Experimental Model of Atrophic Rhinitis in Gnotobiotic Pigs

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To study the pathogenesis of atrophic rhinitis, gnotobiotic pigs ($n = 6$) were inoculated intranasally with a sterile sonicate of a toxigenic strain of *Bordetella bronchiseptica* (0.16 mg of protein per ml) at 5 days of age, and they were then inoculated intranasally with 1 ml (5,250 CFU/ml) of a live, toxigenic strain of *Pasteurella multocida* at 7 days of age. Pigs were necropsied at 2, 5, 9, 14, 21, and 28 days postinoculation; those pigs necropsied after 5 days had developed turbinate atrophy. Other gnotobiotic pigs received the following inoculation protocols: (i) a sterile sonicate of a nontoxigenic strain of *B. bronchiseptica* (0.2 mg of protein per ml), followed by toxigenic *P. multocida* ($n = 4$); (ii) toxigenic *P. multocida* alone ($n = 7$); (iii) diluent (sterile tryptose broth) ($n = 2$); (iv) the sterile sonicate of toxigenic *B. bronchiseptica* alone ($n = 2$); or (v) the sterile sonicate of a nontoxigenic strain of *B. bronchiseptica* alone ($n = 2$). Turbinate atrophy did not occur in the latter groups except for one pig inoculated with only toxigenic *P. multocida*. These studies show that turbinate atrophy occurs in pigs given the toxigenic *B. bronchiseptica* sonicate and then given live, toxigenic *P. multocida*. This experimental regimen is a useful model for (i) studying the pathogenesis of atrophic rhinitis and (ii) testing vaccine strategies.

Severe lesions of atrophic rhinitis are often associated with toxigenic strains of group D *Pasteurella multocida*. It has been suggested that colonization of the turbinates by *Bordetella bronchiseptica* results in weakening of the mucosal barrier which allows the growth of *P. multocida* (8). Recently, Chanter et al. (2) have suggested that the toxin of *B. bronchiseptica* responsible for dermonecrosis may enhance the growth of toxigenic strains of *P. multocida* on the nasal turbinate mucosa.

Previously, we demonstrated that a toxigenic strain of *P. multocida* can colonize the tonsillar crypts of gnotobiotic pigs (1). From this and the results of studies demonstrating the poor ability of *P. multocida* to adhere to epithelial cells, we proposed that toxigenic strains may not need to colonize the turbinate mucosa to cause turbinate atrophy (5, 15). Instead, bacteria may elaborate toxin into tonsil crypts, where it is taken up by the lymph or blood. The purpose of this study was to determine (i) whether the combination of a sterile sonicate of a toxigenic strain of *B. bronchiseptica* and live *P. multocida* results in turbinate atrophy and could thus serve as a model of experimental atrophic rhinitis and (ii) whether the sonicate enhances the growth of *P. multocida* on the turbinates or tonsils.

MATERIALS AND METHODS

Animals. Three sets of piglets derived by cesarean section from three different sows were kept in gnotobiotic isolators and remained free from outside contaminants throughout the experiment. Pigs were fed a sterile diet of SPF Lac (Pet Ag, Hampshire, Ill.) and monitored daily for inappetence or lethargy. To monitor for contamination, rectal swabs were cultured on aerobic and anaerobic media at weekly intervals and at termination.

Inocula. Sterile, filtered sonicates of toxigenic *B. bronchiseptica* (strain P-4609) and nontoxigenic *B. bronchiseptica* (strain P-4607) were prepared as follows. Bacteria grown for 18 h on horse blood agar plates were suspended in phosphate-buffered saline. The suspensions were adjusted to a density equivalent to that of a McFarland 10 nephelometer standard and sonicated at 80% power for 30 s at 4°C with a Branson 185 Sonifier (Branson Instruments, Danbury, Conn.). The sonicated material was centrifuged at 12,500 × *g*, and the supernatant material was filtered through a 0.22-μm-pore-size filter (Millipore Corp., Bedford, Mass.). Protein concentrations of the filtered sonicates were determined by the Bradford method, using bovine serum albumin as a standard. Filtered sonicate of strain P-4609 contained 160 μg of protein per ml of phosphate-buffered saline, while filtered sonicate of strain P-4607 contained 200 μg of protein per ml of phosphate-buffered saline. The levels of toxicity, biochemical characterizations, and geographical origins of these strains were determined previously (17, 18).

