average D_{100kg} was 143 days (± 17). The Pm-T treated pigs needed 5 days more to reach a BW of 100 kg ($P < 0.006$) compared with the controls (Table 4). Exposure to adverse conditions during a 5 week period after weaning caused a delay of 10 days in D_{100kg} ($P < 0.001$). Interaction between climatic environment and Pm-T challenge for this trait tended towards significance ($P < 0.089$). The difference in D_{100kg} between Pm-T treated and control pigs was 3 days at the good environment and 12 days at the adverse environment.

Food intake - During the fattening period (wk 7-15) FI was similar for Pm-T treated and control pigs (1.59 and 1.57 kg/d/pig, respectively) (Figure 1). Until week 13, FI of

pigs from the adverse conditions remained lower than of pigs coming from the good environment (Figure 2). The first-mentioned pigs consumed 320 g/d less during the fattening period ($P < 0.004$) than the latter.

DISCUSSION

The Pm-T administration induced symptoms of *in situ* infection with toxigenic *Pasteurella multocida*. Clinical cases were not observed. No evidence was found that the low ambient temperature with draught periods aggravated (or reduced) AR nose lesions, induced by the challenge model. While in practice, the severity of an AR-outbreak can be controlled to a great extent on most farms by improving climatic and social environment (Smith, 1983; Robertson *et al.*, 1990). The major difference between the studies can be marked as *Pasteurella multocida* field-infection with clinical cases versus Pm-T induced subclinical AR. Suggesting that the improvements in climatic environment as referred to by the above mentioned research workers, might have had a greater impact on the colonization possibilities and concomitant toxin production of the bacterium species on the mucous membrane, than on the passage of the toxin through this membrane. The Pm-T, whether produced by Pm^+ on the spot or applied experimentally, might reach the underlying bony tissues in any case.

Although nasal damage caused by Pm^+ or Pm-T is said to be irreversible in several studies (de Jong, 1985; Rutter, 1985; Foged *et al.*, 1987), in the present study partial regeneration of the conchae appeared in the Pm-T treated pigs. Whereas our exposure models aimed to induce subclinical AR, the other studies were performed with challenge-exposure models aimed at clinical status to establish pathogenic effects or to evaluate vaccines. When the conchae have disappeared completely, regeneration is impossible. The difference between challenged and control pigs in our study, was still present at the end of the fattening period (Table 4). In control animals regeneration of the conchae was not found. Thus it is not likely that the regeneration was caused by regrowth of the conchae after *Bordetella bronchiseptica* infection.

AR is said to be an important cause of economic losses through retarded growth rates, medication costs and inability to sell (breeding-) stock. De Jong (1985) mentioned that especially severely affected animals showed a 5-20% reduction in growth. Growth retardation can be caused by a lower food intake, a lower availability of nutrients and/or by an increased maintenance requirement (heat production) of exposed (affected) animals. Our findings show that the reduction in growth was the outcome of a lower food intake (30 g/d) rather than of a changed partitioning of energy (metabolism) in affected pigs

(Figure 1). The maintenance requirement (heat production), efficiency and metabolizability of the dietary energy (ME:GE) were not significantly changed by the applied Pm-T treatment. The reductions food intake and concomitantly weight gain occurred about one week after the challenge and progressed over time (Figure 1).

This outcome confirms the assumption of Smith (1983) that AR pigs convert food to meat as well as their contemporaries. He thought it likely that affected pigs had a depressed food intake, caused by a poor appetite, due to a possible loss of taste and sense of smell by damaged nasal tissues, or by irritation of the mucosal membrane by dust particles. In clinically diseased pigs differences in food intake, weight gain and metabolism might be more pronounced. On the other hand, Pm-T might cause only a local increase in metabolism, which might be too low to measure. The relation between individual rate of food intake, feeding strategy and severity of AR deserves further investigation in order to diminish economic losses in case of disease outbreaks.

Pigs housed under the adverse condition had less energy available for production than pigs in the good condition, due to their increased maintenance requirement (Table 2). The first two weeks, the piglets in the adverse environment showed a higher HP. The pigs, however, did not respond to the lower temperature by elevating food intake to meet the extra energy demand (Figure 2). Regulation of food intake may attribute to maintain homeothermy. Why an increase in food intake did not take place is not clear. Both control and challenged pigs at the adverse environment had a decreased food intake compared with pigs kept at 25°C. The food intake might be lowered due to reduced physical activity by huddling. This way, the pigs can reduce heat loss to the environment and adapt to their climatic environment. On the other hand, at the adverse conditions applied, pigs of this age (5 weeks) may not have the capacity to pick up heat loss by increasing food intake. Verhagen (1987) observed a delayed response in increasing FI after cold exposure in grower pigs of 10 weeks old.

Pigs could cope with the subclinical disease state as induced in this study, and reach 100 kg live weight with a delay of 3 days compared with their contemporaries. Exposure to adverse environmental conditions, however, during a 5 week period at young age, affected the performance later on as grower pig; Those pigs needed, on average, 10 days more to reach 100 kg live weight. Especially pigs which had to cope with both the Pm-T challenge and the adverse climatic treatment had a prolonged fattening period; 12 days longer to reach 100 kg live weight compared with the challenged pigs coming from the good environment (Table 4).

Thus it is concluded that the effects of Pm-T challenge and climatic treatment are greatly independent of each other. The adverse climatic treatment as applied in the

current study, did not aggravate severity of AR symptoms induced by our challenge model. The performance of the piglets was affected by climatic treatment,due to lower amount of energy available for production. The Pm-T administration caused a lower food intake with concomitantly growth reduction which seemed to be dependent on the development of nasal damage. Metabolizability and maintenance requirements were not changed. By controlling the climatic environment of pigs, the economic losses due to poor performance can be minimized.

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Chapter 4.2

EFFECTS OF *PASTEURELLA MULTOCIDA*-TOXIN INDUCED ATROPHIC RHINITIS ON HEAT PRODUCTION AND ACTIVITY OF PIGLETS KEPT UNDER DIFFERENT CLIMATIC CONDITIONS

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EFFECTS OF *PASTEURELLA MULTOCIDA*-TOXIN INDUCED ATROPHIC RHINITIS ON HEAT PRODUCTION AND ACTIVITY OF PIGLETS KEPT UNDER DIFFERENT CLIMATIC CONDITIONS

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Abstract

Effects of artificially induced moderate atrophic rhinitis symptoms on level and changes in heat production and activity were determined in piglets kept under different climatic conditions. Eight groups of 30 pigs each, housed in one of two climatically controlled respiration chambers, were exposed to a 2 x 2 factorial arrangement of treatments: challenge with 0 or 13 μg of *Pasteurella multocida*-Toxin (Pm-T)/ml, and two climatic environments (good: 25°C, or adverse: 15°C with draught periods). The Pm-T challenge reduced ($P < 0.05$) day averages of total (HP) and activity-related heat production (H_{ar}). The response to Pm-T treatment was similar in both climatic environments. Differences in the heat production and activity caused by the climatic treatment declined ($P < 0.001$) with time - with acclimation to the environment. Analyses of HP, H_{ar} and activity-free heat production in 12 2-h periods showed a biphasic activity rhythm. Both treatments affected ($P < 0.05$) level of HP and H_{ar} in several of the 2-h periods, but the biphasic rhythm was not altered. Day averages of H_{ar} showed a disposition to be differently affected ($P < 0.068$) by Pm-T challenge in the climatic treatments dependent on duration of exposure. This interaction effect appeared to originate from the periods between 1500 and 2100 ($P < 0.001$). Therefore, it might be wise to distinguish between overall effects (day means) on total, activity related and activity free heat production, and effects within a day (e.g. 2-h means). Pm-T treatment seemed to suppress the general state of well-being of pigs, reducing pigs' activity and food intake. By reducing its activity, the piglets seemed to compensate the lower food intake, the lower amount of energy available for production.

Keywords: *Pasteurella multocida* toxin, Atrophic Rhinitis, Climatic Environment, Heat Production, Physical Activity, Piglets

INTRODUCTION

Progressive atrophic rhinitis (AR) is a disease of the proximal respiratory tract that may affect pigs. Toxin-producing strains of *Pasteurella multocida* (Pm^+) cause AR-specific turbinate lesions (de Jong and Nielsen, 1990). Intranasal challenge of pigs with Pm^+-

derived toxin (Pm-T) can artificially induce AR; pathogenicity depends on the applied dose (van Diemen *et al.*, 1994; Foged *et al.*, 1987). Aerial conditions, management factors and hygiene are involved in the epidemiology of AR (Robertson *et al.*, 1990; Smith, 1983).

Animals need to maintain a steady state in their internal environment irrespective of their external surroundings (Curtis, 1983). Ambient temperature may affect level and variation in heat production within and between days (Verhagen, 1987). Activity of pigs has to be considered also because activity is related to heat production (Verhagen, 1987). When a pig experiences an infection, its reaction to adverse climatic conditions might be different from that of non-infected animals (Verhagen, 1987; Noyes *et al.*, 1988). Low ambient temperature and draught are a commonplace in the climatic environment of pigs under practical conditions (Tielen, 1988). The effects of AR in relation to climatic environment on level and changes in metabolic rate within and between days is unknown.

In this study, therefore, influences of atrophic rhinitis in relation to climatic environment on level and changes in heat production and activity of pigs both between and within days were investigated.

MATERIALS AND METHODS

Animals, Housing and Feeding

Eight groups of 30 Large White (GY) pigs each were studied in a 2 × 2 factorial arrangement of treatments: two levels of Pm-T challenge-exposure, and two different climatic environments. Two groups were studied at each combination of treatments. The experimental period was composed of an adaptation period of 5 to 7 days and an exposure period of 5 wk (35 d) in climatically controlled respiration chambers. The 1st d of the exposure period was defined d 0.

Pigs were purchased from four commercial farms that had obtained a 'Pm^+-free' certificate of the Animal Health Service in The Netherlands (de Jong, 1985). Two by two, groups were brought to the experimental facilities. Upon arrival, these 60 pigs were randomly allocated to one of two groups and placed in one of two large, open-circuit, indirect climatically controlled respiration chambers (Verstegen *et al.*, 1987). Within each chamber, two pens of 9 m^2 each were available (15 pigs/pen). On d 0, the pigs (gilts and boars) were 39 ± 5 d old, and weighed 9.1 ± 2.3 kg. Mean initial body weight was similar between treatment groups. Lights were on from 0700 to 1900.

Pigs were fed a pelleted weaner diet ad libitum by self-feeders and had free access to water. Food contained 16.7 kJ of gross energy (GE) per gram and 17% crude protein.

Food intake was measured daily. Results on energy metabolism measurements and performance will be described elsewhere (van Diemen *et al.*, 1995).

Treatments

For each group, at arrival and until d 0, ambient temperature in chambers was 25°C, relative humidity was maintained between 65 and 70% and air velocity was below 0.2 m/s. On d 0, groups were assigned to one of two climatic treatments (CT); a 'good' environment or an 'adverse' environment. At the 'good' environment, the ambient temperature was thermoneutral (25°C) and air velocity was below 0.2 m/s. The 'adverse' environment consisted of an ambient temperature below thermoneutrality (15°C) combined with daily draught periods. Four daily draught-periods were applied, one at daytime: 1300 to 1500, and three during the night: 2100 to 2300, 0100 to 0300 and 0500 to 0700. During each draught-period air velocity within the chamber was increased to 0.6 m/s and temperature of the air stream was lowered by three degrees compared with the ambient temperature in the chamber. The air stream was applied intermittently (4 min on and 4 min off) similar to Verhagen (1987). Between draught-periods, the air velocity was below 0.2 m/s. Exposure to the adverse conditions started on d 0 and lasted throughout the 35-d exposure period.

Atrophic rhinitis-like symptoms were induced with the Pm-T challenge-exposure model described by van Diemen *et al.* (1994) (Chapter 3). This 3-day challenge-exposure model was aimed to cause moderate (subclinical) disease-symptoms, 5 weeks post challenge. Factors related to the mucosal system of the turbinates that may have a positive or negative effect on the disease can be studied. In short, all animals were pretreated with a 1%-acetic acid solution in water (0.5 ml/nostril), 3 d before a 3-d intranasal challenge with Pm-T (derived from Pm^+-strain 5/05097-1 type D, Intervet International BV, Boxmeer, The Netherlands). Applied daily challenge-dose was 13 μg of Pm-T/ml Phosphor Buffered Saline solution (PBS) or 0 μg of Pm-T/ml PBS (control pigs), 0.5 ml/nostril. Challenge treatment started on d 0.

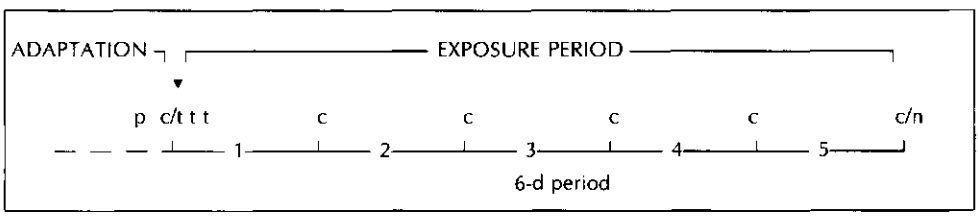

Figure 1 - Plan of study. ▾: start climatic treatment; p: HAc pre-treatment; t: Pm-T challenge; c: collection day (weigh pigs, clean pens, sample for nitrogen and energy balance); n: necropsy of 6 piglets per group.

Experimental Routine

The 35-d exposure period was divided into five subsequent 6-d periods separated by a collection day used to weigh pigs, clean pens, and to sample for nitrogen and energy balance (Figure 1). Rectal temperature was measured twice each 6-d period.

During the 6-d periods, the gaseous exchange of oxygen and carbon dioxide of a group of pigs within a chamber was determined continuously as described by Verstegen *et al.* (1987). Data on these gaseous exchanges were used to calculate heat production (HP) according to the formula of Brouwer (1965) in successive 9-min intervals. On the collection days, HP was not measured.

Physical activity was measured by an ultrasound burglar device above each pen (Wenk and van Es, 1976). Quantitative estimates of activity of the pigs per pen were recorded during successive 3-min intervals and stored as 9-min means, matching with heat production intervals. The relation between 9-min values of heat production and activity was determined for each group and for each day separately, according to Equation [1]. Activity in both pens within a chamber was used.

$$HP_{ijkl} = \mu + ß_1 \cdot X_{1i} + ß_2 \cdot X_{2i} + L_j + D_k + e_{ijkl} \quad [1]$$

where HP_{ijkl} = heat production in $kJ/kg^{0.75}$ per 9-min; X_{1i}, X_{2i} = activity counts of 9-min periods (i) of the ultrasound device in pens 1 (X_1) and 2 (X_2); $ß_1$, $ß_2$ = regression coefficients of heat on activity counts; L_j = fixed effect of Lights on (j = 1) or off (j = 2); D_k = effect of Draught on (k = 1) or off (k = 2); e_{ijkl} = error term.

Activity-related heat production (H_{ar}, $kJ/kg^{0.75}$) per 9-min interval was subsequently obtained according to the following:

$$H_{ar} = b_1 \cdot X_{1i} + b_2 \cdot X_{2i} \quad [2]$$

Where b_1, b_2 = estimates of regression coefficients ($ß_1$,$ß_2$) out of Equation [1]. Activity-free heat production (H_{af}, $kJ/kg^{0.75}$) was calculated as total heat production per 9-min interval minus activity-related heat production per interval. For each 6-d period, data on HP, H_{af}, and H_{ar} of 4 d were used in analyses. Data of HP, H_{af}, and H_{ar} on rectal temperature days (2 d per 6-d period) were omitted from the data set because of disturbance of the pigs and potentially disturbed measurements.

At the end of the exposure period (d 35), six pigs per group were slaughtered (stunned and bled) to observe AR characteristics. Progression of AR was defined by the grade of conchae atrophy after cross-sectioning the snout between the first and second premolar tooth. Atrophy of the ventral conchae (VCA) was graded from 0 (complete

conchae) to 4 (total atrophy) and of the dorsal conchae (DCA) from 0 to 3 (total atrophy) (de Jong, 1985). The average of both nostrils was used in calculations.

Statistical Analyses

The effects of Pm-T challenge, climatic treatment, day, and their interactions on total heat production (HP), activity related (H_{ar}) and activity free heat production (H_{af}) were tested by means of a F-test (SAS, 1989) using below mentioned Equation [3]. Daily values of traits within groups were taken as repeated measurements.

$$Y_{ijkl} = \mu + PmT_i + CT_j + (PmT \times CT)_{ij} + e_{1,ijk} + ß*day_l + \text{interactions} + e_{2,ijkl} \qquad [3]$$

where Y_{ijkl} = HP, H_{ar} or H_{af} at challenge-exposure i, and climatic treatment j, in group k, day l; PmT_i = the effect of Pm-T challenge-exposure i (i = 1,2); CT = the effect of climatic treatment j (j = 1,2); $e_{1,ijk}$ = error term 1, which represents the random effect of group k within PmT and CT combination (k = 1,2); day_l = day number l (l = 1,..,35); ß = regression coefficient of trait on day number; $e_{2,ijkl}$ = error term 2. The effects of Pm-T challenge, climatic treatment and their interaction were tested against error term 1. The effects of day and potential interaction terms were tested against error term 2.

To study whether the effects of Pm-T challenge and climatic treatment on HP, H_{af}, and H_{ar}, were influenced by the time of day, the day was arbitrarily divided into 12 periods of 2 h each (2-h periods), starting at 0700. Averages for each of the defined periods were analyzed with Equation [3].

Table 1 - Least square means (± SEM[a]) and significance level of daily weight gain (WG, $g \cdot d^{-1} \cdot pig^{-1}$), food intake (FI, $g \cdot d^{-1} \cdot pig^{-1}$), total (HP), activity-related (H_{ar}), and activity-free (H_{af}) heat production ($kJ \cdot kg^{0.75} \cdot d^{-1}$) as influenced by Pm-T treatment (μg/ml) and climatic treatment (CT)

	Pm-T		CT			P-value[b]		
	0	13	good	adverse	SEM	PmT	CT	PmT×CT
WG	444	427	479	393	18.2	0.545	0.030	0.959
FI	723	693	746	670	38.4	0.613	0.232	0.988
HP	751	725	737	740	11.2	0.178	0.868	0.761
H_{ar}	180	162	168	175	5.5	0.076	0.432	0.724
H_{af}	571	563	569	565	9.5	0.606	0.784	0.579

[a]SEM between treatment means.
[b]In these separate columns the effect of Pm-T treatment (PmT), climatic treatment (CT), and their interaction (PmT×CT) on the depicted traits are given.

RESULTS

General

All pigs treated with Pm-T showed nose damage. Mean VCA scores (SEM) were 1.96 (± 0.14) and 3.29 (± 0.12) for control and Pm-T challenged pigs respectively ($P < 0.008$). Mean DCA score was 0.29 (± 0.09) for control and 1.69 (± 0.18) for challenged pigs ($P < 0.03$). Effect of climatic treatment on conchae atrophy was not found.

In Table 1, daily gain and food intake are given. Pm-T treated animals consumed 30 g/d less and gained 17 g/d less weight than their contemporaries. The effect of Pm-T treatment on both traits increased as time progressed. In the last 6-d period, the effect of Pm-T treatment on daily gain and food intake was more severe in the 'adverse' environment (45 g/d and 64 g/d reduction, respectively) compared with the 'good' environment (31 g/d and 44 g/d reduction, respectively) ($P < 0.1$). Results on performance and partitioning of energy will be described elsewhere (van Diemen *et al.*, 1995).

Heat Production

Total (HP), activity related (H_{ar}), and activity free (H_{af}) heat production are stated in kiloJoule per kilogram of metabolic weight per day ($kJ \cdot kg^{-0.75} \cdot d^{-1}$).

Day Averages - Day averages of HP and H_{af} were not affected by PmT, CT, nor by their interaction (Table 1). H_{ar} of the Pm-T treated pigs was 18 $kJ \cdot kg^{-0.75} \cdot d^{-1}$ lower than of the control pigs ($P < 0.076$). All heat production traits increased with increasing day number ($P < 0.001$) in any treatment. The Pm-T treatment caused, with increasing day number, a lower increase in HP ($P < 0.01$) as well as in H_{af} ($P < 0.044$). This treatment effect started approximately 6 d after challenge (Figure 2A). The interaction of Pm-T with day number on H_{ar} tended to be different for both climatic treatments ($P < 0.068$). In the 'adverse' environment, the challenged pigs spent less heat on activity in the last 14 days compared with the Pm-T treated pigs in the 'good' environment. In time, climatic treatment affected ($P < 0.001$) both HP and H_{ar} (Figure 2B). These traits were raised during the first 14 days and lowered during the last 14 days in the adverse environment as compared with the good environment (Figure 2B).

Two-hour Periods - Analyses of HP, H_{ar}, and H_{af} in 12 2-h periods showed a biphasic activity rhythm within a day (Figure 3A and B). Just after 'lights on' (0700) the first peak of activity was seen, followed by a relatively quiet period. In the afternoon a second, more pronounced peak of activity ('playtime') appeared, which decreased rapidly after 'lights out' (1900) and stayed low during the night. The main peak in activity-free heat production occurred after 'lights out'. This pattern remained throughout the exposure period for all treatment groups.

