Effect of Ovalbumin Aerosol Exposure on Colonization of the Porcine Upper Airway by Pasteurella multocida and Effect of Colonization on Subsequent Immune Function

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Seventy-three piglets were weaned at 1 week of age, randomly assigned to 10 groups (A to J), accommodated in stainless steel exposure chambers, and exposed continuously to a controlled environment containing aerosolized ovalbumin. The concentrations of ovalbumin dust were as follows (milligrams per cubic meter): A and F, 16.6; B and G, 8.4; C and H, 4.2; D and I, 2.1; E and J, 0. At weekly intervals, the pigs were bled via venipuncture and anesthetized for nasal lavage and tonsilar biopsies performed for subsequent bacteriologic analysis. At 2 weeks of age, the pigs in groups A to E were challenged with toxigenic Pasteurella multocida (10⁶ CFU pig⁻¹), and at 6 weeks of age, the pigs were euthanatized. At postmortem, the extent of turbinate atrophy was assessed on the snout sections by using a morphometric index. Exposure to aerial ovalbumin resulted in a dose-dependent increase in serum antimultocida immunoglobulin G (IgG; P < 0.001) and serum antimultocida IgA (P < 0.001). Exposure also caused a significant increase in the numbers of P. multocida organisms isolated from the upper respiratory tract (P < 0.001) and a corresponding increase in turbinate atrophy, as judged by the morphometric index (P < 0.001). Concurrent challenge with P. multocida and ovalbumin resulted in a significant decrease in both the IgG and IgA responses to ovalbumin (P < 0.001). These results show that ovalbumin exposure increases pig susceptibility to P. multocida colonization and that toxigenic P. multocida modifies the serum IgG and IgA responses to ovalbumin in the pig. Both of these effects may enhance the virulence of this respiratory pathogen and so influence the pathogenesis of atrophic rhinitis in pigs.

Atrophic rhinitis is an upper respiratory tract disease of pigs characterized clinically by atrophy and degeneration of the bony and cartilaginous structures of the snout, in particular, the nasal turbinate and septum. In severe cases, this leads to visible shortening or twisting of the snout (12). In addition to these local effects, the disease is also associated with a decreased rate of weight gain and an increased time to market (6). The etiology and pathogenesis of this disease are multifactorial, involving interactions between primary bacterial pathogens and inhaled environmental pollutants. Toxigenic strains of Bordetella bronchiseptica and Pasteurella multocida types A and D have been established as primary etiological agents by experimental studies (10, 20, 26, 28). Epidemiological studies have also shown that severe turbinate atrophy predisposes young pigs to pneumonia (9, 37).

Experimentally, purified P. multocida toxin induces turbinate atrophy when aerosolized into the nasal cavity or injected into the subcutis, muscle, or peritoneum (6). The more severe, naturally occurring form of the disease is known as progressive atrophic rhinitis and is attributed specifically to colonization of the pig nasal cavity by toxigenic strains of P. multocida (25, 28, 31). However, the failure to reproduce the clinical disease consistently in experimental studies without depriving pigs of passive immunity, i.e., withholding colostrum (24), or pretreat-
MATERIALS AND METHODS

Animals. Seventy-three minimal-disease pigs were derived from Large White sows obtained from the Institute of Animal Health, Compton Laboratory, Newbury, England. The pigs were weaned onto a dry commercial ration (given twice daily) at 1 week of age and randomly assigned to 10 experimental groups (A to J). Before the experiment commenced, all of the pigs were screened for the presence of *P. multocida* by nasal lavage with 10 ml of phosphate-buffered saline (PBS) as described by Chanter and Rutter (6). The recovered fluid was cultured overnight at 37°C on 5% horse blood agar plates with added antibiotics (29).

Exposure chambers. All groups of pigs were housed in 1.4-m³ stainless steel Rochester exposure chambers built to the design of Timbrell et al. (32). The chambers were run at ~1000 LPM, giving an air change rate of 60 air changes per hour. The aerosolized material was introduced into the chamber via a HEPA filter, and, after traversing the chamber, was vented via a HEPA filter to prevent the release of biological material. Within the chamber, the air was maintained at a temperature of 25 ± 1°C and a relative humidity of 50 ± 5%.