P. multocida (group D, strain P4148) was grown on dextrose starch agar for 18 h and suspended in phosphate-buffered saline. Suspensions were adjusted to a density equivalent to that of a McFarland 1 nephelometer standard. The standardized suspension was used for appropriate dilutions, and the final inoculum had 5,250 CFU/ml as determined by plate counts.

Experimental design. Gnotobiotic pigs were assigned to one of five intranasal inoculation protocols (Table 1): (i) sterile sonicate of the toxigenic strain of *B. bronchiseptica* (0.5 ml per nostril) at 5 days of age, followed by live *P. multocida* (group D, strain P4148) (5,250 CFU/ml; 0.5 ml per nostril) at 7 days of age (pigs 1 to 6); (ii) sterile sonicate of the nontoxigenic strain of *B. bronchiseptica* at 5 days of age, followed by live *P. multocida* at 7 days of age (pigs 7 to 10); (iii) live *P. multocida* at 7 days of age (pigs 11 to 16); (iv) diluent (sterile tryptose broth) at 7 days of age (pigs 17 and 18); (v) sterile sonicate of toxigenic *B. bronchiseptica* (pigs 19 and 20); or (vi) sterile sonicate of nontoxigenic *B. bronchiseptica* (pigs 21 and 22). All pigs were held upright, and the inoculum was dropped into each nostril opening with a 1-ml syringe.

Pigs were killed at various times postinoculation (2 to 28 days) by an intravenous pentobarbital overdose, and tur-

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TABLE 1. Turbinate lesions and CFU of *P. multocida* from tissue recovered from gnotobiotic pigs inoculated intranasally by six different protocols

Pig no.	Inoculum protocol no.[a]	Days after inoculation	Subjective rating of turbinate lesion	TPR[b]	Turbinate area ratio[c]	10^3 CFU of *P. multocida*/g of following tissue			
						Turbinate	Tonsil	Trachea	Lung
1	1	2	None	1.01	0.39	4	1,666	1,875	<1
2	1	5	Mild	0.99	0.20	25	194	14	1
3	1	9	Moderate	0.85	0.20	0	220	135	7
4	1	14	Marked/severe	0.80	0.16	7	24,785	<1	<1
5	1	21	Marked	0.43	0.08	8	39,258	6	11
6	1	28	Severe	0.40	0.06	27	215	21	5 (5,045)[d]
7	2	2	None	1.10	0.36	0	1,266	577	0
8	2	5	None	1.02	0.42	6	440	1	0
9	2	9	None	1.08	0.37	0	0	0	0
10	2	14	None	1.04	0.52	2	1,794	25	17
11	3	2	None	1.15	0.47	2	3,100	3	0
12	3	5	None	1.05	0.37	44	48	36	0
13	3	9	None	1.14	0.37	0	320	2	0
14	3	14	Severe	0.72	0.11	4	43,733	3	6
15	3	21	None	1.27	0.36	1	3,217	220	34
16	3	28	None	1.28	0.36	3	83	0	0
17	4	21	None	1.14	0.41	0	0	0	0
18	4	28	None	1.42	0.38	0	0	0	0
19	5	16	None	1.23	0.40	ND[e]	0	ND	ND
20	5	23	None	1.16	0.48	ND	0	ND	ND
21	6	16	None	1.32	0.38	ND	0	ND	ND
22	6	23	None	1.10	0.33	ND	0	ND	ND

[a] Protocols are as follows (days refer to time after treatment): 1, sterile sonicate of toxigenic *B. bronchiseptica* at 5 days and *P. multocida* (5,250 CFU/ml) at 7 days; 2, sterile sonicate of nontoxigenic *B. bronchiseptica* at 5 days and *P. multocida* (5,250 CFU/ml) at 7 days; 3, no sonicate primer at 5 days and *P. multocida* (5,250 CFU/ml) at 7 days; 4, no sonicate primer at 5 days and sterile diluent (tryptose broth) at 7 days; 5, sterile sonicate of a toxigenic strain of *B. bronchiseptica* at 5 days only; 6, sterile sonicate of a nontoxigenic strain of *B. bronchiseptica* at 5 days of age only.

[b] TPR were determined by previously described techniques (6). A score of <1.0 suggests moderate to severe turbinate atrophy.