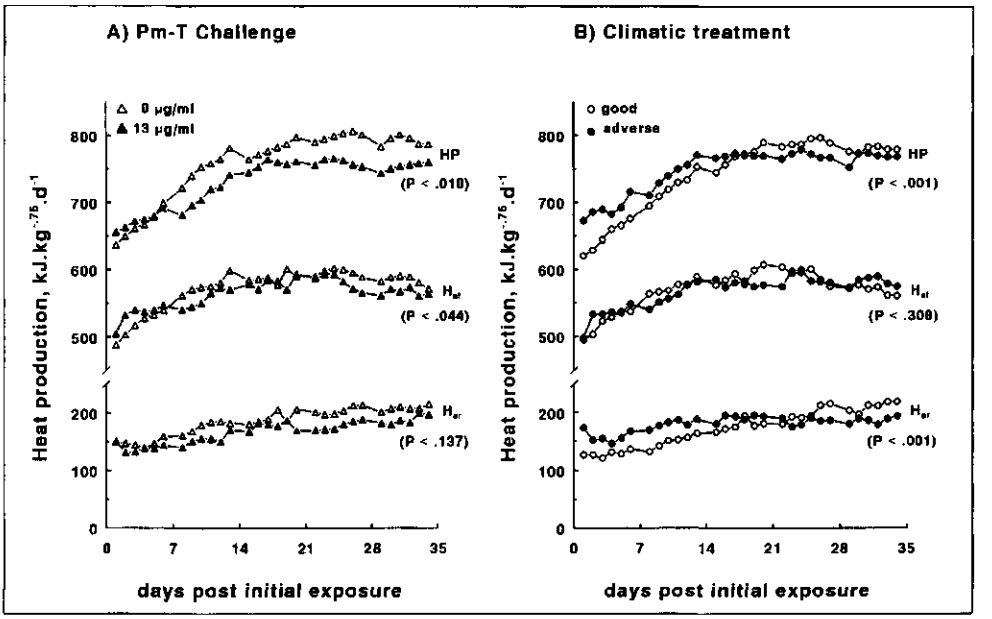

Figure 2 - Day averages (and significance level of interaction) of total (HP), activity-related (H_{ar}) and activity-free (H_{af}) heat production during 35-d exposure period, A) within Pm-T challenge treatment, and B) within climatic treatment.

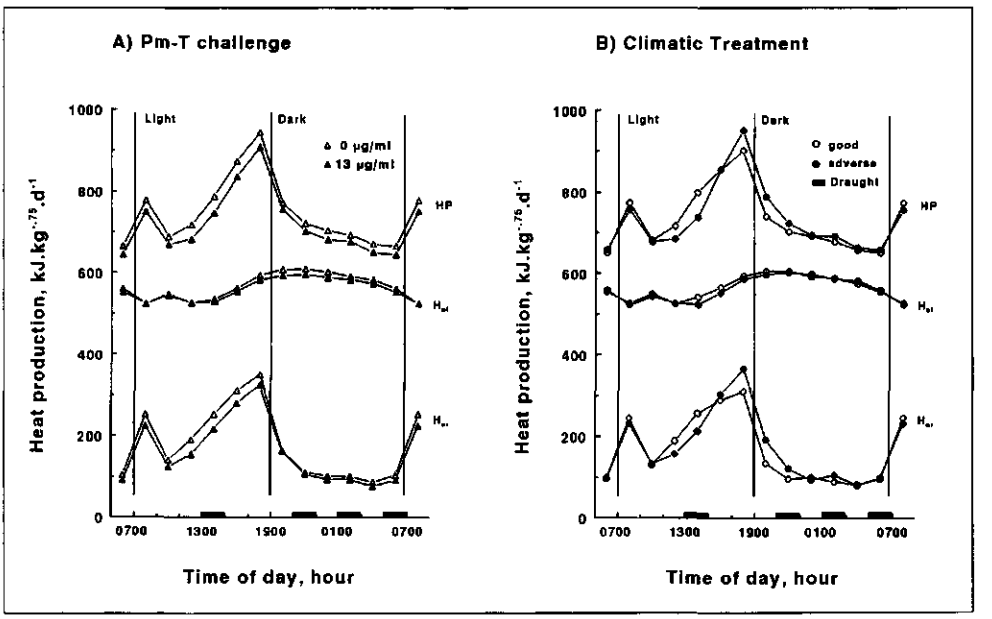

Figure 3 - Daily rhythm in total (HP), activity-free (H_{af}) and activity-related (H_{ar}) heat production (2-h means) as influenced by A) Pm-T challenge and B) climatic treatment.

All 2-h period means of HP were lower in Pm-T treated groups than in controls, although not significantly (Figure 3A). The Pm-T treatment reduced H_{ar} in most periods (Table 2). This effect was significant in the periods just before and after 'lights on' at 0700, in the first afternoon period (1300 to 1500), and after midnight from 0100 to 0300 (Table 2). Climatic treatment affected H_{ar} in the afternoon draught period (1300 to 1500), from the 'playtime' peak (1700 to 1900) up to and including the second nocturnal draught period (1700 to 0300) with an exception for the period between the nocturnal draught periods (2300 to 0100) (Table 2). Compared with the groups in the good environment, H_{ar} was 44 $kJ \cdot kg^{0.75} \cdot d^{-1}$ lower during the afternoon draught period, whereas H_{ar} was 25, respectively, 16 $kJ \cdot kg^{0.75} \cdot d^{-1}$ increased during the first, respectively, second nocturnal draught period, in the groups in the adverse environment (Figure 3b). Effects of interaction between PmT and CT treatment on HP and H_{ar} were not found. The 2-h means of H_{ar} were not affected by Pm-T challenge, CT, nor by their interaction.

With increasing day number, the reducing effect of Pm-T challenge on 2-h means of HP was especially found during day-time periods (data not shown). Interaction effect between Pm-T treatment and day number occurred only on H_{ar} during the 'playtime' peak (1700 to 1900) (Table 2).

Table 2 - Least square means (± SE[a]) and significance level of two-hour averages of activity-related heat production (H_{ar}, $kJ \cdot kg^{-0.75} \cdot d^{-1}$)within Pm-T treatment ($\mu g$/ml) and climatic treatment (CT) using Equation [3]

	Pm-T		CT			P-value[b]				
period	0	13	good	adverse	SEM	PmT	CT	d×PmT	d×CT	d×PmT×CT
0700-0900	253	225	245	232	5.0	*	ns	ns	***	ns
0900-1100	140	124	131	133	10.1	ns	ns	ns	*	ns
1100-1300	191	155	189	157	11.3	†	ns	ns	***	ns
1300-1500 D[c]	253	217	257	213	8.6	*	*	ns	***	ns
1500-1700	311	280	289	303	19.5	ns	ns	ns	***	*
1700-1900	351	325	310	366	12.6	ns	*	*	***	***
1900-2100	164	162	134	192	7.7	ns	**	ns	***	**
2100-2300 D[c]	111	106	96	121	2.7	ns	**	ns	***	ns
2300-0100	101	93	100	94	2.3	†	ns	ns	***	ns
0100-0300 D[c]	101	93	89	105	1.7	*	**	ns	***	ns
0300-0500	87	76	82	81	3.2	†	ns	ns	**	ns
0500-0700 D[c]	104	91	96	99	2.5	*	ns	ns	***	ns

[a]SE between treatment means.
[b]In these separate columns the effect of Pm-T treatment (PmT), climatic treatment (CT), and interactions with day number (d) in the depicted periods are given as resulted from Equation [3]. P-value of PmT × CT ns, and of d *** in all periods. (ns $P > .1$; † $P < .1$; * $P < .05$; ** $P < .01$; *** $P < .001$).
[c]Draught periods in the 'adverse' environment.

All 2-h means of H_{ar} were affected by interaction of CT with day number. The adverse CT raised the heat production traits in the first two 6-d periods ($P < 0.05$), followed by a small reduction, comparable with in the day averages (Figure 2B). As time progressed, climatic treatment affected the 2-h means of H_{af} from 0700 to 1700 and the 2-h means of HP from the afternoon draught period (1300) until 0500. Interaction of both treatments with day number affected HP and H_{ar} during the 'playtime' peak period (1700 to 1900) and H_{ar} also in the periods before and after this peak (1500 to 2100).

In Figure 4, the actual data and lines drawn from regression of HP and H_{ar} on day number are shown for the 'playtime' peak. In the good environment, a level difference in HP ($\pm$ 47 kJ·kg$^{0.75}$·d^{-1})occurred between Pm-T treated and control groups over the 35-d exposure period (Figure 4A). Whereas in the 'adverse' environment, HP of the Pm-T challenged groups increased less with increasing day number than did the control groups (Figure 4B). In the last 6-d period, HP of Pm-T treated groups was equal in both climatic environments. The activity-related heat production of Pm-T treated pigs in the adverse environment stayed behind compared with their controls (Figure 4B). While in the good environment difference in H_{ar} were mainly present in the second and third week after initial exposure (Figure 4A).

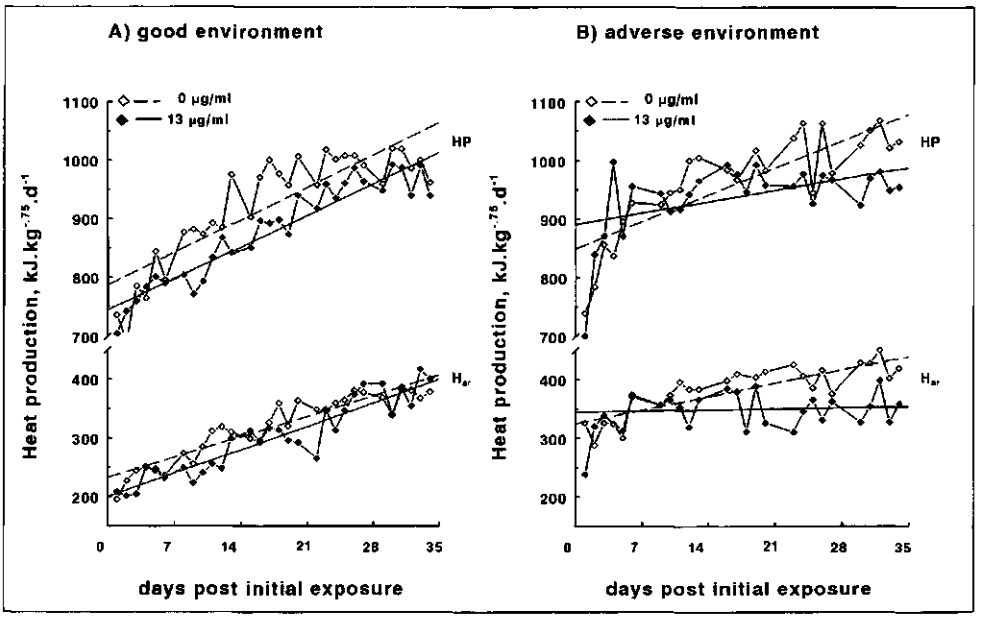

Figure 4 - Effect of interaction between Pm-T challenge, climatic treatment and day number on total (HP) and activity-related (H_{ar}) heat production during the 'playtime' peak (1700 to 1900). A) Controls (0 μg of Pm-T/ml) and Pm-T challenged (13 μg/ml) in 'good' environment, and B) Controls and Pm-T challenged in 'adverse' environment. Lines drawn from regression of 2-h means of HP and H_{ar} on day number.

DISCUSSION

Total heat production of pigs is composed of the maintenance requirement (related to metabolic body size), activity, and heat production associated with the synthesis of fat and protein. Level and variation in heat production within and between days may be affected by climatic environment. It is known that in cold environments, the maintenance requirement will increase, due to a rise in convective heat loss (Mount *et al.*, 1980). As a consequence, pigs have to generate more heat to maintain relative constant (homoeothermic) body temperature.

A single environmental stimulus (e.g. cold air), can effectively reduce resistance to disease-causing organisms in swine, like *Actinobacillus pleuropneumoniae* (Verhagen, 1987), or killed Aujeszky's virus vaccine (Noyes *et al.*, 1988). In this study, subclinical atrophic rhinitis was artificially induced with *Pasteurella multocida* derived toxin in pigs kept in a 'good' environment or an 'adverse' environment. The Pm-T treated animals responded similar in both climatic environments. No evidence was found that the low ambient temperature with draught periods aggravated AR nose lesions induced by our challenge model. Main effects of Pm-T challenge, climatic treatment and their interaction on heat production or physical activity were not found. As time progressed, the Pm-T challenge caused a reduction in heat production traits compared with the controls.

Difference in HP and H_{ar} between the Pm-T treated and control pigs occurred approximately one week after initial challenge, and remained as time progressed. This difference coincided with the moment the challenged pigs started to sneeze and showed nasal discharge. First pathological signs of atrophic rhinitis can be found then. Moreover, at the same time, the FI of these pigs dropped behind compared with the control pigs. Apparently, Pm-T induced effects have a one week incubation time to become measurable.

The pigs in this study reacted similarly to the lower ambient temperature with the higher air velocity periods (draught) as described for 10-wk-old pigs (Mount *et al.*, 1980; Verhagen, 1987). During the first two 6-d periods, our piglets in the adverse environment showed an increased HP and H_{ar} after which they adapted to their climatic environment. When using the definition of acclimation of Verhagen (1987) - acclimation is the time after which exposure to a climatic treatment does no longer affect heat production or activity - the pigs were acclimated to the adverse climatic treatment at the end of the second 6-d period. This is comparable to the studies of Verhagen (1987), where acclimation was established 10 days after initial exposure.

The pigs did, however, not use the ad libitum feeding as an additional source of thermal regulation. On the contrary, a reduction in food intake was seen (77 $g \cdot d^{-1} \cdot pig^{-1}$) in the 'adverse' environment (van Diemen *et al.*, 1995). Why this reduction in food intake did take place is not clear. Both control and challenged pigs at the adverse environment had a decreased food intake compared with pigs kept at 25°C. Thus the Pm-T administration is not likely to be the reason. Pigs of this age (5 weeks) may not have the capacity to pick up heat loss by increasing food intake at the adverse conditions as applied. Verhagen *et al.* (1987) observed a delayed response in increasing FI after cold exposure in fattening pigs of 10 weeks old.

Results of the present study showed that the ad libitum fed pigs had two peaks in heat production within a day, corresponding with the endogenous biphasic activity rhythm (Alternanstype) within a time period of 8 to 10 h as described by Schrenk (1981). He stressed that light was the actual timegiver ('Zeitgeber'), stimulating activity. The circadian rhythm was led by a rhythmic excretion of hormones of the hypothalamus-pituitary system. Split into 2-h periods, both Pm-T challenge and climatic treatment affected level of HP and H_{ar} in several of those periods, but the biphasic rhythm was not altered.

Three draught-periods were chosen to take place during the night because the normal level of metabolism and activity is low during the night (Schouten, 1986). The other draught-period was applied in the afternoon at the beginning of 'playtime', with an increasing level of metabolism and activity. It was expected that specific effects on metabolic rate and activity, if any, would be present in these periods.

The reaction of the pigs to the draught was not the same in each period. During the afternoon draught period, the activity-related heat production was significantly lower, and during the first two nocturnal periods the H_{ar} was significantly higher than in the good environment. The other draught period did not affect the trait. The different reaction of the pigs in H_{ar} to the afternoon draught is possibly due to another mechanism related to thermal demand than during the nocturnal draught periods. The pigs in the 'adverse' environment huddle to prevent heat loss, whereas the pigs in the 'good' environment begin to be active. Active individuals will have concomitant an increased heat production. At night, when the activity is low, the lower ambient temperature and the increased air velocity (draught) may have disrupted the huddling behavior of the pigs. Thus causing increased heat loss and heat production, compared with the 'good' environment.

All differences between the 2-h means of the heat production traits of the piglets caused by the climatic treatment declined with time - with acclimation to the environment. The reducing effect of Pm-T challenge was also affected by time of day. The effect on H_{ar} is a direct level difference between control and challenged piglets, especially

in the periods after midnight. The effect of challenge treatment on HP and H_{af}, on the other hand, progressed over time and was mainly found during day-time periods. With increasing day number, day averages of activity-related heat production showed a disposition to different effects of Pm-T challenge in both climatic treatments. When analyzing 2-h periods, this interaction effect on H_{ar} appeared to originate mainly from afternoon period, around and during the 'playtime' peak. Therefore, it might be wise to distinguish between overall effects (day means) on total, activity related and activity free heat production, and effects within a day (e.g. 2-h means). The thermal demand of a diseased pig might be fluctuated differently within a day, than that of a healthy pig. The relation between activity, heat production and AR is not clear and remains to be sorted out. By doing so one has to take in account the biphasic circadian rhythm in heat production and activity of pigs, which is led by rhythmic excretion of hormones of the hypothalamus-pituitary system (Schrenk, 1981). It is possible that this hormonal system dismisses the Pm-T effect by other mechanisms (e.g. behaviour) to balance heat production and heat loss and thus maintain a constant core temperature in the body.

IMPLICATIONS

Our findings, sofar, show that the effect of *Pasteurella multocida*-toxin (Pm-T) challenge on heat production is mainly expressed by a decreased activity. The effects of both treatments seemed greatly independent of each other. The reaction of the pigs to the treatments was influenced by the time of day. Therefore, it might be wise to distinguish between overall effects (day means) on total, activity related and activity free heat production, and effects within a day (2-h means). The toxin seemed to suppress the general state of well-being of pigs, reducing pigs' activity and food intake. By reducing its activity, the piglets compensated the lower food intake, the lower amount of energy available for production. This way the induced subclinical atrophic rhinitis did not cause substantial growth retardation in our experiments.

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Chapter 5

IMMUNOLOGICAL ASPECTS

Chapter 5.1

IMMUNE RESPONSES OF PIGLETS TO PASTEURELLA MULTOCIDA TOXIN AND TOXOID

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IMMUNE RESPONSES OF PIGLETS TO *PASTEURELLA MULTOCIDA* TOXIN AND TOXOID

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Abstract

Experimental atrophic rhinitis (AR), serum antibody titres and *in vitro* lymphoproliferation to *Pasteurella multocida*-derived toxin (Pm-T) were studied in piglets. Specific immune responses to Pm-T and Pm-T induced conchae atrophy were compared with AR immunity. This immunity was initiated by the Nobi-VAC® AR-T vaccine administered at various times with respect to Pm-T challenge. Animals challenged with Pm-T developed conchae atrophy, but no antibodies nor cellular immune responses to Pm-T were detected. Vaccination 3 weeks before Pm-T challenge protected pigs against breakdown of nasal bony tissues. This protection was accompanied by an increase of serum antibodies and *in vitro* lymphoproliferation to Pm-T. Animals vaccinated 10 days before or after Pm-T challenge also had antibodies and cellular immune responses. However, these animals developed AR. *In vitro*, Pm-T appeared mitogenic for quiescent (non-immune) peripheral lymphocytes and Concanavaline A stimulated lymphocytes from some pigs. These *in vitro* lymphoproliferative responses could be partly abrogated by the addition of monomorphic anti-swine major histocompatibility complex class II DQ and DR specific monoclonal antibodies. We conclude that Pm-T is poorly immunogenic *in vivo* and does not initiate a protective Pm-T specific immune response. Pigs were protected from AR by vaccination, but protection was dependent on the timing of vaccine administration. We speculate that Pm-T modifies the immune response such that the response is not directed towards the toxin but to an unidentified component in the nose of piglets.

Key words: *Pasteurella multocida*-toxin, Atrophic Rhinitis, immune response, mitogenicity

INTRODUCTION

Pasteurella multocida derived toxin (Pm-T) initiates the pathogenic processes of atrophic rhinitis (AR), leading to irreversible destruction and reabsorption of nasal bony tissues in pigs (Martineau-Doizé *et al.*, 1990; Williams *et al.*, 1990; de Jong, 1991; van Diemen *et al.*, 1994). The latter can be reliably diagnosed by visual examination of turbinate atrophy in snout sections from slaughtered pigs (Collins *et al.*, 1989). AR can be induced artificially by intranasal challenge of young piglets with Pm-T. Pathogenicity

caused by intranasally administered Pm-T depended on the dose applied (Frymus *et al.*, 1986; van Diemen *et al.*, 1994).

Detection of antibodies to Pm-T would be singular proof of contact or infection with toxigenic *Pm* strains (Schimmelpfennig and Jahn, 1988). Clinical symptoms, however, do not relate to a detectable humoral immune response to Pm-T (Frymus *et al.*, 1986; Bording Jensen and Riising, 1988; Foged *et al.*, 1989; de Jong, 1991). Sera from naturally affected pigs lack toxin neutralising antibodies or show a late and hardly detectable humoral response. This suggests that Pm-T does not initiate a (protecting) response. Heat or formaldehyde treatment, however, can convert the low immunogenic toxin into a high immunogenic toxoid, that initiates antibodies to Pm-T (Bording *et al.*, 1990). The toxoid is commonly applied as vaccine (Voets, 1988; Kobisch and Pennings, 1989).

The mechanism(s) by which Pm-T initiates or mediates the breakdown of bony tissues in piglets is unknown. Chanter (1990) suggests that the toxin upsets the balance between bone formation and resorption in favour of a net resorption. The duration of toxin production of *Pm* strains may be short. Therefore bacterial products in the nasal mucosa of pigs affected by AR may influence bone metabolism. The influence may not be a direct toxic effect but can be indirect through mediators produced by immune cells (Ueberschär *et al.*, 1983; Pedersen and Elling, 1984). As yet, little is known of cellular immunity in AR-affected pigs.

The purpose of this study was to elucidate the role of the immune system of piglets in relation to AR pathology. Therefore, immunity-initiating characteristics of Pm-T and toxoid after primary and secondary Pm-T challenge, were studied in relation to pathology. Also, the relationship between the time of vaccination and protective humoral and cellular immune responses and pathology was studied. We attempted to identify mechanisms underlying the lack of conventional immune responses of pigs to Pm-T, and their possible consequences with respect to AR.

MATERIALS AND METHODS

Animals

The first group of animals studied (Experiment 1) were Large White (Groot York (GY)) × Dutch Landrace (DL) crossbred piglets. In Experiments 2 and 3, DL piglets were studied. For each experiment, animals were obtained from one farm with a '*Pm*$^+$ free' certificate (Animal Health Service, Deventer, Netherlands). Within experiments, littermates were equally distributed over treatments. In all experiments, the first day of challenge was defined as Day 0. The average age of the piglets on Day 0 was 37 days, 44 days and 33 days, respectively for Experiments 1, 2 and 3. Piglets were fed ad libitum

a commercial diet containing 16.7 kJ gross energy (GE) g^{-1} and 17% crude protein. They had free access to water. All animals were intranasally pretreated with diluted acetic acid (1% Aa in water, 0.5 ml/nostril), three days before treatment. At the end of each experiment all animals were killed (stunned and bled) in a slaughterhouse.

