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Contributory and Exacerbating Roles of Gaseous Ammonia and Organic Dust in the Etiology of Atrophic Rhinitis

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Pigs reared commercially indoors are exposed to air heavily contaminated with particulate and gaseous pollutants. Epidemiological surveys have shown an association between the levels of these pollutants and the severity of lesions associated with the upper respiratory tract disease of swine atrophic rhinitis. This study investigated the role of aerial pollutants in the etiology of atrophic rhinitis induced by *Pasteurella multocida*. Forty, 1-week-old Large White piglets were weaned and divided into eight groups designated A to H. The groups were housed in Rochester exposure chambers and continuously exposed to the following pollutants: ovalbumin (groups A and B), ammonia (groups C and D), ovalbumin plus ammonia (groups E and F), and unpolluted air (groups G and H). The concentrations of pollutants used were 20 mg m^{-3} total mass and 5 mg m^{-3} respirable mass for ovalbumin dust and 50 ppm for ammonia. One week after exposure commenced, the pigs in groups A, C, E, and G were infected with *P. multocida* type D by intranasal inoculation. After 4 weeks of exposure to pollutants, the pigs were killed and the extent of turbinate atrophy was assessed with a morphometric index (MI). Control pigs kept in clean air and not inoculated with *P. multocida* (group H) had normal turbinate morphology with a mean MI of 41.12% (standard deviation [SD], ± 1.59%). In contrast, exposure to pollutants in the absence of *P. multocida* (groups B, D, and F) induced mild turbinate atrophy with mean MIs of 49.65% (SD, ±1.96%), 51.04% (SD, ±2.06%), and 49.88% (SD, ±3.51%), respectively. A similar level of atrophy was also evoked by inoculation with *P. multocida* in the absence of pollutants (group G), giving a mean MI of 50.77% (SD, ±2.07%). However, when *P. multocida* inoculation was combined with pollutant exposure (groups A, C, and E) moderate to severe turbinate atrophy occurred with mean MIs of 64.93% (SD, ±4.64%), 59.18% (SD, ±2.79%), and 73.30% (SD, ±3.19%), respectively. The severity of atrophy was greatest in pigs exposed simultaneously to dust and ammonia. At the end of the exposure period, higher numbers of *P. multocida* bacteria were isolated from the tonsils than from the nasal membrane, per gram of tissue. The severity of turbinate atrophy in inoculated pigs was proportional to the number of *P. multocida* bacteria isolated from tonsils ($r^2 = 0.909$, $P < 0.05$) and nasal membrane ($r^2 = 0.628$, $P < 0.05$). These findings indicate that aerial pollutants contribute to the severity of lesions associated with atrophic rhinitis by facilitating colonization of the pig's upper respiratory tract by *P. multocida* and also by directly evoking mild atrophy.

Atrophic rhinitis is an upper respiratory tract disease of pigs characterized by degeneration of the bony and cartilaginous structures of the nasal cavity, which in severe cases can result in twisting and shortening of the pig's snout (9). The disease is attributed to the colonization of the pig's upper respiratory tract by toxigenic strains of *Bordetella bronchiseptica* and/or *Pasteurella multocida* (21, 25, 26, 29). The more severe form of the disease is associated specifically with *P. multocida* and is known as progressive atrophic rhinitis. Historically, atrophic rhinitis has proved difficult to reproduce in experimental studies, and consequently, most studies have used pigs deprived of passive immunity by withholding of colostrum (20) or with nasal cavities pretreated with a chemical irritant prior to microbial challenge (22). The etiology and pathogenicity of this disease are multifactorial, involving interactions between primary bacterial pathogens and environmental pollutants (3, 17, 18).

Pigs housed in intensive production systems are continuously exposed to high concentrations of aerial pollutants in the form of dust and noxious gases. The dust is derived largely from the pigs' integument, feed, and excrement and includes viable and dead microorganisms and their associated endotoxins (3, 23). Pollutant gases include ammonia and hydrogen sulfide which are generated during the microbial degradation of the animals' excrement (3). A correlation between the severity of lesions associated with atrophic rhinitis and the concentration of aerial pollutants in the buildings in which the pigs were reared has been demonstrated in epidemiological studies (3, 23). However, as yet the full implications of exposure to aerial pollutants for the incidence and severity of porcine respiratory disease are unclear (11, 19, 28). The study reported here investigated the effects of ammonia and organic dust, both individually and in combination, on the severity of atrophic rhinitis induced by experimental challenge with a toxigenic strain of *P. multocida* type D. Also described is the relationship between the extent of turbinate atrophy and the number of *P. multocida* bacteria colonizing the tonsils and nasal membranes.

