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The C-Terminal Domain Is Essential for Protective Activity of the *Bordetella pertussis* Adenylate Cyclase-Hemolysin

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The adenylate cyclase-hemolysin of *Bordetella pertussis* consists of a cell-invasive N-terminal adenylate cyclase domain linked to a C-terminal RTX hemolysin containing extensive glycine-rich repeats. The toxin is an essential virulence factor required in the initial stages of infection. Adenylate cyclase-hemolysin was also shown to be a potent vaccinating antigen inducing protection against *B. pertussis* colonization of the mouse respiratory tract. This protective activity depends on a posttranslational fatty-acylation modification. We used a set of deletion derivatives of the recombinant adenylate cyclase-hemolysin to localize the protective epitopes on the 1,706-residue toxin. We show that specific anti-adenylate cyclase-hemolysin antibodies present in the sera of *B. pertussis*-infected mice and humans are directed predominantly against the modification-and-repeat portion of the toxin, contained in the last 800 residues of the adenylate cyclase-hemolysin. These antibodies appear to recognize conformational epitopes present only in a structure formed by the intact C-terminal half of the toxin. There was no correlation between the capacity of the truncated adenylate cyclase-hemolysin derivatives to induce both toxin-neutralizing antibodies upon immunization of mice and protective immunity. However, only the truncated proteins which were recognized by the sera of infected mice and humans and which had their last 800 residues intact had the capacity to induce protection of mice against colonization by *B. pertussis*. This indicates that the structure of the modification-and-repeat region of adenylate cyclase-hemolysin is critical for its protective activity.

The adenylate cyclase-hemolysin (AC-Hly) is an essential virulence factor required in the early stages of respiratory tract colonization by the agent of whooping cough, *Bordetella pertussis* (14, 17, 30, 31, 43). AC-Hly is a 1,706-residue member of the RTX family and is endowed with both invasive adenylate cyclase (AC; cytotoxic) and hemolytic (pore-forming) activities (3, 12, 38, 39, 45). The toxin has a 400-residue N-terminal AC domain (34, 35), which penetrates target cells and catalyzes the formation of supraphysiologic amounts of intracellular cyclic AMP (cAMP), altering cellular functions (10, 15, 25, 27). This cytotoxic activity of AC-Hly is required specifically for lethality, for creation of pulmonary lesions during infection by *B. pertussis* in vivo, and for in vitro induction of apoptosis of alveolar macrophages (30, 32, 43). The C-terminal moiety of AC-Hly (last 1,300 residues) is required for delivery of the AC domain into target cells and exhibits the typical features of RTX proteins (11, 23, 24, 44). This region contains a hydrophobic domain (residues 385 to 913) involved in the hemolytic (pore-forming) activity of AC-Hly (4), a glycine- and aspartate-rich repeat domain (residues 913 to 1612) characteristic of all RTX toxins and involved in calcium binding (7, 36), and a C-terminal secretion signal (13, 16, 41). Like all RTX toxins, AC-Hly is synthesized as an inactive protoxin which is converted to an active toxin by a posttranslational activation dependent on the product of an accessory gene, *cyaC* (2, 26, 28). This activation has been shown recently to be a palmitoylation of the ε-amino group of lysine 983 of AC-Hly (22).

AC-Hly has been shown to be an important protective antigen against bacterial colonization by a murine respiratory model (18, 20, 21). Both active and passive immunizations with AC-Hly significantly shorten the period of bacterial colonization of the mouse respiratory tract (9, 21). Furthermore, anti-AC-Hly antibodies are detected in the sera of *B. pertussis*-infected children and adults (1, 19). We have shown recently that the CyaC-mediated fatty acylation of AC-Hly is essential not only for its toxic activity but also for its protective activity. In fact, the *cyaC* gene product confers protective activity on recombinant AC-Hly produced in *Escherichia coli* (5). However, it remained unclear whether the palmitoylated lysine 983 is itself an essential component of the protective epitope(s) of AC-Hly, whether the palmitoylation of this residue induces structural changes in the toxin molecule which are essential for correct presentation of protective epitopes located elsewhere in the molecule, or both. In the present study, we have used the murine respiratory model and different truncated constructs of the recombinant AC-Hly (r-AC-Hly) to approach the localization of the protective epitopes of AC-Hly. Our results indicate that the protective epitope(s) of AC-Hly is most likely conformational and dependent on the integrity of the 800-residue C-terminal repeat portion of AC-Hly.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Virulent *B. pertussis* 18323 was grown on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood (BG medium) at 36°C for 72 h and again for 24 h. Subcultures in liquid medium were performed in Stainer-Scholte medium (42) for 20 h at 36°C to an optical density at 650 nm of 1.0.

