INFECTION AND IMMUNITY, Feb. 1990, p. 352–357
0019-9567/90/020352-06$02.00/0

Vol. 58, No. 2

Identification of a 68-Kilodalton Outer Membrane Protein as the Major Protective Antigen of *Bordetella bronchiseptica* by Using Specific-Pathogen-Free Piglets

MARYLENE KOBISCH[1] AND PAVEL NOVOTNY[2]*

Station de Pathologie Porcine, Ploufragan, France,[1] and Department of Molecular Biology, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, United Kingdom[2]

Received 1 August 1989/Accepted 30 October 1989

Maternal antibody to an outer membrane 68-kilodalton (kDa) protein of *Bordetella bronchiseptica* was shown to be protective in experiments on specific-pathogen-free piglets. After challenge with *B. bronchiseptica*, 100% ($n = 19$) control piglets from nonimmunized sows developed pneumonia, coughing, and sneezing, and 74% of the animals developed severe atrophic rhinitis. In 12 piglets from a sow immunized with 68-kDa protein, pneumonia occurred only in 34% of offspring, coughing was reduced, the duration of coughing bouts was shortened, and severe atrophic rhinitis occurred in one animal only (8%). The difference in the occurrence of atrophic rhinitis and of pneumonia in immunized and nonimmunized offspring was statistically significant ($P < 0.05$). Sera of protected piglets had high titers (enzyme-linked immunosorbent assay) of antibodies that showed a high specificity for the 68-kDa protein isolated from *B. bronchiseptica*, whereas their reactivity with an analogous 69-kDa protein isolated from *Bordetella pertussis* was low or absent. The 68-kDa protein of *B. bronchiseptica* appeared to be the major protective antigen in *B. bronchiseptica* infection; however, isolated protein alone did not induce such a solid protection, as observed in a previous study after the application of an effective whole cell vaccine.

From the investigation of piglet sera from sows immunized with efficient and inefficient atrophic rhinitis vaccines, it was possible to identify an antigen of *Bordetella bronchiseptica* that appears to be important for protection. Antibodies to this *B. bronchiseptica* 68-kilodalton (kDa) outer membrane protein can be detected in relatively high titers in protected piglets, whereas the titer is low or absent in nonprotected piglets (15). The variability of vaccine efficacy was explained by the fact that the 68-kDa antigen is expressed only under balanced growth conditions; slight changes in *B. bronchiseptica* fermentation lead to its disappearance from the outer membrane (12, 15). A naturally occurring mutant lacking the 68-kDa protein is unable to protect piglets when used as a vaccine and is also unable to induce pathological changes in infected piglets (12).

In experiments on mice that were either aerosol infected with a virulent strain of *B. bronchiseptica* or infected by contact with aerosol-infected mice when kept in the same cage, it was demonstrated that passively administered monoclonal antibody BB05, specific for the 68-kDa protein, was able to prevent death from pneumonia and development of atrophic rhinitis. Another monoclonal antibody, BB07, specific for a nonoverlapping epitope of the same protein, did not show such properties (11). In addition, purified 68-kDa protein used to immunize pregnant mice was able to promote protection of the offspring; i.e., the vaccine prevented death and development of atrophic rhinitis. The clearance of *B. bronchiseptica*, however, from the lungs of baby mice in these experiments was not as fast as that observed after immunization of mothers with a whole cell vaccine (13). To confirm these data we investigated the protective effect of the isolated 68-kDa protein in experiments on piglets, in which the *B. bronchiseptica* infection occurs naturally. Since analogous proteins were detected in *Bordetella pertussis* and in *Bordetella parapertussis* with molecular masses of 69 and 71 kDa, respectively (12), the results may be of importance in the elucidation of protective properties of this family of bordetella-specific antigens.

* Corresponding author.

MATERIALS AND METHODS

Vaccine. The vaccine used was immunopurified and isoelectrofocused 68-kDa protein of *B. bronchiseptica* prepared as previously described (15). After purification, the protein was tested for the presence of other antigens by immunoelectroblotting and enzyme-linked immunosorbent assay (results not shown) with specific monoclonal antibodies (BB05 and BB07) (11) and piglet sera from a previous study that yielded (i) sera with a very high titer against lipopolysaccharide and no titer with the 68-kDa protein and (ii) sera which reacted strongly with outer membrane proteins (12, 15). The presence of adenylate cyclase enzyme, which as a rule copurifies with the 68-kDa protein, was determined as previously described (12); each 1 mg of the 68-kDa protein used as a vaccine converted 1.08 nM ATP per min. Assuming that the turnover number of the *Bordetella* adenylate cyclase is 27,000 and that its mass is 40 kDa (20), then the actual concentration of the enzyme present in each mg of the 68-kDa protein was 1.6 μg. The preparation did not show detectable biological activity (adenylate cyclase toxin activity) (4); i.e., it was unable to generate cyclic AMP in S49 cells.