Experimental design

EXPERIMENT 1 - Nineteen GY x DL crossbred piglets were allotted to one of five treatment groups. Group 1 (control) consisted of five animals which were intranasally challenged with phosphate-buffered saline (PBS) on 3 consecutive days (0.5 ml per nostril); Groups 2 and 3 consisted of 4 piglets each intranasally challenged with 13 μg ml^{-1} Pm-T in PBS on 3 consecutive days (0.5 ml per nostril, Van Diemen *et al.*, 1994). Additionally, Group 3 animals received a single secondary Pm-T challenge (13 μg ml^{-1}, 0.5 ml per nostril) on Day 22. Groups 4 and 5 consisted of 3 animals each intramuscularly vaccinated on Day 0 with 2 ml Nobi-VAC® AR-T (Intervet International BV, Boxmeer, Netherlands). This vaccine contains toxoid. On Day 22, Group 5 animals were challenged once with Pm-T (13 μg ml^{-1}, 0.5 ml per nostril). Blood samples were drawn on Day -3, 28, 35 and 42 for serum analyses. Heparinised blood samples were drawn once a week over a 6 week period for lymphocyte isolation.

EXPERIMENT 2 - Twenty-eight DL piglets, allotted to one of five treatment groups (Groups 6-10), were used. Three days after pretreatment with acetic acid, 20 piglets received the intranasal Pm-T challenge (Van Diemen *et al.*, 1994). Of these challenged piglets, five were vaccinated with 2 ml Nobi-VAC® AR-T 10 days before the Pm-T challenge (Day -10) (Group 8). Five pigs (Group 9) were vaccinated simultaneously with the Pm-T challenge (Day 0) and another five pigs (Group 10) were vaccinated 10 days after Pm-T challenge (Day 10). The other five Pm-T challenged piglets (Group 7) were not vaccinated. The remaining eight piglets which served as controls (Group 6) were intranasally challenged with PBS on Day 0, 1 and 2. The latter two groups served respectively as positive (AR^+, Group 7) and negative (AR^-, Group 6) controls for pathogenesis. Depending on immunity, pathology in vaccinated groups was expected to be directed towards the negative control in case of protection or towards the positive control in case of AR symptoms.

From all animals, blood samples were drawn for serum analyses before treatment, and thereafter weekly for an 8 week period. Heparinised blood samples were drawn once a week for lymphocyte isolation.

EXPERIMENT 3 - For the third experiment, 30 DL piglets were purchased. Three days after pretreatment, 15 piglets (Group 12) were challenged intranasally with Pm-T (Van Diemen *et al.*, 1994) and 15 piglets (Group 11) served as non Pm-T challenged control group (PBS). For serum analyses, blood samples were drawn from all animals before treatment, and weekly over a 6 week period. Heparinised blood samples were drawn 3 days before treatment, and on Days 14, 31 and 82 for lymphocyte isolation.

A survey of treatment groups is given in Table 1.

Table 1 - Least-Square Mean (SE) of ventral and dorsal conchae atrophy scores (VCA and DCA) and change in brachygnathia superior (cBS) per treatment group.

Experiment	Treatment group	n	VCA	DCA	cBS
1	1 Control	5	1.30(0.4)	0	-
	2 Pm-T challenge	4	2.00(0.4)	0.50(0.4)	-
	3 Pm-T + secondary Pm-T	4	2.50(0.4)	1.25(0.5)	-
	4 Vaccination	3	1.16(0.5)	0	-
	5 Vaccination + Pm-T	3	1.00(0.5)	0	-
2	6 Control (AR^-)	8	1.44(0.2)^a	0.06(0.2)^a	1.38(0.6)^a
	7 Pm-T challenge (AR^+)	5	2.60(0.3)^b	0.60(0.3)^{ab}	3.60(0.8)^b
	Vaccination:				
	8 10 days before Pm-T	5	2.40(0.3)^b	0.30(0.3)^{ab}	2.00(0.8)^{ab}
	9 simultaneous with Pm-T	5	2.80(0.3)^b	0.90(0.3)^b	3.00(0.8)^{ab}
	10 10 days after Pm-T	5	2.30(0.3)^b	0.70(0.3)^{ab}	3.20(0.8)^{ab}
3	11 Control	15	1.39(0.1)^a	0.18(0.1)^a	1.86(0.5)^a
	12 Pm-T challenge	15	2.87(0.1)^b	1.10(0.1)^b	5.67(0.5)^b

^{a,b}Within columns, different superscripts indicate a significant difference ($P < 0.05$) between treatment groups within an experiment.

Response characteristics

Progression of AR was defined, post mortem, by the grade of conchae atrophy after cross-sectioning the snout between the first and second premolar tooth. The method of grading described by De Jong (1985) was used; ventral conchae atrophy (VCA) graded from 0 (no lesions) to 4 (total atrophy) and dorsal conchae atrophy (DCA) from 0 (no lesions) to 3 (total atrophy). The average of both nostrils was used in calculations. The challenge model used, aimed at an intermediate grade of conchae atrophy (van Diemen *et al.*, 1994), VCA grade 2-2.5 and DCA grade 1, i.e. subclinical AR symptoms. In Experiments 2 and 3, the brachygnathia superior (BS) was measured in millimetres on Day 0 (BS_0) and on Day 35 (BS_{35}). The change in BS (cBS) over the interval was used in calculations (van Diemen *et al.*, 1994).

Immune characteristics

Humoral response - Pm-T specific *in vivo* antibody titres were determined routinely by enzyme-linked immunosorbent assay (ELISA). Serial dilutions of serum were applied to toxin-coated wells of a microtitre plate. After incubation for 1 h at 37°C and subsequent washing, wells were incubated for 1 h with a 1:2000 diluted peroxidase conjugated goat anti-swine antibody (GASw/IgG_{H+L}-PO, Kpl, Gaithersburg, MD, USA) followed by tetramethylbenzidine (TMB, Sigma T2885, USA) as substrate. Colour formation was stopped after 10 minutes with 2.5 N sulphuric acid. All absorbances were expressed relative to the absorbance of a standard positive control serum obtained from a vaccinated animal.

Cellular response - Pm-T specific *in vitro* cellular immunity was determined by lymphocyte stimulation test (LST). Peripheral blood leucocytes (PBLs) were obtained from heparinised blood using Ficoll density gradient centrifugation. PBLs were tested for proliferation in the presence of either 5 μg ml^{-1} Concanavaline A (ConA), 1 and 10 μg ml^{-1} Pm-T, heat inactivated Pm-T (1 h, 60°C, toxoid) or combinations of both Pm-T and ConA in RPMI tissue medium containing 10% foetal calf serum and antibiotics. The cultures ($4 \cdot 10^5$ PBLs per well), set up in triplicate, were incubated 3 days at 37°C, 5% CO_2 in a humidified atmosphere. Eighteen hours before harvest, 0.5 μCi of methyl-^{3}H-thymidine (^{3}H, Amersham Nederland BV, Houten, Netherlands) was added. ^{3}H uptake was determined with a Beckman ß-scintillation counter. Results were expressed as mean counts per minute (cpm). Stimulation Indices (*SI*) were calculated as:

SI = cpm in antigen stimulated cultures / cpm in unstimulated cultures

A *SI* > 2 was regarded as positive. Dose response series of Pm-T on PBLs were performed to ascertain the lowest mitogenic concentration of the Pm-T.

To study whether the mitogenic nature of Pm-T was related to major histocompatibility complex (MHC) class II dependent antigen presentation, either a 1:4000 diluted monomorphic monoclonal antibody with specificity to swine MHC class II DQ (MSA_3, obtained from Dr J. Lunney, Beltsville, USA) or to swine MHC class II DR (TH16ß, obtained from Dr W.C. Davis, Pullman, USA) antigens were added to *in vitro* cultures of control piglets. The cultures were incubated with Pm-T or ConA. The effect of anti-class II monoclonal antibodies (mAb) on Pm-T or ConA proliferation was calculated as:

$$SI = cpm_{Pm\text{-}T(ConA) + mAb} / cpm_{mAb}$$

Statistical analysis

The results of each experiment were statistically analyzed for effect of treatment using a one-way analysis of variance per test date (Statistical Analysis Systems Institute (SAS), 1985). Subsequently, pairwise comparisons within test date were performed between control and experimental groups using least-square mean differences at the overall 0.05 level of significance. Correlations between response and immune characteristics were analyzed by method of Pearson's partial correlation (SAS, 1985).

RESULTS

Response characteristics

Ventral and dorsal conchae atrophy scores (VCA and DCA, respectively) and changes in brachygnathia superior (cBS) are shown in Table 1. No clinical AR symptoms occurred.

In the first experiment no significant differences in VCA between treatment groups were found. The ventral conchae of animals in both Pm-T treated groups (Groups 2 and 3), however, were more atrophied than those of control (Group 1) or vaccinated animals (Groups 4 and 5). This difference tended towards significance. Only the dorsal conchae of the Pm-T challenged animals (Groups 2 and 3) were affected ($P < 0.05$). The secondary Pm-T challenge (Group 3) showed a tendency towards additional dorsal atrophy ($P < 0.1$). The vaccinated and Pm-T challenged (Group 5) animals did not develop conchae atrophy symptoms.

In Experiment 2 all Pm-T treated groups (Groups 7 - 10) had a higher VCA score than the negative control (Group 6) ($P < 0.05$). The vaccinated animals were situated in between in order of vaccination moment, i.e. before (Group 8), simultaneously with (Group 9), or after (Group 10) Pm-T challenge. No differences were found between the positive control (Group 7) and the vaccinated groups (Groups 8-10). The DCA scores of the simultaneously vaccinated and challenged piglets (Group 9) were significantly ($P < 0.05$) more affected than those of the control piglets (Group 6). With respect to cBS, the positive control (Group 7) differed significantly from the negative control (Group 6) ($P < 0.05$).

In Experiment 3, the ventral and dorsal conchae of the Pm-T challenged piglets (Group 12) were affected more than those of the control group (Group 11) ($P < 0.001$). Also, BS changed more in the challenged than in the control piglets ($P < 0.001$).

Immune characteristics

EXPERIMENT 1 - *General characteristics of immune responses and the protecting capacity of toxoid.*

Humoral response - Vaccinated animals (Groups 4 and 5) mounted an anti Pm-T serum antibody response about 4 weeks post-vaccination (Day 28). Treatment effect was found on Day 28 and Day 42 ($P < 0.05$). On Day 28 this effect was caused by a rise in antibodies in the Pm-T challenged vaccinated piglets (Group 5), and on Day 42 in both vaccinated groups (Groups 4 and 5) ($P < 0.05$). On Day 35, treatment tended towards significance ($P < 0.10$) because of a large standard deviation. No differences in antibody response were found between the non-challenged and challenged vaccinated groups (Groups 4 and 5). None of the piglets treated with a primary (Group 2) or secondary Pm-T challenge (Group 3) developed detectable levels of anti Pm-T serum antibodies. No correlation was found between antibody titres and response characteristics.

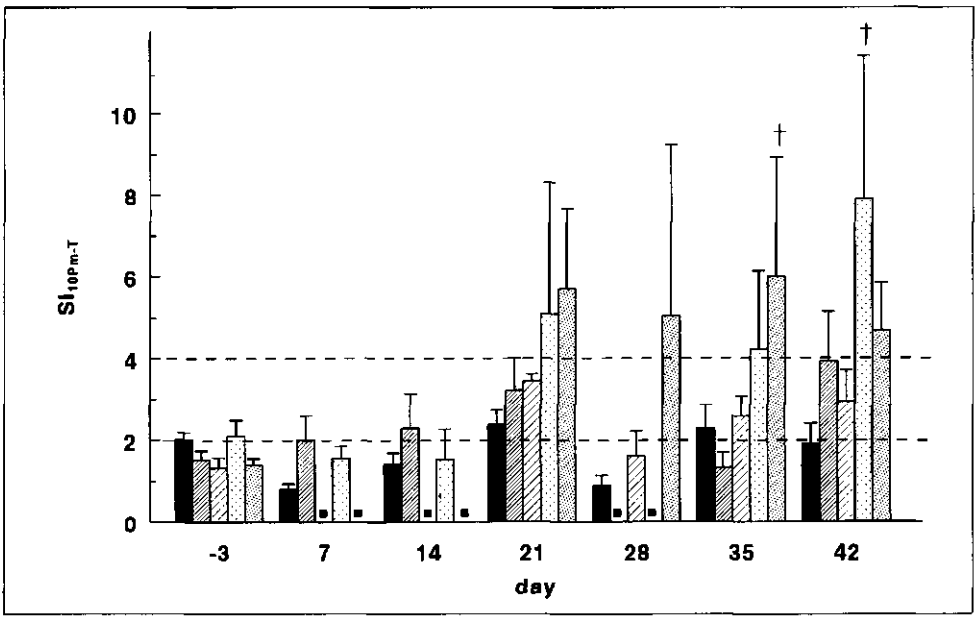

Figure 1 - Stimulation indices of cultures stimulated with 10 μg ml^{-1} Pm-T (SI_{10Pm-T}) per treatment group in Experiment 1. Solid, control; close cross-hatched, Pm-T challenge; cross-hatched, Pm-T + secondary Pm-T; dots, vaccination; close dots, vaccination + Pm-T; solid squares, not done; dagger, $P < 0.10$ difference. The cpm of unstimulated cultures ranged between 87 and 427.

Cellular response - In Figure 1, *SI* to 10 μg ml^{-1} Pm-T are given. The cpm of unstimulated cultures ranged between 87 and 427. In the lymphocyte stimulation test before treatment, the PBLs of five out of 18 piglets tested showed proliferation in the presence of Pm-T ($2 \leq SI_{10Pm-T} \leq 4$). Throughout this experiment, control animals (Group 1) maintained this level of proliferation. No difference in lymphocyte stimulation by Pm-T was seen between the controls (Group 1) and the primary (Group 2) or the secondary

Pm-T challenged animals (Group 3). Heat-inactivated toxin (toxoid) did not stimulate PBLs ($SI_{toxoid} = 1$). The vaccinated piglets (Groups 4 and 5) mounted a cellular response to Pm-T and toxoid 21 days post-vaccination ($SI_{10Pm\text{-}T} \geq 5$). On Days 35 and 42, the PBLs of vaccinated animals tended ($P < 0.10$) to be more stimulated in the presence of Pm-T and toxoid than PBLs of control (Group 1) or Pm-T treated (Group 2) animals. The PBLs of vaccinated animals with (Group 5) or without (Group 4) Pm-T challenge did not differ in response to Pm-T.

Furthermore, ConA responses from PBLs of all animals were elevated in the presence of Pm-T, $SI_{ConA+Pm\text{-}T}/SI_{ConA}$ ranged between 1 and 3. This elevated ConA response remained absent in the presence of toxoid. No difference was found between treatment groups. The application of a higher dose of Pm-T to ConA cultures gave more proliferation as compared with ConA alone. In the tests performed with PBLs of control (compared with all) animals, 0.1 μg ml^{-1} Pm-T gave more proliferation in 15% (13%) of the cases, whereas the addition of 10 μg ml^{-1} Pm-T to ConA cultures gave more proliferation in 65% (57%) of the tests performed.

In vitro proliferation of non-immune lymphocytes in the presence of Pm-T indicated a moderate mitogenic activity of the Pm-T. Approximately 40% of tests performed with PBLs of control animals (Group 1) in the presence of Pm-T resulted in an $SI_{10Pm\text{-}T}$ that ranged between 2 and 5. The PBLs of Pm-T challenged animals (Groups 2 and 3) showed similar proliferation.

The responses to all antigens in the lymphocyte stimulation assay varied between test dates. No partial correlation was found between response characteristics and lymphocyte stimulation.

EXPERIMENT 2 - *Relationships between humoral and cellular immunity induced by vaccination and AR pathology.*

Humoral response (Figure 2) - None of the piglets treated solely with Pm-T (Group 7) developed detectable levels of anti Pm-T serum antibodies compared with control animals (Group 6). From about Day 32 onwards, a treatment effect was present ($P < 0.05$). Animals vaccinated before (Group 8) as well as after (Group 10) Pm-T challenge mounted an anti Pm-T antibody response. This response was still increased at the end of the experiment. The timing of vaccination, however, with respect to Pm-T challenge influenced the swiftness of antibody formation. Both groups responded at about Day 32. This was 42 days post-vaccination for animals challenged 10 days after vaccination (Group 8) and 22 days post-vaccination for animals challenged 10 days before vaccination (Group 10).

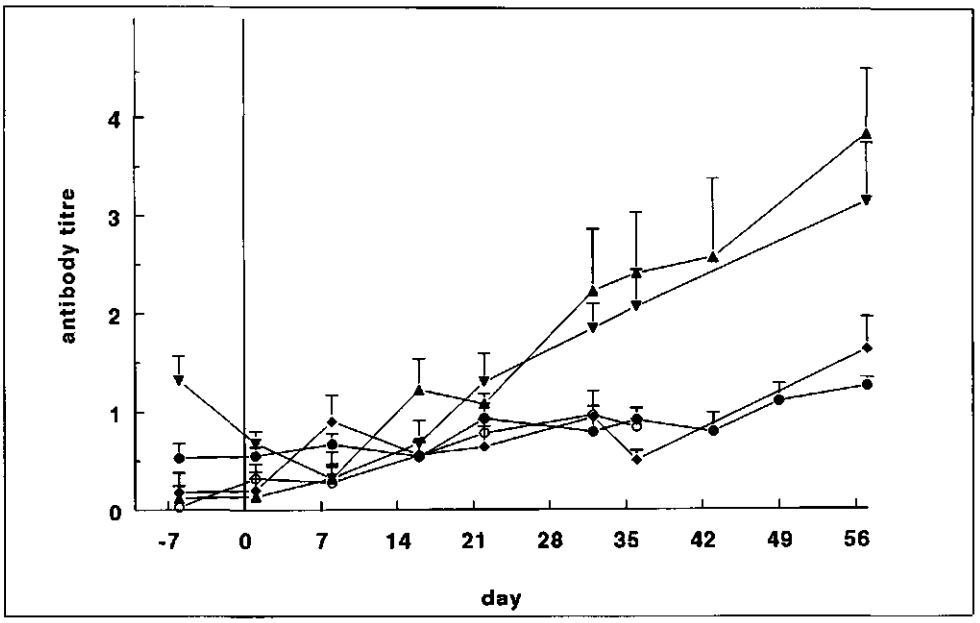

Figure 2 - Pm-T specific antibody titres per treatment group in Experiment 2. •, control (AR^-); ○, Pm-T challenge (AR^+). Vaccination: ▼, 10 days before Pm-T; ◆, simultaneous with Pm-T; ▲, 10 days after Pm-T.

On Day 56, the simultaneously vaccinated and challenged piglets (Group 9) had not (yet) developed anti Pm-T antibodies. Both groups vaccinated before (Group 8) or simultaneously with (Group 9) Pm-T challenge showed a tendency ($0.05 < P < 0.1$) towards a negative correlation ($r = -0.87$ and $r = -0.83$ respectively) between VCA and antibody titre on Day 56.

Cellular response (Figure 3) - The animals vaccinated on Day -10 (Group 7) mounted an anti Pm-T cellular response on Day 16 (26 days post-vaccination). Mean *SI* was 4.37 or 7.60, respectively, for the addition of 1 μg ml^{-1} and 10 μg ml^{-1} Pm-T to cultures (mean $cpm_{unstimulated}$ = 498). Animals vaccinated on Day 10 (Group 10) mounted a cellular response on Day 22 (12 days post-vaccination). Mean $SI_{1Pm\text{-}T}$ was 6.31 and mean $SI_{10Pm\text{-}T}$ was 10.39 (mean $cpm_{unstimulated}$ = 465). These levels of proliferation remained until the end of the experiment. The simultaneously vaccinated and challenged piglets (Group 9) were tested on Days 22 and 57. On both days, two of five animals had an $SI_{Pm\text{-}T}$ of about 5. The toxin stimulated non-immune lymphocytes in approximately 30% and 60% of the tests performed in the presence of 1 μg ml^{-1} and 10 μg ml^{-1} Pm-T ($SI > 2$, mean $cpm_{unstimulated}$ = 492). The value of $SI_{Pm\text{-}T}$ did not exceed 5. This proliferation remained absent in the presence of heat-inactivated Pm-T (toxoid). The non-vaccinated Pm-T challenged animals (Group 7) showed proliferation similar to the control animals. The

SI_{ConA} of non-vaccinated challenged animals was reduced compared with other treatment Groups. These differences, however, were not significant.