The plasmids used for production of r-AC-Hly and its truncated derivatives in *E. coli* have already been described elsewhere (5, 29, 40) (detailed schemes of the plasmids will be provided upon request to P. Sebo). These plasmids allow production of the different proteins (see Fig. 1) in the presence of the activating protein CyaC. *E. coli* XL-1 Blue (Stratagene) strains harboring the respective plasmids were grown at 37°C on 2× YT medium (1.6% Bacto tryptone, 1% Bacto yeast extract, 85 mM NaCl) containing 100 mg of ampicillin per liter to an

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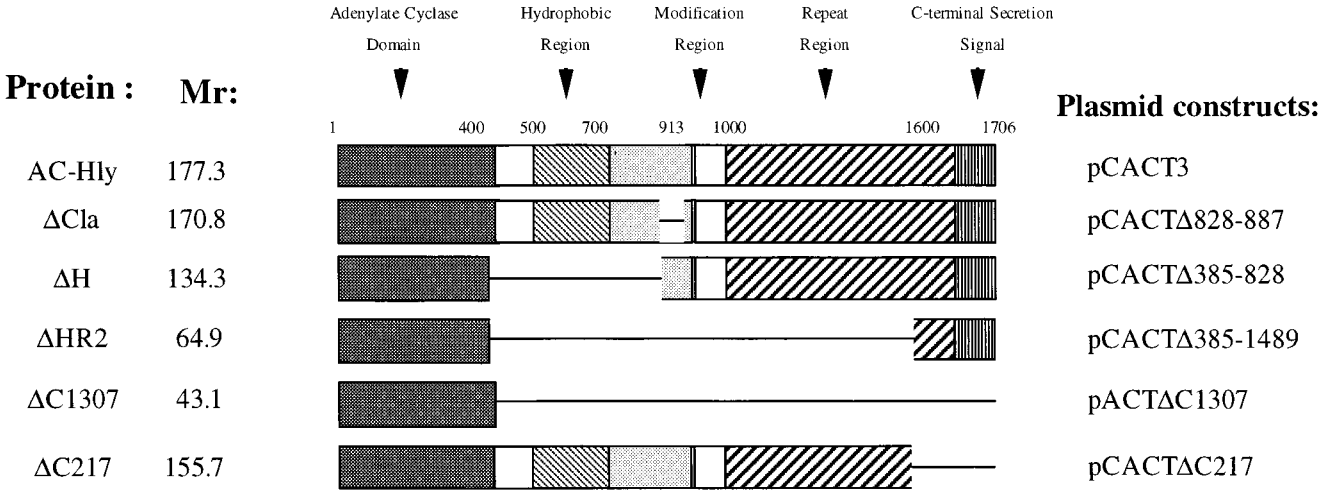

FIG. 1. Schematic representation of the used truncated AC-Hly proteins. The construction of the plasmids and production of the corresponding truncated proteins were described previously (29, 40, 41). The numbers in the column labelled Mr indicate relative molecular masses (in thousands) of the toxins calculated from their deduced amino acid sequences. The numbers that follow the symbol Δ in the name of the plasmid are the numbers of the first and the last amino acids of the deleted parts of the *cyaA* reading frame. The numbers that follow the symbol ΔC represent the missing C-terminal residues. The *cyaA* alleles are coexpressed with the *cyaC* gene under the control of the transcriptional and translational initiation signals of the *lacZ* gene identical to that on pCACT3 (5). ΔC1307 was produced from pDIA 5240 (pACTΔC1307) in the presence of *cyaC* expressed in *trans* from a compatible plasmid, pPS4C (5, 40).

optical density at 600 nm of 0.5 to 0.7 and induced for production of AC-Hly by isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) for an additional 4 h (5).