Finally, the preparation was freeze-dried in the presence of 20% sucrose as a protective medium. The ampoules were stored at 4°C until used; each contained an equivalent of 1 mg of protein. Before injection the protein was reconstituted in phosphate-buffered saline.

Animals. Five primiparous sows (Large White breed), originating from an experimental, hysterectomy-derived herd, were reared in a pathogen-free environment (2). Ten weeks before the expected farrowing date, they were housed

in individual compartments and supplied with filtered air to exclude any contact with pathogenic organisms. The drinking water was filter sterilized, and the feed was sterilized by irradiation (4×10^4 Gy by Institute National des Radioelements; Complexe Industriel, Merides 6220, Fleurus, Belgium).

During the pregnancy one sow (no. 50041) was immunized by two intramuscular injections of 2 mg of 68-kDa protein 6 weeks apart (8 and 2 weeks before expected farrowing) alternately behind the left and right ears. The other sows (no. 50038, 50039, 50037, and 50045) were kept as controls.

Challenge infection. Thirty-one piglets (12 from immunized sow 50041 and 19 from non-immunized sows 50038 and 50039) were challenged by intranasal instillation of a 0.2-ml suspension of *B. bronchiseptica* on days 2, 3, 4, and 5 of life. Thirteen piglets from nonimmunized sows 50037 and 50045 were left unchallenged.

The challenge strain used, strain 5, was isolated from a piglet with atrophic rhinitis. Before use it was grown for 18 h at 37°C on Bordet-Gengou agar and suspended for challenge in nutrient broth to a density of 10^9 cells per ml.

Clinical evaluation. Body temperature and clinical signs, such as frequency of coughs and sneezes, were recorded daily over 10-min observation periods for each group of piglets at the same time of day. Colostrum of each sow was obtained during the delivery, synchronized by medication (Prostaglandins Planat, Distrivet SA, France). Sera of sows and piglets were collected weekly during the whole experimental period. At the end of the experiment all animals that did not die were sacrificed (barbiturate anaesthetic Nesdonal [Specia, France], intravenously), i.e., sows 4 weeks after the farrowing and piglets at the age of 4 weeks. The lungs and nasal labyrinth of each animal were examined macroscopically. The lungs either appeared normal (0) or had sporadic loci (+), changes in part of the lobulus (++), or changes of the whole or several lobuli (+++) with characteristics of bronchopneumonia. Macroscopic changes of the nasal labyrnth were similarly graded: congestion and/or hyperplasia of conchae (+), partial regression of conchae (++), and almost complete disappearance of conchae from one or both sides of the septum (+++). For microscopic examination the tissues were fixed (decalcified in case of bones), and paraffin sections were prepared and stained with haematoxylin-eosin. For bacterial counts the turbinates were swabbed with 2 ml of phosphate-buffered saline (pH 7.2), and 50-μl samples were dropped onto blood-agar plates for colony counts. For the determination of colony counts in lungs, the surface of the inflamed tissue was cauterized, the tissue was dissected, and after squeezing with forceps 50-μl volumes of exudate were aspirated.

Antibodies in sera were determined by enzyme-linked immunosorbent assay as reported previously (15), with the following modifications. The 68-kDa protein was the same as that used for immunization. All the sera dilutions were twofold and prepared in U plates, eight for reference serum and four for test sera. Serum dilutions started from 1/24 to 1/200, depending on the outcome of preliminary tests. All of the dilutions were then transferred to flat-bottom antigen-coated plates (Linbro, Titertec; Flow Laboratories, United Kingdom). All of the liquid handling was automated with a Cetus Pro-Pette instrument (Perkin-Elmer Ltd., United Kingdom), and the optical density readings (Titertec Multiscan MC; Flow Laboratories, United Kingdom) were converted into units by the parallel-line bioassay method (10) with a computer program kindly provided by D. G. Burstyn (Center for Drugs and Biologics, Bethesda, Md.). Most of the sera were evaluated by parallel line or, occasionally, by a single-point method (mostly in low-titer sera). Reference pig serum globulin containing 600 arbitrary units per ml was used as described previously (15).

