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 (10^6 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 antiovalbumin immunoglobulin G (IgG; P < 0.001) and serum antiovalbumin 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.

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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 turbinates 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, inefficient feed conversion, and 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 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 pretreating the pigs’ nasal cavities prior to challenge with a chemical irritant such as acetic acid (26) supports the clinical impression that external factors, in particular, atmospheric pollutants, are necessary to the pathogenesis of progressive atrophic rhinitis as seen in commercial pig houses. We have previously established a clear synergistic role for gaseous ammonia in the etiology of P. multocida-induced atrophic rhinitis (18). We have also demonstrated that aerosolized ovalbumin predisposes young pigs to P. multocida colonization, which results in lesions typical of progressive atrophic rhinitis (16, 17). The epidemiological study of Baekbo (2) supports this observation and shows that relatively small aerial dust concentration changes can significantly increase the incidence and severity of atrophic rhinitis.

The seminal observation that infection with microorganisms may decrease the immune response to a different pathogen was made in 1908 by von Pirquet, who showed that the tuberculin reaction, a typical expression of cell-mediated immunity, was depressed in patients with measles (35). This depressed reaction also applies to other clinical infections such as pertussis (4), typhus (38), scarlet fever (22), influenza (3), and brucellosis (1).

There are some data that suggest that toxin derived from P. multocida modulates the humoral immune response to atrophic rhinitis vaccine containing P. multocida toxoid (34). The objective of the present study was to explore the role of organic dusts in the etiology and pathogenesis of progressive atrophic rhinitis by observing (i) the effect of exposing pigs to an organic dust, ovalbumin, on their susceptibility to P. multocida colonization and (ii) whether the humoral immune response to aerial ovalbumin exposure was compromised by P. multocida colonization of the upper respiratory tract.
**Materials and Methods**

**Animals.** Seventy-three minimal-disease piglets were derived from Large White sows obtained from the Institute of Animal Health, Compton Laboratory, Newbury, England. The piglets were weaned onto a dry commercial ration (given twice daily) within 2 weeks 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 10–12 kPa, giving an air exchange rate of 60 air changes per hour. A negative pressure of 20 mm Hg was applied to the chamber, and, after traveling through a HEPA filter, the air was vented into a chimney. A positive pressure of 10 mm Hg was applied to the chamber via a HEPA filter to prevent the release of biological material. Within the chamber, the air was maintained at a temperature of 25 ± 1.0°C and a relative humidity of 50 ± 5%.

In 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 inoculum.** *P. multocida* LFB3, a toxigenic isolate from a clinical case of atrophic 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 Robinson’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 aerobically 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 rpm 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 grade V ovalbumin (Freund incomplete adjuvant) and at 5 weeks of age with grade V ovalbumin (Freund complete adjuvant). The serum from the pigs was collected at 10 weeks of age. A positive IgA standard was raised by intramuscular 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.

**Bacteriology.** Biopsy tissue samples were weighed and then homogenized in 2 ml of PBS at full speed in an Ultra-Turrax T25 Homogenizer (M. J. Patterson Scientific Ltd., Luton, United Kingdom). The homogenates were diluted 1:10 and 1:100 in brain heart infusion broth and plated onto blood agar and 7% sheep blood agar. Biopsy tissue homogenates 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.

**Snout snouts.** After fixation in 10% formalin, the snouts were sectioned transversely at 5-mm intervals with a band saw. Snout sections at the level of the second premolar teeth were placed under a dissecting microscope (MBS; Wild, Heerbrugg, Switzerland) and linked to a computerized image analysis system (VIDAS IMT, Analytical Measuring Systems, Oxford, United Kingdom). A morphometric index (MI) of atrophy was calculated based on the ratio of the open area of the nasal cavity to that of the ventral turbinate (12).

