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Virulence of *Bordetella bronchiseptica*: Role of Adenylate Cyclase-Hemolysin

PASCALE GUEIRARD[1,2] AND NICOLE GUISO[1]*

Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15,[1] and Laboratoire Virbac, 06511 Carros Cedex,[2] France

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***Bordetella bronchiseptica* is a pathogen of laboratory, domestic, and wild animals and sometimes of humans. In the present study some characteristics of the virulence of *B. bronchiseptica* isolates of different origin were studied. All isolates had similar phenotypes, similar bacteriological characters, and synthesized adenylate cyclase-hemolysin, filamentous hemagglutinin and pertactin but not pertussis toxin. These isolates, however, differed in their ability to express dermonecrotic toxin and to cause a lethal infection, but no correlation was found with the human or animal origin of the isolates. The fact that the most virulent isolate did not express dermonecrotic toxin suggests that this toxin does not play an important role in the virulence of the bacteria in the murine model. After infection with virulent *B. bronchiseptica* a very early synthesis and a persistence of anti-adenylate cyclase-hemolysin and anti-filamentous hemagglutinin antibodies were observed in the sera of infected mice, suggesting a persistence of the bacteria or of its antigens. *B. bronchiseptica* adenylate cyclase-hemolysin was purified and was shown to be a major protective antigen against *B. bronchiseptica* infection. Furthermore, we showed that its immunological and protective properties were different from that of *B. pertussis* adenylate cyclase-hemolysin, confirming that *Bordetella* species are immunologically different.**

Bordetella bronchiseptica is a pathogen of laboratory, domestic, and wild animals and sometimes of humans (8, 20, 37, 46). This bacterium causes kennel cough in dogs, atrophic rhinitis in piglets, and bronchopneumonia in rabbits and guinea pigs (20). In humans, evidence suggests that *B. bronchiseptica* may be encountered as a commensal or colonizer of the human respiratory tract and rarely as a pathogen but may act as a predisposing factor for respiratory disease (16, 20, 45). *B. bronchiseptica* is closely related to *Bordetella pertussis*, the agent of whooping cough, as shown by DNA hybridization (25), multilocus enzyme electrophoresis (34), and sequence analysis (3).

Research, mainly focused on *B. pertussis*, has identified some factors involved in the virulence of the bacteria. These factors include filamentous hemagglutinin (FHA), fimbriae, and pertactin, which play a role in the adhesion of the bacteria (13, 28, 39, 40); pertussis toxin (PTX), the A-B type toxin responsible for many biological effects (44); dermonecrotic toxin (DNT) and tracheal cytotoxin, responsible for the destruction of the ciliated cells (15, 17, 30); and adenylate cyclase-hemolysin (AC-Hly), the RTX toxin which is able to disrupt host cellular functions by penetrating into the cells and by increasing, after activation by calmodulin, intracellular cyclic AMP concentration (14, 22). Expression of these factors, with the exception of tracheal cytotoxin, is under the control of the *Bordetella* virulence gene (*bvg*) locus, a two-component sensory transduction system (5). Two mechanisms of regulation have been characterized. One is called antigenic modulation since there is repression or activation of the synthesis of virulence factors depending on growth conditions; the other is called phase variation and is dependent on mutations resulting in *vir* variants which no longer express any of the virulence genes, irrespective of environmental conditions. The variants arise at a frequency of $1/10^3$ to 10^6 bacteria (32).

All factors involved in *B. pertussis* virulence seem to be also synthesized by *B. bronchiseptica* (9, 15, 17, 18, 33), with the exception of PTX (4, 31). The regulation of the expression of these factors is similar to that in *B. pertussis* (5). The exact role of these factors in the pathogenesis of *B. bronchiseptica* infection is not well documented with the exception of that of pertactin. The structural gene for this outer membrane protein has been cloned and sequenced (29). It has been shown that protection against *B. bronchiseptica*-mediated atrophic rhinitis correlates with the presence of pertactin (36), and maternal antibodies to pertactin were protective in experiments with specific-pathogen-free piglets (36). However, the isolated protein alone did not induce effective protection (36). The other factors are probably involved in *B. bronchiseptica* virulence, as in *B. pertussis* virulence. However, some important differences have to be noted between *B. bronchiseptica* and *B. pertussis*: (i) *B. bronchiseptica* is urease positive and motile, two characters not found in *B. pertussis*; (ii) *B. bronchiseptica* is able to survive in the environment and to grow in lake water (38), suggesting diverse ecological niches for this bacterium; and (iii) *B. bronchiseptica* may be invasive and cause bacteremia in immunocompromised humans (10, 23). For the last reasons, studies are necessary to determine which factors are involved in the pathogenesis of this bacterium.