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MATERIALS AND METHODS

Animals. Forty minimal-disease Large White piglets were derived from five sows from an atrophic-rhinitis-free herd at the Institute for Animal Health. The piglets were weaned at 1 week of age, randomly assigned to eight groups of five animals (designated A to H), and group housed in separate Rochester exposure chambers (30). All pigs were screened initially for the presence of *P. multocida*

and *B. bronchiseptica* by collecting nasal lavage as described by Chanter et al. (5) and culturing samples on selective media (see below).

Exposure chambers. Each group of pigs was housed in a 1.4-m^3 stainless steel Rochester exposure chamber built to the design of Timberell et al. (30). Air entered each chamber via a high-efficiency particulate air filter and after traversing the chamber was vented via a high-efficiency particulate air filter to prevent the release of biological material. The chambers were operated at negative atmospheric pressure (−70 kPa), giving 60 air changes per h. Within the chambers, the air temperature was maintained at 30 ± 1.0°C and at a relative humidity of 50% ± 5%.

Pollutant generation and monitoring. Ovalbumin (Sigma Chemicals Ltd., Poole, United Kingdom) was air milled to an aerodynamic particle size of <5 μm (Glen Cresten Ltd., Stanmore, United Kingdom) and sterilized to medical grade by gamma irradiation. The particulate aerosol was generated by a rotating brush powder dispersion generator (RGB1000; Palas, Karlsruhe, Germany) supplied with dried compressed air at 20 kPa and introduced into the chambers housing groups A, B, E, and F via the air inlet pipes. The concentration of dust in a chamber was adjusted to a total dust concentration of 20 mg m^{-3} by altering the delivery rate of the dust generator. Ammonia from a cylinder containing compressed gas was introduced into the chambers housing groups C, D, E, and F via the air inlet pipes, and the concentration was adjusted with an individual flow tube for each chamber (BOC Special Gases, London, United Kingdom).

The concentration of ovalbumin dust in the chambers was characterized and measured twice daily for 2-h periods with Institute of Occupational Medicine respirable and total dust samplers (Negretti Automation Ltd., Aylesbury, United Kingdom) fitted with nucleopore filter papers (Costar UK Ltd., High Wycombe, United Kingdom). A Rion airborne particle counter (KC-01A; Lynjay Services, Worthing, United Kingdom) was used to characterize the size distribution of the ovalbumin aerosol. Ammonia concentrations were measured twice daily with gas diffusion tubes (Ammonia 5/a 20501; Draeger, Lübeck, Germany).

Bacteria. A toxigenic isolate of *P. multocida*, strain LFB3, from a clinical case of atrophic rhinitis in a British pig (26), was supplied by the Institute for 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. Prior to use, the organism was cultured overnight on 5% horse blood agar at 37°C. A single colony was taken from the plate and inoculated into 10 ml of brain heart infusion broth which was incubated overnight at 37°C and used to inoculate the pigs. The numbers of organisms in the broth were enumerated by a modified Miles and Misra technique (6).

Experimental protocol. The pigs were exposed continuously to the following pollutant regimens: groups G and H, filtered air; groups C and D, ammonia; groups A and B, ovalbumin dust; groups E and F, ammonia and ovalbumin dust. One week after exposure commenced, the pigs in groups A, C, E, and G were given a bilateral intranasal inoculation of 9×10^7 CFU of *P. multocida* in 1 ml of brain heart infusion broth, while those in groups B, D, F, and H received 1 ml of filter-sterilized spent brain heart infusion broth from an overnight culture of *P. multocida*. At the end of the pollutant exposure period, all pigs were killed by injection of sodium pentobarbitone into the brachial vein.