Sera from children used for the determination of cross-reactivity were convalescent sera from cases of culture-confirmed whooping cough and were kindly provided by G. D. W. McKendrick (Clinical Department, Wellcome Research Laboratories). The 69-kDa protein was isolated from *B. pertussis* 2992 by a method identical to that used for the isolation of the 68-kDa protein of *B. bronchiseptica*.

RESULTS

Clinical results. Neither increase of rectal temperature nor reaction in the injection site was observed after vaccination. Postmortem histological examination did not show any changes in the site of antigen injection.

Four piglets from two nonvaccinated sows (50038 and 50039) died of pneumonia between days 6 and 17 of life. One piglet from the vaccinated sow died for no obvious reasons on day 9 of life. Rectal temperature of infected piglets did not exceed 40°C. Coughing developed after approximately a week, whereas sneezes occurred 4 to 5 days after the challenge (Fig. 1). Coughing and sneezing occurred in repeating bouts, with occasional dyspnea and with diminishing intensity to the end of the experiment. Coughs in piglets from vaccinated sows ceased by day 19, 4 days earlier than in nonimmunized piglets (Fig. 1A). The average number of coughs per 10-min observation period in piglets from the vaccinated sow was lower than that in infected piglets from the nonvaccinated sows, whereas the frequency of sneezes was affected very little if at all by the immunization (Fig. 1B). No clinical signs were observed in noninfected control piglets.

Weight gains are illustrated in Table 1. Experimental infection had an effect on the mean values, but due to the different sizes of the litters the values are not suitable for statistical evaluation.

Postmortem results. The results of postmortem examinations are in Table 2, and the grades of destruction to turbinates are illustrated in Fig. 2. Differences in occurrence of pneumonia and nasal changes between piglets from vaccinated and nonvaccinated sows were statistically significant at the 5% level (test of independence by the chi-square method). Two of the challenged piglets from nonvaccinated sows died of pneumonia on days 6 and 9 of life, this represents too short a period for the development of nasal changes. None of the piglets developed prognatism or deformation of the snout. Nonchallenged piglets from nonvaccinated sows showed no macroscopic abnormalities.

Histopathology. Infected piglets from nonvaccinated sows suffered from interstitial pneumonia with mononuclear, prevalently peribronchial infiltration (with distinctive presence of macrophages) and with small foci of alveolar confluence (emphysemalike). Suppurative alveolitis was rather rare, and there were no other pyogenic changes. In the later stages the lesions were characterized by atelactasis and an organized cicatrized fibrosis, which mostly occurred interlobularly or peribronchially. Atrophic rhinitis in these animals was characterized by hyperplasia of the superficial nasal epithelium. In many cases the hyperplasia disappeared; where it persisted, considerable metaplasia of epithelial cells was seen. There was inflammation of the chorion and modification of the osseous lamellae, particularly at the apical ends of the conchae, and the inflammation was