[c] Turbinate ratio scores were determined by dividing the turbinate surface area by the surrounding air space surface area. Numbers listed are portions of air space occupied by turbinate tissue.

[d] A focal area of pleuritis had 5,045 CFU/g.

[e] ND, not determined.

binate atrophy was scored subjectively as minimal, mild, moderate, marked, or severe. Weighed specimens of turbinate, tonsil, trachea, and lung tissue were ground in saline with a Ten Broeck grinder. Numbers of CFU of *P. multocida* per gram of tissue were determined by plating serial dilutions of homogenates on blood agar plates. *P. multocida* was identified by colony morphology, iridescence after growth on dextrose starch agar, biochemical fermentation of selected colonies, and a membrane lift procedure. The membrane lift procedure was used according to previously described techniques (13). Briefly, a circular piece of nylon membrane was lightly applied to a blood agar plate to lift colonies incubated for 18 h. The membranes were washed, a peroxidase-labeled monoclonal antibody to the *P. multocida* toxin was applied to the membrane, and the labeled colonies were identified with a 4-chloronaphthol solution.

Histopathology. Specimens of turbinate, tonsil, trachea, and lung tissue were fixed in 10% neutral buffered formalin for 24 h. Turbinates were decalcified in EDTA Decalcifying Solution (Baxter Scientific Products, McGaw Park, Ill.) for an additional 24 h after fixation. Sections were stained with hematoxylin and eosin. By using sections of turbinates, the surface areas of the turbinates and of the nasal air spaces surrounding the turbinates (periturbinate air spaces) and the turbinate perimeter ratios (TPR) (6) were measured with a Zeiss SEM-IPS image analysis system (Zeiss-Kontron; IBAS version 2.10). Sections were photographed with a Sony 3CCD color video camera with a Fujinon zoom lens. The internal scaling feature of the image analysis software was calibrated for the image under investigation. The tur-

binate surface area was divided by the nasal periturbinate air space surface area (Table 1) in order to quantify turbinate lesions to supplement our subjective scores. The ratios measure the portion of the air space surface area occupied by turbinate tissue. Pigs with turbinate atrophy have decreased turbinate surface area, increased nasal air space, and, therefore, smaller ratios than control pigs.

RESULTS

Control pigs and inoculated pigs that lacked turbinate atrophy appeared clinically normal throughout the experiment. Those pigs with moderate to severe turbinate atrophy, however, had small statures, rough hair coats, mildly distended abdominal walls, and weak squeals. At 5 days postinoculation, pigs that had undergone the toxigenic *Bordetella* sonicate-*P. multocida* protocol (pigs 2 to 6) developed at least mild turbinate atrophy (Table 1; Fig. 1). Histologically, turbinates were characterized by thin, irregular fragments of bone trabeculae surrounded by increased numbers of fibroblasts, large osteoclasts, and occasional necrotic osteoblasts (Fig. 2). There were no significant infiltrates of inflammatory cells. One pig (no. 1) that was killed 2 days after inoculation with *P. multocida* did not develop turbinate atrophy.

Pigs given the sonicate of the nontoxigenic *B. bronchiseptica* and live *P. multocida* (pigs 7 to 10) did not develop any discernible turbinate lesions by either subjective scoring or morphometry. Pigs that received *P. multocida* alone (no. 11 to 16) (i.e., with no sonicate primer) did not develop tur-
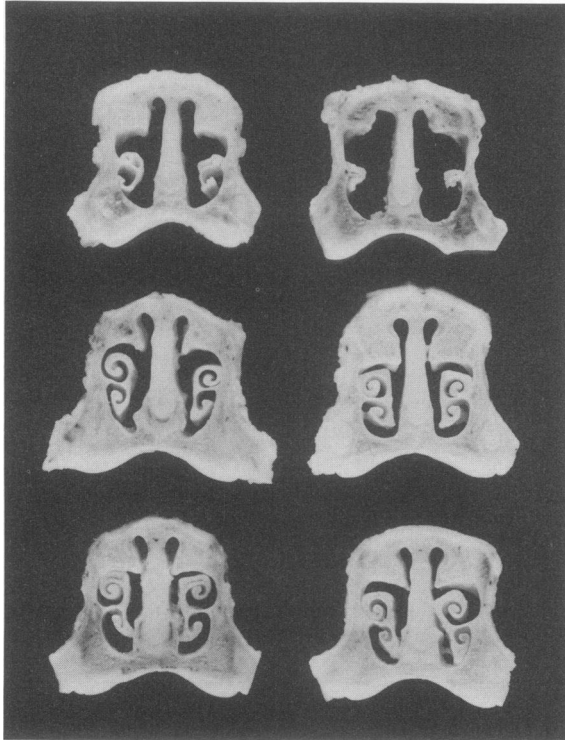
FIG. 1. Nasal turbinates of pigs 5, 6, and 15 to 18. In the top two turbinate sections, those of pigs 5 and 6, only small amounts of irregular turbinate tissue are present within moderately enlarged airway spaces.