Necropsy. Samples from the tonsils and nasal membranes (at the caudal extremity of the dorsal conchea) were taken for bacteriological analysis. The presence of macroscopic lesions was noted, and the snouts were removed by cutting transversely with a band saw at the level of the second premolar and fixed in 10% neutral buffered formalin for 1 week.

Bacteriology. Tissue samples collected at necropsy were weighed and homogenized in phosphate-buffered saline with a 15-mm-diameter glass homogenizer (Fisons Scientific, Loughborough, United Kingdom). The homogenate was diluted in phosphate-buffered saline with a 10-fold dilution series and cultured on 5% horse blood agar containing neomycin, cycloheximide (Acti-Dione), and bacitracin (27) for *P. multocida* and Bordet-Gengou medium containing furaltadone, penicillin, streptomycin, and spectinomycin (24) for *B. bronchiseptica*. Following incubation at 37°C, presumptive *P. multocida* organisms were counted after 24 h and presumptive *B. bronchiseptica* organisms were counted after 48 h, and the identities of representative colonies were confirmed with API 20E and API 20NE test strips (API-bioMerieux, Basingstoke, Hampshire, United Kingdom), respectively.

Snout sections. After fixation, the pig snouts were sectioned transversely at 5-mm intervals with a band saw. Radiographs of the sections were taken with GRE film (Kodak Ltd., London, United Kingdom) in Min R cassettes with an exposure of 42 kV and 6.4 mA/s. The radiographic images of the snout sections were analyzed with a computerized image analysis system (VIDS III; Analytical Measuring Systems, Cambridge, United Kingdom). A morphometric index (MI) of atrophy was calculated from the ratio of the open area of the nasal cavity to that of the ventral turbinate (10).

Histology. Transverse sections of the pigs' snouts at the level of the second premolar teeth were prepared and stained either with Harris' hematoxylin and eosin or by the periodic acid-Schiff technique. Subjective assessments were made of the thickness of the nasal epithelium and the area of the submucosa occupied by glandular tissue. In addition, any morphological or inflammatory changes within the epithelium, submucosa, and ossified turbinate core were noted and scored subjectively.

Statistics. Statistical analysis was performed by general linear model of variance (GLM) and simple regression analysis (RA) with a statistical computer software package (Minitab, release 10.51; Minitab Inc., State College, Pa.).

RESULTS

Clinical signs. Throughout the study, all animals retained a healthy appetite and exhibited normal behavior patterns. Clinical signs of disease were restricted to sporadic sneezing by some of the pigs which had been exposed to pollutants and inoculated with *P. multocida* (groups A, C, and E).

Pollutant exposure. In the chambers to which pollutants had not been added, the levels of total dust and ammonia remained undetectable throughout the study, by the methods stated. The concentrations of dust in the chambers housing groups A, B, E, and F were maintained at 20 mg m^{-3} (standard deviation [SD], ±6.0 mg m^{-3}) total mass and 5 mg m^{-3} (SD, ±0.7 mg m^{-3}) respirable mass. Analysis of the aerosol particle sizes showed that 97% of the ovalbumin particles had an aerodynamic diameter of less than 5 μm. The concentration of ammonia in the chambers housing groups C, D, E, and F was maintained at 50 ppm (SD, ±4.3 ppm).

Macroscopic findings. The severity of clinical lesions as quantified by MI is summarized in Table 1. The pigs which were in clean air and free of *P. multocida* (group H) had normal snout morphology with a mean MI of 41.12% (SD, ±1.59%). The pigs which were in clean air and inoculated with *P. multocida* (group G) had a mean MI of 50.77% (SD, ±2.07%), a value significantly higher than that of group H ($P < 0.05$) (GLM). The pigs which had been exposed to ovalbumin, ammonia, and a combination of both but had not received an inoculum of *P. multocida* (groups B, D, and F, respectively) had mean MIs of 49.65% (SD, ±1.96%), 51.04% (SD, ±2.06%), and 49.88% (SD, ±3.51%), respectively, indicating a statistically significant degeneration of the nasal turbinates compared to those of the control group, group H ($P < 0.05$) (GLM). The pigs which had been exposed to ovalbumin, ammonia, and a combination of both and had also received an inoculum of *P. multocida* (groups A, C, and E, respectively) had mean MIs of 64.93% (SD, ±4.64%), 59.18% (SD, ±2.79%), and 73.30% (SD, ±3.19%), respectively. These values are all significantly higher than those of the corresponding pollutant exposure groups B, D, and E, respectively, in which the pigs were free of *P. multocida* ($P < 0.05$) (GLM). These results show that exposure to aerial pollutants caused a marked increase in the severity of clinical lesions associated with atrophic rhinitis. The extent of this increase was greatest in group E, which had been exposed concurrently to dust and ammonia and also inoculated with *P. multocida*. It is notable that the increase in MI for group E was approximately equal to the sum of the increases in the two inoculated groups exposed to ammonia or dust alone (groups A and C).