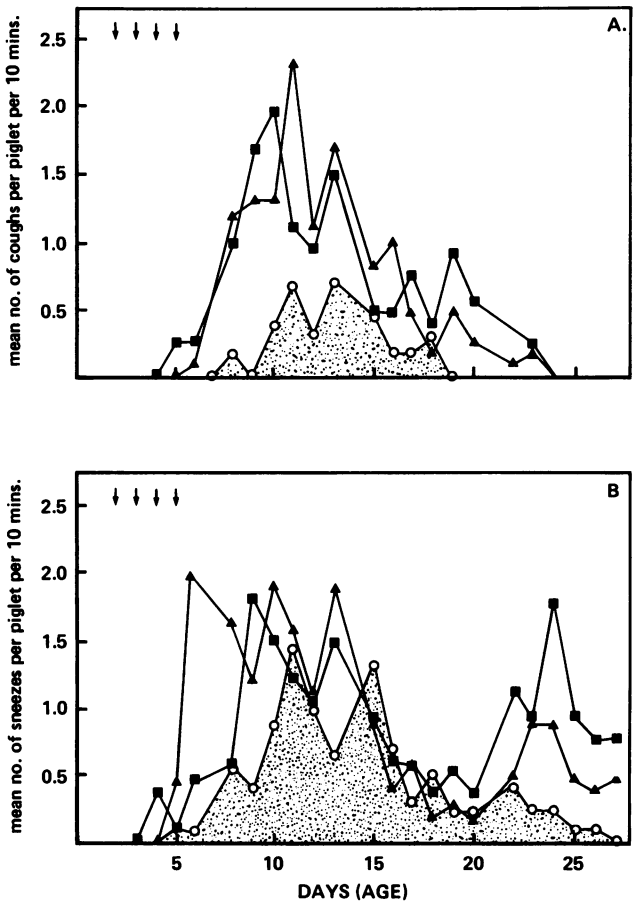

FIG. 1. (A) Mean frequency of coughs per piglet per 10-min observation period on the days indicated. The reason for the lower frequency at day 12 is unknown. (B) Mean frequency of sneezes per piglet per 10-min observation period on the days indicated. Piglets were from vaccinated sow 50041 (○) and nonvaccinated sows 50038 (■) and 50039 (▲). Arrows indicate the days of the challenge. The area under the data from the piglets from vaccinated sow 50041 has been shaded to aid the visual interpretation.

characterized by mononuclear infiltration of the submucosa. The other bones of the nasal cavity were not affected. In animals past the acute stage, masses of osteoclasts were visible.

Among piglets from vaccinated sows, only four animals had discrete pulmonary lesions. With the exception of one animal, nasal changes were also discrete. The osseous skeleton of the conchae appeared normal; the only apparent changes were inflammatory foci in the nasal mucosa. No abnormalities were observed in noninfected control piglets.

TABLE 1. Mean weights of piglets at the end of the experiment

Piglets	Sow (no.)	*n*	Mean wt[a] (g)
Challenged	Immunized (50041)	11	225
	Nonimmunized (50038)	10	210
	Nonimmunized (50039)	5	262
Healthy	Nonimmunized (50037)	9	247
	Nonimmunized (50045)	4	320

[a] Overall mean weights were 227 g for nonimmunized challenged piglets and 264 g for healthy piglets.

Microbiological findings. *B. bronchiseptica* was detected in the nasal cavity of piglets from nonvaccinated sows and from the vaccinated sow for the whole observation period. The colony counts per 50-μl sample in both instances were higher than 50 colonies. In piglets that died during the experiment the lung counts were also high, i.e., >50 CFU per 50-μl sample, and remained so in piglets sacrificed at the end of the experiment. In piglets from the vaccinated sow, *B. bronchiseptica* was always present in the nasal cavity, with colony counts similar to those in the nonimmunized animals, but at the end of the observation period the colony counts from the lungs of immunized animals decreased (one to three colonies per 50-μl sample); i.e., clearance from lungs appeared faster in immunized animals. The nasal counts seemed unaffected by immunization with the 68-kDa antigen, a situation that was also observed when whole cell preparations of *B. bronchiseptica* were used as the vaccine (5, 15).

Serology. A measurable but low titer (70 U/ml) of antibodies against the 68-kDa protein developed in the immunized sow after the first injection of the antigen. The titer increased after the second injection, was particularly high in serum samples taken during parturition (600 U/ml), and rose further (>10,000 U/ml) during the lactation period. In nonvaccinated and control sows the titers remained below the level of significance (i.e., 25 U/ml). The average titers in the sera of piglets from the vaccinated sow and those from infected piglets from nonvaccinated sows are shown in Fig. 3. It is evident that immunized sows provided the piglets with a high dose of immunoglobulin G antibodies. In infected piglets from nonimmunized sows, the active antibody response due to the challenge infection started to appear toward the end of the observation period.

To establish the specificity of the pig antibodies, we compared the reactivity of piglet sera with both the 68-kDa protein of *B. bronchiseptica* and an analogous protein (69 kDa) isolated from *B. pertussis*; at the same time we tested the reactivity of sera from children (whooping cough convalescents) against both these antigens. Surprisingly, the sera from piglets reacted strongly with the *B. bronchiseptica*-derived 68-kDa protein antigen and weakly or not at all with the 69-kDa protein derived from *B. pertussis*; the sera from children reacted with both equally well (Fig. 4). The reason for this effect is not yet known. However, both of these antigens react equally well with available monoclonal antibodies prepared against them (data not shown).