In the present study, using a murine respiratory model, some characteristics of the virulence of *B. bronchiseptica* were studied. First, we analyzed respiratory and systemic responses to antigens of *B. bronchiseptica* following infection of mice with virulent and avirulent *B. bronchiseptica*. The results revealed a very early synthesis and a persistence (more than 70 weeks) of anti-AC-Hly and anti-FHA antibodies in the sera of mice infected with virulent bacteria, whereas antipertactin antibodies were detected much later after infection and did not persist as long as the anti-AC-Hly and anti-FHA antibodies. Secondly, *B. bronchiseptica* AC-Hly was purified and its enzymatic, immunological, and protective properties were examined. Our results indicate

* Corresponding author.

TABLE 1. Bacterial strains used in this study

Strain	Virulence[a]	Origin	Source or reference
B. pertussis			
Tohama	Vir^+	Human	38
18323	Vir^+	Human	32
B. bronchiseptica			
9.73H+	Vir^+	Rabbit	This study
9.73H−	Vir^-	Rabbit	This study
LAPR	Vir^+	Rabbit	This study
5	Vir^+	Piglet	This study
11	Vir^+	Dog	This study
12	Vir^+	Piglet	This study
PRE	Vir^+	Human	This study
DEL	Vir^+	Human	This study
REM	Vir^+	Human	This study
SEI	Vir^+	Human	This study
GAN	Vir^+	Human	This study

[a] Virulence was determined by the ability of the different strains to produce hemolysis on BGA medium after isolation from humans or animals and was confirmed in the murine respiratory model.

that AC-Hly plays an important role in *B. bronchiseptica* virulence and is a major protective antigen against *B. bronchiseptica* infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table 1. Bacteria were grown on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood (BGA) at 36°C for 48 h and again for 24 h. Subculturing in liquid medium was done in Stainer-Scholte medium (42) for 20 h at 36°C, until the optical density measured at 650 nm reached 1.0.

9.73H− is an avirulent phase variant isolated after several passages on Stainer-Scholte medium and selected for its inability to produce hemolysis on BGA medium.

For Western blot (immunoblot) analysis, bacteria grown on BGA were resuspended in saline at a concentration of 2×10^{10} CFU/ml, diluted in Laemmli buffer, and boiled for 15 min (27).

Protein purification. *B. pertussis* and *B. bronchiseptica* AC-Hlys were purified from the bacteria after urea extraction by using a calmodulin affinity column, as described previously (21, 23). The enzyme preparations were stored in 8 M urea in 50 mM Tris hydrochloride (pH 8)–0.2 mM $CaCl_2$ at −20°C. All preparations were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and had less than 0.0001% endotoxin as determined by the *Limulus* amoebocyte lysate assay. The antisera raised against purified *B. pertussis* or *B. bronchiseptica* AC-Hly did not recognize purified FHA, PTX, or pertactin (data not shown).

AC, invasive, and hemolytic activity assays. AC activity was measured as previously described (26). One unit of AC activity corresponds to 1 μmol of cyclic AMP formed per min at 30°C, pH 8.0. Invasive (internalized AC) and hemolytic activities of AC-Hly were determined at 37°C, using washed sheep erythrocytes (10^9/ml) as described previously (7). Protein concentrations were determined by the method of Bradford (11).

Dermonecrotic activity assay. Dermonecrotic activity was assayed by the method of Nagano et al. (35). Mice were depilated and injected intradermally with 0.1 ml of a bacterial suspension of each isolate (3×10^9 CFU/ml); a necrotic lesion more than 5 mm in diameter observed 72 h after injection defined a positive reaction.

Immune sera. Groups of 10 4-week-old female BALB/c mice received subcutaneously 10 μg of purified FHA, purified detoxified PTX, or purified AC-Hly adsorbed on aluminium hydroxide four times at 3-week intervals. Mice were bled 7 days after the last injection. The specificity of polyclonal antibodies was checked by Western blotting using purified antigens and a whole *B. pertussis* bacterial suspension.