Bacteriological findings. No *B. bronchiseptica* bacteria were isolated from any pigs prior to, or at the end of, the study. No *P. multocida* bacteria were detected in any pigs prior to inoculation, nor at the end of the study in uninoculated pigs. The numbers of *P. multocida* bacteria isolated at the end of the study are summarized in Table 1. All four groups of pigs inoculated with *P. multocida* had significantly higher numbers of this organism per gram of tonsil than per gram of nasal membrane ($P < 0.05$). Pigs exposed to one or more pollutants in combination with *P. multocida* (groups A, C, and E) had significantly higher numbers of *P. multocida* bacteria per gram of both the tonsils and the nasal membranes, compared to group G, which had been kept in filtered air only ($P < 0.05$). Analysis of the results in Table 1 shows significant relationships

TABLE 1. Mean MIs (percent) and mean numbers of *P. multocida* bacteria isolated from the tonsils and nasal membranes of pigs in groups A to H

Group	Treatment			MI (%)		*P. multocida* bacteria isolated (log_{10} CFU g^{-1})			
						Tonsils		Nasal cavities	
	P. multocida[a]	Ovalbumin[b]	NH_3[c]	Mean	SD	Mean	SD	Mean	SD
H	−	−	−	41.12	1.59				
G	+	−	−	50.77[d]	2.07	4.46	0.16	3.18	0.23
B	−	+	−	49.65[d]	1.96				
D	−	−	+	51.04[d]	2.06				
F	−	+	+	49.88[d]	3.51				
A	+	+	−	64.93[d]	4.64	6.98[d]	0.32	4.02[d]	0.14
C	+	−	+	59.18[d]	2.79	5.45[d]	0.19	4.30[d]	0.15
E	+	+	+	73.30[d]	3.19	7.22[d]	0.14	4.32[d]	0.17

[a] *P. multocida* challenge at 9×10^7 CFU per pig.
[b] Ovalbumin exposure at 5 mg m^{-3}.
[c] Ammonia exposure at 50 ppm.
[d] $P < 0.05$.

between MI and the number of *P. multocida* bacteria isolated from the tonsils ($r^2 = 0.909$, $P < 0.05$) (RA) and, to a lesser extent, between MI and the number of *P. multocida* bacteria isolated from the nasal membranes ($r^2 = 0.628$, $P < 0.05$) (RA).

Histological findings. Exposure to ovalbumin dust in the absence of *P. multocida* (group B) had no discernible effect on the histological appearance of the nasal mucosa. In contrast, exposure to ammonia (group D) evoked a number of distinctive histological changes, including epithelial hyperplasia, goblet cell hypoplasia, and a mild inflammatory cell infiltrate within the epithelium and adjacent submucosa. Hyperplasia was characterized by a marked thickening of the epithelium and an increase in the number of cells constituting this pseudostratified layer. The hyperplasia was most pronounced on the ventral and dorsal extremities of the dorsal and ventral turbinates, respectively. Goblet cell hypoplasia was characterized by a marked reduction in the numbers of goblet cells and a decrease in the amount of mucus stored within individual goblet cells. The hypoplasia was most apparent in the epithelium overlying the nasal septum and palatine bone, where a mixed inflammatory cell infiltrate was noted. This was characterized by an increase in the numbers of lymphocytes and the presence of intraepithelial lymphocytes within the submucosa adjacent to the epithelium. Regions of the epithelium overlying the dorsal and ventral turbinates were grossly distorted by numerous microcysts containing small amounts of necrotic cell debris. The bony core of the ventral turbinates had a normal histological appearance; however, an increase in the number of cells within the periostium surrounding the osseous core of the ventral turbinate was apparent. Analysis also revealed a significant increase in the number of osteoclasts per unit area of spicular bone ($P < 0.05$) (GLM) (data not shown). In pigs exposed simultaneously to ovalbumin dust and ammonia (group F), the histological appearance was similar to that evoked by exposure to ammonia alone; however, in regions of gross epithelial hyperplasia a decrease in the number and length of cilia was noted in several samples.