DISCUSSION

Under the conditions described herein, the experimental *B. bronchiseptica* infection of nonimmunized animals resulted in 21% mortality, development of the clinical signs (coughs, sneezing, and pneumonia) in 100% of the animals, and changes in the nasal skeleton in 74% of cases. These results are comparable to those observed after the monovalent *B. bronchiseptica* infection described previously (5, 7, 8, 15). Since this model appears to be consistent and suitable for an assay of the efficacy of whole cell vaccines, we felt justified in using it in the evaluation of the protective effect of antibodies specific for a single protein derived from the outer membrane of *B. bronchiseptica*. The 68-kDa protein antibodies conferred significant protection (reduction of pneumonia to 34%, reduction in the frequency and duration of coughs), and severe atrophic rhinitis was noted in one piglet only (8%). These data strongly suggest that the 68-kDa protein is a significant protective antigen of *B. bronchiseptica*. The 68-kDa protein of *B. bronchiseptica* has been

TABLE 2. Results of postmortem examination[a]

Origin	Piglets			No. of piglets with pneumonia graded as follows:				No. of piglets with the following changes in turbinates:			
	No. born	No. challenged	No. dead	0	+	++	+++	0	+	++	+++
Nonimmunized sows 50038 and 50039	19	19	4	0	2	3	14	4[b]	1	1	13
Immunized sow 50041	12	12	1	8	3	1	0	1	8	2	1
Nonimmunized sows 50037 and 50045	13	0	0	0	0	0	0	0	0	0	0

[a] See Materials and Methods for explanations of grading systems.
[b] Two piglets died soon after the challenge, before changes of turbinates could develop.

previously demonstrated in association with adenylate cyclase activity (12). However, the concentration of adenylate cyclase enzyme in the preparation used in these experiments was too low (1.6 μg per 1 mg of the 68-kDa protein) to contribute significantly as an immunizing antigen. Nevertheless, the isolated 68-kDa protein appears to be less effective than whole cell vaccines, which are able to prevent totally the destructive effect on the nasal skeleton (15). There may be several reasons for this. The isolated protein may lack the adjuvant effect of the whole cell, its configuration may have been partially damaged by the isolation process, or it may need the presence of another, so far undefined, supportive antigen. Several important virulence factors have been suggested, such as pili (1) and dermonecrotic toxin (17), but their role in protection is unknown. We do not agree with the observation of Lugtenberg et al. (9), who suggested that lipopolysaccharide is a protective antigen against *B. bronchiseptica* infection. In a previous study (15), the most efficient whole cell vaccines did not induce high titers of anti-lipopolysaccharide antibodies, whereas the vaccines that did not protect induced anti-lipopolysaccharide antibodies almost exclusively and in very high titers. The assumption of the importance of the lipopolysaccharide in protection by Lugtenberg et al. (9) was based upon the phase III lipopolysaccharide present in strain 53453, which is evidently *vir* and lacks several factors, including the 68-kDa protein (12, 15).

During *B. bronchiseptica* infection, the organism actively multiplies in the respiratory tract and remains detectable for at least a month after exposure. This also has been observed by others (3). Characteristically, the whole layer of the mucosa is infiltrated by lymphocytes and macrophages, whereas polymorphs are scarce. The tissue is apparently removed as a consequence of housekeeping immune processes. The presence of osteoclasts is abundant at the end of the disease, when the acute process has finished. The inflammatory lymphocytic infiltration with mature lymphocytes is common to all *Bordetella* diseases (16) and cannot be caused by the lymphocytosis-promoting factor (pertussis toxin) because neither *B. bronchiseptica* nor *B. parapertussis* produces it (21).

We are well aware that under the conditions maintained during our experiments *B. bronchiseptica* infection does not lead to the development of the severe nasal lesions, such as prognatism and distortion of snouts, which are observed in experimental *B. bronchiseptica* infection in association with toxigenic *Pasteurella multocida* type D (6, 7a, 18, 19) and which resemble the naturally occurring disease. *B. bronchiseptica* infection predisposes the host to infection with other agents (1). A prerequisite for the destructive atrophic rhinitis by *B. bronchiseptica* in piglets is the infection of young animals a few days after their birth. This is the period during which the build-up of the nose skeleton proceeds at an almost embryonic rate, with the balance of the clastic and blastic processes regulated in the favor of the latter. The most interesting observation with the monovalent *B. bronchiseptica* infection model in newborn specific-pathogen-free piglets is the fact that severe changes to turbinates were observed in piglets investigated 4 to 5 weeks after the challenge. However, when half the littermates were left and investigated at their slaughter weight, the turbinates in most of them could be seen to have regenerated almost to normal, even though the mucosa may still harbor *B. bronchiseptica*. It seems that the pathophysiological process leading to the destruction of turbinates is (completely?) reversible even in the presence of bacteria. It is tempting to speculate that the *Bordetella* disease affects the regulatory processes involved in nasal skeleton development in some as yet unknown