Electrophoresis and immunoblotting methods. SDS-PAGE was performed on ready-to-use 8 to 25% polyacrylamide gels in the PhastSystem (Pharmacia). After electrophoresis the proteins were transferred from polyacrylamide gels to Hybond C-Super membranes (Amersham). After a blocking step, the membranes were incubated with 10^{-3} serum dilutions at 4°C overnight. Immunochemical detection was performed using horseradish peroxidase-labelled sheep anti-mouse immunoglobulins and an enhanced chemiluminescence system (ECL; Amersham), which has several advantages, including high sensitivity (the first serum dilution that can be used is 10^{-3} and not 10^{-1} or 10^{-2}) and speed (a maximum of 30 min for revelation of the reaction). In this detection system, blackening of the X-ray film is proportional to the light emission from the sample. In order to compare results obtained with different sera, the treated membranes were exposed to X-ray film for the same amount of time. For all immunoblotting experiments performed in this study the membranes were exposed to X-ray film for 6 s, 1 min, and 10 min. Detection of the immune complex was classified as follows: after 6 s, +++; after 1 min, ++; after 10 min, +; no detection, −.

Vaccines. *B. bronchiseptica* grown on BGA medium was resuspended in saline and heat killed (20 min at 56°C). Whole-cell vaccine suspensions were diluted in saline to 5×10^8 CFU per dose and adsorbed on aluminium hydroxide (250 μg/ml, final concentration) prior to use. After elimination of urea by using Sephadex G-25, AC-Hly preparations were adsorbed on aluminium hydroxide at a 250-μg/ml final aluminium concentration. The final concentration of AC-Hly varied between 3 and 15 μg per immunization depending on the experiment.

Active immunizations. Female 3- to 4-week-old BALB/c mice (CERJ, St. Berthevin, France) were injected subcutaneously with 250 μl of whole-cell vaccine or AC-Hly twice at a 2-week interval. Controls were injected with 250 μl of control buffer containing aluminium hydroxide. In order to assess the presence of circulating antibodies, mice were bled 1 week after the last injection. The respiratory sublethal infections were performed 2 weeks after the second immunization.

Intranasal infection of mice. *B. pertussis* and *B. bronchiseptica* were grown on BGA medium as described above. Bacteria were resuspended and diluted in 1% Casamino Acids and then serially diluted to provide challenge inoculum dilutions to evaluate the 50% lethal doses (LD_{50}). For respiratory infection, 50 μl of bacterial suspension was injected intranasally into groups of 10 3- to 4-week-old female Swiss mice (CERJ). The LD_{50} for the challenge inocula were determined by recording daily the number of dead mice during 30 days.

Sublethal challenges were performed by intranasal injections of 50 μl of bacterial suspensions. Infected mice were sacrificed by cervical dislocation 1 h after exposure (at time

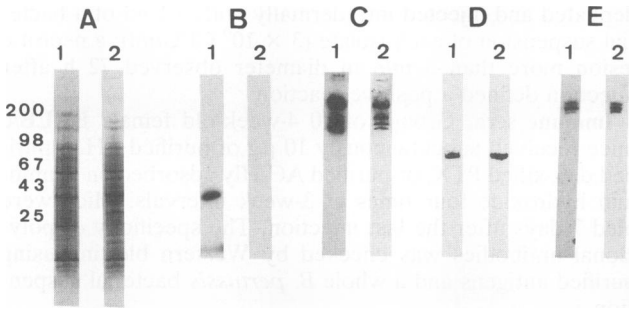

FIG. 1. Characterization of PTX, FHA, pertactin, and AC-Hly in bacterial suspensions of *B. pertussis* and *B. bronchiseptica*. One microliter (500 ng of total proteins) of *B. pertussis* (lane 1) or *B. bronchiseptica* (lane 2) bacterial suspension was submitted to SDS–8 to 25% PAGE, and proteins were stained with Coomassie blue (A) or were transferred to Hybond C-Super membranes after electrophoresis. The membranes were incubated with polyclonal sera raised against *B. pertussis* detoxified PTX (B), *B. pertussis* FHA (C), *B. pertussis* pertactin (D), or *B. pertussis* AC-Hly (E). The immunochemical detection was performed with horseradish peroxidase-labelled sheep anti-mouse immunoglobulins, using the ECL detection system from Amersham.

designated day 0) and at various days thereafter (four to six mice per time point). Lungs were removed and homogenized in saline with tissue grinders. Dilutions of lungs homogenates were plated on BGA, and CFU were counted after 3 days of incubation at 36°C. All experiments were performed three times, and they gave consistent results.