Pigs in groups A to D and F to I were exposed to various concentrations of aerial ovalbumin from 1 to 6 weeks of age. Ovalbumin dust (Sigma) was generated by a Palas RGB-1000 rotating-brush powder dispersion generator (Palas), split and diluted to the required total dust concentrations, and introduced via the inlet pipe to respective chambers. The concentration of aerial ovalbumin (in milligrams per cubic meter) in the chambers was determined weekly by using an Institute of Occupational Medicine inspirable personal samples (Negretti Automation Ltd.) and Nuclepore filters (Costar). Daily, the ovalbumin levels were monitored with a Rion KC 01A particle counter (Lynjay Services Ltd., Worthing, West Sussex, United Kingdom). This instrument also measured the aerodynamic particle sizes of the dust.

Bacterial inoculation. *P. multocida* LFB3, a toxicogenic isolate from a clinical case of atopic rhinitis in a British pig (20), was supplied by the Institute of Animal Health, Compton Laboratory. The isolate was stored at ~70°C in brain heart infusion broth containing Robertson’s cooked meat granules and 5% glycerol.

Immediately prior to use, *P. multocida* LFB3, was cultured overnight under aerobic conditions on 5% horse blood agar at 37°C. A single colony from this plate was used to inoculate 10 ml of brain heart infusion broth, which was incubated overnight at 37°C. The number of organisms in the broth was determined by a modified method of Miles and Misra (8). This broth was centrifuged at 11,800 × g for 10 min and washed with 37°C PBS three times; the cells were then resuspended in PBS at the required concentration for animal inoculation.

Experimental protocol. The experiment was designed to challenge pigs with chronic exposure to five different concentrations of inhaled ovalbumin dust with and without inoculation with *P. multocida*. Pigs were randomly divided into 10 groups. Groups A to E were inoculated at 2 weeks of age with 10⁸ CFU of *P. multocida* LFB3 in a 0.5-ml dose (suspended in PBS) per nostril. Groups F to J were treated with 0.5 ml of PBS per nostril. Groups were exposed to ovalbumin dust (85% of the particles had an aerodynamic diameter above 5 μm) from weeks 1 to 6 as follows (milligrams per cubic meter ± 10%): A and F, 16.6; B and G, 8.3; C and H, 4.1; D and I, 2.1; E and J, 1.229 per group; sizes were as follows: A to D and F to I, 6 pigs each; E, 7 pigs; J, 18 pigs.

At weekly intervals, all pigs were bled via venipuncture and anesthetized with 20-mg kg⁻¹ intravenous thiopentone (Vet Drug Ltd.) and a tonsilar biopsy was taken with additional 0.05% lidocaine (Fortis Health Care Ltd., Berkshire, United Kingdom). While the animal was under anesthesia and in dorsal recumbency, nasal lavage was performed by using a 50-ml catheter-tipped syringe with a 1-ml automatic pipette tip attached. Ten milliliters of PBS was injected into the nasal cavity to that of the ventral turbinate (12). Right and the wash was collected from the left nostril. At 6 weeks, the pigs were euthanatized by intravenous injection of sodium pentabarbitone and subjected to postmortem examination.

Necropsy. Postmortem examination was done immediately after death, and any macroscopic signs of disease were noted. The snout was removed by making a transverse cut just in front of the eyes (21) with a band saw and fixed by immersion in 10% neutral buffered formalin for 1 week.

Antibody detection. The blood samples were left to clot at room temperature for 1 h and then centrifuged at 4,000 × g for 10 min. The serum was decanted and stored at ~20°C until the end of the experiment, when the samples were subjected to two enzyme-linked immunosorbent assays (ELISAs) (anti-ovalbumin immunoglobulin G [IgG] and anti-ovalbumin IgA). All reagents were diluted in PBS with added 0.05% Tween 20 (PBST) (pH 7.4), apart from the ovalbumin used to coat the plate and the alkaline phosphatase substrate, which were diluted in carbonate-bicarbonate buffer (pH 9.8). The 96-well plates (Greiner Ltd.) used for the ELISAs were thoroughly washed (six times) with PBST between ELISA steps.