Assays of AC, hemolytic, and cytotoxic activities. AC activities were measured as described previously (33). One unit of AC activity corresponds to 1 μmol of cAMP formed per min at 30°C and pH 8.0. Hemolytic and cytotoxic activities of AC-Hly were determined, at 37°C, with washed sheep erythrocytes (10^9/ml) as described previously (3). Protein concentrations were determined by the method of Bradford (8).

AC inhibition assays. Purified *B. pertussis* AC-Hly was incubated at 0.1 U/ml in 50 mM Tris-HCl (pH 7.6)–0.2 mM $CaCl_2$–0.1% Nonidet P-40 with various 100-fold-diluted serum samples for 18 to 20 h at 4°C, and the postincubation AC activity of the samples was measured. AC activity upon incubation with serum from mice immunized with aluminum hydroxide only was taken as 100% activity (0% inhibition). The percent inhibition of AC activity was calculated as follows: % inhibition = 100% − [(100% × postincubation activity)/(control activity)].

Hemolytic activity inhibition assays. One unit of toxin and 5 μl of the different sera were mixed in 1 ml of 10 mM Tris-HCl (pH 8)–2 mM $CaCl_2$–150 mM NaCl–1 μM bovine brain calmodulin and preincubated for 20 min at 4°C. Washed sheep erythrocytes (10^9) were then added, and the remaining hemolytic activity was determined upon incubation for 3 h at room temperature. The unlysed erythrocytes were pelleted by centrifugation at 2,000 rpm (Jouan GR 4.11), and the optical density of the released hemoglobin in the supernatants was measured at 541 nm. Hemolytic activity of toxin incubated with sera from mice immunized only with aluminum hydroxide was taken as 100% activity value (0% inhibition). Sheep erythrocytes incubated without toxin were used as a control of nonspecific lysis. The percent inhibition of hemolytic activity was calculated as follows: % inhibition = 100% − [(100% × postincubation activity)/(control activity)].

Cytotoxic activity inhibition assays. One unit of toxin and 5 μl of the different sera were preincubated at 4°C for 20 min in a total volume of 1 ml of a mixture of 10 mM Tris-HCl (pH 8), 2 mM $CaCl_2$, 150 mM NaCl, 5 mM glucose, 1 mg of bovine serum albumin per ml, and 1 μM bovine brain calmodulin. Then, 10^9 washed sheep erythrocytes were added, and the incubation was continued at 37°C for 30 min. To stop the toxin activity, 50 μl of the erythrocyte suspension was injected into 1 ml of boiling 50 mM sodium acetate (pH 5.2) and heated at 100°C for 5 min. The amount of cAMP formed in the lysed erythrocytes was determined by a standard enzyme-linked immunosorbent assay (ELISA) method (37). Toxin incubated at 4°C was used as a negative control of cytotoxicity. Activity of toxin incubated with serum from mice immunized with aluminum hydroxide only was taken as 100% activity (0% inhibition). The percent inhibition cytotoxic activity was calculated as follows: % inhibition = 100% − [(100% × postincubation activity)/(control activity)].

Electrophoresis and immunoblotting methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on ready-to-use 8 to 25% polyacrylamide gels for the PhastSystem (Pharmacia), and the separated proteins were electrotransferred from polyacrylamide gels to Hybond C-Super membranes (Amersham). After blocking, membranes were incubated at a 10^{-3} dilution with polyclonal sera at 4°C overnight. The immunochemical detection was performed with horseradish peroxidase-labelled sheep anti-mouse immunoglobulins and an enhanced chemiluminescence system (ECL-Amersham).