Analysis of respiratory antibodies. At different time intervals after intranasal infection, mice were anesthetized with chlorobutanol and their tracheas were cannulated with a piece of polyethylene tubing. Sterile saline (0.5 ml) was gently instillated into the lungs and withdrawn two times. The bronchoalveolar lavage fluids were centrifuged, and the presence of anti-AC-Hly, anti-FHA, and antipertactin antibodies was immediately analyzed by Western blotting with a 10^{-2} dilution. We checked that the presence of antibodies in bronchoalveolar lavage fluid was not due to blood contamination.

RESULTS

Immunological detection and regulation of synthesis of *B. bronchiseptica* FHA, pertactin, and AC-Hly. As shown in Fig. 1, polyclonal antibodies specific to purified *B. pertussis* FHA, pertactin, or AC-Hly recognized the corresponding factors in *B. bronchiseptica* bacterial suspensions. This indicates that *B. pertussis* and *B. bronchiseptica* synthesize factors that cross-react immunologically. However, *B. pertussis* anti-PTX antibodies did not recognize any protein (Fig. 1B), confirming the nonexpression of this factor by *B. bronchiseptica* (4, 26). As shown in Table 2, all *B. bronchiseptica* isolates analyzed in this study synthesized AC-Hly, FHA, and pertactin but their ability to produce DNT varied. As expected, AC-Hly, FHA, and pertactin were not detected in a bacterial suspension of the *bvg* mutant 9.73H− isolated after many subcultures in Stainer-Scholte synthetic medium of the isolate 9.73H+ (Table 2). The expression of these different factors varied under different growth conditions: they were not detected in bacterial suspensions of *B. bronchiseptica* cultures incubated at 22°C or in the presence of $MgSO_4$ or nicotinic acid (data not shown). These results confirmed that the modulation of the expression of these factors is similar in *B. pertussis* and *B. bronchiseptica* (5).

Virulence of *B. bronchiseptica* in a murine respiratory model. The ability of different *B. bronchiseptica* isolates to cause a lethal infection in 3- to 4-week-old mice was examined. As shown in Table 2, the different isolates were able to cause a lethal infection, whereas the avirulent mutant was not, even at a dose higher than 10^9 CFU. However, the LD_{50} of the different isolates varied from 5×10^3 to 1×10^8 CFU without any correlation with the human or animal origin of the isolate.

Persistence of *B. bronchiseptica* after sublethal infection. The ability of the virulent *B. bronchiseptica* 9.73H+ to induce a persistent infection was compared with that of the *bvg* mutant 9.73H−. As shown in Fig. 2, the 9.73H+ strain

TABLE 2. Expression of AC-Hly, FHA, pertactin, and DNT and virulence of the different *B. bronchiseptica* isolates

Strain	AC[a]	Hly[b]	AC-Hly[c]	FHA[c]	Pertactin[c]	DNT[d]	LD_{50}[e]
B. pertussis							
Tohama	30	+	+	+	+	+	8×10^7
18323	120	+	+	+	+	+	2×10^6
B. bronchiseptica							
9.73H+	60	+	+	+	+	+	3×10^6
9.73H−	0	−	−	−	−	−	$>10^9$
LAPR	35	+	+	+	+	−	2×10^6
5	45	+	+	+	+	ND[f]	5×10^5
11	40	+	+	+	+	+	2×10^6
12	34	+	+	+	+	ND	2×10^6
PRE	56	+	+	+	+	+	5×10^6
DEL	66	+	+	+	+	−	5×10^3
REM	50	+	+	+	+	−	10^8
SEI	30	+	+	+	+	−	3×10^5
GAN	11	+	+	+	+	ND	4×10^6

[a] AC activity (milliunits per milliliter) assayed in bacterial suspension.
[b] Hemolysin activity detected on BGA plates.
[c] AC-Hly, FHA, and pertactin detected by Western blot.
[d] Dermonecrotic activity was determined as described in Materials and Methods.
[e] LD_{50} determinated after intranasal infection of 4-week-old mice.
[f] ND, not done.