The antigen used for both ELISAs was grade V ovalbumin (Sigma), which was used to coat 96-well plastic plates at a concentration of 1 mg ml⁻¹ and left overnight at 4°C. After washing, serum samples were diluted with PBST and pipetted in 0.1-ml volumes into wells of a row A, where they were diluted by tripling dilution columnwise and incubated at 4°C overnight. For the IgG ELISA, an anti-pig IgG-alkaline phosphatase conjugate (1:10,000) raised in rabbits and affinity purified (Sigma) was used and incubated for 4 h at 4°C, after which the alkaline phosphatase substrate (1 mg ml⁻¹ in 100 mM diethylbarbituric acid, 50 mM glycine, and 1 mM MgCl₂, pH 9.8) was added to each well. The plates were incubated at 4°C, followed by an anti-mouse IgG-alkaline phosphatase conjugate (1:8,000) (Sigma) which was incubated again overnight at 4°C. Finally, the alkaline phosphatase substrate (1 mg ml⁻¹) (Sigma) was added. At this stage, all plates were left to incubate for approximately 10 min and read with a Multiskan Bichromatic plate reader (Labsystems Ltd.) at 405 and 495 nm. The data were then analyzed by using a computerized ELISA analysis package (ELISANALYSIS, release 5.53; J. H. Peterman, Birmingham, Ala.), and each serum sample was expressed as ELISA units (EU) by reference to a standard curve.

A positive IgG control and standard was produced in a pig injected at 3 weeks of age with grade V ovalbumin (Freund incomplete adjuvant) and at 5 weeks of age with grade V ovalbumin (Freund complete adjuvant). The serum from the pig was collected at 10 weeks of age. A positive IgA standard was raised by intranasal inoculation of a lactating sow. Milk from the unweaned sow was collected, clarified by ultracentrifugation, and stored at ~20°C. The IgG and IgA responses to ovalbumin exposure were calculated by subtracting the EU value at week 0 from the EU value at week 6 of the experiment.

**RESULTS**

**Clinical signs.** Throughout the experiments, all animals retained a normal appetite and exhibited behavior consistent with good health. Clinical signs of disease were restricted to sporadic sneezing by some pigs in the groups inoculated with toxicogenic *P. multocida*.

**MI and bacteriological findings.** There was a direct linear correlation between the MI and the cumulative number of *P. multocida* organisms isolated per pig over the course of the experiment. Snout wash samples, fixed in 10% formalin, and the snout samples collected at intervals with a band saw were isolated on blood agar and were subjected to postmortem examination.

**Morphometric analysis.** The results were analyzed using a general linear model of variance (GLM) and regression analysis (RA). Both analyses were performed by a statistical computer software package (Minitab, release 10.51; Minitab Inc., State College, Pa.).

**Results.** Statistical analyses were performed using a general linear model of variance (GLM) and regression analysis (RA). Both analyses were performed by a statistical computer software package (Minitab, release 10.51; Minitab Inc., State College, Pa.).
nificantly lower than the MIs of all of the corresponding groups challenged with *P. multocida*, which ranged from 54.63% (SD, ±2.43%) in group E to 74.23% (SD, ±2.12%) in group A (*P*, 0.001 [GLM]).

**Serum antibody.** All pigs exposed to ovalbumin dust in the absence of *P. multocida* showed a significant increase in the level of serum IgG (*P*, 0.001 [GLM]) and IgA (*P*, 0.001 [GLM]) to ovalbumin at the end of the experiment (Fig. 3 and 4), and this response was dose dependent (*R*² = 0.858, *P* < 0.001 [RA], and *R*² = 0.539, *P* < 0.001 [RA], respectively). Pigs infected with *P. multocida* and concurrently exposed to ovalbumin showed significantly lower (effectively no) IgG and IgA responses to ovalbumin (*P* < 0.001 [GLM]) compared to exposure to ovalbumin alone (Fig. 3 and 4). Levels of antiovalbumin IgG and IgA in pigs exposed to *P. multocida* plus ovalbumin (groups A to D) were not significantly higher than in pigs exposed to neither (group E; GLM) (Fig. 3 and 4).