Production and purification of AC-Hly. *B. pertussis* AC-Hly, *E. coli*-produced r-AC-Hly, and the different recombinant truncated proteins were extracted from the bacteria with urea and purified on calmodulin affinity columns as described previously (21). The enzyme preparations were stored in 8 M urea in 50 mM Tris-HCl (pH 8)–0.2 mM $CaCl_2$ at −20°C. All preparations were analyzed for purity by SDS-PAGE.

Active immunization. Purified preparations of r-AC-Hly and of the truncated proteins were adsorbed at 60 μg/ml on aluminum hydroxide (1 mg/ml). For active immunization, female 3- to 4-week-old BALB/c mice (CERJ, St. Berthevin, France) were injected subcutaneously with 15 μg of protein antigen twice at a 2-week interval. Controls received only buffer with aluminum hydroxide. Mice were bled 1 week after the last injection to assess the presence of circulating antibodies. The respiratory sublethal infection was performed 2 weeks after the second immunization.

Mouse intranasal infection. *B. pertussis* was grown on BG medium for 48 h as described above, and the bacteria were resuspended in 1% Casamino Acids. Sublethal challenge was performed by intranasal injections of 50 μl of bacterial suspension. Infected mice were sacrificed by cervical dislocation 1 h after exposure (at time designated day 0) and at various days thereafter (six mice per time point). The lungs were removed aseptically and homogenized in saline with tissue grinders. Dilutions of lung homogenates were sampled on BG medium, and CFU were counted after 3 days of incubation at 36°C. All experiments were performed at least twice and gave consistent results.

Preparation of immune sera. To obtain sera from infected mice, 10 female (4-week-old) BALB/c mice were infected intranasally with 2×10^5 virulent *B. pertussis* organisms as described previously (20). The mice were bled 14 days after infection or at indicated days thereafter.

The polyclonal mouse sera raised against the truncated AC-Hly derivatives were collected 1 week after the second injection of mice with the respective antigen.

The sera of infected humans were prepared by pooling polyclonal immune sera, collected early after the beginning of the cough from 10 selected nonvaccinated infants infected by *B. pertussis*. These infants were older than 8 months, to exclude the presence of maternal antibodies, and younger than 2 years of age, so that their past clinical histories could be known (19).

RESULTS

Immunological properties of r-AC-Hly and its truncated variants. We have previously shown that CyaC-mediated modification of *B. pertussis* AC-Hly is essential for its protective activity (5). It was important to determine whether the modification is itself part of a linear protective epitope or whether the modification induces a conformational change in the toxin which is required for presentation of protective epitopes. Therefore, we have examined the immunological and protec-

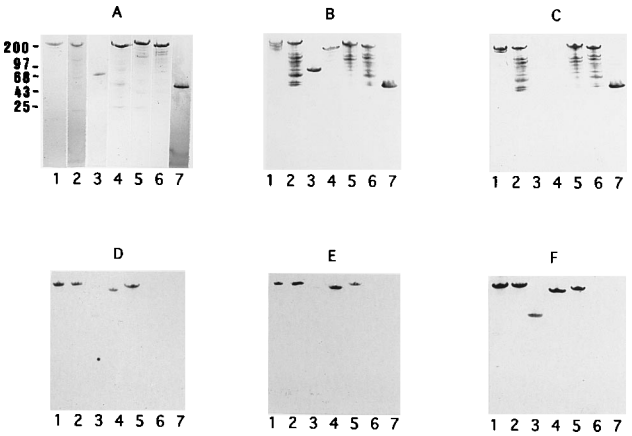

FIG. 2. Characterization of *B. pertussis* and r-AC-Hly. Two hundred nanograms each of purified *B. pertussis* and r-AC-Hly was subjected to SDS-PAGE (8 to 25% polyacrylamide), and the proteins were either stained with Coomassie blue (A) or transferred to a Hybond C-Super membrane and incubated with anti-r-AC-Hly pooled sera from 20 mice, 22 days postvaccination (B), with monoclonal antibodies specific to *B. pertussis* AC-Hly (C), with a pool of sera from infected infants (culture confirmed) (D), with serum of *B. pertussis* 18323-infected mice collected 2 weeks after infection (E), or with serum of *B. pertussis* 18323-infected mice collected 2 months after infection (F). The immunodetection was performed with peroxidase-labelled rabbit anti-mouse antibodies. Lanes 1, *B. pertussis* AC-Hly; 2, r-AC-Hly; 3, ΔHR2; 4, ΔH; 5, ΔCla; 6, ΔC217; 7, ΔC1307. The numbers on the left of the gel in panel A indicate molecular weight markers (in thousands).

tive properties of a set of truncated r-AC-Hly proteins which are represented schematically in Fig. 1.