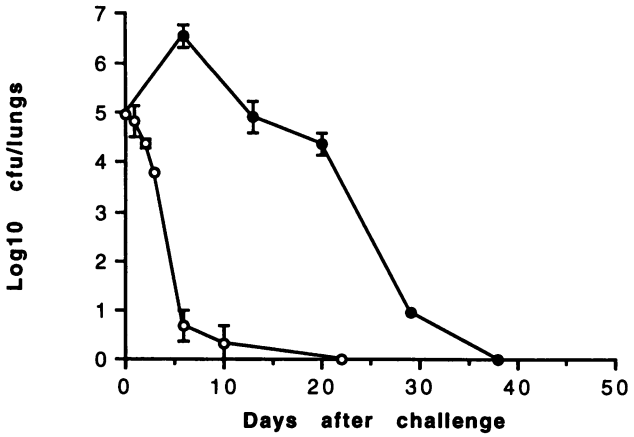

FIG. 2. *B. bronchiseptica* 9.73H+ and 9.73H− colonization of the lungs of mice. Three- to four-week-old mice were challenged intranasally with 10^5 CFU of *B. bronchiseptica* 9.73H+ (●) or 10^5 CFU of *B. bronchiseptica* 9.73H− (○). The plots show the geometric means ± standard errors (bars) for six mice per time point.

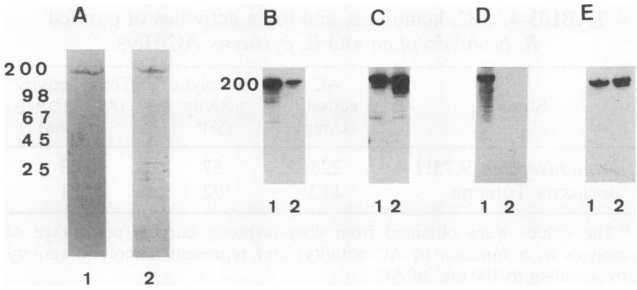

FIG. 3. Immunological properties of *B. bronchiseptica* and *B. pertussis* AC-Hly. Two hundred nanograms of purified *B. pertussis* AC-Hly (lane 1) or *B. bronchiseptica* AC-Hly (lane 2) was submitted to SDS–8 to 25% PAGE, and proteins were stained with Coomassie blue (A) or transferred to Hybond C-Super membranes and incubated with specific anti-*B. bronchiseptica* AC-Hly serum (B), specific anti-*B. pertussis* AC-Hly serum (C), serum from *B. pertussis* Tohama-infected mice (D), or serum from *B. bronchiseptica* 9.73 H+-infected mice (E). The immunodetection was performed with peroxidase-labelled sheep anti-mouse immunoglobulins, using the ECL detection system from Amersham.

was able to adhere and multiply in the lungs of the mice and persisted for at least 30 days. The *bvg* mutant, which did not express any of the known virulence factors, was not able to adhere and multiply and was cleared within 6 days.

Respiratory and systemic immune responses after infection with live *B. bronchiseptica*. After intranasal infection of mice with 10^5 CFU of *B. bronchiseptica* 9.73H+, respiratory tract and serum immunoglobulins directed against AC-Hly, FHA, and pertactin were analyzed. As shown in Table 3, anti-AC-Hly and anti-FHA antibodies could be detected 2 and 3 weeks after infection, respectively, in the sera of infected mice and they persisted for more than 70 weeks after infection. Furthermore, anti-AC-Hly and anti-FHA antibodies could be detected in bronchoalveolar lavage fluids of infected mice 5 and 13 weeks following the infection. Antipertactin antibodies were detected between 12 and 22 weeks after infection in serum; they never persisted for more than 8 to 12 weeks (Table 3) and were not detected in the bronchoalveolar lavage fluids. Similar results were obtained with of all the other isolates.

No circulating antibody could be detected after infection with 10^5 CFU of the *bvg* mutant (data not shown).

Purification of *B. bronchiseptica* AC-Hly. *B. pertussis* Tohama and *B. bronchiseptica* 9.73H+ AC-Hlys were purified by affinity chromatography using a calmodulin affinity column (21). As shown in Fig. 3A, the purified preparations contained a major polypeptide of 200 kDa corresponding to AC-Hly and several fragments. The fact that these fragments were recognized by monoclonal antibodies specific for the AC domain of *B. pertussis* AC-Hly (data not shown) indicates that they were proteolytic fragments still containing the calmodulin-binding AC domain and copurified with AC-Hly on calmodulin-agarose. These proteolytic fragments had already been detected in *B. pertussis* and *B. bronchiseptica* bacterial suspension samples collected at the end of the culture before any treatment, even before centrifugation.