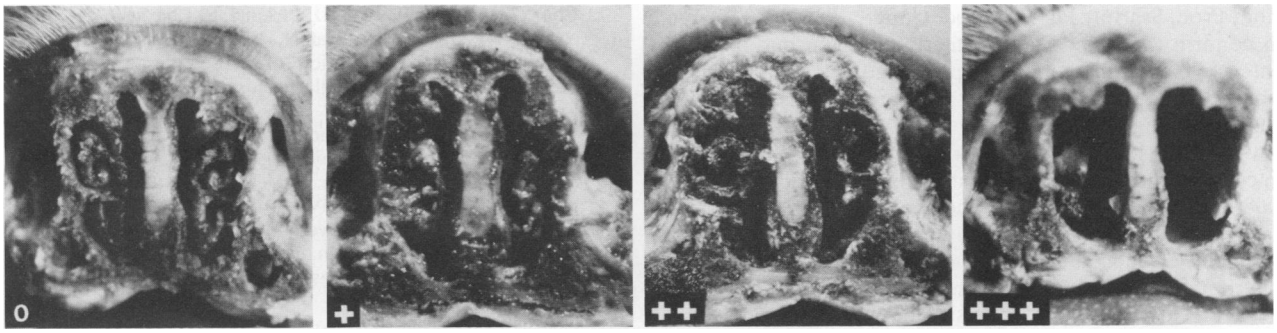

FIG. 2. Grading of the changes to the turbinates after an experimental *B. bronchiseptica* infection. Snout sections of piglets sacrificed 4 weeks after the challenge: normal snout (○), congestion and/or hyperplasia of conchae (+), partial regression of conchae (++), almost complete disappearance of conchae (+++).

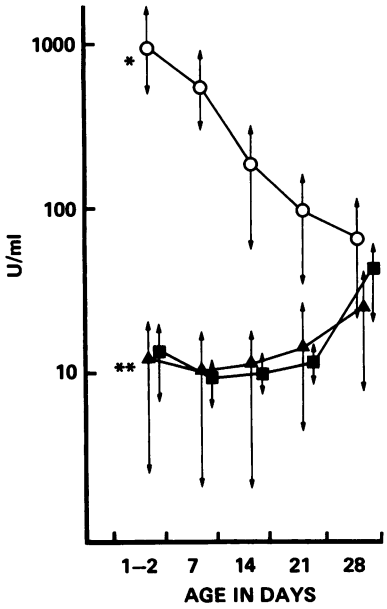

FIG. 3. Anti-68-kDa protein antibody titers in sera from challenged piglets from immunized sow 50041 (○) and nonimmunized sows 50038 (■) and 50039 (▲) as determined by enzyme-linked immunosorbent assay. Bars represent standard errors of samples in the groups. Asterisks represent antibody titers of colostrum from the corresponding sows. Samples were taken 1 or 2 days after birth, at regular weekly intervals, and after sacrifice.

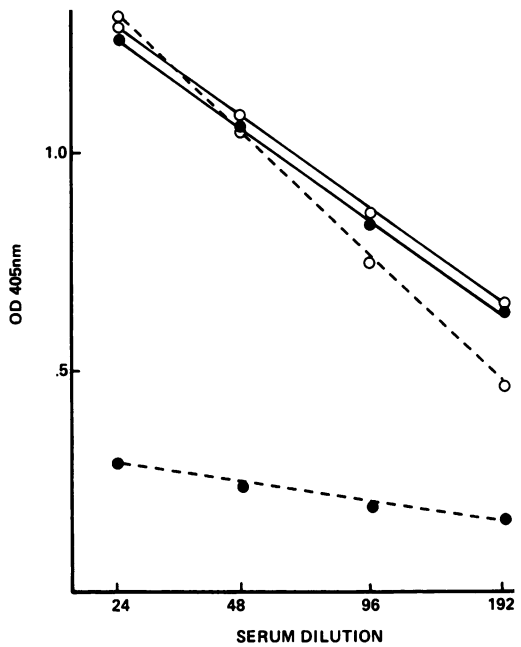

FIG. 4. Comparison of enzyme-linked immunosorbent assay readings (optical density [OD] at 405 nm) of sera from a piglet (●) and from a child (○). Sera were reacted with 68-kDa protein of *B. bronchiseptica* (——) and the 69-kDa protein of *B. pertussis* (– – –). Two plates coated with one or the other antigen were allowed to react with dilutions of five serum samples each from piglets and children (whooping cough convalescents) and finally reacted with corresponding conjugates. All of the tested sera reacted alike, but for the graphical representation only data from one sample each from a piglet and from a child giving similar optical density readings with the 68-kDa protein are shown.