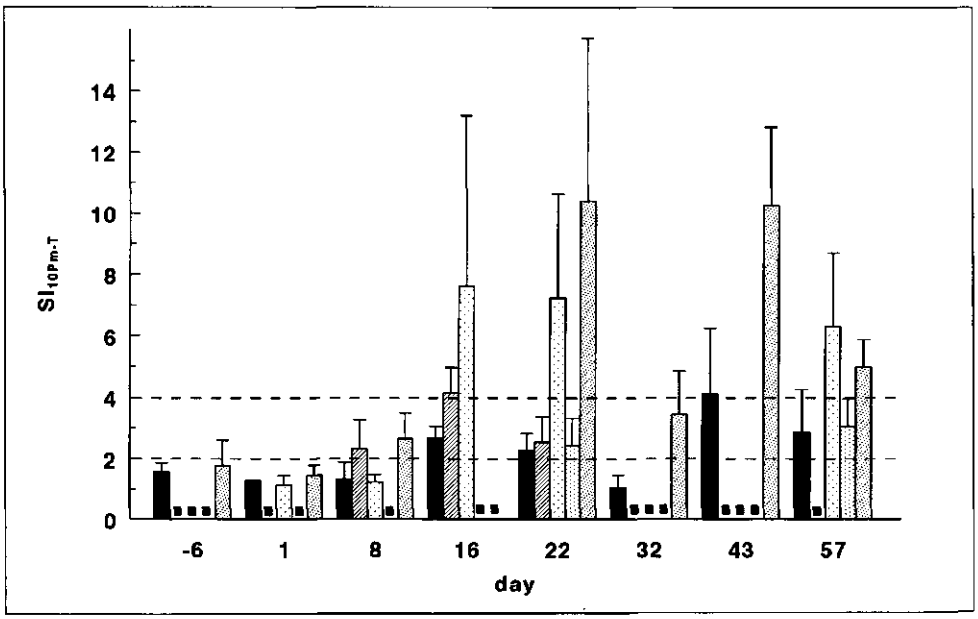

Figure 3 - Stimulation indices of cultures stimulated with 10 μg ml^{-1} Pm-T (SI_{10Pm-T}) per treatment group in Experiment 2. Solid, control (AR); close cross-hatched, Pm-T (AR$^+$). Vaccination: widely spaced dots, 10 days before Pm-T; medium spaced dots, simultaneous with Pm-T; closely spaced dots, 10 days after Pm-T; solid square, not done. The cpm of unstimulated cultures ranged between 148 and 1027.

The application of 1 μg ml^{-1} Pm-T to ConA cultures elevated the SI_{ConA} of the control (compared with all) animals in 47% (44%) of the tests performed. The application of 10 μg ml^{-1} Pm-T increased the SI_{ConA} in 69% (72%) of cases. No differences between treatment groups were found. The appli-cation of toxoid to ConA cultures did not elevate the response. No partial correlation was found between response characteristics and lymphocyte stimulation.

EXPERIMENT 3 - *Mechanisms underlying the mitogenic activity of Pm-T on non-immune PBLs.*

Humoral response - None of the piglets treated with Pm-T (Group 12) developed detectable levels of anti Pm-T antibodies.

Cellular response - Three days before Pm-T treatment, 29 animals were tested for lymphocyte stimulation. The cpm of unstimulated cultures ranged between 157 and 1365 and averaged 476 (in three animals the cpm exceeded 1000). The addition of 1 μg ml^{-1}

Pm-T gave stimulation ranging between 0.6 and 28.1. In 25 cases, $SI_{1Pm\text{-}T}$ was higher than 2, in 11 cases even higher than 5. Addition of 10 μg ml^{-1} gave stimulation in the same range as $SI_{1Pm\text{-}T}$. In 23 cases, the $SI_{10Pm\text{-}T}$ was higher than 2, of which 14 had $SI_{10Pm\text{-}T}$ of over 5. At Day 14 post-challenge, PBLs of six control (Group 11) and 14 Pm-T challenged (Group 12) animals were tested. All animals showed mitogenic proliferation ($SI > 2$) in the presence of 1 μg ml^{-1} Pm-T. The addition of an anti-swine MHC class II DQ monoclonal (MSA_3) partially reduced mitogenic proliferation in 70% of the cases. The degree of reduction ranged between 13 and 69% (average 32% and 41% for control and Pm-T challenged animals, respectively).

Table 2 - Lymphocyte stimulation indices on Day 31 of none-immune PBLs (control piglets, Group 11) after the addition of 1 μg ml^{-1} Pm-T ($SI_{1Pm\text{-}T}$) or 1 μg ml^{-1} Pm-T and anti-swine MHC class II DQ ($SI_{Pm\text{-}T+DQ}$) or DR monoclonal ($SI_{Pm\text{-}T+DR}$). Counts per minute (cpm) of unstimulated cultures (–), and of unstimulated cultures in the presence of DQ or DR monoclonal antibodies.

Piglet No.	$SI_{1Pm\text{-}T}$			SI_{ConA}			cpm		
	–	+DQ	+DR	–	+DQ	+DR	–	+DQ	+DR
8	12.7	2.5	1.3	42.1	15.3	9.5	159	657	797
15	10.8	2.7	6.4	85.8	17.1	19.6	233	836	337
26	14.4	3.5	3.8	29.5	9.7	10.0	346	852	612
30	9.2	4.9	8.3	25.1	13.1	28.9	280	386	135
36	2.7	5.6	1.4	47.9	18.2	25.5	148	142	158
4	0.8	2.4	1.1	11.1	18.9	19.6	833	658	495
13	1.3	2.3	3.9	6.2	12.1	7.6	1541	840	1030
48	0.6	1.4	0.9	8.3	11.8	10.0	1274	479	435

At Day 31, five of eight non-challenged animals (Group 11) showed a mitogenic response in the presence of 1 μg ml^{-1} Pm-T. Four piglets had a $SI_{1Pm\text{-}T}$ of over 5. MSA_3 reduced the $SI_{1Pm\text{-}T}$ (and SI_{ConA}) of these 4 cases more than 50% (Table 2). Adding an anti-swine MHC class II DR monoclonal (TH16ß) also gave reduction in these cases but less than MSA_3. In cases where no mitogenicity to Pm-T occurred, MSA_3 and TH16ß stimulated PBLs to proliferate. In one case, slight proliferation occurred in the presence of Pm-T ($SI_{1Pm\text{-}T} = 2.7$), MSA_3 gave extra proliferation ($SI_{1Pm\text{-}T} = 5.6$) while TH16ß reduced the $SI_{1Pm\text{-}T}$ to 1.4.

At Day 82, 12 control animals (Group 11) were tested for mitogenic response to 1 μg ml^{-1} Pm-T. The cpm of unstimulated cultures ranged between 149 and 684 (average 243). Pm-T stimulated the non-immune lymphocytes in ten cases (mean $SI_{1Pm\text{-}T} = 3.95$). The addition of MSA_3 reduced this proliferation with about 40%, ranging between 17 and 66%. In eight cases, SI_{ConA} was reduced by about 40%, in the same range as $SI_{1Pm\text{-}T}$. In one case, MSA_3 stimulated proliferation, in the other cases no reduction or stimulation occurred.

The lowest mitogenic concentration of the Pm-T was in the 10 and 50 ng ml^{-1} range (Figure 4). This comes down to 1 – 5 ng Pm-T on $4 \cdot 10^5$ PBLs.

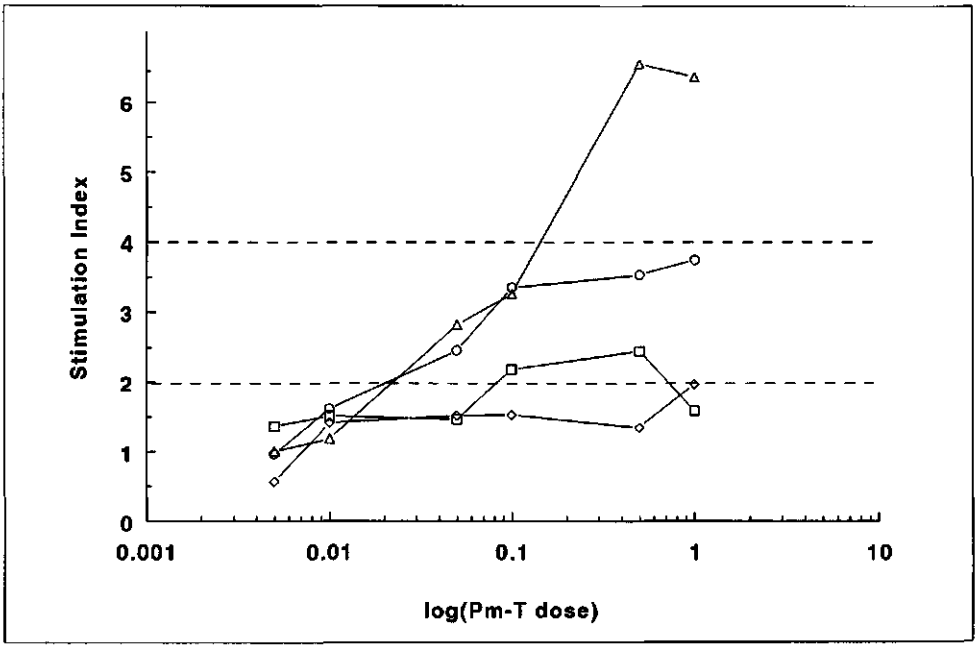

Figure 4 - Stimulation indices of cultures of four control piglets of Experiment 3 (◇, No.1; △, No.2; O, No.3; □, No.4) stimulated with dose series of Pm-T.

DISCUSSION

Intranasal administration of Pm-T induced subclinical symptoms of in situ infection with toxigenic *Pm* strains as previously reported (van Diemen *et al.*, 1994). In none of the three experiments, however, were these symptoms accompanied by a detectable systemic humoral and cellular response to Pm-T (Groups 2, 7, 12). Also, after secondary Pm-T administration (Group 3), no anti-toxin immune responses were found. No Pm-T antibodies were found in 'swabs' of nasal mucosae from the Pm-T challenged groups (data not shown).

However, *Pm* toxoid vaccinated animals (Groups 4 and 5) mounted both humoral and cellular responses to Pm-T after 3 – 4 weeks. These anti Pm-T responses protected animals from the annihilative effects of a subsequent Pm-T challenge. Thus, in principle, cells of the immune system can be sensitised by vaccination with *Pm* toxoid. These cells can recognise and react to Pm-T, as illustrated by the elevated *in vitro* proliferation and antibody titres to Pm-T from vaccinated animals.

Animals vaccinated 10 days before (Group 8) or after (Group 10) Pm-T challenge developed humoral and cellular responses to Pm-T. Antibodies were detectable 42 or 22 days post-vaccination (Day 32, Figure 2). Proliferation occurred 26 or 12 days post-vaccination (Day 16 and 22, Figure 3) for both groups, respectively. In spite of these anti Pm-T responses, the noses of the animals were affected to the same extent as non-vaccinated Pm-T treated animals (Group 7). Thus, vaccination 10 days before or after Pm-T challenge did not prevent or diminish pathology. In the animals vaccinated simultaneously with Pm-T challenge (Group 9), no detectable systemic humoral and/or cellular responses to Pm-T were found. The Pm-T challenge seemed to slow down (vaccination before challenge) or abrogate (simultaneous vaccination and challenge) the immune responses to Pm-T initiated by the toxoid in the vaccine. The Pm-T specific immune responses in the second experiment could not inhibit an irreversible process. This process was initiated by the Pm-T and led to pathology. These data suggest that when the immune response has not sufficiently built up, the devastating process will not be slowed down or stopped. The effect of Pm-T on pig noses seemed to be a non-reversible process with an all-or-nothing effect. Apparently, nose damage can develop in the presence of antibodies to Pm-T and anti Pm-T T cells *in vivo*.

The humoral and cellular immune responses of vaccinated animals showed that in principle the immune system can recognise Pm-T, whether or not this results in protective immunity. It remains to be determined, however, why no detectable responses to Pm-T appear without prior vaccination with toxoid.

In our model, pigs were challenged on 3 consecutive days with a relatively low dose of Pm-T. Clinical symptoms/pathology progressed gradually during weeks. The duration of toxin production of pathogenic *Pm* strains in the nose under 'field conditions' may be short. Therefore, Pm-T in the nasal mucosa may influence bone metabolism. The influence may not be a direct toxigenic effect, but can be mediated through unidentified products of immune cells (Ueberschär *et al.*, 1983; Pedersen and Elling, 1984; Frymus *et al.*, 1986). Pm-T is an extremely potent mitogen for several cell types of different mammals (Rozengurt *et al.*, 1990; Williams *et al.*, 1990). In the present experiments, Pm-T appeared mitogenic for non-immune quiescent and ConA-stimulated PBLs of some piglets. PBLs of both the GY × DL crossbred and DL purebred responded. Similar results were found when PBLs of purebred GY pigs were tested (data not published). This proliferation can probably be attributed to CD4+ T cells. Fluorescence Activated Cell Sorter (FACS) analysis of PBLs incubated with the toxin showed increased numbers of CD2+ and CD4+ cells (data not shown). Animals in the Pm-T challenged groups showed proliferation similar to the control animals. This proliferation depended on dose

and did not differ before or after Pm-T challenge. Thus, the cause of proliferation was probably the mitogenic activity of the toxin and built-up 'memory'.

Heat-inactivation abrogated the mitogenic activity of Pm-T. This indicated that the mitogenic capacity may not rest on lipopolysaccharide components regularly present in endotoxins from Gram-negative bacteria. The monomorphic anti-MHC class II DQ and DR monoclonal antibodies could partly block the *in vitro* lymphoproliferative activity of Pm-T. If no Pm-T induced proliferation occurred, addition of the monoclonal antibodies could induce proliferation. This suggested that the mitogenic activity of Pm-T is based on stimulation of T cells through the MHC class II antigens on the cell membrane of antigen presenting cells. The results of the tests on mitogenicity and blocking with MSA_3 or TH16ß were diverse. This might be due to the fact that no inbred lines were used in this research.

Recently, a specific class of antigens which either belong to the endotoxins of Gram-negative bacteria, mycoplasmas, or viral antigens has been reviewed. These, so called 'superantigens' are potent T cell mitogens and do not induce conventional humoral and/or cellular immune responses. In murine models and in humans, they differ from 'normal' antigens such that they 'nonspecifically' activate T cells without the conventional processing by antigen presenting cells. The superantigens bind MHC class II molecules on antigen presenting cells and are then presented to T cells with certain subsets of T cell receptor ß chains. This may result ad random, either in activation of (preferentially autoimmune) T cells, followed by a rise of (auto)antibodies, or in death/inactivation of the responding T cells, leaving holes in the T cell repertoire (Acha-Orbea and Palmer, 1991; Cole and Atkin, 1991). A 'genetic' diversity in subsequent immune responses between MHC compatible individuals was attributed to the repertoire of $V_ß$ T cell receptor genes (Cole and Atkin, 1991; Coffin, 1992). Whether Pm-T acts as a superantigen in pigs remains to be determined. However, the lack of a detectable anti-toxin immune response in all Pm-T treated animals (Groups 2, 3, 7 and 12), while Pm-T can be recognised by immune cells, the irreversible pathogenicity in the presence of immune cells and the mitogenicity of Pm-T, make it tempting to speculate that Pm-T modifies the immune response. The response may not be directed towards the *Pasteurella multocida* toxin but to an unidentified component in the nose of piglets.

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Chapter 5.2

EFFECT OF *PASTEURELLA MULTOCIDA*-TOXIN ON THE IMMUNE SYSTEM OF PIGLETS

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Submitted

EFFECT OF PASTEURELLA MULTOCIDA-TOXIN ON IN VIVO IMMUNE RESPONSES OF PIGLETS

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Abstract

Effects of intranasal administration of *Pasteurella multocida*-toxin (Pm-T) on cellular and T-cell dependent antibody responses of piglets were studied in a 3 by 2 factorial arrangement of treatments: three levels of intranasal challenge with Pm-T (either once, or on 3 consecutive days, or no Pm-T challenge), and with or without simultaneous immunization with a 'cocktail' containing Keyhole Limpet Haemocyanin (KLH), Ovalbumin (OA) and Tetanus Toxoid (TT). Total Ig, IgG, and IgM antibody formation as determined by ELISA revealed that Pm-T treatment decreased but did not abrogate the humoral response to KLH, advanced and decreased the humoral response to OA, whereas the response to TT was not affected. *In vivo* cellular immunity to OA was accelerated and enhanced, but the cellular response to TT was increased or decreased depending on applied Pm-T treatment. The results indicate that Pm-T toxin does not suppress immunity to T-cell dependent antigens. This suggests that the lack of detectable immune responses to Pm-T in Pm-T challenged pigs may not be based on non-specific suppression of the cellular immune system.

Key words: *Pasteurella multocida*-toxin, immune response, T-cell dependent antigens, pigs

INTRODUCTION

Pasteurella multocida-toxin (Pm-T) is the causing agent of atrophic rhinitis (AR), which is characterized by irreversible destruction and reabsorption of nasal bony tissues in piglets (de Jong, 1985; van Diemen *et al.*, 1994a). In field outbreaks of AR, no antibodies to Pm-T were found (de Jong, 1985), and also (experimental) intranasal administration of Pm-T does not evoke detectable Pm-T-specific humoral nor cellular immune responses in pigs (Foged *et al.*, 1987; van Diemen *et al.*, 1994b). The reasons for the lack of systemic and local immunity in pigs to Pm-T are unknown.

Pigs can be protected against Pm-T-initiated nasal breakdown by vaccination 3 weeks before challenge with inactivated Pm-T, 'Pm-toxoid' (Kobisch and Pennings, 1989; van Diemen *et al.*, 1994b). Although anti-Pm-T immune cells were present, nose damage was, however, found in pigs vaccinated 10 days before or after challenge (van Diemen *et al.*,

1994b). Furthermore, pigs simultaneously challenged with Pm-T and vaccinated with Pm-toxoid failed to respond to Pm-toxoid (van Diemen *et al.*, 1994b), suggesting that Pm-T affected cellular immunity.

Recently, we developed a 3-day challenge-exposure model with Pm-T that was aimed to cause moderate (subclinical) disease-symptoms at 5 weeks post challenge (van Diemen et *al.*, 1994a). This enabled to study the effects of Pm-T on *in vivo* cellular and T-cell dependent humoral immunity in pigs that had been challenged intranasally with Pm-T and simultaneously sensitized systemically with various (non-related) T-cell dependent antigens.

MATERIALS AND METHODS

Animals, housing and feeding

Sixty Dutch Landrace piglets were obtained from one farm with a 'Pm$^+$ free'-certificate issued by the Animal Health Service in The Netherlands (De Jong, 1985). Upon arrival at the experimental facilities, the piglets were randomly allocated to one of 6 treatment groups and housed in 2.75 x 2.20 m pens, 5 piglets per pen. About 30% of the floor was covered with slats. Ambient temperature was maintained at 25°C.

At the start of the treatments, the piglets (gilts and boars) were 38 $\pm$ 1.5 days old, and weighed 9.7 $\pm$ 1.3 kg. Piglets were fed a pelleted weaner diet *ad libitum* by self-feeders and had free access to water. Food contained 16,7 kJ of gross energy (GE) per gram and 17% crude protein. All animals were intranasally pre-treated, on Day -3, with a 1% acetic acid solution in water, 0.5 ml in each nostril (Van Diemen *et al.*, 1994a).

Experimental routine

The experimental period was composed of a preliminary period of 7 days and an exposure period of 5 weeks. The first day of the exposure period was defined as Day 0.

In two treatment groups (*Group 3-0* and *Group 3-1*), subclinical AR-like symptoms were induced with the Pm-T challenge model as described by Van Diemen *et al.* (1994a). This 3-day challenge-exposure model was aimed to cause moderate (subclinical) disease-symptoms, 5 weeks post challenge. The applied daily challenge-dose was 0.5 ml of 13 μg ml^{-1} Pm-T in phosphate buffered saline (PBS) in each nostril. The Pm-T challenge started on Day 0. The second pair of treatment groups were challenged only once with 13 μg ml^{-1} Pm-T, 0.5 ml in each nostril (*Group 1-0* and *Group 1-1*). This treatment was chosen to compare the effects of Pm-T *in vivo* with *in vitro* stimulation of lymphocytes where Pm-T is only once added to the cultures. The last pair of groups served as control

groups (*Group 0-0* and *Group 0-1*). They were challenged similarly with 0 μg Pm-T ml^{-1} PBS.

On Day 0, of each pair of treatment groups, one group was intramuscularly immunized with 2 ml antigen cocktail (*Group 0-1*, *Group 1-1*, and *Group 3-1*). The cocktail contained 1 mg Keyhole limpet haemocyanin (KLH), 4 mg ovalbumin (OA) and 15 lf tetanus toxoid (TT) in a 1:1 PBS and Freund's incomplete adjuvant solution. The other groups were treated similarly with the PBS-adjuvant solution (*Group 0-0*, *Group 1-0* and *Group 3-0*).

Atrophic rhinitis characteristics

The presence of brachygnathia superior (BS) was measured in mm on Day 0 and on Day 35 in all animals. The change in BS (cBS) over this 35-d period was used as disease characteristic (Van Diemen *et al.*, 1994a).

On Day 38 after challenge, all piglets were necropsied (stunned and bled) to observe AR characteristics. Progression of AR was defined by the grade of conchae atrophy after cross-sectioning the snout between the first and second premolar tooth (De Jong, 1985); atrophy of the ventral conchae (VCA) was graded from 0 (no lesions) to 4 (total atrophy) and of the dorsal conchae (DCA) from 0 (no lesions) to 3 (total atrophy). The average of both nostrils was used in calculations (Van Diemen *et al.*, 1994a).

Immune characteristics

Before treatment, and at weekly intervals, bloodsamples were collected.

Humoral response - KLH, OA, TT and Pm-T specific *in vivo* antibody titres were determined routinely by either one- or two step indirect ELISA. Shortly, serial dilutions of serum were applied to antigen-coated wells of a microtitre-plate. After incubation for 1 h at 37°C, and subsequent washing, either a one (IgG, total Ig) or two step (IgM) conjugation followed. The one step assay consisted of incubation for 1 h with a 1:2000 diluted peroxidase (PO) conjugated rabbit antibody directed to swine IgG_{Fc} (RASw-IgG_{Fc}/PO, Nordic, Tilburg, The Netherlands) or IgG_{H+L} (RASw-IgG_{H+L}/PO, Kpl, Gaithersburg, MD, USA) for IgG and total Ig respectively. The two step assay consisted of incubation for 1 h with 1:6000 diluted mouse anti-swine IgM monoclonal antibody (MASw-IgM, ID-DLO, Lelystad, The Netherlands) and 1 h incubation with 1:500 PO-conjugated rabbit anti-mouse antibody (RAM/PO, Dakopatts, Glostrup, Denmark). After washing, tetramethylbenzidine (TMB, Sigma) was added as a chromogen. Colour formation was stopped after 10 minutes with 2.5 N sulphuric acid. All absorbances were

expressed relatively to the absorbance of a standard positive control serum obtained from a vaccinated animal.