As shown in Table 4, both *B. bronchiseptica* 9.73H+ and *B. pertussis* Tohama AC-Hlys possessed AC, hemolytic, and invasive activities that were similar or not significantly different. However, their immunological properties were different. As shown in Fig. 3B, specific anti-*B. bronchisep-*

TABLE 3. Serum and respiratory antibodies to AC-Hly, FHA, and pertactin after infection with live *B. bronchiseptica* 9.73 H+

Wk after infection	Titer[a] of the indicated antibodies in:					
	Serum			Bronchoalveolar lavage fluid		
	Anti-AC-Hly	Anti-FHA	Antipertactin	Anti-AC-Hly	Anti-FHA	Antipertactin
0	−	−	−	−	−	−
1	−	−	−	−	−	−
2	+	−	−	−	−	−
3	++	+	−	−	−	−
4	++	+	−	ND[b]	ND	ND
5	+++	++	−	++	++	−
13	+++	+++	+	++	++	−
23	+++	+++	+	ND	ND	ND
42	+++	+++	−	ND	ND	ND
55	+++	+++	−	ND	ND	ND
72	++	+++	−	ND	ND	ND

[a] Detection of antibodies was performed using Western blotting and ECL (Amersham) as described in Materials and Methods. Detection of the immune complex was classified as follows: after 6 s, +++; after 1 min, ++; after 10 min, +; no detection, −.

[b] ND, not done.

TABLE 4. AC, hemolytic, and toxin activities of purified *B. bronchiseptica* and *B. pertussis* AC-Hlys

Strain	AC activity (U/mg)	Hemolytic activity (%)[a]	Toxin activity[b] (AC internalization)
B. bronchiseptica 9.73H+	223	57	93
B. pertussis Tohama	112	92	91

[a] The values were obtained from dose-response curves (percentage of hemolysis as a function of AC activity) and represent hemolytic activity corresponding to 100 mU of AC.

[b] Sheep erythrocytes were incubated for 30 min at 37°C with purified AC-Hly at a final concentration of 40 mU of AC per ml, as described in Materials and Methods. Internalized AC activity is expressed as picomoles of cyclic AMP per min per 5×10^8 cells.

tica AC-Hly antibodies recognized in a Western blot *B. bronchiseptica* and *B. pertussis* AC-Hlys. Similarly, specific anti-*B. pertussis* AC-Hly antibodies recognized both enzymes (Fig. 3C). However, sera of *B. pertussis* Tohama-infected mice collected 30 days after the beginning of the infection recognized the *B. pertussis* AC-Hly but not the *B. bronchiseptica* AC-Hly (Fig. 3D), whereas sera of *B. bronchiseptica* 9.73H+-infected mice collected 30 days after infection recognized both enzymes (Fig. 3E). These results indicate that antisera specific to purified protein, but not sera from convalescent mice, cross-reacts. Thus, these two enzymes appear to be immunologically different.

Protective efficacies of *B. bronchiseptica* AC-Hly, *B. pertussis* AC-Hly, and *B. bronchiseptica* whole-cell vaccine against *B. bronchiseptica* infection. The protective efficacy of *B. bronchiseptica* AC-Hly was evaluated in the murine respiratory model and compared with that of *B. bronchiseptica* whole-cell vaccine. After two immunizations with *B. bronchiseptica* AC-Hly, anti-AC-Hly antibodies were detected in both bronchoalveolar lavage fluids and sera of the immunized mice. Two weeks after the last immunization, infection with a sublethal dose of virulent *B. bronchiseptica* 9.73H+ was performed. As can be seen in Fig. 4, in the group of mice immunized with *B. bronchiseptica* AC-Hly, the bacteria did not adhere or multiply in the lungs of infected mice. On day 3 after infection there was already a 5-log difference in the number of bacteria in the lungs between the group of mice immunized twice with 15 μg of purified *B. bronchiseptica* AC-Hly and the group of control mice immunized with aluminium hydroxide alone.