fashion. Where there is predominant mononuclear infiltration of the inflamed tissue, the 68-kDa protein, which does not have a direct effect on the tissue (i.e., does not possess necrotic or pyogenic properties) (11), may have an effect on a hypothetical regulatory mechanism of the host, leading to the destruction of conchae. The anti-68 kDa protein antibodies formed during this prolonged infection apparently have a neutralizing effect on the 68-kDa protein activity, whereby the inhibitory regulatory events controlling turbinate development are switched off, thus allowing for the regeneration of the conchae. We are pursuing this hypothesis in further experiments.

Our results confirm further the etiological role of *B. bronchiseptica* in the genesis of atrophic rhinitis and are in agreement with the observations of Kume et al. (K. Kume, T. Nakae, and T. Yoshikawa, Proc. 9th Int. Pig Vet. Soc. Congr., 1986, p. 223) and Oyamada et al. (T. Oyamada, T. Yoshikawa, H. Yoshikawa, M. Shimizu, T. Nakai, and K. Kume, Proc. 9th Int. Pig Vet. Soc. Congr. 1986, p. 235). We also confirm that candidate protective antigen may be pinpointed by screening sera from protected and nonprotected animals (12, 15) and that the atrophic rhinitis model in aerosol-challenged mice (11, 13, 14) is a valid model for the study of the disease on the laboratory scale.

We find the exclusive reactivity of piglet antibodies with the 68-kDa protein intriguing. The analogous 69-kDa protein of *B. pertussis*, which is similarly controlled by the *vir* locus, has recently been cloned and sequenced (2a). Preliminary data from Southern blot experiments show a difference in DNA-DNA hybridization intensity between the genes encoding the 68-kDa proteins; analysis of these differences may provide the reasons for the high specificity of piglet antibodies for the 68-kDa protein.

ACKNOWLEDGMENTS

The assistance of P. Morvan, A. Labbe, R. Cariolet, M. Le Menec, M. Lagadic, and N. Baker is greatly appreciated. We also thank D. G. Burstyn for making his computer program available, Ann Cleverly for statistical evaluation, and I. Charles for useful discussions. We thank T. Silva for typing the manuscript.

LITERATURE CITED

1. **Bemis, D. A., and S. A. Wilson.** 1985. Influence of potential virulence determinants on *Bordetella bronchiseptica*-induced ciliostasis. Infect. Immun. **50**:35–42.
2. **Cariolet, R.** 1986. Bilan de dix annees d'utilisation de porcs exempts d'organismes pathogenes specifiques (EOPS) a la Station de Pathologie Porcine de Ploufragan. Journ. Rech. Porcine Fr. **18**:321–330.
2a. **Charles, I. G., G. Dougan, D. Pickard, S. Chatfield, M. Smith, P. Novotny, P. Morrissey, and N. Fairweather.** 1989. Molecular cloning and characterization of protective outer membrane protein P.69 from Bordetella pertussis. Proc. Natl. Acad. Sci. USA **86**:3554–3558.
3. **Gois, M., H. J. Barnes, and R. F. Ross.** 1983. Potentiation of turbinate atrophy in pigs by long-term nasal colonisation with *Pasteurella multocida*. Am. J. Vet. Res. **44**:372–378.
4. **Hewlett, E. L., A. A. Weiss, J. K. Crane, R. D. Pearson, H. J. Anderson, G. A. Myers, W. S. Evans, L. L. Hantske, D. Smith, and M. J. Cronin.** 1986. Bordetella extracytoplasmic adenylate cyclase: actions as a bacterial toxin. Dev. Biol. Stand. **61**:21–26.
5. **Kobisch, M.** 1983. Passive protection against experimental infection with *Bordetella bronchiseptica*: efficacy evaluation of 7 vaccines, p. 177–192. *In* K. B. Pedersen and N. C. Nielson (ed.), Atrophic rhinitis of pigs. Commission of European Com-