**DISCUSSION**

Pigs reared in intensive production systems are continuously exposed to dusts which are principally organic in nature and originate largely from the animals’ feed and integument. Epidemiological studies have demonstrated a link between dust exposure.
exposure and nasal turbinate scores (2, 27). Ovalbumin is an organic dust, novel to the pigs in this study, which can be milled to a specific range of particle sizes; hence, it has been used in inhalation studies (15). The aerial dust concentrations of 2.1 to 16.6 mg m\(^{-3}\) used in this study cover the upper range of dust concentrations measured in commercial piggeries (5).

In this study, the pigs were presented with the antigen (ovalbumin) in the form of a respirable aerosol which will have come into contact with the upper respiratory tract, conjunctiva, and mucosa of the gastrointestinal tract. The upper respiratory tract has been shown to be an important site for the deposition of organic dust, which has a profound influence on the colonization kinetics of \textit{P. multocida} (17). Ovalbumin was used in this study to replicate the predominately organic nature of dusts in swine confinement buildings. Furthermore, it is an organic food substance antigenically distinct from pig feed and available as a sterile powder which is easily milled to a size suitable for inhalation. The data presented in this study demonstrate that ovalbumin dust at the maximal exposure concentration (16.6 mg m\(^{-3}\)) increased the pigs’ susceptibility to \textit{P. multocida} colonization of the upper respiratory tract at both the tonsil and nasal cavity (\(P < 0.001\)) (Fig. 2). This concentration gave the highest yield of \textit{P. multocida} from both sites in the upper respiratory tract and resulted in the severest turbinate damage (Fig. 1). This implies increased susceptibility to \textit{P. multocida} colonization. Potentially, this could be achieved by one or more mechanisms, e.g., (i) modulation of the local immune system, lowering its effectiveness against the bacteria; (ii) disruption of the integrity of the host’s mucosal surfaces; (iii) alteration of the competing commensal flora; or (iv) a direct effect on the viability and/or growth of the pathogen.

The extent of colonization of the tonsil with \textit{P. multocida} was proportional to the extent of turbinate damage (\(R^2 = 0.968\), Fig. 1). Hamilton et al. (18) observed a similar effect when studying interactions between \textit{P. multocida} and inhaled ammonia. There is a significant increase in the number of \textit{P. multocida} organisms isolated from the nasal cavity (\(R^2 = 0.921\), Fig. 1), which has a quadratic relationship with the MI. However, because the numbers of \textit{P. multocida} organisms isolated are expressed as CFU per milliliter of recovered fluid from the nasal lavage, it is not possible to calculate the concentration of \textit{P. multocida} in the recovered mucus alone. Therefore, it is possible that the relationship between the number of \textit{P. multocida} organisms isolated from the nasal lavage and the MI is influenced by the unmeasured proportion of mucus recovered in the PBS lavage. It is not clear which is the more important site (tonsil or nasal cavity) for toxin absorption, but previous studies suggest that local absorption of toxin may be indirectly mediated through unidentified products of immune cells in the upper respiratory tract mucosa (14, 26). Therefore, both sites may have equal importance in the absorption of the toxin but one site may be more important than the other in the downstream processing of the toxin.

A dose-dependent relationship was found between the concentration of ovalbumin to which the pigs were exposed and the increase in serum IgG and IgA measured at the end of the experiment (\(R^2 = 0.858\) and 0.539, respectively) (Fig. 3 and 4). However, at all dust levels, pigs challenged with toxigenic \textit{P. multocida} showed a much lower immune response to ovalbumin (i.e., increase in serum IgG and IgA) than those not challenged with \textit{P. multocida}, (\(P < 0.001\), Fig. 3 and 4). No statistically significant difference was observed in the magnitude of the apparent decrease in immunoglobulin levels among groups A to E (pigs challenged with \textit{P. multocida}).

The strain of \textit{P. multocida} used in this study produces a dermotoxin which has several biological properties, ranging from nasal turbinate atrophy to pronounced hyperplasia of the transitional epithelium of the urethra and bladder (6). Nakai et al. (23) observed poor immunogenicity of the toxin during infection of pigs, and Williams et al. (36) observed lymphopenia when the toxin was injected parenterally. These findings suggest that the toxin is cytotoxic to lymphocytes, which renders the pig less able to initiate an effective response to foreign antigens, which may include invading bacteria on the mucosa of the respiratory tract.