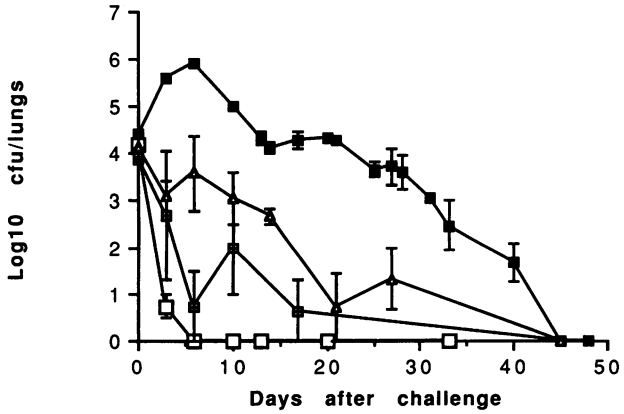

FIG. 4. Protective activities of *B. bronchiseptica* AC-Hly against *B. bronchiseptica* lung colonizations. Mice 3 to 4 weeks old were immunized twice, at a two-week interval, with 15 (□), 10 (⊞), or 3 (△) μg of *B. bronchiseptica* AC-Hly adsorbed on aluminium hydroxide or with buffer containing aluminium hydroxide alone as a control (■). They were infected intranasally two weeks later with 10^5 CFU of *B. bronchiseptica*. The plots show the geometric means ± standard errors (bars) for four mice per time point.

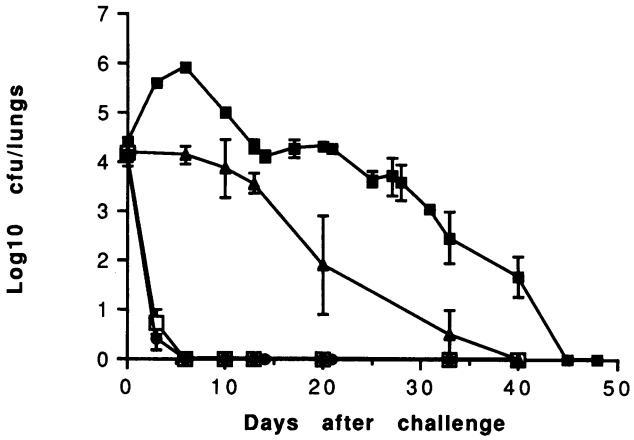

FIG. 5. Protective activities of *B. bronchiseptica* AC-Hly, *B. pertussis* AC-Hly, and *B. bronchiseptica* whole-cell vaccine against *B. bronchiseptica* lung colonization. Mice 3 to 4 weeks old were immunized twice at a two-week interval with 15 μg of *B. bronchiseptica* AC-Hly (□), 15 μg of *B. pertussis* AC-Hly (▲), or *B. bronchiseptica* whole-cell vaccine (●) or with buffer containing aluminium hydroxide alone as a control (■). They were infected intranasally 2 weeks later with 10^5 CFU of *B. bronchiseptica* 9.73H+. The plots show the geometric means ± standard errors (bars) for six mice per time point.

The protective activity of *B. bronchiseptica* AC-Hly was maximal after two immunizations with 15 μg of this enzyme. This efficacy decreased when immunizations were performed with lower doses (Fig. 4). Two immunizations with 15 μg of purified *B. bronchiseptica* AC-Hly protected mice as well as two immunizations with *B. bronchiseptica* whole-cell vaccine (Fig. 5). Surprisingly, the group of mice immunized twice with 15 μg of purified *B. pertussis* AC-Hly, which protected against *B. pertussis* infection (24), were not protected against *B. bronchiseptica* colonization of the lungs (Fig. 5).

DISCUSSION

In the present study, isolates of *B. bronchiseptica* of different origins (human or animal) were analyzed. It was not possible to differentiate these isolates by culture or bacteriological or phenotypical characters. They all synthesized factors such as AC-Hly, FHA, or pertactin antigenically related to *B. pertussis* factors. In each isolate the regulation of expression of these factors was similar to that of the *B. pertussis* factors. However, their ability to produce DNT and to cause a lethal infection in mice varied. The fact that the most virulent isolate did not produce DNT suggests that this factor does not play an important role in the virulence of *B. bronchiseptica* in the murine model. No correlation was found between the virulence of the isolates and their animal or human origin.