Inoculation with *P. multocida* in the absence of pollutants (group G) evoked mild inflammatory changes within the epithelium and adjacent submucosa and degenerative changes to the osseous core of the ventral turbinate. When *P. multocida* inoculation was combined with pollutant exposure, the severity of these changes increased substantially. Inflammatory changes were most marked in pigs from groups exposed to ammonia (groups C and E). In these pigs, the mixed cell inflammatory response was concentrated within the submucosa adjacent to the epithelium and around the ducts from the submucosal glands. This inflammation extended into the epithelium, which also contained numerous microabscesses packed with polymorphonucleated cells and necrotic debris. Changes in the osseous core of the ventral turbinate included an increase in the concentration of cells in the periostium and a loss of intramembranous bone at the core of the turbinates, characterized by a loss of bony spicule and a marked influx of fibrous connective tissue. All of the changes described for pigs inoculated with *P. multocida* were most pronounced in those animals exposed simultaneously to ammonia and ovalbumin dust (group E).

DISCUSSION

Pigs reared in intensive commercial conditions are continuously exposed to high levels of aerial pollutants in the form of dusts and noxious gases (3, 7, 23). The effect of these pollutants on the health and productivity of young growing pigs is largely unknown. The study described here investigated the effects of pollutant exposure on the severity of the upper respiratory tract disease of pigs progressive atrophic rhinitis. Ovalbumin powder was used as the particulate aerosol because, although not a normal constituent of piggery dust, it is an organic food substance antigenically distinct from pig feed and is available as a powder easily milled for aerosolization. Ammonia was selected as the pollutant gas because of its association with intensive pig production and its known irritant effect on mucous membranes (14). The pollutant exposure levels used in this study were higher than those encountered normally in buildings used for the intensive rearing of pigs (31). Typically, exposure ranges for the United Kingdom and The Netherlands are 5.1 to 18.2 ppm of ammonia and 0.63 to 2.61 mg of dust m^{-3} (31). However, under a very low ventilation rate, such as occurs during extremely cold weather, levels in excess of those used in this study have been reported (4).

The current study revealed that prolonged exposure to ovalbumin dust, gaseous ammonia, or a combination of the two evoked a mild level of turbinate atrophy in the absence of the

pathogenic organisms associated with atrophic rhinitis. The severity of atrophy was similar in all three cases, with no evidence of a cumulative effect of exposure to dust and ammonia simultaneously. The clinical significance of this atrophy is unclear, since the pigs were killed at a much younger age than they would have been commercially; however, the presence of turbinate atrophy after only 5 weeks of exposure clearly indicates the detrimental effects of prolonged pollutant exposure and concurs with the findings of an earlier study in which the effect of ammonia alone was investigated (17). It is unclear from the current study whether the observed atrophy occurred as a direct consequence of pollutants interacting with the tissues of the nasal cavity or as a result of a synergistic effect on a pathogen hitherto not associated with atrophic rhinitis. Further studies are needed to clarify this point; nevertheless, this finding may provide an explanation for the reported occurrence of mild turbinate atrophy in pigs from herds shown to be free of *B. bronchiseptica* and *P. multocida* (16).

Previous studies by others have reported tracheitis, rhinitis, and a range of histopathological changes affecting the mucous membranes of pigs' upper respiratory tracts following prolonged pollutant exposure but found no evidence of gross changes in nasal architecture (7, 12, 13). In contrast, the findings of the current study revealed clear evidence of turbinate atrophy following pollutant exposure. The explanation for the disparity between these findings is unclear; however, it is probable that the use of the MI based on image analysis of the pigs' nasal cavities enabled subtle changes in architecture that might have been overlooked with a purely subjective system of evaluation to be detected. In addition, differences in the ages and breeds of pigs used in the studies may account for some of the disparity.