Cellular response - Ovalbumin- and tetanus toxoid-specific *in vitro* cellular immunity was determined by a whole blood lymphocyte stimulation test (LST). One to ten diluted heparinized whole blood samples were tested for proliferation in the presence of either 15 μg ml^{-1} OA or 5 μg ml^{-1} TT in RPMI culture medium containing antibiotics. Numbers of peripheral blood leucocytes (PBLs) per ml were determined with a Coulter Counter® ZM (Coulter Electronics LTD, Luton, UK).

Mitogenic activity of Pm-T on PBLs (Van Diemen *et al.*, 1994b) was determined by LST on isolated PBLs, obtained from heparinized blood samples using Ficoll density gradient centrifugation. PBLs ($4*10^5$ per well) were tested in the presence of 1 μg ml^{-1} Pm-T in RPMI containing 10% foetal calf serum and antibiotics.

All cultures, set up in triplicate, were incubated 4 days at 37°C, 5% CO_2 in humidified atmosphere. Eighteen hours before harvest, 0.5 μCi of methyl-^{3}H-thymidine (^{3}H, Amersham, Bucks, UK), was added. ^{3}H uptake was determined with a Beckman ß-scintillation counter. Results were expressed as mean counts per minute (cpm). Stimulation Indices (*SI*) were calculated as: *SI* = cpm in antigen stimulated cultures / cpm in unstimulated cultures. A *SI* > 1.5 was regarded as positive.

Statistical analysis

Traits were statistically analyzed for the effect of Pm-T challenge, cocktail vaccination and their interaction using a two-way analysis of variance per testdate (SAS, 1989). Subsequently, pairwise comparisons within testdate were performed between treatment groups using least square mean differences at the overall 0.05 level of significance. Correlation between parameters of (subclinical) disease and immune characteristics were analyzed by method of Pearsons partial correlation (SAS, 1989).

RESULTS

Atrophic rhinitis characteristics

Ventral and dorsal conchae atrophy scores (VCA and DCA) and change in brachygnathia superior (cBS) per treatment group are shown in Figure 1. No clinical AR symptoms occurred. All pigs treated with Pm-T on 3 consecutive days were significantly more severely affected with respect to VCA, DCA, and cBS ($P < 0.0001$) than the not Pm-T challenged control pigs (Figure 1). The pigs challenged only once with Pm-T did not differ in ventral or dorsal conchae atrophy nor in cBS from the control pigs.

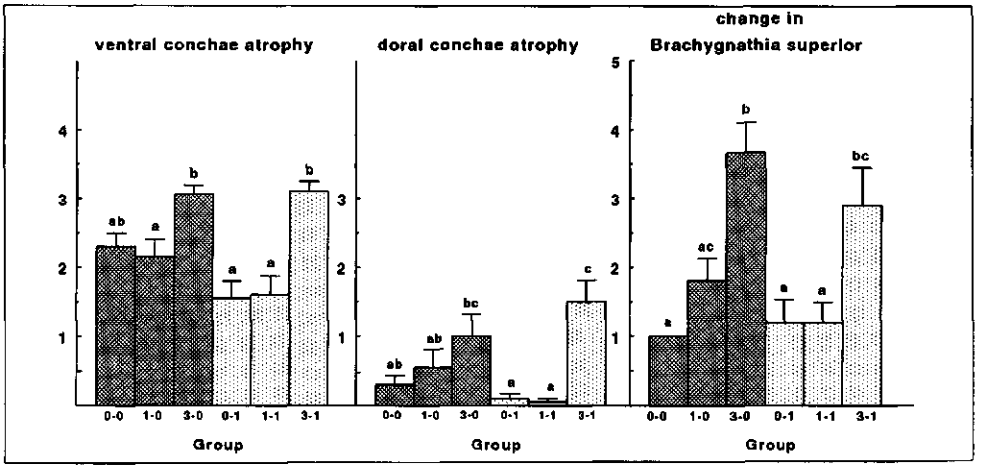

Figure 1 - Ventral and Dorsal Conchae Atrophy scores and change in Brachygnathia superior (mean ± SEM) per treatment group. Cross-hatched, no cocktail immunization (*Groups 0-0, 1-0* and *3-0* respectively); dots, cocktail immunization (*Groups 0-1, 1-1* and *3-1* respectively), a,b: Different characters indicate pairwise significant difference, $P < 0.05$.

The administration of the antigen-cocktail had a significantly decreasing effect ($P < 0.02$) on the VCA scores, which were 2.08 for the antigen-challenged group and 2.50 for the non-antigen challenged group, respectively. The simultaneous administration of other antigens tended to affect the DCA scores differently, depending on the number of Pm-T doses administered ($P < 0.07$). The DCA scores of the pigs of *Group 0-1* and *Group 1-1* were decreased compared with their non-antigen treated contemporaries, while the pigs of *Group 3-1* had an increased score compared to the pigs in *Group 3-0*.

Immune characteristics

Humoral response - In general, pigs immunized with the cocktail consisting of KLH, OA, and TT mounted systemic antibody responses directed to KLH, OA and TT, which were measured from one week post vaccination on.

Keyhole limpet haemocyanin - The total antibody response to KLH of the Pm-T-challenged groups (*Group 1-1* and *Group 3-1*) were lower than the non-Pm-T treated (control-) group (*Group 0-1*) (Figure 2a). On Days 17, 24 and Day 31, this reduced response was significant for *Group 1-1* ($P < 0.05$). Within the groups which had not received KLH, *Group 1-0* had a lower anti-KLH total Ig titre than *Group 0-0*.

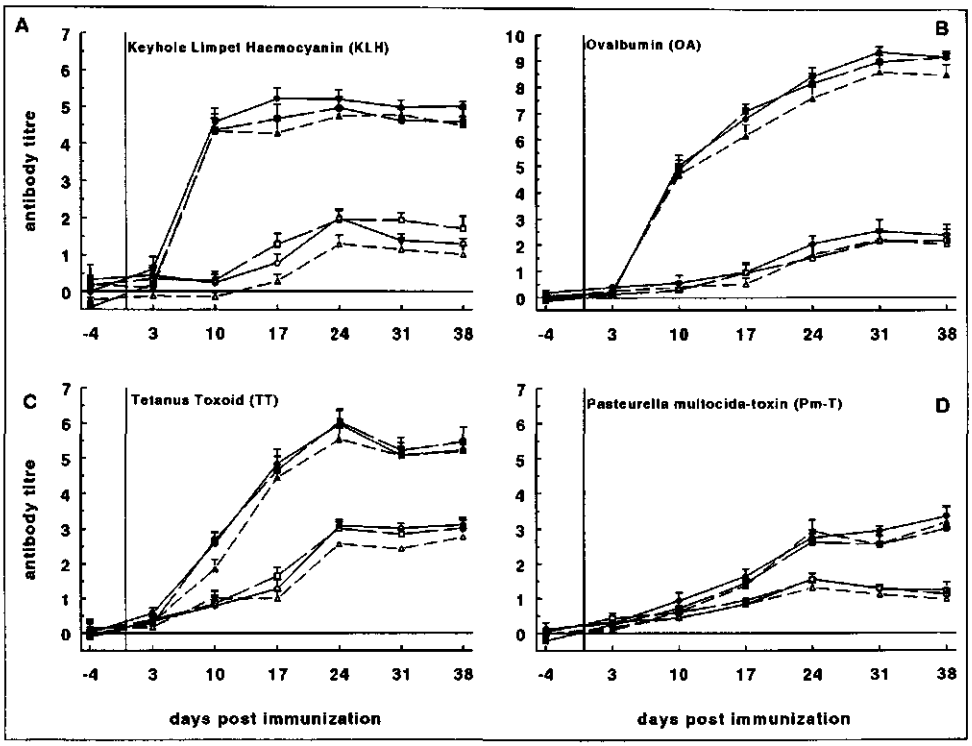

Figure 2 - Antigen specific total Ig titres (mean ± SEM) per treatment group. A) KLH, B) OA, C) TT and D) Pm-T. ○: *Group 0-0*, ●: *Group 0-1*, △: *Group 1-0*, ▲: *Group 1-1*, □: *Group 3-0*, ■: *Group 3-1*.

On day 10, a maximum anti-KLH IgM response was found for all antigen-treated groups (Figure 3a). From day 17 onwards anti-IgM responses were similar in all groups. The anti-KLH IgG response of *Group 0-1* pigs increased on Day 10 and remained high throughout the observation period (Figure 4a). The response of the pigs in *Group 3-1* increased simultaneously, and decreased slightly towards the end of the exposure period. The response of the *Group 1-1* pigs was lower compared with the *Group 0-1* pigs. On Day 17, 24 and 38 this decrease tended towards significance ($P < 0.1$). This suggested that the lower total antibody response to KLH was due to a lower IgG response.

Ovalbumin - The total Ig response was equal for *Group 0-1* and *Group 3-1*. Though not significantly, a lower response was found in *Group 1-1* (Figure 2b). The IgM response to OA increased throughout the experimental period. Difference between *Group 0-1* and *Group 3-1* was not found (Figure 3b). Both groups treated once with Pm-T (*Group 1-1* and *Group 1-0*) showed a lower response than their control contemporaries (*Group 0-1* and *Group 0-0*).

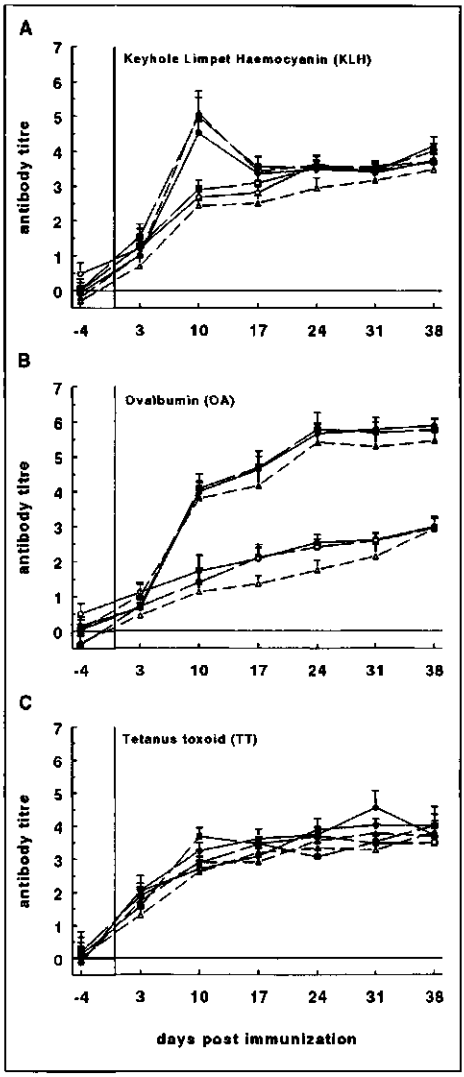

Figure 3 - Antigen specific IgM titres (mean ± SEM) per treatment group. A) KLH, B) OA, and C) TT. ○: *Group 0-0*, ●: *Group 0-1*, ▵: *Group 1-0*, ▲: *Group 1-1*, □: *Group 3-0*, ■: *Group 3-1*.

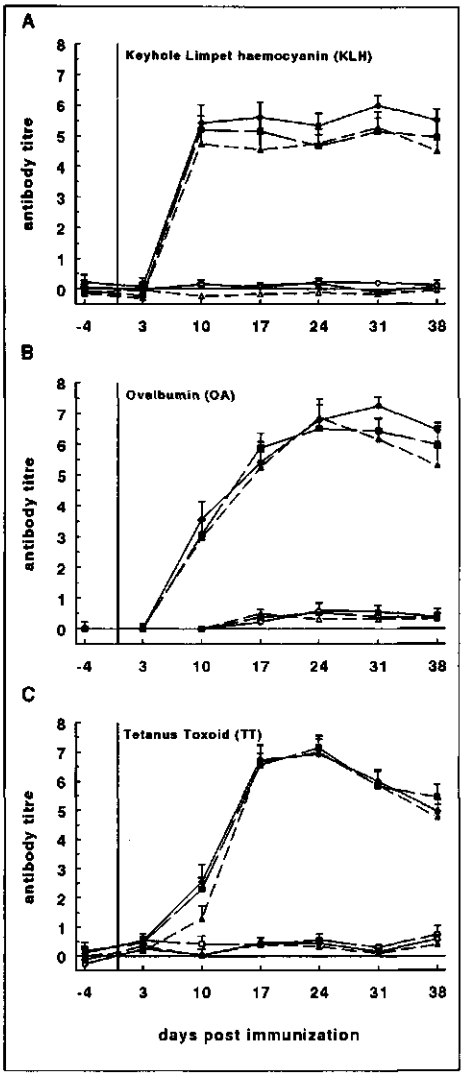

Figure 4 - Antigen specific IgG titres (mean ± SEM) per treatment group. A) KLH, B) OA and C) TT. ○: *Group 0-0*, ●: *Group 0-1*, ▵: *Group 1-0*, ▲: *Group 1-1*, □: *Group 3-0*, ■: *Group 3-1*.

From Day 10 onwards, *Group 0-1* pigs mounted an anti-OA IgG response with the highest response at Day 31 (Figure 4b). Similar responses were found in the other OA-sensitized groups, although in *Group 1-1* pigs the maximum response occurred earlier (Day 24) and appeared lower, whereas the response of the *Group 3-1* pigs was broader and lower.

Tetanus Toxoid - The total antibody responses to TT were similar for all three TT-sensitized groups. All groups showed a peak at Day 24 (Figure 2c). The response of the *Group 1-1* pigs, however, was slower and remained lower than the *Group 0-1* pigs until Day 31. No higher anti-TT IgM responses were found in TT sensitized groups as compared to non-TT sensitized groups (Figure 3c). The IgG responses to TT were similar in all TT sensitized groups (Figure 4c). Although pigs of *Group 1-1* had a slower IgG response to TT than pigs from the other cocktail groups (Figure 4c).
Pm-T - Low, but significantly elevated total antibody titers to Pm-T were found in all groups sensitized with the mixture of KLH, OA, and TT (Figure 2d).

Cellular response - The effect of a single or repeated (3 days) exposure of pigs to Pm-T on cellular immunity *in vitro* to OA and TT was measured.

Ovalbumin - The proliferation of lymphocytes of all groups in the presence of OA are shown in Figure 5a. The *Group 0-1* responded from Day 10 onwards ($SI_{OA} > 1.5$) and peaked at day 31 ($SI_{OA} = 3.86$). The cellular response to OA of the pigs treated three times with Pm-T (*Group 3-1*) started later and peaked sooner and more pronounced (Day 17, $SI_{OA} = 5.38$) with a large variation between individual pigs, compared with the *Group 0-1* pigs. The *Group 1-1* pigs mounted their cellular response to OA at Day 10, and peaked at Day 24 ($SI_{OA} = 5.92$). A large variation between animals was found (Figure 5a).

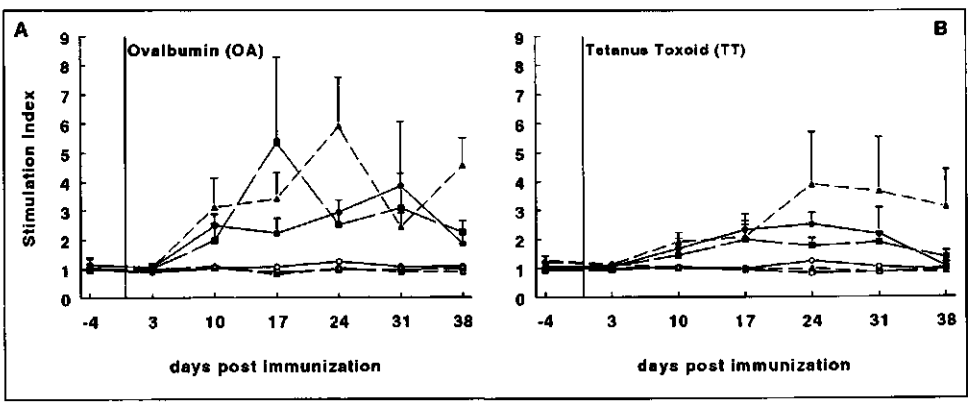

Figure 5 - Stimulation indices (mean ± SEM) of cultures stimulated with A) 15 μg ml^{-1} OA or B) 5 μg ml^{-1} TT, per treatment group per testday. ○: *Group 0-0*, ●: *Group 0-1*, △: *Group 1-0*, ▲: *Group 1-1*, □: *Group 3-0* ■: *Group 3-1*. The mean cpm of unstimulated cultures averaged 76 (range: 45 and 111 cpm).

Tetanus Toxoid - The cellular response to TT of *Group 0-1* occurred from Day 10 up till Day 31 ($SI_{TT} > 1.5$) with a peak at day 24 ($SI_{TT} = 2.51$, Figure 5b). In the group of pigs treated three times with Pm-T (*Group 3-1*), the response was delayed and remained

lower, and was without a clear peak compared with the response of *Group 0-1*. The PBLs of *Group 1-1* pigs started to proliferate in the presence of TT on Day 10, which was comparable with *Group 0-1* pigs. However, whereas of the latter group the SI_{TT} peaked at Day 24, the SI_{TT} of *Group 1-1* increased to 3.90 ($P < 0.001$), and remained high until the end of the experiment. The SE of the SI_{TT} increased simultaneously.

Pm-T - In approximately 47% of the pigs tested, the non-immune lymphocytes proliferated in the presence of Pm-T ($SI_{Pm\text{-}T} > 1.5$, mean $cpm_{un} = 204$). No partial correlations between mitogenic activation of T-cells by Pm-T, and AR-specific pathology were found.

DISCUSSION

Pasteurella multocida-toxin (Pm-T) initiates the pathogenic processes of atrophic rhinitis (AR). This results into irreversible destruction and reabsorption of nasal bony tissues in pigs (de Jong, 1987; van Diemen *et al.*, 1994a). No detectable levels of Pm-T-specific antibodies are found in pigs challenged with Pm-T (Foged *et al.*, 1987; van Diemen *et al.*, 1994b), and also in field outbreaks of AR, no antibodies to Pm-T have been found (de Jong, 1985). Also in the present study, intranasal Pm-T challenge did not induce a cellular nor humoral immune response *in vivo*, as reported previously (Frymus *et al.*, 1986; Foged *et al.*, 1989; van Diemen *et al.*, 1994b). The lack of immune responses to Pm-T may be caused by the inability of T cells to recognise Pm-T. However, previously we found that T cells recognising Pm-T were present in pigs vaccinated with Pm-toxoid prior to Pm-T challenge. Pm-T was reported to be mitogenic *in vitro* for several cell types (Frymus *et al.*, 1986; Foged *et al.*, 1989; Rozengurt *et al.*, 1990), and also for naive and lectin-stimulated porcine T cells *in vitro* (Van Diemen, 1994b). This 'nonspecific' activation of T cells by Pm-T was individually restricted, and could be partly abrogated by monomorphic anti-swine MHC class II DQ and DR specific monoclonal antibodies (van Diemen *et al.*, 1994b). These observations suggested that Pm-T may affect cellular immunity by interference with antigen presentation via MHC class II antigens. Pigs simultaneously challenged with Pm-T and vaccinated with Pm-toxoid failed to respond to Pm-toxoid (van Diemen *et al.*, 1994b). *In vivo*, Pm-T may be presented to T cells in such a way, that T cells responding to Pm-T and possibly to other antigens are inactivated.

The present results indicated that a single or repeated treatment of pigs with Pm-T did not cause a 'non-antigen specific' suppression of immune responses *in vivo* to various T-cell dependent antigens. Challenge with Pm-T affected but did not abrogate the *in vivo* immune response against OA, KLH or TT. This was especially true for pigs which were

challenged only once with Pm-T. Though not always significantly, the animals treated once with Pm-T showed lower total antibody responses to KLH, OA and TT, which seemed to rest on the lower IgG responses to these antigens. No clear effects were found on cellular immune responses to these antigens *in vitro*. The present results indicated 1) that the lack of detectable immune responses to Pm-T probably does not depend on a 'general non-specific' suppression of cellular immunity, and 2) that challenge with a dose of Pm-T, which ultimately results in a breakdown of nasal bony tissues does not uniformly affect immune responses to various T-cell dependent antigens.
A low, but significant increase of antibodies binding to Pm-T were found in pigs sensitized with a mixture of KLH, OA, and TT, irregardless of Pm-T sensitization. As yet, we have no explanation for this phenomenon. The increase in antibodies binding Pm-T, however, did not protect group 3-1 animals from nose damage, although in these animals a lower VCA score was found.

The relationship(s) between Pm-T and porcine T cells deserve further study. Bacterial endo- and exotoxins can act as so called 'superantigens' in mouse and man. These antigens nonspecifically activate the cellular immune response, such, that ad random activation of preferably autoimmune T cells with concomitant rise of (auto)antibodies can occur. On the other hand, death or inactivation of the responding T cells may leave holes in the T-cell repertoire of the individual (Cole and Atkin, 1991; Coffin, 1992). The lack of immune responses to Pm-T *in vivo*, and the individual restriction of the mitogenic effect of Pm-T on porcine immune cells *in vitro* (van Diemen *et al.*, 1994b), therefore urges studies on the expression of T-cell receptor V-beta families (Cole and Atkin, 1991; Coffin, 1992) in affected and non-affected pigs.