binate atrophy, except for pig 14, in which turbinate atrophy was severe. Turbinates of pigs receiving only the sterile sonicates (no. 19 to 22) or sterile broth (no. 17 and 18) remained normal in size and, histologically, lacked inflammatory cell infiltrates (Table 1). Area ratio scores of turbinate surface area/air space surface area (air space occupied by turbinate) were decreased (≤ 0.20 for area scores) for those pigs (no. 2 to 6 and no. 14) with subjective scores of turbinate atrophy (Table 1). The ratio scores of the remaining pigs (no. 1, 7 to 13, and 15 to 22) were >0.32. The TPR scores were <1.0 for pigs 2 to 6 and pig 14 and >1.0 for all other pigs. A score of <1.0 suggests moderate to severe turbinate atrophy (6).

The largest numbers of bacteria per gram of tissue were generally isolated from tonsil tissue (Table 1). *P. multocida* was not isolated from tonsil or any other tissue from pig 9. The largest numbers of *P. multocida* isolated from tonsil tissue were associated with severe turbinate atrophy and were seen in pigs 4, 5, and 14. Relatively smaller numbers of *P. multocida* were isolated from trachea, turbinate, and lung tissue; bacteria isolated from these tissues varied greatly in number and were sometimes not isolated. Pig 6 had severe turbinate atrophy and relatively low numbers of *P. multocida* in cultured tonsil tissue; however, this pig had focal pleuritis in the right cranial lung lobe that contained relatively large numbers of *P. multocida*.

P. multocida was the only bacterium isolated from inoculated pigs. All of the isolated *P. multocida*, tested by the membrane lift technique with monoclonal antibody to the dermonecrotic toxin, actively produced toxin.
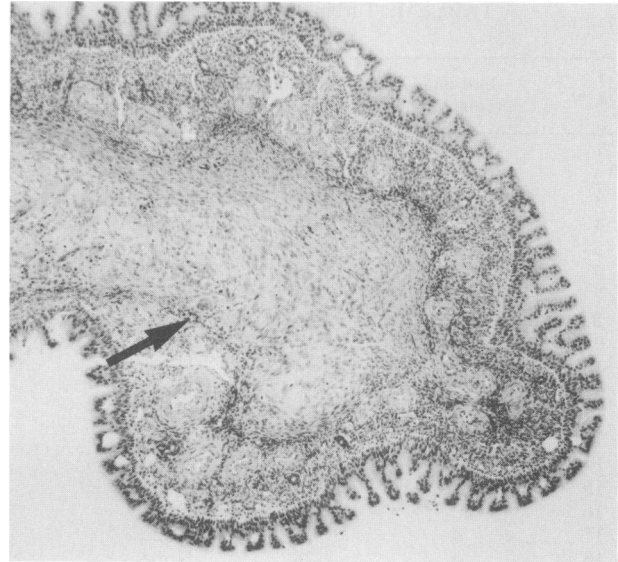

FIG. 2. Nasal turbinate of pig 4. The turbinate is small and irregular, and only remnants of trabecular bone remain. The trabeculae are replaced by extensive fibrosis (arrow).

DISCUSSION

We feel that we have developed a consistent and reproducible experimental model of atrophic rhinitis. This model can be used for studies on the pathogenesis of turbinate atrophy and for vaccine testing. It may be possible to improve this model by using a semipurified or highly purified *Bordetella* toxin. A purified toxin would remove lipopolysaccharides, polysaccharides, and other bacterial components present in the crude sonicate that may influence the experimental outcome. It also would demonstrate whether the effects seen in this study are indeed caused by toxin or another, yet undefined, virulence factor. The absence of turbinate atrophy in one pig (no. 1) was probably due to the short time span (2 days) postinoculation with *P. multocida*.