When pollutant exposure was combined with intranasal instillation of *P. multocida*, it evoked turbinate degeneration more severe than that seen in pigs either exposed to pollutants alone or inoculated with *P. multocida* and kept in unpolluted air. Pollutant exposure substantially increased the number of *P. multocida* bacteria colonizing the tonsils and the nasal mucosa. The severity of atrophy was greatest in pigs which had been exposed to dust and ammonia in conjunction with *P. multocida*. The synergistic effect of ammonia on colonization of the pig's upper respiratory tract by *P. multocida* has been reported previously; however, we believe that the synergistic effect evoked by exposure to dust in combination with *P. multocida* and the cumulative effect of exposure to more than one pollutant to be novel findings (17).

Analysis revealed a close correlation between the severity of turbinate atrophy, represented by MI, and the number of *P. multocida* bacteria per gram of nasal membrane and tonsil (Table 1). An association between the number of *P. multocida* bacteria colonizing the tonsil and the severity of turbinate atrophy has been previously reported (5), and the tonsil has been proposed as a preferred site for *P. multocida* colonization and toxin absorption (1). The findings of the current study indicate that exposure to aerial pollutants facilitates colonization of the pig's upper respiratory tract by *P. multocida*, thereby contributing to the severity of the clinical condition progressive atrophic rhinitis. The cumulative effect evoked by combined pollutant exposure suggests that dust and ammonia facilitate *P. multocida* colonization by independent mechanisms. Further support for this theory comes from the observation that the detrimental effects of these pollutants on the morphology of the nasal turbinates in the absence of *P. multocida* was not cumulative.

The precise mechanism by which pollutants facilitate colonization of the pig's upper respiratory tract by *P. multocida* is unclear. Aerial pollutants have been shown to compromise lower respiratory tract defenses by reducing mucociliary clearance and by interfering with the clearance and phagocytosis of bacterial cells (2, 15). However, little is known about the effects of aerial pollutants on the mucosal defenses of the nasal cavity, which, in addition to being exposed to the greatest concentration of pollutants, has to protect the host from a microbial flora containing a number of potential pathogens. Histopathological examination of the tissues of the nasal cavities of pigs exposed to pollutants revealed several distinct pathological changes. These were found to be consistent with those reported by others and included mild inflammatory infiltrates within the epithelium and submucosa, hyperplastic and pathological changes to the nasal epithelium, and goblet cell hyperplasia (7, 8, 12–14, 17). It is highly likely that these changes would impair the defenses of the upper respiratory tract by altering the consistency of mucus and the rate of mucociliary clearance. It is unclear to what extent these changes facilitate colonization of the pig's upper respiratory tract by *P. multocida*. Other factors which need to be considered in this context include the effects of pollutant exposure on the composition and population density of the commensal flora, the effects of pollutants on the nutrients which could support microbial growth at this site, and the possibility of pollutants triggering virulent pathogenic mechanisms.

In conclusion, pollutant exposure in the form of either ovalbumin dust, gaseous ammonia, or a combination of the two contributes to the degenerative changes associated with atrophic rhinitis in two distinct ways: firstly, by directly impairing the development of the nasal turbinates in the young growing pig, and secondly, by facilitating colonization of the pig's upper respiratory tract by *P. multocida*. Of these two effects, the latter is probably the more important; however, the former may explain the occurrence of mild turbinate atrophy reported in herds deemed to be free of the primary etiological agents *B. bronchiseptica* and *P. multocida*. In the pigs inoculated with *P. multocida*, the exacerbating effect of the two pollutants used in this study was cumulative, with the greatest severity of lesions occurring when pigs were exposed simultaneously to dust and ammonia. This study highlights the detrimental effects of pollutant exposure on the health of pigs according to an endemic disease model. Further studies are needed to enable tolerance limits for pollutant exposure to be established in order to maximize the health, welfare, and productivity of pigs.

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