Modulation of the immune system with \textit{P. multocida} has also been observed in an epidemiological study which looked at the isolation of \textit{B. bronchiseptica} and \textit{P. multocida} from the nasal cavities of piglets (13). This study found that total immunoglobulin concentrations in serum (IgG, IgA, and IgM) decreased in proportion to an increase in the concentration of \textit{P. multocida} isolated per piglet (CFU per piglet) over a 6-week period. The authors did not comment on this observation. It is, however, consistent with our own observations (Fig. 3 and 4).

Experimental studies on gnotobiotic pigs have shown that crude \textit{P. multocida} toxin is a poor immunogen but that when the toxin is made inactive (toxoid), it becomes a potent vaccine (6). This implies that active immunity is seen in pigs when the toxin is given in its biologically inactive state (by intranasal vaccination) but not its biologically active state (by natural infection). A recent study (34) found that a commercially available \textit{P. multocida} vaccine imparted protection against atrophic rhinitis with a subsequent rise in antibodies to \textit{P. multocida}. However, when animals were vaccinated simultaneously with a \textit{P. multocida} toxin challenge, no detectable systemic humoral or cellular response to \textit{P. multocida} toxin was found. Challenge with active \textit{P. multocida} toxin before vaccination appeared to slow down the immune responses initiated by the toxoid in the vaccine; when vaccination and challenge were simultaneous, the immune response was abolished.

The findings of the current study show that colonization of the upper respiratory tract with toxigenic \textit{P. multocida} (and consequent exposure to \textit{Pasteurella} toxin) suppresses the humoral immune response to inhaled and/or ingested ovalbumin. This systemic modulation of the immune system may be an important (and as yet undocumented) virulence factor in this complex multifactorial disease. Indeed, this effect may have more far-reaching implications for other opportunistic bacterial infections in pigs and other farm animals intensively housed in polluted environments. Progressive atrophic rhinitis can severely impair growth rate and food conversion efficiency in commercial pig herds (6, 33). However, it is not clear why such a localized condition can have such a prolonged systemic effect. One possibility is that it exacerbates the effects of concurrent or subsequent infections, such as enzootic pneumonia. Atrophic rhinitis and enzootic pneumonia (mycoplasma-induced respiratory disease complex) are clearly quite distinct conditions, and the majority scientific opinion is that there is no mechanistic or epidemiological relationship between the two (30). However, Cowart et al. (9) found that in individual pigs, an increasing atrophic rhinitis score was related to an increasing pneumonia score at 8 weeks but not at 6 months of age. This finding is consistent with the study by Chanter and Rutter (6) of the colonization kinetics of toxigenic \textit{P. multocida} following experimental inoculation. This showed that \textit{P. multocida} colonized the nasal cavity in large numbers at 3 weeks of age but fell to below 10% at 2 months of age. Thus, \textit{P. multocida}-induced immune modulation may occur early in life and wane when the pig ages. Clinical evidence indicates that enzootic pneumonia can occur as early as 2 to 8 weeks of age (19). This would coincide with the time of maximal immunosuppres-
sion due to \textit{P. multocida} although not with the time the effects of turbinate atrophy are most apparent.

In conclusion, exposure of young pigs to ovalbumin predisposed the nasal cavity and the tonsil to colonization by \textit{P. multocida} in a dose-dependent manner. Colonization of the upper respiratory tract with \textit{P. multocida} was directly proportional to the severity of turbinate atrophy, as judged by the MI. Challenge with \textit{P. multocida} profoundly suppressed the humoral immune response induced by chronic exposure to ovalbumin dust. On the basis of these observations, we propose a new hypothesis which may partly explain the roles of \textit{P. multocida} and respirable dusts in the etiology and pathogenesis of ill thrift due to endemic respiratory diseases in housed pigs. Organic dusts predispose the upper respiratory tract to colonization with \textit{P. multocida}. Colonization with \textit{P. multocida} induces immunomodulation, which may not only increase the severity of atrophic rhinitis but also predispose the pig to enzootic pneumonia and similar, more chronically debilitating infections of the lower respiratory tract.

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