munities report EUR 8643N. Office for Official Publications of the European Communities, Luxenbourg.
6. **Kobisch, M.** 1986. Mise au point d'un modele experimental associant *B. bronchiseptica* et *P. multocida* dans le controle de vaccines contre la rhinite atrophique du porc. J. Rech. Porcine France **18**:341–347.
7. **Kobisch, M., M. Lagadic, M. Le Menec, and K. Irgens.** 1984. Infection a *B. bronchiseptica* et rhinite atrophique chez le porc. Evaluation de differente methodes de diagnostic. Rec. Med. Vet. **6**:573–579.
7a. **Kobisch, M., and A. Pennings.** 1989. An evaluation in pigs of Nobi-Yac AR and an experimental atrophic rhinitis vaccine containing P. multocida DNT-toxoid and B. bronchiseptica. Vet. Rec. **124**:57–61.
8. **Kobisch, M., J. P. Tillon, and G. Perrin.** 1979. Pathologie respiratoire du porc: determination du pouvoir pathogene experimental d'une souche de *Bordetella bronchiseptica*. Rec. Med. Vet. **155**:225–232.
9. **Lugtenberg, B., R. Van Boxtel, R. Van Den Bosch, M. De Jong, and P. Storm.** 1984. Biochemical and immunological analyses of the cell surface of *Bordetella bronchiseptica* isolates with special reference to atrophic rhinitis in swine. Vaccine **2**:265–273.
10. **Manclark, C. R., B. D. Meade, and D. G. Burstyn.** 1986. Serological response to *Bordetella pertussis*, p. 338–394. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), Manual of clinical laboratory immunology, 3rd ed. American Society for Microbiology, Washington, D.C.
11. **Montaraz, J. A., P. Novotny, and J. Ivanyi.** 1985. Identification of a 68-kilodalton protective protein antigen from *Bordetella bronchiseptica*. Infect. Immun. **47**:744–751.
12. **Novotny, P., A. P. Chubb, K. Cownley, and J. A. Montaraz.** 1985. Adenylate cyclase activity of a 68,000-molecular-weight protein isolated from the outer membrane of *Bordetella bronchiseptica*. Infect. Immun. **50**:199–206.
13. **Novotny, P., A. P. Chubb, K. Cownley, J. A. Montaraz, and J. E. Beesley.** 1985. Bordetella adenylate cyclase: a genus specific protective antigen and virulence factor. Dev. Biol. Stand. **61**:27–41.
14. **Novotny, P., and K. Cownley.** 1987. Infection de porcelet par *Bordetella bronchiseptica*. Rec. Med. Vet. **163**:431–438.
15. **Novotny, P., M. Kobisch, K. Cownley, A. P. Chubb, and J. A. Montaraz.** 1985. Evaluation of *Bordetella bronchiseptica* vaccines in specific-pathogen-free piglets with bacterial cell surface antigens in enzyme-linked immunosorbent assay. Infect. Immun. **50**:190–198.
16. **Olsen, L. C.** 1975. Pertussis. Medicine **54**:427–469.
17. **Roop, M. R., II, H. P. Veit, R. J. Sinsky, Sandra P. Veit, E. L. Hewlett, and E. T. Kornegay.** 1987. Virulence factors of *Bordetella pertussis* associated with the production of infectious atrophic rhinitis and pneumonia in experimentally infected neonatal swine. Infect. Immun. **55**:217–222.
18. **Rutter, J. M.** 1983. Virulence of *Pasteurella multocida* in atrophic rhinitis of gnotobiotic pigs infected with *Bordetella bronchiseptica*. Res. Vet. Sci. **34**:287–295.
19. **Rutter, J. M., and X. Rojas.** 1982. Atrophic rhinitis in gnotobiotic piglets: differences in the pathogenicity of *Pasteurella multocida* in combined infections with *Bordetella bronchiseptica*. Vet. Rec. **110**:531–535.
20. **Shattuck, R. L., D. J. Oldenburg, and D. R. Storm.** 1985. Purification and characterization of a calmodulin-sensitive adenylate cyclase from *Bordetella pertussis*. Biochemistry **24**: 6356–6362.
21. **Weiss, A. A., and E. L. Hewlett.** 1986. Virulence factors of *Bordetella pertussis*. Annu. Rev. Microbiol. **40**:661–686.