Others have been able to reproduce turbinate atrophy by (i) dual infections with *B. bronchiseptica* and *P. multocida*, (ii) infection with *P. multocida* following intranasal acetic acid treatment, (iii) intradermal or subcutaneous injections with the purified *P. multocida* toxin, (iv) intranasal aerosol with the purified toxin, and (v) intranasal inoculation of pigs with high numbers of *B. bronchiseptica* and *P. multocida* (2, 3, 7, 8, 14, 20). In this model, we were able to use small numbers ($<10^5$) of *P. multocida* in our inoculum in combination with the sonicate primer to induce turbinate atrophy, whereas other studies generally used larger numbers ($>10^6$). The TPR is currently considered the best morphometric procedure in determining the degree of turbinate atrophy (6). The TPR scores of <1.0 in this study are in agreement with the 1.0 cutoff suggested by Collins et al., where undetectable or mild turbinate atrophy is seen with scores >1.0 and moderate to severe turbinate atrophy is seen with scores <1.0 (6).

Because of the limited number of pigs used in this study, we were unable to determine whether the *Bordetella* sonicate influenced the growth of *P. multocida* on the turbinate. The *Bordetella* sonicate may not alter *P. multocida* growth, but it may directly affect bone cells. The *Bordetella* toxin impairs the differentiation of osteoprogenitor cells to osteo-

blasts in vitro, and the *Pasteurella* toxin induces osteoclasis in situ (7, 10, 14). Thus, the *Bordetella* toxin may inhibit bone synthesis and the *Pasteurella* toxin may induce bone and cartilage resorption. The *Bordetella* sonicate used in this study may have influenced the elaboration of the *Pasteurella* toxin, but this is unlikely since the sonicate was instilled 2 days prior to inoculation with *P. multocida*.

The fact that dramatically smaller numbers of bacteria were isolated from the turbinates may downplay the traditional hypothesis that toxin elaboration must occur on the turbinate mucosa. Instead, turbinate atrophy was associated with large numbers of *P. multocida* in the tonsils in some pigs, which may suggest that tonsil colonization may be important in the development of atrophic rhinitis. We have previously shown that the tonsils are an important site for colonization by a toxigenic strain of *P. multocida* (1). On the other hand, the few toxigenic *P. multocida* organisms present on the turbinate mucosa may be all that are needed to induce turbinate atrophy. The purified toxin is very potent (4), and a subcutaneous injection of just 0.2 µg/kg of body weight can result in turbinate atrophy in young gnotobiotic pigs (personal observation). Field studies that quantify the number of toxigenic *P. multocida* organisms per gram of turbinate and tonsil tissue from (i) healthy pigs in rhinitis-free herds and (ii) pigs with atrophic rhinitis are needed to better characterize the role of tonsil colonization in the pathogenesis of the disease.

Since the sonicate of the nontoxigenic strain of *B. bronchiseptica* was not associated with turbinate atrophy or with large numbers of *P. multocida* in the tonsils, these nontoxigenic strains may have only a small role in the development of atrophic rhinitis or in the colonization and replication of *P. multocida*. Other studies have also shown that nontoxigenic strains of live *B. bronchiseptica* are not associated with turbinate atrophy (2).

The severe turbinate atrophy in pig 14, which was given only *P. multocida* and no primer, cannot be explained. In any event, the localization of bacteria in this pig was similar to the localization in those pigs given toxigenic sonicate; i.e., large numbers were isolated from the tonsils and lesser numbers were isolated from the turbinates. The pleuritis that developed in pig 6 is similar to the pleuritis reported by diagnosticians (16). One Japanese study showed that group D strains of *P. multocida* are most often associated with lung abscesses in pigs, whereas group A strains are associated with pneumonia (11).

Our model can be used to test various vaccines under experimental conditions. In contrast to other models using live *B. bronchiseptica* or intranasal acetic acid in combination with *P. multocida*, our model incorporates virulence factors (i.e., toxin) from *B. bronchiseptica* without actual growth and colonization by the organism. A toxoid vaccine developed in our laboratory by using the purified *P. multocida* toxin protects rats from challenge (19) and can be tested by using the *Bordetella* sonicate-live *P. multocida* protocol. A similar toxoid, in combination with *B. bronchiseptica* bacterin, protects conventionally reared pigs from atrophic rhinitis (9, 12).

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