After *B. bronchiseptica* infection we observed a very early synthesis of anti-AC-Hly antibodies. These antibodies were detected in the sera and also in bronchoalveolar lavage fluids of infected mice. For this reason, *B. bronchiseptica* AC-Hly

was purified and was shown to possess AC, hemolytic, invasive, and protective activities, as does *B. pertussis* AC-Hly. However, we showed that *B. bronchiseptica* and *B. pertussis* AC-Hlys were immunologically different. In fact, sera from *B. pertussis*-infected mice recognized the *B. pertussis* AC-Hly 200-kDa polypeptide and its proteolytic fragments but failed to recognize *B. bronchiseptica* AC-Hly, whereas sera from *B. bronchiseptica*-infected mice recognized both the *B. pertussis* and the *B. bronchiseptica* AC-Hly 200-kDa polypeptide. This indicates that anti-AC-Hly antibodies synthesized after *B. pertussis* and *B. bronchiseptica* infection are directed against different epitopes: anti-AC-Hly antibodies synthesized after *B. bronchiseptica* infection are directed against *B. pertussis* and *B. bronchiseptica* AC-Hly common epitopes, but anti-AC-Hly antibodies synthesized after *B. pertussis* infection are directed against an epitope(s) specific to *B. pertussis* AC-Hly. This epitope(s) is important for protection, since the protective activity of *B. pertussis* AC-Hly against *B. bronchiseptica* infection is much lower than that of *B. bronchiseptica* AC-Hly. All of these results extend our previous data obtained for *B. pertussis* and *B. parapertussis* (24) to the third *Bordetella* species and confirm that *Bordetella* species are immunologically different.

Immunizations with *B. bronchiseptica* AC-Hly prevent *B. bronchiseptica* initial colonization. Furthermore, the AC-Hly protective activity was similar to that of the whole-cell vaccine, suggesting that this enzyme is a major protective antigen against *B. bronchiseptica* infection.

We have observed an early synthesis of anti-AC-Hly and anti-FHA antibodies in the sera of infected mice. These antibodies persisted for at least 70 weeks after infection. This persistence of antibodies may be due to the persistence of viable *Bordetella* organisms or to the persistence of *Bordetella* antigens. In a recent study, Ambaugh et al. (2), examined the possibility of persistent *B. pertussis* and showed that the latest time point at which they were able to culture bacteria was 8 weeks after infection. However, they could detect *B. pertussis*-specific DNA by polymerase chain reaction in 37.5% of the mice at 26 weeks after infection. Furthermore, recent studies have shown that *B. pertussis* can be internalized by various cells in vitro (19, 43), *B. pertussis* can be associated with alveolar macrophages in vivo (12), and *B. bronchiseptica* can be isolated several times from the same patient, suggesting a persistence of the bacteria (20a). Thus, all of these results suggest a persistent low level of *Bordetella* infection which may contribute to the observed long-lived antibody response. If *B. bronchiseptica*, as *B. pertussis*, persists in the host, PTX is not the factor responsible for this persistence, since *B. bronchiseptica* does not produce this factor. Our observation that antipertactin antibodies were detected a long time after infection may be explained by a delayed synthesis of pertactin as compared with FHA synthesis. In vivo, synthesis of FHA may occur first, inducing a synthesis of anti-FHA antibodies, and then, in order to escape the host immune response, synthesis of another adhesin such as pertactin may occur. Such a delayed synthesis of some *B. pertussis* virulence factors has already been demonstrated in vitro by Scarlato et al. (41).

A mutant unable to express *bvg*-activated gene products such as AC-Hly, FHA, and pertactin was unable to induce an infection and to colonize the respiratory tract of the mice, indicating that these factors are necessary for *B. bronchiseptica* to initiate infection. However, Akerley et al. (1) have preliminary results showing that an antibody response to flagella, whose expression is negatively controlled by the *bvg* locus, accompanies guinea pig colonization by *B. bronchiseptica* (1). This result, if confirmed, suggests that flagella are expressed in vivo during infection. Preliminary results suggest that a *bvg*-repressed gene product, whose function is unknown, may also play a role in bacterial colonization by *B. pertussis* (6).

Our observation that anti-AC-Hly and anti-FHA antibodies are detected earlier than antipertactin antibodies may either reflect differences in the amounts of virulence factors made or their inherent immunogenicity. However, one can suppose that antigenic modulation may occur during infection: first, the bacteria express AC-Hly, which disrupts host cellular functions, such as those of alveolar macrophages, in order to escape the first line of host defense and to initiate the infection; secondly, the bacteria express adhesins such as FHA and/or pertactin in order to adhere and multiply in the respiratory tract of the host; thirdly, antigenic modulation occurs, and the bacteria express factors which were initially repressed, such as flagella or *bvg*-repressed gene products, in order to escape the host immune response and to persist longer in the host. Furthermore, our model proposes that AC-Hly is a major protective antigen against *B. bronchiseptica* infection since it may be the factor required to initiate the infection.

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