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Chapter 5.3

THE ROLE OF T CELLS IN ATROPHIC RHINITIS - A PILOT STUDY

P.M. van Diemen, W. Pellikaan, G. de Vries Reilingh and H.K. Parmentier

THE ROLE OF T CELLS IN ATROPHIC RHINITIS - A PILOT STUDY

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Abstract

Attempts were made to unravel the role of T cells during the early phase of atrophic rhinitis (AR) experimentally induced by *Pasteurella multocida* toxin (Pm-T). In the first pilot experiment, AR was studied in piglets treated with the immuno-suppressant Cyclosporin A (CyA). Treatment with CyA affected the proportions of peripheral T cell subsets. Symptoms of AR appeared to be less pronounced in these pigs, but no significant differences between CyA treated and control pigs were found for any parameter of AR studied. Pm-T was mitogenic for non immune lymphocytes *in vitro* for most but not all animals. Disease symptoms of AR *in situ* could not be initiated by syngeneic transfer of peripheral white leucocytes sensitized *in vitro* with Pm-T. However, a significant positive relation was found between the number of T cells returned and the change of brachygnathia superior. Although the role of T cells during the early phase of AR could not be established, the latter result urges further studies on putative involvement of T cells in the development of AR.

Key words: *Pasteurella multocida* toxin, immunosuppression, Cyclosporin A, T cell, pigs

INTRODUCTION

The causative agent of AR in pigs, *Pasteurella multocida* toxin (Pm-T) appeared to be mitogenic for naive and Concanavalin A (ConA) stimulated peripheral white blood cells of pigs (van Diemen *et al.*, 1994b; Chapter 5.2). The mitogenic activity of Pm-T could be abrogated by the addition of antibodies directed to MHC class II antigens (van Diemen *et al.* 1994b). As is true in naturally infected pigs, no antibodies nor sensitized T cells directed to Pm-T are found in pigs which develop symptoms of AR after experimental administration of Pm-T. The role of T cells in the (lack of) protection to Pm-T is largely unknown. A possible relation may exist between the mitogenic effect of Pm-T on porcine T cells on the one hand, and the lack of immune responses to Pm-T, and the individual variability of (sub)clinical AR symptoms on the other hand.

In case of T-cell mediation, immuno-suppressant treatment is expected to abrogate or reduce AR lesions. Therefore, in the first experiment, effects of suppressing cellular immunity on the development and severity of AR lesions were studied. In the second experiment, the potency of peripheral white leucocytes (PBL) stimulated *in vitro* with Pm-

T to induce specific nose damage was studied. Specific nose damage is expected in case of T-cell mediation.

EXPERIMENT 1

The effect of the putative immunosuppressive agent Cyclosporin A (CyA) on Pm-T initiated nose damage in pigs, and the proportion of peripheral T-cell subsets were studied.

Experimental design

Thirty DL piglets were purchased from a farm with a '*Pm*$^+$-free' certificate (Animal Health Service, Deventer, Netherlands) and housed in a climate respiration chamber used as an isolator. Littermates were equally divided into 2 groups, a control and CyA treated group. Within these groups, pigs were randomly allotted to either Pm-T treatment (n = 10, Pm-T) or no treatment (n = 5, NON).

The CyA treatment (5 mg kg^{-1}) started one week before and continued for another two weeks after Pm-T challenge. CyA dissolved in ethanol/PBS (1:4) was administrated once a day subcutaneously. Pigs were weighed twice a week to adapt the amount of CyA to the individual body weight. Control pigs received only the ethanol/PBS solvent.

Pm-T treatment was conducted according to the challenge model developed by van Diemen *et al.* (1994a). In short, three days after pretreatment with acetic acid, 20 piglets were intranasally challenged with 13 μg ml^{-1} Pm-T in PBS on 3 consecutive days (0.5 ml per nostril). The pigs in the NON groups were intranasally challenged with PBS. First day of challenge was defined Day 0.

For analyses, blood samples were drawn from all animals before treatments and weekly over a 5 week period. Percentages of T-cell phenotypes in the blood were determined by Fluorescence Activated Cell Sorter (FACS) analysis (Joling *et al.*, 1994).

EXPERIMENT 2

The potency of PBL stimulated *in vitro* with Pm-T to induce AR characteristics *in situ* was studied.

Experimental design

Nineteen GY × DL crossbred piglets, 4-5 weeks old, were used. PBL were obtained from heparinised blood samples using Ficoll density gradient centrifugation. The PBL were incubated with 1 μg ml^{-1} Pm-T for 4 days at 37°C, 5% CO_2 in humidified atmosphere. Subsequently, cells were washed and returned to the respective 14 donor piglets through an ear vein (T-cell group). The remainder five pigs served as control

group. Number of PBL returned ranged between $18*10^6$ and $62*10^6$ (mean $38*10^6$). The day of returning the cells was defined as Day 0.

AR characteristics

At the end of both experiments (day 35) all pigs were stunned and bled in a slaughterhouse. Progression of AR was examined, post mortem, by the grade of ventral and dorsal conchae atrophy (VCA and DCA) after cross-sectioning the snout between the first and second premolar tooth (De Jong, 1985). The turbinate perimeter ratio (TPR) was derived by digitizing photographs of cross-sections (Collins *et al.*, 1989). The change in Brachygnathia superior (cBS) between onset and end of the experiment was measure determined.

In both experiments, mitogenic activity of Pm-T on PBL (Van Diemen *et al.*, 1994b) was determined by lymphocyte stimulation test. Isolated PBL ($4*10^5$ per well) were tested in the presence of 1 μg ml^{-1} Pm-T in RPMI containing 10% foetal calf serum and antibiotics. The cultures, set up in triplicate, were incubated 4 days at 37°C, 5% CO_2 in humidified atmosphere. Eighteen hours before harvest 0.5 μCi of methyl-^{3}H-thymidine (^{3}H) was added. ^{3}H uptake was determined with a Beckman ß-scintillation counter. Results were expressed as mean counts per minute (cpm). Stimulation Indices (*SI*) were calculated as: *SI* = cpm in stimulated cultures / cpm in unstimulated cultures. A *SI* > 1.5 was regarded as positive.

RESULTS AND DISCUSSION

EXPERIMENT 1

The effects of CyA treatment of pigs on parameters of AR are summed up in Table 1. No distinct differences in VCA, DCA or cBS between the two Pm-T challenged groups were found. However, the reduction in severity in TPR between CyA treated and non-treated pigs tended towards significance ($P < 0.1$). A negative relation ($r^2 = -0.67$, $P < 0.05$) was found between VCA and TPR. No increase in severity of AR was found either in CyA-treated pigs. The high 'background' level in VCA of the non Pm-T treated (NON) groups may have been caused by (ubiquitous present) *Bordetella bronchiseptica*, which can induce moderate nose lesions (de Jong, 1985; Rutter, 1985). In this respect, the slight increase in VCA, DCA, and cBS in the CyA treated NON pigs was noteworthy (Table 1).

Considering our results, CyA treatment before and early after Pm-T challenge did not clearly affect symptoms of AR. It is possible that the amount of CyA in the blood had not reached a sufficient level to suppress cellular immunity in pigs. On the other hand, Pm-T was mitogenic for pig lymphocytes *in vitro*, thus it cannot be excluded that the

suppression of T cells is 'overruled' by an activation of T cells due to the mitogenic activity of Pm-T.

Table 1 - Pilot study on immunosuppression. Comparison of mean AR characteristics by treatment group.

Variable	Control		Cyclosporin A		SEM
	NON	Pm-T	NON	Pm-T	
n	5	9	4	10	
VCA	1.70[a]	2.72[b]	2.13[a]	2.65[b]	0.20
DCA	0.10[a]	1.00[b]	0.38	0.75	0.39
TPR	0.81[c]	0.66[d]	0.81	0.77	0.07
cBS (mm)	1.80[a]	4.11[b]	2.75[a]	3.90[b]	0.57
SI_{Pm-T}	4.00	3.60	2.39	4.02	0.90

Different row superscripts indicate pairwise, significant differences or trends; [a,b] $P<0.05$, [c,d] $P<0.1$.

Cyclosporin A was given preference over the immunosuppressive agent dexamethasone. CyA acts selectively on primary cellular immune responses and leaves secondary responses intact (Anonymous, 1985 and 1988). Flaming *et al.* (1994) reported that pigs seem remarkably resistant to immunosuppression by dexamethasone treatment. That treatment resulted in only mild alterations in porcine immune functions even at a dose that is 150 times higher than the dose that is consistently immunosuppressive in cattle (Roth and Kaeberle, 1981 cited by Flaming *et al.*, 1994). In the current experiment, the humoral immune system seemed undisturbed, i.e. the applied CyA treatment did not affect the percentage of B cells in the blood.

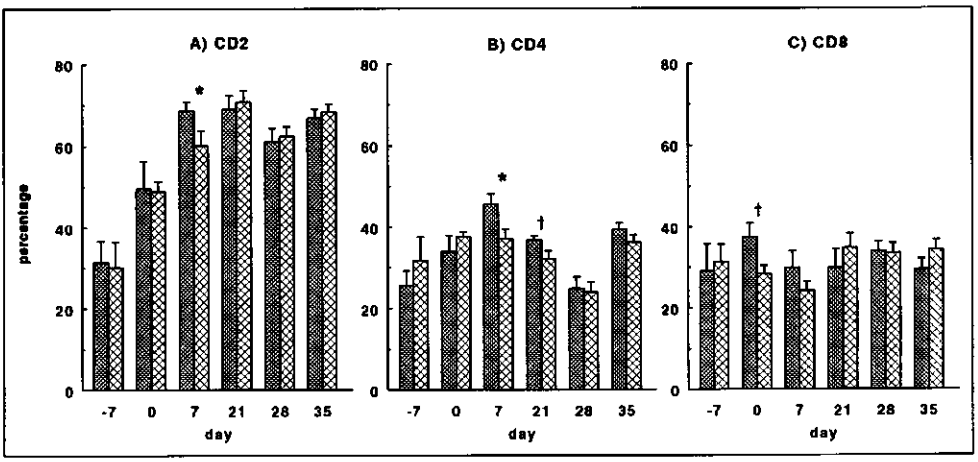

Figure 1 - Proportions of A) CD2, B) CD4, and C) CD8, in Pm-T challenged pigs. Close cross hatched: control; cross hatched: Cyclosporin A. (*: $P < 0.05$; †: $P < 0.1$).

At day 7, the total T cell (CD2) pool was significantly lower in the CyA treated pigs ($P < 0.05$, Figure 1A). In the period after CyA treatment (from Day 10 onwards), proportions of CD2 were similar in both groups. After Pm-T treatment at Day 0, percentages of CD4 increased only in pigs not treated with CyA. At day 7, the percentage of CD4 (T_{helper}) was significantly lower in CyA-treated pigs ($P < 0.05$, Figure 1B). This difference was still present on day 21 ($P < 0.07$). The proportion of CD8 ($T_{suppressor}$) showed a trend downwards in the CyA pigs at Day 0 and later on (Figure 1C). A large diversity in immune parameters between individuals was found. As in humans, (Hu, 1994), the CD8 positive cells appeared to respond earlier (day 0) to the CyA treatment than CD4 cells. Thus, CyA treatment seemed to affect porcine cellular immune functions, although the method of application and dose of CyA used may require optimization.

EXPERIMENT 2

In Table 2 characteristics of AR of pigs which received syngeneic PBL sensitized *in vitro* with Pm-T and control pigs are given. Pigs which received non-stimulated PBL were not included in this pilot experiment. No differences in parameters of nose damage between the treatment and control groups were found. A positive relation ($r^2 = 0.70$, $P < 0.006$) occurred between the numbers of PBL (T cells) returned and the change in brachygnathia superior (cBS). This suggested that the number of cells returned might have been too low to induce detectable nose damage by Pm-T sensitized T cells.

Table 2 - Pilot study on *in vitro* Pm-T stimulated T cells. Comparison of mean AR characteristics by treatment group.

Variable	control	SEM	T cell	SEM
n	5	-	14	-
VCA	1.10	0.24	1.32	0.15
DCA	0	0	0	0
TPR	0.74	0.04	0.68	0.03
cBS (mm)	0	0.55	0.36	0.32
$SI_{Pm\text{-}t}$	2.34	0.42	2.47	0.47

CONCLUSION

Although the application method and dose of CyA used may not have been optimal to suppress cellular immunity in pigs, a decrease of T (helper) cells early after Pm-T challenge did not result in either a significant increase, nor a significant decrease of AR symptoms. Thus, the role of T cells during the early phase of AR remained obscure. On

the other hand, data of the second experiment suggested an involvement of (T) cells in the initiation of AR, but the number of PBL returned may have been too low to induce detectable nose damage. In conclusion, the data of both pilot experiments urges further research on the immune mechanisms operating during the early phase of AR.

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Chapter 6

GENERAL DISCUSSION

GENERAL DISCUSSION

INTRODUCTION

In the pig production industry, high-density rearing ensures that virtually every animal will be exposed to (potentially lethal) infectious agents through direct or aerial contacts (Dietert *et al.*, 1994). This is irrefutably true for infections of the upper respiratory tract, where a direct contact between the animal and its environment exists. In bacterial diseases, such as atrophic rhinitis, environmental management can be pivotal in host disease-resistance. Environmental factors represent a ubiquitous, yet frequently manageable, category of components that can influence performance and disease susceptibility or resistance (Verhagen, 1987; Scheepens, 1991). The climatic environment of animals exerts a considerable influence on immune status, particularly on the overall capacity to mount immune responses and to protect the host from disease (Kelley, 1985; Verhagen, 1987; Kreukniet *et al.*, 1990). With respect to atrophic rhinitis, knowledge of environmental and animal factors on disease progression, is lacking.

The development of a challenge model to mimic the disease facilitated studies on atrophic rhinitis. Two areas of interest in atrophic rhinitis have been described in this thesis. First, the impact of climatic environment on the severity of disease symptoms, and the effects of *Pasteurella multocida* toxin (Pm-T) on energy metabolism, production traits, heat production and activity of piglets were determined. Second, investigations after the role of the immune system in atrophic rhinitis-pathogenesis have been conducted with emphasis on mechanisms underlying the lack of conventional (classic) immune responses to Pm-T. The performed research, as well as prospective research are discussed here and, finally, consequences and implications of the proposed concept are outlined.

INDUCTION OF ATROPHIC RHINITIS

Experimental model

When ciliary function of the nasal mucosa is normal and mucus is removed, airborne micro-organisms and dust can be cleared. Once adhered to the nasal epithelium, a bacterium can avoid clearance by the mucociliary transport system. The ability of toxigenic *Pasteurella multocida* (Pm^+, serotype D), however, to adhere to the upper respiratory tract epithelial cells is poor (Frymus *et al.*, 1986; Jacques, 1987), but Pm^+ has affinity for mucus (Letellier *et al.*, 1991). When ciliary activity is impaired and the mucus stagnant or absent, colonization with toxigenic Pm^+ on the conchal mucous membrane, and concomittant production of its toxin (Pm-T), will be enhanced. Also the clearance

mechanisms of the upper respiratory tract in the pig may be overloaded, when the rate of bacterial invasion or the rate of bacterial multiplication exceeds the rate of muco-ciliary transport and clearance (Letellier *et al.*, 1991). The developed AR challenge model (Chapter 3) operates independently of the colonizing ability of the bacterium by using Pm-T, but it needs impaired ciliary function.

Both acetic acid (under experimental conditions) and *Bordetella bronchiseptica* (*Bb*) (under experimental and natural conditions) are known as predisposing factors to the nasal infection with toxigenic *Pm*. They share the ability to cause ciliostasis and stagnation of nasal mucus (Gagné and Martineau-Doizé, 1993; Nielsen and Rosendal, 1994). Since a *Bb*-infection is ubiquitous in most commercial pig units, it is hard to obtain conventionally raised *Bb*-free piglets. Even by treating sow and litter with antibiotics, an infection could not be prevented (data not published). The degree of mucosal irritation by *Bb* also depends on maternal immunity against the bacterium. For the standardization of the model, acetic acid pretreatment was, therefore, preferred over *Bb* inoculation. This pretreatment will at least cause a minimum degree of mucosal irritation in all animals. In a pilot study it was found that pretreatment with acetic acid reduced the snout lesions caused by *Bb* (data not published).

The results of the conducted experiments indicate that our model mimics the pathogenic effect of *in vivo* infection with toxin-producing *Pm* strains. The induced disease characteristics are dose-dependent. However, it has to be kept in mind that dose dependency is relative, whereas the 'amount' of Pm-T that penetrates the respiratory epithelium is unknown. The ventral conchae are affected first, the dorsal conchae atrophy (DCA) developed slower and occurred with the higher ventral conchae atrophy (VCA) scores (Chapter 3). It remains to be established whether the AR-symptoms caused by administration of Pm-T are based on a mechanism similar to that attributable to *in vivo* infection with Pm^+ strains. Concerning the nose damage, the difference in outcome between the Pm-T challenged and control animals in the model was fairly constant over experiments. All Pm-T treated piglets developed turbinate lesions. The base level of nose damage (control pigs), however, varied between experiments. Both Done (1985) and Goodwin *et al.* (1990) mentioned the problem of higher snout scores without epidemiological or bacteriological evidence to indicate that the pigs were experiencing active AR. They stated that all pigs with AR have conchae atrophy, but not all pigs with conchae atrophy have actually AR. The higher snout scores were associated with *Bordetella bronchiseptica*- and non-toxigenic *Pasteurella multocida*-infections. Further association with other diseases influencing bone formation, with unsatisfactory environmental conditions, like dust or concentration of ammonia, and with recurring husbandry problems, like overstocking, are mentioned (Done, 1985; Goodwin *et al.*, 1990). In our

piglets, the higher base level in nose scores might have had a bacteriological cause since both *Bordetella bronchiseptica* and non-toxigenic *Pasteurella multocida* were present, as demonstrated through nasal swabs. Within an experiment, however, the difference in VCA and DCA scores between the Pm-T challenged and control pigs was solely attributable to Pm-T administration. The severity of the induced lesions were, nevertheless, moderate (subclinical), thus enabling us to study factors which may have a positive or negative effect on the disease symptoms. Clinical cases were not observed.

Parameters

In this thesis, the lesions were graded at five weeks post challenge with the method described by De Jong (1985). This method is based on changes in shape of the conchae. The ventral conchae are evaluated in 9 classes and the dorsal conchae in 7 classes. This system of scoring was refined enough to distinguish between control and challenged pigs (t-test). However, to detect nuances in lesions between challenged piglets in different treatment groups, the grading system seemed not sensitive enough. It was felt that next to changes in shape also the loss in extent should be accounted for. Therefore, in two experiments morphometric values (turbinate perimeter, TP and turbinate perimeter ratio, TPR) were derived by digitizing photographs of cross-sections according to Collins *et al.* (1989).

Although TPR is said not to be influenced by pig age or breed (Collins *et al.*, 1989), in our experiments, the TPR differed considerably between experiments. The TPR of non Pm-T treated 10-wk-old DL pigs was 1.15 (± .09) and 0.81 (± .06) respectively in the 2 experiments. Within experiments, the differences between TPR of control and Pm-T treated pigs were significant (t-test). The TPR is valuable as a less subjective, and possibly more sensitive, measure of atrophic rhinitis within an experiment than VCA/DCA, providing parametric data suitable for quantitative analysis. For comparison between experiments, however, caution is needed, since the TPR seemed affected by factors from the 'outside', just as VCA and DCA.

The position of the upper jaw with respect to the lower jaw (Brachygnathia superior, BS) reflects the shortening of a snout in AR. This AR characteristic is the only indicator for impairment of the nose, measured in a living pig. Measured once, however, BS is a breed- and litter-associated characteristic in herds without the disease (Groenland, 1984; Rutter 1985; Chapter 3). When measuring the same animal a second time after a five week interval, the change in Brachygnathia superior (cBS) between the measurements was only dose associated (Chapter 3). Notwithstanding a degree difference in malapposition, the breeds used in our experiments reacted similarly to the applied Pm-T doses. The cBS seems routinely applicable as AR characteristic in practice by measuring BS of piglets at

4-5 and 10 weeks of age. If used consequently, cBS can possibly be used as simple indicator for the presence of AR. When the average cBS of a herd rises, this herd can be subjected to bacteriological testing. Subjective effects of the observer cannot be excluded (Groenland, 1984), but measurements by one person should produce an accurate change over the time interval.

In conclusion, the developed challenge-exposure model enables studies on factors involved in the multifactorial etiology of AR in pigs. The characteristics used to measure AR are satisfactory, but can be refined. As will be discussed further on, the model allowed judgement of factors (positive or negative) related to the mucosal system of the turbinates and did not largely impaired animal welfare. With the same challenge model, on the other hand, clinical disease symptoms can be induced after an adjustment of the Pm-T dose (Chapter 3). For investigating effectiveness of medication or vaccination, the induction of clinical pathogenesis might be the correct method.

ATROPHIC RHINITIS AND CLIMATIC ENVIRONMENT

Because of a direct contact between the animal and its environment in the respiratory tract, the local defence mechanism is prone to be affected by environmental conditions. Several non-contagious factors such as air quality, ammonia and dust are thought to be of importance in herd outbreaks of progressive atrophic rhinitis (Hamilton *et al.*, 1993). Factors such as low ambient temperature, draught and low relative humidity are known to hamper the local defence system of the respiratory tract of pigs (Verhagen, 1987; Kreukniet *et al.*, 1990). Especially factors which relate to the mucosal system of the turbinates may compromise the mucociliary clearance and facilitate bacterial colonization. Several of those factors seem to have seasonally conditioned effects on AR, although such effects are not clearly established (Smith, 1983; Goodwin, 1988; Kavanagh, 1994). Recurring husbandry problems such as overstocking, have been associated with higher snout scores (Goodwin, 1988). And, in practice, the severity of an AR-outbreak can be controlled to a great extent on farms by improving climatic environment and housing conditions (Smith, 1983; Goodwin, 1988). In Chapter 4, interaction between AR and climatic environment in weanling pigs was studied. As 'adverse' environment, ambient temperature below thermal neutrality (below the lower critical temperature, Chapter 3, Figure 1) with four draught-periods was applied. No evidence was found, however, that the low ambient temperature with draught periods aggrevated AR nose lesions induced by our challenge model (Chapter 4.1). Even slightly less severe conchae atrophy was noticed. This indicates that the passage of Pm-T through the mucous

membrane seemed not affected or reduced rather than enhanced. Based on this study, it is probable that the improvements in climatic environment as referred to by the above mentioned research workers, have had an impact on the colonization possibilities and concomitant toxin production of the bacterium on the mucous membrane. The Pm-T, whether produced by Pm^+ on the spot or applied experimentally, might reach the underlying bony tissues in any case. This implicates that in studies on the role of climatic conditions on the severity of AR, effects on the colonizing ability of the Pm^+ should be reckoned with. For this type of research, a challenge model using the *Pasteurella multocida* bacterium should be applied.