**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 toxigenic *P. multocida*.

**MI and bacteriological findings.** There was a direct linear correlation between the MI and the cumulative number of *P. multocida* organisms isolated from the nasal cavity (Fig. 1). There was no significant difference in the cumulative total number of *P. multocida* organisms isolated from the nasal cavity between groups B, C, and D and groups A and F (the group that was inoculated with *P. multocida* but not exposed to ovalbumin dust) (GLM). However, Fig. 1 does suggest a tendency for both the cumulative numbers of *P. multocida* organisms and the MI to increase with the concentration of ovalbumin dust (from groups E to A) and fits a quadratic equation ($y = 0.011x^2 - 1.229x + 37.055$). The difference is further illustrated by Fig. 2. The increase in the cumulative numbers of *P. multocida* organisms isolated from the nasal cavity was significantly greater in group A than in groups B, C, and D ($P < 0.001$, GLM). Furthermore, a significantly greater cumulative number of *P. multocida* organisms was isolated in group E from both the nasal cavity ($R^2 = 0.965$) and the tonsil ($R^2 = 0.963$) compared to the dust concentration. Groups F to J (pigs not challenged with *P. multocida*) had a mean MI of 48.4% (standard deviation [SD], ±4.43%), which was sig-
significantly 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 and the prevalence of respiratory diseases in pigs. The results of this study further support the hypothesis that dust exposure can affect the immune response to ovalbumin, as evidenced by the significant increase in serum IgG and IgA levels in pigs exposed to ovalbumin alone, and the suppression of these responses in pigs exposed to *P. multocida* plus ovalbumin. The dose-dependent effect observed in the serum antibody responses suggests that the intensity of dust exposure can influence the magnitude of the immune response.

**FIG. 1.** Relationship between the cumulative total number of *P. multocida* organisms isolated from the tonsil (C), linear regression, *y* = 0.027x + 1.232 (*R*² = 0.968) and nasal cavity (C; quadratic regression, *y* = 0.010x² - 1.228x + 37.055 (*R*² = 0.921)) over the course of the experiment plotted in relation to the MI at the end of the experiment.

**FIG. 2.** Cumulative total number of *P. multocida* organisms isolated from the tonsil (C; linear regression, *y* = 0.027x + 1.232 (*R*² = 0.968)) and nasal cavity (C; quadratic regression, *y* = 0.010x² - 1.228x + 37.055 (*R*² = 0.921)) over the course of the experiment plotted in relation to the mean ovalbumin dust concentration in the air.

**FIG. 3.** Mean IgG responses of pigs to exposure to inhaled ovalbumin dust from 1 to 6 weeks of age, with (open bars, groups A to E) and without (closed bars, groups F to J) challenge with *P. multocida*. Mean concentrations of ovalbumin in air are shown above the respective exposure groups. Mean IgG responses to ovalbumin exposure were calculated by subtracting the EU value at week 0 from the EU value at week 6. Error bars denote the SD (when not shown, the SD is less than 3).

**FIG. 4.** Mean IgA responses of pigs to exposure to inhaled ovalbumin dust from 1 to 6 weeks of age, with (open bars, groups A to E) and without (closed bars, groups F to J) challenge with *P. multocida*. Mean concentrations of ovalbumin in air are shown above the respective exposure groups. Mean IgA responses to ovalbumin exposure were calculated by subtracting the EU value at week 0 from the EU value at week 6. Error bars denote the SD (when not shown, the SD is less than 3).
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 colon-
ization 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 dem-
Onstrate that ovalbumin dust at the maximal exposure concen-
tration (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 concentra-
tion gave the highest yield of \textit{P. multocida} from both sites in
the upper respiratory tract and resulted in the severest turbi-
inate 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 ammo-
nia. 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. mul-
tocida} 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 down-
stream processing of the toxin.

A dose-dependent relationship was found between the con-
centration 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 ovalbu-
mín (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 magni-
tude 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 lymphope-
nia when the toxin was injected parenterally. These findings
suggest that the toxin is cytotoxic to lymphocytes, which ren-
ders 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 immuno-
globulin concentrations in serum (IgG, IgA, and IgM) de-
creased 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
\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 avail-
able \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 multifactorial
important (and as yet undocumented) virulence factor in this
complex multifactorial disease. Indeed, this effect may have
more far-reaching implications for other opportunistic bacte-
rial 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 con-
current or subsequent infections, such as enzootic pneumonia.
Atrophic rhinitis and enzootic pneumonia (mycoplasma-in-
duced 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. mul-
tocida} 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. multo-
cida}-induced immune modulation may occur early in life and
wane when the pig ages. Clinical evidence indicates that enzo-
tic 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 *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 *P. multocida* in a dose-dependent manner. Colonization of the upper respiratory tract with *P. multocida* was directly proportional to the severity of turbinate atrophy, as judged by the MI. Challenge with *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 *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 *P. multocida*. Colonization with *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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