However, next to a direct effect on the local defence system against respiratory disorders, indoor climatic conditions also exert an influence on immune status of the pig. Diarrhoea, coughing and sneezing as well as haemorrhagic ear lesions are reported to be more pronounced in pigs kept under poor indoor climatic conditions (Scheepens, 1991). Even if pigs appear healthy and do not seem to have depression of growth, immune function can be impaired (Noyes *et al.*, 1988). Immune assessment relative to environmental-immune interactions can enhance the efficiency of the production operation and optimize the welfare of the animals during the production cycle (Dietert *et al.*, 1994). In the performed study, climatic treatment started at the first day of Pm-T challenge. Experimental work with *Actinobacillus pleuropneumoniae* (*App*) (Verhagen, 1987; Kreukniet *et al.*, 1990) revealed namely, that in young growing pigs the time of occurrence of climatic stress influenced the effect of a challenge. When the adverse conditions occurred after the *App* challenge, incidence and severity (mortality and morbidity) increased. When the low temperature occurred before challenge, clinical symptoms were related to the duration of exposure before challenge.

The characteristic conchae atrophy of AR, at the other hand, was not accompanied by a detectable systemic humoral immune response to Pm-T (Chapters 3 and 4). Thus, effects of environmental factors on specific immune responsiveness (antibodies) could not be measured. This lack of conventional (classic) immune responses to Pm-T, stimulated us to study the role of the immune system in atrophic rhinitis in the second part of the project (Chapter 5).

When an animal experiences disease (e.g. *App*), the maintenance requirement will increase due to fever and an activated immune system (Verstegen *et al.*, 1987). Efficiency of production will dwindle. In case of *App*, the first week after experimental inoculation the diseased pigs lost weight (Verhagen, 1987). This weight loss could mainly be attributed to an increased maintenance requirement (fever) and partly to a reduced food intake. The adverse climatic treatment delayed and prolonged the rectal temperature

increase in diseased pigs (Verhagen, 1987). Moreover, a higher mortality and a different antibody level was noted due to the adverse environment.

The effects of Pm-T (AR) on performance were less dramatic (16 g/d weight gain) and increased over time. The growth retardation seemed to be dependent on the development of nasal damage. Growth reduction up till 80 g/d for the most severe cases of AR have been reported (Dominick and Rimler, 1986). In our experiments described in Chapter 4, however, subclinical AR was induced and clinical cases were not observed. With the development of the challenge model, growth of pigs with more severe conchae atrophy was more (negatively) affected than that of pigs with no or slight conchae atrophy (Chapter 3). The reduction in growth was the outcome of a lower food intake (30 g/d) rather than of a changed partitioning of energy (metabolism) in affected pigs. The maintenance requirement (heat production), efficiency and digestability were not changed by the Pm-T treatment (Chapter 4.1). The reductions in food intake and concomittantly weight gain occurred about one week after the challenge and progressed over time. In experiments conducted with Dutch landrace (DL) pigs, similar reductions were found.

These observations confirm the assumption of Smith (1983) that AR pigs convert food to meat as well as their contemporaries. He thought it likely that there is a correlation between the severity of AR and the rate of food intake. A depressed food intake can be caused by a poor appetite, due to a possible loss of taste and sense of smell by damaged nasal tissues, or by irritation of the mucosal membrane by dust particles. Therefore, the relation between individual rate of food intake, feeding strategy and severity of AR deserves further investigation in order to diminish economic losses in case of disease outbreaks. On the other hand, it is possible that Pm-T causes only a local increase of the metabolism. The effects on local metabolism may be too low to be measured.

Exposure to cold with draught increases the maintenance requirement in pigs, which can be partly compensated by a reduced activity at daytime (extended 'midday nap') and between draught periods in healthy pigs (Verhagen, 1987; Chapter 4.2). The *App*-infected pigs could not compensate this inceased demand by behaviour (e.g. huddling), their heat production (HP) increased in the adverse environment (Verhagen, 1987). This increase was at the cost of efficiency of production. Differences as found between AR and control piglets in the good or the adverse climatic treatment were similar (Chapter 4.2). The reaction, however, of the pigs to the *Pasteurella multocida*-toxin (Pm-T) challenge on heat production traits was influenced by the time of day and was largely independent of climatic treatment (Chapter 4). The effect of the challenge on heat production is mainly expressed by a decreased activity. Only during the activity peak in the afternoon interaction with climatic treatment was found. It might be wise to distinguish between

overall effects (day means) on total, activity related and activity free heat production, and effects within a day (2-h means). The toxin seemed to suppress the general state of well-being of pigs, reducing pigs' activity and food intake. By reducing its activity, the piglets compensated the lower food intake, the lower amount of energy available for production. This way the induced subclinical atrophic rhinitis did not cause substantial growth retardation in our experiments. It is very well possible that clinically diseased pigs will not be able to reduce the effects of lower energy intake by behavioural responses.

The Pm-T treated pigs seemed to respond to the challenge by means of a small temporary rise (0.2°C) in rectal temperature, suggesting that Pm-T acts systemically. However, this rise was not supported by a synchronous increase in heat production. On the contrary, the Pm-T challenge had a reducing effect on heat production and activity, which was affected by time of day. The relation between rectal temperature, heat production and AR is not clear and remains to be sorted out. By doing so one has to take into account the biphasic circadian rhythm in heat production and activity of pigs, which is led by rhythmic excretion of hormones (e.g. thyrotropin) of the hypothalamus-pituitary system (Schrenk, 1981). It is possible that this hormonal system dismisses a Pm-T effect by other mechanisms to balance heat production and loss (e.g. behaviour), and thus maintains a constant core temperature in the body.

The major objective of the conducted experiments was to evaluate the effects of climatic environment on AR in relation to productivity under field-like conditions. Summarized, Pm-T-induced AR-lesions were not affected by the applied climatic treatment. The toxin treatment mainly suppressed pigs' food intake and activity. And a systemic response to the presence of Pm-T was evoked as a small reduced weight gain. The effects of the challenge as well as of climatic treatment on performance traits, and heat production seemed greatly independent of each other. By reducing its activity, the piglets seemed to compensate the lower food intake, the lower amount of energy available for production.

ATROPHIC RHINITIS AND THE IMMUNE SYSTEM

Resistance to infectious diseases rests on the activation of the innate (a-specific) and acquired (specific) immune system. Whereas the innate immune system of the respiratory tract against invading pathogens is composed of the mucociliary transport system together with phagocytic cells, the specific immunity rests on specific recognition and binding of (non-self) antigens (pathogens) by cell receptors. This is followed by a cascade of mechanisms which inactivates the pathogen. Simultaneously, 'memory' for the specific

pathogen is established. Such, that in case of renewed contact with the pathogen a fast and efficient specific immune response will follow. These mechanisms, however, do not always act adequately: infectious diseases are still widespread in man and (production) animals. Much effort is given to the unraveling of mechanisms involved in immune responses and applying them in disease prevention via vaccination or zootechnical intervention.

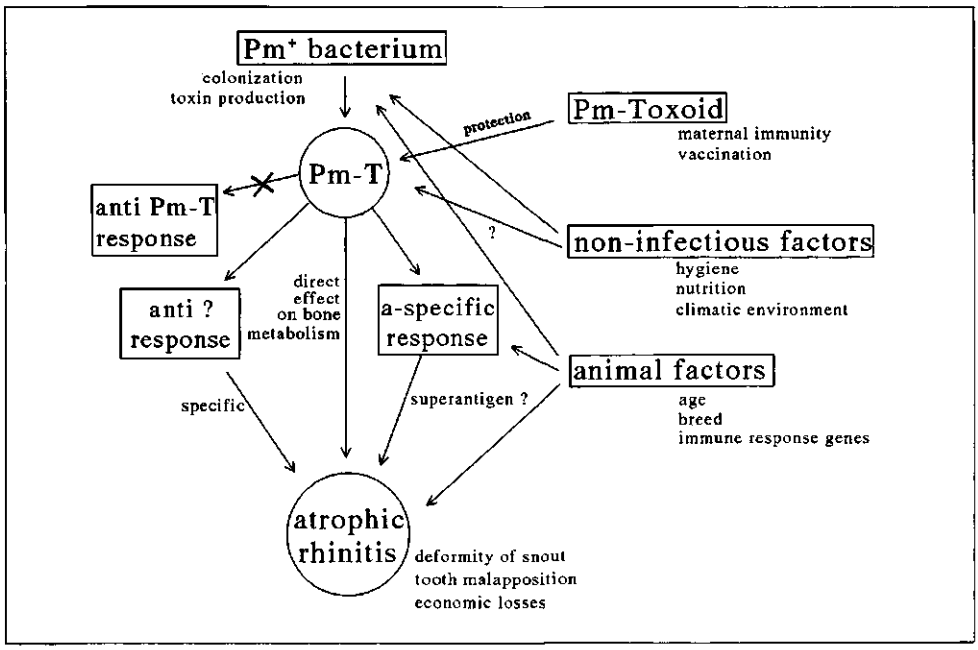

Figure 1 - Relations and mutual interference between Pm-T and the immune system: a hypothetical model.

In Figure 1, (possible) relations and mutual interference between the immune system of the pig and Pm-T are schematically outlined. Via the mucosa of the respiratory system, Pm-T appears to be a poor immunogen (Foged *et al.*, 1987; Rutter, 1988; Chapters 3-5). Serum titres of antibodies against Pm-T were not detectable (Figure 1). The reason why such immune response was absent was not clear and pleaded for further research. Especially because intramuscular or intraperitoneal administration of Pm-T cause - next to nose lesions - a strong antibody response (Rutter, 1985; Foged *et al.*, 1987).

Parenteral immunisations with preparations of Pm-T or detoxified Pm-T have been used for the production of sera with both high anti Pm-T titres and anti Pm-T monoclonal antibodies (Foged *et al.*, 1987). Thus, in principle, cells of the immune system can be sensitised to Pm-T by vaccination with Pm-toxoid (Figure 1). These cells can recognize

and react to Pm-T as was illustrated by the elevated *in vitro* proliferation and antibody titres to Pm-T in vaccinated animals (Chapter 5.1). It was found, however, that despite the presence of Pm-T binding antibodies, nose damage can develop. When the immune response has not sufficiently been build up, the devastating process will not be slowed down nor stopped (Chapter 5.1). Protection was dependent on the timing of vaccine administration. It was possible to observe pathogenesis developing and a 'protective' immune response (antibodies and T cells) next to each other. Surprisingly, pigs simultaneously vaccinated with Pm-toxoid and challenged with Pm-T, failed to develop anti Pm-T immune responses (Chapter 5.1). It could not be established whether this lack of reactivity to Pm-toxoid was due to idiotypic interference of Pm-T with presentation of Pm-toxoid, or 'nonspecific' binding of Pm-T to MHC class II molecules. Also vaccination could not eradicate infection with the AR pathogenic *Pm*$^+$ (Smelt, 1989). Apparently, nose damage can develop in the presence of antibodies to Pm-T and anti-Pm-T T cells *in vivo*.

With respect to the involvement of the immune system, 'atrophic rhinitis' can be based on several fundamentally different mechanisms (Figure 1), which eventually may involve different types of treatment or management.

First, intranasally applied Pm-T acts locally, directly on osteoclast precursors with specific preference for the nasal turbinate bones because of the structure and high natural turnover (Dominick and Rimler, 1988; Martineau-Doizé *et al.*, 1990). The (local) immune system does not become involved or was not able to recognize the Pm-T. The latter is not likely since protective antibodies and T cells to Pm-T can develop after injecting the Pm-T.

Second, atrophic rhinitis may result from a functional immune system (mis)directed to an unidentified component in the nose of the pig itself. Instead of the toxin, the effector mechanism of the immune system causes the damage. The influence on the turbinate bones might be directed via immune cells or through mediators produced by immune cells (Pedersen and Elling, 1984; Martineau-Doizé *et al.*, 1990).

And third, atrophic rhinitis may result from Pm-T actively or passively misleading the immune system. Pm-T might have immunomodulatory effects on various lymphoid cell populations (Chapter 5.1, mitogenic effect), with the lack of immune responses to Pm-T, in solely Pm-T challenged pigs resulting from the way of presentation of Pm-T to T cells. Pm-T might be presented to T cells in such a way, that T cells responding to Pm-T and possibly to other antigens are (in)activated. This might lead to subsequent non-antigen specific (in)activation of T cells, as was repeatedly found for bacterial proteins in murine models (Misfeldt, 1990; Cole and Atkin, 1991). The last two mentioned mechanisms may

result in hypersensitivity or autoimmune reactions in the nasal environment to 'self' antigens. Such antigens remain to be determined.

Although the mode of action of Pm-T in atrophic rhinitis is not yet unriddled, an interaction between the immune system of the pig and Pm-T is established. As mentioned before, this interaction is not conventional, i.e. no classic humoral nor cellular immunity to Pm-T is built, even though the Pm-T molecule is immunogenic (Foged, 1992). However, Pm-T was reported to be mitogenic *in vitro* for some cultured fibroblasts (Rozengurt *et al.*, 1990), and for naive and lectin-stimulated porcine T cells *in vitro*. This 'nonspecific' activation of T-cells by Pm-T could be partly abrogated by monomorphic anti-swine major histocompatibility complex (MHC) class II DQ and DR specific monoclonal antibodies (Chapter 5.1). The effect of Pm-T on immune cells was found to be individually restricted. This proliferation can probably be attributed to CD4+ T cells. Fluorescence Activated Cell Sorter (FACS) analysis of PBL incubated with the toxin showed increased numbers of CD2+ and CD4+ T cells. Heat-inactivation abrogated the mitogenic activity of Pm-T. These observations suggested that a possible way by which Pm-T affects the cellular immunity may be interference with antigen presentation through MHC class II antigens. *In vivo*, Pm-T might be presented to T cells in such a way, that T cells responding to Pm-T and possibly other antigens are (in)activated. Furthermore, pigs simultaneously challenged with Pm-T and vaccinated with Pm-toxoid failed to respond to Pm-toxoid (Chapter 5.1). Challenge with Pm-T did affect the *in vivo* immune response against various T-cell dependent antigens (Ovalbumin, Keyhole Limpet Haemocyanin and Tetanus Toxoid) in a dose dependent fashion, but did, however, not abrogate these immune responses (Chapter 5.2). In this respect, the lack of detectable immune responses to Pm-T probably does not depend on a 'general non-specific' suppression of cellular immunity. Challenge with Pm-T, which ultimately results in the specific nose damage does not affect immune responses to various T-cell dependent antigens. The relationship(s) between Pm-T and porcine T cells deserve further attention.

Bacterial endo- and exotoxins can act as so called 'superantigens' in mouse and man. 'Superantigens' are a specific class of antigens which belong either to the endotoxins of Gram negative bacteria, mycoplasmas, or viral antigens (Figure 2). They cause disease, are potent T-cell mitogens and do not induce conventional humoral and/or cellular immune responses (Misfeldt, 1990). In murine models and in humans, they differ from 'normal' antigens such that they 'nonspecifically' activate T cells (or B cells) without the conventional processing by antigen presenting cells (Figure 2). The superantigens bind MHC class II molecules on antigen presenting cells and are then presented to T cells (or B cells) with certain subsets of T-cell receptor beta chains. This

may result either in activation of (preferentially autoimmune) T cells, with concomitant rise of (auto)antibodies, or in death/inactivation of the responding T (B) cells, leaving holes in the T-cell repertoire of the individual (Acha-Orbea and Palmer, 1991; Cole and Atkin, 1991; Coffin, 1992). 'Genetic' diversity in subsequent immune responses between MHC compatible individuals depended on the expression of certain T-cell receptor V-beta families (Cole and Atkin, 1991; Coffin, 1992).

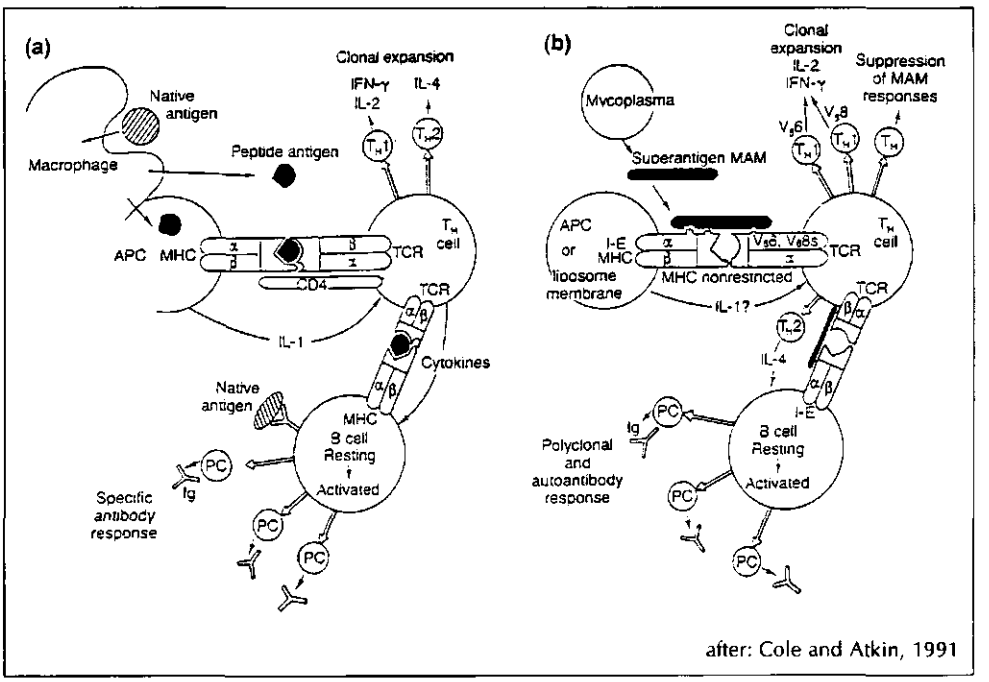

Figure 2 (a) 'Normal' antigens are processed by antigen-presenting cells (APC) and are re-expressed on the APC surface in the antigen groove of the MHC molecule. (b) MAM and other superantigens are released by microorganisms in a form that binds directly to MHC molecules without processing.

The mechanisms behind the non-specific activation of porcine lymphocytes by Pm-T, and the differential effects of Pm-T on the immune response to T-cell dependent antigens need further research. Considering the characteristics of Pm-T in AR (Chapter 5.1; Chapter 5.2) and the preliminary studies described in Chapter 5.3, a T cell involvement in the pathogenic process of AR in Pm-T treated pigs is not unlikely. Especially the lack of immune responses to Pm-T *in vivo*, and the individual restriction of the mitogenic effect of Pm-T on porcine immune cells *in vitro*, urges studies on the expression of T-cell receptor V-beta families in affected and non-affected pigs. Knowledge of the relation between the swine MHC (SLA) types, T-cell receptor V-beta's and severity of AR might be enlightening. Differences in sensibility of the nasal bone tissue and receptors on cells

may explain the age-related affection and differences found between breeds, lines (Martineau *et al.*, 1988) or individuals. A complex relationship between the immune system and the persistence of infection seems present in some strains of pigs (Smith, 1983).

Nevertheless, it is still possible that all *in vitro* features of Pm-T have nothing to do with the *in vivo* processes of AR specific bone damage, simply because no contact is made between the immune system and Pm-T. The cell type(s) directly or indirectly responsible for AR-symptoms and target receptors of Pm-T, together with age-susceptibility, needs further research to broaden insight in the mode of action of Pm-T associated with the nasal breakdown.

It was noticeable that all measured effects of Pm-T treatment, whether temporarily or lasting, seemed to initiate about a week after challenge. This moment coincides with the moment upon which the Pm-T treated pigs started to sneeze and showed nasal discharge. According to Martineau-Doizé *et al.* (1990), this is also the moment when the number of osteoclasts is increased and the atrophy of conchae is started. Pathology of atrophic rhinitis will progress gradually during weeks after the initial challenge.

IMPLICATIONS

Up till now, the most reliable criterion for diagnosis of AR has remained the isolation of toxigenic *Pasteurella multocida* through nasal swabs and tonsil biopts, combined with the visual examination of the turbinates. The best combating strategy is still prevention of the AR pathogenic *Pasteurella* from entering the herd (risk management). And by means of vaccination and/or zootechnical management, economical damage can be controlled in case of a break-out.

Before atrophic rhinitis is 'solved', several gaps in knowledge as mentioned in the previous sections and Chapters need to be clarified. Especially the role of animal factors in the development and severity of AR needs to be elucidated. Among which the reason why only young pigs, in contrast to adult pigs, are sensitive to Pm-T to induce nasal breakdown, and why asymmetrical affection of the nose can occur. Identification of the immune mechanisms underlying AR-pathology will help to increase understanding of the role of the bacterially derived T-cell mitogen (Pm-T) in disease pathogenesis. It might enable development of better therapeutic approaches for intervention in this and possibly other (bacterial) diseases. Assessment of the mechanisms behind the selective bone resorption in AR can produce benefits in two areas. First, via better diagnostic values, insight in the quantitative influence of a single factor on the health status and performance of pigs can be obtained. Differences in susceptibility or resistance to the Pm-

T (AR) between breeds, lines, or individuals can be looked into, and genotype-environment interactions determined. And second, the mechanisms might contribute to the knowledge of mechanisms involved in human bone diseases, with a possible genetic (autoimmune) component.

If atrophic rhinitis really is a disease with the Pm-T triggering T cells to destroy nose tissue, there are possible consequences for pig management in combating this disease. Selective breeding towards less sensitive individuals, families and/or breeds, and early recognition of the disease belongs to the possibilities. For both economical and ethical reasons, improving genetic disease resistance is an attractive preventive measure against infectious diseases in livestock production. The efficiency of production can be enhanced and the welfare of the animals during the production cycle can be optimized. In this perspective, a genetic factor, like T-cell receptor V-beta, is prone to be used in disease resistance. Pig families known to bear a such a risk factor for AR, can be monitored closer as indicator for infection.

CONCLUSIONS

The developed challenge model to induce subclinical atrophic rhinitis with Pm-T enables studies on factors involved in the multifactorial etiology of AR in pigs. The relationship(s) between the severity of disease and (individual) traits such as growth and feed intake and conversion can be studied.

The applied Pm-T suppressed the general state of well-being of pigs, reducing pigs' activity and food intake, and, concurrently, the weight gain. By reducing their activity, the piglets compensated the lower food intake - the lower amount of energy available for production. This way the induced subclinical atrophic rhinitis did not cause substantial growth retardation in our experiments.

The circadian rhythm in heat production (HP) and activity (H_{ar}) of 4 to 10 week old pigs, was not changed by the Pm-T challenge as applied in our model. The level of HP and H_{ar}, however, was more or less reduced dependent on the time of day. Therefore, it is recommendable to distinguish between overall (day) effects on total, activity related and activity free heat production, and effects within a day (e.g. 2-h means) in investigations on climatic demands of an animal.

Atrophic rhinitis is a complex, multifactorial, infectious disease, which is not a simple all-or-nothing phenomenon, neither at individual pig nor at herd level. Variations in response to Pm-T are the rule rather than the exception. The symptoms of atrophic rhinitis are amenable to strategic modulation through vaccination and zootechnical management (e.g. feeding), but these modulations do not eradicate the infectious cause,

the toxigenic *Pasteurella multocida*, from a herd. The role of climatic environment in the severity of atrophic rhinitis, seems primarily expressed in the colonizing ability of the Pm^+ on the mucous membrane, and likely affects the concomitant amount of toxin reaching the bony tissues.

The indistinct role of the immune system in AR needs further elucidation. Although interaction between the immune system of the pig and Pm-T is established, the (immune) mechanisms underlying the specific nasal breakdown are not known. Pathology might result from hypersensitivity or autoimmune (superantigenic) reactions in the nasal environment to 'self' antigens. If, in this respect, a relation between Pm-T and porcine T cells or expression of T-cell receptor V-beta's could be demonstrated, this would be of tremendous help in unravelling the mechanisms involved in AR-pathology.

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Chapter 7

SUMMARY
SAMENVATTING

SUMMARY

In today's pig husbandry, upper respiratory tract infections, such as atrophic rhinitis are common and insidious diseases of swine. They are often considered causes of decreased rate of gain, inefficient feed conversion, and increased time to market, although these parameters do not absolutely correlate with the severity of lesions. Studies investigating the role and effects of toxigenic *Pasteurella multocida* (Pm^+) or its toxin, Pm-T, in atrophic rhinitis under experimental conditions (Chapter 2, review), show that several factors attribute to the severity of observed clinical, pathological or anatomical deformations specific for this disease. Although they are reported to be of importance, the impact of most of them and their relationships with severity of disease and age susceptibility are inadequately understood. Moreover results of studies on atrophic rhinitis conducted from several angles of incidence are hard to compare because various routes, and agents are used to induce AR experimentally. Unequivocal definitions of AR and/or grading systems of conchal lesions have been used.

Consequently to be able to study effects of environmental and animal factors on AR, a standard challenge-exposure model to induce (sub)clinical AR experimentally was required. Chapter 3 of this thesis is focused on the development of such a challenge model. The optimal model to induce subclinical AR appeared to be pretreatment with 1% acetic acid, three days later followed by 13 μg of Pm-T/ml (0.5 ml/nostril/day) on 3 consecutive days. The intranasally administered Pm-T mimicked, dose-dependently the pathogenic effects of *in vivo* infection with toxigenic Pm-strains.

The developed challenge-exposure model enables studies on factors involved in the multifactorial etiology of AR in pigs. The characteristics used to measure AR are satisfactory, but can be refined. The model allowed judgement of factors (positive or negative) related to the mucosal system of the turbinates and did not largely impaired animal welfare.

The fourth Chapter of this study was aimed to establish the impact of climatic environment on the severity of AR-symptoms, and to study the effects of AR (Pm-T) and climatic environment on energy metabolism (heat production), and performance of weaned piglets.

Exposure to adverse climatic conditions as applied in the current study (15°C with draught periods) did not affect the severity of Pm-T induced AR symptoms. Growth retardation caused by Pm-T administration was suggested to be mainly the outcome of a lower food intake, since no change in metabolizability and maintenance requirements were found (Chapter 4.1). No interaction between the challenge and climatic treatments

was seen. The time to market (100 kg body weight) was 3 days longer for Pm-T treated pigs compared with their not treated control contemporaries.

The effects of Pm-T challenge as well as of climatic treatment on heat production traits seemed greatly independent of each other (Chapter 4.2). Day averages in heat production and activity related heat production were reduced by Pm-T challenge in both climatic environments. Within a day, heat production showed an endogenous biphasic activity rhythm (Alternanstype). The reaction of the pigs to the treatments was influenced by the time of day. Suggesting, that differentiation between overall effects (day means), and effects within a day (e.g. 2-h means) on heat production traits might be important.

The major objective of the conducted experiments was to evaluate the effects of climatic environment on AR in relation to productivity under field-like conditions. Summarized, the toxin treatment mainly suppressed pigs' food intake and activity. And a systemic response to the presence of Pm-T was evoked as a small reduced weight gain. By reducing their activity, the piglets seemed to compensate the lower food intake, the lower amount of energy available for production.

The purpose of the fifth Chapter of the study was to elucidate the role of the immune system of piglets in relation to AR pathology. We attempted to identify mechanisms underlying the lack of conventional immune responses of pigs to Pm-T, and their possible consequences with respect to AR.

Pm-T is poorly immunogenic *in vivo* and does not initiate a protective Pm-T specific humoral and/or cellular immune response. Protection by means of vaccination was dependent on the timing of vaccine administration. The pathogenicity is irreversible also in the presence of immune cells. The toxin was found to be mitogenic for naive T cells *in vitro*. The 'nonspecific' activation of T cells by Pm-T could be partly abrogated by monomorphic anti-swine MHC class II DQ and DR specific monoclonal antibodies. The effect of Pm-T on immune cells was found to be individually restricted. This suggested that the mitogenic activity of Pm-T is based on stimulation of T cells through the MHC class II antigens on the cell membrane of antigen presenting cells (Chapter 5.1).

The effects of Pm-T on *in vivo* cellular and T-cell dependent humoral immunity in pigs that had been challenged intranasally with Pm-T and simultaneously sensitized systemically with various (non-related) T-cell dependent antigens were studied in Chapter 5.2. The results of this study indicated 1) that the lack of detectable immune responses to Pm-T probably does not depend on a 'general non-specific' suppression of cellular immunity, and 2) that challenge with a dose of Pm-T, which ultimately results in a breakdown of nasal bony tissues does not uniformly affect immune responses to various T-cell dependent antigens.

In the General Discussion (Chapter 6), the challenge model in relation with the results described in Chapters 4 and 5, and the features and the possible mode of action of the Pm-T are discussed. It was noticeable that all measured effects of Pm-T treatment, whether temporarily or lasting, seemed to initiate about a week after challenge.

Although the mode of action of Pm-T in atrophic rhinitis is not yet unriddled, an interaction between the immune system of the pig and Pm-T is established. Considering the characteristics of Pm-T in AR as found in Chapter 5.1 and Chapter 5.2, supplemented with the preliminary studies described in Chapter 5.3, a T cell involvement in the pathogenic process of AR in Pm-T treated pigs is not unlikely. The speculation was made that Pm-T modifies the immune response such that the response is not directed towards the toxin, but to unidentified component(s) in the nose of piglets, in an autoimmune-like (superantigenic) fashion. Subsequently, consequences and implications of the proposed concept are outlined and discussed.

CONCLUSIONS

The developed challenge model to induce subclinical atrophic rhinitis with Pm-T enables studies on factors involved in the multifactorial etiology of AR in pigs. The relationship(s) between the severity of disease and (individual) traits such as growth and feed intake and conversion can be studied.

The applied Pm-T suppressed the general state of well-being of pigs, reducing pigs' activity and food intake, and, concurrently, the weight gain. By reducing their activity, the piglets compensated the lower food intake - the lower amount of energy available for production. This way the induced subclinical atrophic rhinitis did not cause substantial growth retardation in our experiments.

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severity of atrophic rhinitis, seems primarily expressed in the colonizing ability of the *Pm*$^+$ on the mucous membrane, and likely affects the concomitant amount of toxin reaching the bony tissues.

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SAMENVATTING

In de hedendaagse varkenshouderij, zijn bovenste luchtweg infecties, zoals atrofische rhinitis, veel voorkomende en hardnekkige ziekten bij varkens. Ze veroorzaken vaak een verlaagde groeisnelheid, een inefficiënte voederconversie en een verlengde mestperiode. Deze parameters correleren niet altijd volledig met de ernst van de aandoening. Uit onderzoek naar de rol en effekten van de *Pasteurella multocida* (Pm^+) bacterie en toxine in atrofische rhinitis blijkt dat meerdere faktoren bijdragen aan de ernst van de waargenomen klinische, pathologische of anatomische afwijkingen welke specifiek zijn voor deze ziekte (Hoofdstuk 2). Ofschoon vele faktoren van belang worden geacht, is er weinig bekend van hun impact en hun relatie met leeftijdsgevoeligheid en ernst van de ziekte. Bovendien zijn door verschillende invalshoeken de onderzoeksresultaten moeilijk met elkaar te vergelijken. Een verscheidenheid aan besmettingsroutes en -agentia zijn gebruikt om AR experimenteel op te wekken. Verschillende, niet eensluidende definities van AR en beoordelingsmethoden voor de specifieke neusbot aantastingen zijn gebruikt.

Om effekten van omgevings- en dierfaktoren op AR te kunnen bestuderen, moet atrofische rhinitis derhalve, op een gecontroleerde wijze experimenteel geïnduceerd kunnen worden. In Hoofdstuk 3 van dit proefschrift wordt de ontwikkeling van zo'n besmettingsmodel beschreven. Het optimale (subklinische) model was uiteindelijk voorbehandelen met een 1% azijnzuur oplossing, 3 dagen later gevolgd door een drie-daagse behandeling met 13 μg Pm-T per ml PBS, 0.5 ml per neusgat per dag. De intranasaal toegediende Pm-T bootste, dosis afhankelijk, de pathogene effekten van een *in vivo* besmetting met toxine producerende *Pasteurella multocida* na.

Met het ontwikkelde besmettingsmodel kan de multi-faktoriële aetiologie van AR in varkens bestudeerd worden. De variabelen gebruikt om AR te beoordelen voldoen maar zouden verfijnd kunnen worden. Het model staat beoordeling van faktoren toe welke een positief of negatief effekt in AR kunnen hebben zonder het welzijn ernstig aan te tasten.

In Hoofdstuk 4 is de impact van omgevingsfaktoren op de ernst van AR symptomen bepaald. Tevens zijn de effekten van AR (Pm-T) en omgevingsfaktoren op de energie huishouding (warmte produktie) en productie kenmerken (inclusief afmest periode) van gespeende biggen bestudeerd.

Blootstelling aan niet optimale omgevingscondities zoals in de uitgevoerde studie (15°C met tocht perioden) veranderde de ernst van met Pm-T geïnduceerde AR symptomen niet. Groei achterstand ontstaan door de Pm-T toediening leek voornamelijk veroorzaakt door een verminderde voeropname, aangezien er geen verschillen werden gevonden in metaboliseerbaarheid van voerenergie en in onderhoudsbehoefte (Hoofdstuk

4.1). Interactie tussen beide behandelingen werd niet waargenomen. De klimaatsbehandeling verhoogde de onderhoudsbehoefte (warmteproduktie). De afmestperiode (dagen tot 100 kg lichaamsgewicht) van de Pm-T behandelde varkens was gemiddeld 3 dagen langer dan van niet behandelde leeftijdsgenoten.

De effekten van Pm-T challenge als ook die van omgevingscondities op warmte produktie kenmerken leken grotendeels onafhankelijk van elkaar (Hoofdstuk 4.2). De totale en aktiviteit-gerelateerde warmte produktie gemiddeld per etmaal waren verlaagd in beide omgevingscondities. De warmte produktie binnen een etmaal vertoonde een endogeen bi-fasisch aktiviteitsritme (Alternanstype). De reaktie in warmte produktie kenmerken van de biggen op de behandelingen was afhankelijk van het tijdstip van de dag. Dit suggereert dat het belangrijk kan zijn om in onderzoek naar effekten op warmte produktie kenmerken, onderscheid te maken tussen daggemiddelden en dagdelen.

Het voornaamste doel van de uitgevoerde experimenten was het effekt van omgevingscondities op AR te bepalen in relatie met de produktiviteit onder praktijk-achtige condities. Samengevat kan gezegd worden dat de toxine behandeling voornamelijk de voeropname en de aktiviteit van de biggen verlaagde. Een systemische respons op de aanwezigheid van het Pm-T leek een iets verlaagde groei. De biggen leken de lagere voeropname - de lagere hoeveelheid energie beschikbaar voor produktie - te compenseren door hun aktiviteit te verlagen.

Het doel van het vijfde Hoofdstuk van het onderzoek was het in kaart brengen van de rol van het immuun systeem in de AR pathogenese. Gepoogd is die mechanismen te identificeren, waardoor er geen conventionele immuun respons tegen Pm-T optreedt, samen met hun mogelijke consequentie voor AR.

In vivo, is Pm-T laag immunogeen, het wekt geen beschermende, Pm-T-specifieke humorale en/of cellulaire immuun respons op. De effektiviteit van een 'beschermende' vaccinatie was afhankelijk van het tijdstip van vaccineren ten opzichte van het tijdstip van besmetting. De destructieve werking van Pm-T is onomkeerbaar, ook in de aanwezigheid van specifieke immuun cellen (Hoofdstuk 5). *In vitro*, is het toxine mitogeen voor naïeve T cellen. Deze 'niet-specifieke' aktivatie van T cellen door Pm-T kon deels worden afgeblokt door monomorfe anti-varken MHC klasse II DQ en DR specifieke monoklonale antilichamen. Het effekt van Pm-T op de immuun cellen was individu gebonden. Dit wekt de suggestie dat de mitogene werking van Pm-T gebaseerd is op stimulatie van T cellen via MHC klasse II antigenen op de celmembraan van antigeen presenterende cellen (Hoofdstuk 5.1).

In Hoofdstuk 5.2 is het effekt van Pm-T op de *in vivo* cellulaire en T cel afhankelijke humorale immuunrespons bestudeerd van biggen die met Pm-T intranasaal behandeld

waren alsmede simultaan met verschillende (niet verwante) antigenen (KLH, OA, TT) systemisch gesensibiliseerd waren. De resultaten uit deze studie geven aan 1) dat het gebrek aan een detekteerbare immuunrespons tegen Pm-T waarschijnlijk niet afhangt van een 'algemene niet-specifieke' onderdrukking van de cellulaire immuniteit en 2) dat de challenge met een dosis Pm-T, welke uiteindelijk resulteert in neusaantasting, de immuunrespons tegen verschillende T cel afhankelijke antigenen niet uniform beïnvloed.

In Hoofdstuk 6 (General Discussion) worden het besmettingsmodel in relatie met de resultaten uit de Hoofdstukken 4 en 5, de karakteristieken en de mogelijke werkingswijze van het Pm-T bediscussieerd. Het was opvallend dat alle waargenomen effekten van Pm-T behandeling, hetzij tijdelijk hetzij blijvend, ongeveer één week na challenge schenen te ontstaan.

Ofschoon het 'hoe veroorzaakt Pm-T atrofische rhinitis' nog niet ontraadseld is, is een interactie tussen het immuun systeem van het varken en Pm-T vastgesteld. Gebaseerd op de resultaten uit de Hoofdstukken 5.1 en 5.2, aangevuld met de vooronderzoeken beschreven in Hoofdstuk 5.3, is de betrokkenheid van T cellen in de pathogene processen van AR in Pm-T behandelde varkens niet onwaarschijnlijk. Gespeculeerd wordt dat het Pm-T de immuun respons dusdanig aanpast dat de respons niet meer gericht is op het toxine zelf, maar op niet-geïdentificeerde componenten in de snuit van een big op autoimmuun-achtige (superantigene) wijze.

CONCLUSIE

Faktoren betrokken bij de multi-faktoriële aetiologie van AR in varkens kunnen bestudeerd worden met het ontwikkelde AR-challenge model, waarbij subklinische atrofische rhinitis met Pm-T wordt geïnduceerd. Verbanden tussen de ernst van de ziekte en (individuele) kenmerken, zoals groei en voeropname kunnen onderzocht worden.

Het toegediende Pm-T verlaagde de aktiviteit en voeropname van de biggen en, bijgaand hun groei. Door hun aktiviteit te verlagen, compenseerden de biggen hun lagere voeropname, de lagere hoeveelheid energie dat beschikbaar was voor produktie. Zodoende veroorzaakte de geïnduceerde subklinische atrofische rhinitis in onze experimenten geen substantiële groei vertraging.

De Pm-T challenge veranderde het 24-uurs ritme in warmte produktie en aktiviteit van 4 tot 10 weken oude biggen niet. Het niveau van HP en H_{ar} was echter meer of minder verlaagd, afhankelijk van het tijdstip van de dag. Het is daarom aanbevelingswaardig om bij onderzoek naar de temperatuurbehoefte van een dier onderscheid te maken tussen effekten binnen een dag of tussen dagen.

Atrofische rhinitis is een complexe, multi-factoriële ziekte, welke noch op dierniveau noch op bedrijfsniveau een simpel alles-of-niets fenomeen vertoond. Variatie in reactie op Pm-T is eerder de regel dan de uitzondering. Via vaccinatie en zoötechnisch management kan atrofische rhinitis in de hand worden gehouden; dit soort maatregelen verwijderen echter niet de eigenlijke oorzaak, de toxigene *Pasteurella multocida*, van het bedrijf. De rol van omgevingscondities op de ernst van AR, lijkt voornamelijk tot uitdrukking te komen op de kolonisatie mogelijkheden van de bacterie op het neusslijmvlies en, mogelijk, op de hoeveelheid toxine dat het botweefsel bereikt.

De onduidelijke rol van het immuun systeem in AR heeft verdere opheldering nodig. Ofschoon interactie tussen het immuun systeem van het varken en Pm-T is vastgesteld, zijn de (immuun) mechanismen verantwoordelijk voor de specifieke bot afbraak niet bekend. Pathogenese kan ontstaan uit hypersensitiviteits- of autoimmune (superantigene) reakties tegen 'eigen' antigenen in de neus. Als, in dit opzicht, een relatie tussen Pm-T en varkens T cellen of expressie van T cel receptor V-beta's aangetoond kan worden, zou dit een enorme hulp zijn bij het ontrafelen van de mechanismen betrokken bij AR-pathologie.

LIST OF PUBLICATIONS

Diemen PM van, Jong MF de, Vries Reilingh G de, Hel W van der, and Schrama JW (1994a) Intranasal administration of *Pasteurella multocida* toxin in a challenge-exposure model used to induce subclinical signs of atrophic rhinitis in pigs. *Am J Vet Res* **55**: 49-54.

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Diemen PM van, Jong MF de, and Rijke E (1992) A dose-response model to induce subclinical atrophic rhinitis in pigs by intranasal administration of *Pasteurella multocida* toxin. The 12th International Pig Veterinary Society Congress, 17-20 August 1992, The Hague, The Netherlands: 167 (abstract)

Diemen PM van, Vries Reilingh G de, Rijke E, and Parmentier HK (1992) Studies in conventional piglets on the immunity-initiating characteristics of *Pasteurella multocida* toxin and *Pasteurella multocida* derived toxoid. The 12th International Pig Veterinary Society Congress, 17-20 August 1992, The Hague, The Netherlands: 178 (abstract)

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Hel W van der, and Diemen PM van (1992) Effect of ambient temperature and draught on energy metabolism of piglets challenged with *Pasteurella multocida*-Toxin. The 12th International Pig Veterinary Society Congress, 17-20 August 1992, The Hague, The Netherlands: 539 (abstract)

CURRICULUM VITAE

Pauline Maria van Diemen werd op 28 maart 1963 geboren te Amsterdam. In 1981 behaalde zij het Gymnasium-ß diploma aan het St. Nicolaas Lyceum te Amsterdam en begon in september van dat jaar de studie Zoötechniek (N-20) aan de toenmalige Landbouw Hogeschool te Wageningen. In augustus 1988 sloot zij haar studie af met als hoofdvakken Veehouderij (Gezondheids- en Ziekteleer) en Veefokkerij en met als bijvak Pluimveeteelt. Tevens behaalde zij haar Onderwijsbevoegdheid.

Na 3 maanden gewerkt te hebben als toegevoegd onderzoekster by de sectie Gezondheidsleer en Reproduktie, werd ze aangesteld als Assistent in Opleiding op het AR-project van deze sectie. De resultaten van het genoemde project zijn beschreven in dit proefschrift. Tijdens deze aanstelling werden verschillende cursussen gevolgd, te weten 'Immunologie voor gevorderden' te Amsterdam, 'Proefdierkunde' te Utrecht en 'Organisatie en begeleiding van het afstudeervak' te Wageningen.