These proteins were produced in *E. coli* in the presence of the CyaC protein by use of the pCACT or pDIA plasmids, to allow the CyaC-mediated fatty-acylation of the constructs having the modification site. As shown in Fig. 2A, the purified preparations of all proteins contained a major polypeptide corresponding to the expected molecular weight. These proteins were probed by Western blotting (immunoblotting) for recognition by various sera. As shown in Fig. 2B, the serum raised against r-AC-Hly recognized r-AC-Hly as well as the AC-Hly purified from *B. pertussis* and all truncated r-AC-Hly-derived proteins. As can be seen in Fig. 2A, the full-length purified polypeptide preparations of AC-Hly, r-AC-Hly, ΔCla, and ΔH truncated proteins also contained several fragments which were recognized by a polyclonal serum raised against purified r-AC-Hly (Fig. 2B). A monoclonal antibody (Fig. 2C) specific to the AC domain of AC-Hly (9) also recognized these fragments of AC-Hly, ΔCla, and ΔC217, showing that they are C-terminally-truncated proteolytic fragments containing the AC domain which copurified on calmodulin-agarose with AC-Hly. However, the monoclonal antibody did not recognize the ΔH or ΔHR2 proteins, which lack residues 385 to 828 and 385 to 1489, respectively, but recognized the ΔC1307 protein. This indicates that the monoclonal antibody is directed against the region of the molecule located between amino acids 385 and 400. Alternatively, amino acids 385 to 400 may also be necessary for maintaining the conformation of an epitope(s) which is located at another region(s) in the ΔC1307 molecule.

Remarkably, in contrast to the specific anti-r-AC-Hly serum, the pooled sera from infected children failed to recognize the AC domain of AC-Hly (ΔC1307; Fig. 2D, lane 7), the protein lacking the last 217 residues of AC-Hly (ΔC217; Fig. 2D, lane 6), or the protein containing the last 217 residues but lacking the hydrophobic modification regions and a major part of the repeat regions of AC-Hly (ΔHR2; Fig. 2D, lane 3). However, these human sera recognized *B. pertussis* AC-Hly and r-AC-Hly (Fig. 2D, lanes 1 and 2) and the two truncated proteins possessing intact modification-and-repeat regions (last 800 residues) of AC-Hly (Fig. 2D, lanes 4 and 5). An identical recognition pattern (Fig. 2E) was obtained with the serum of mice infected with the *B. pertussis* 18323 reference strain and collected early after infection (14 days). The sera collected later after infection (35 days) recognized the C-terminal part of AC-Hly present in the ΔHR2 protein (Fig. 2F, lane 3), whereas they still failed to recognize the AC domain (Fig. 2F, lane 7) and the ΔC217 protein lacking only the last 217 residues (Fig. 2F, lane 6). These results suggest that anti-AC-Hly antibodies synthesized after infection by *B. pertussis* are directed predominantly against the C-terminal modification-and-repeat region of AC-Hly (last 800 residues). However, these data do not exclude the possibility that the anti-AC-Hly antibodies are directed against conformational epitopes in other regions of the molecule which are folded correctly only when the C-terminal end of the molecule is intact. Furthermore, neither the ΔC217 protein which lacks the last 217 residues of AC-Hly nor the ΔHR2 protein which contains the last 217 residues was recognized by the human and mouse sera collected early after infection (2 weeks), suggesting that these polyclonal sera recognize a particular structure of the repeat region of AC-Hly which is ablated by any of the two nonoverlapping deletions. However, ΔHR2 was recognized by mouse sera collected later after infection (2 months).

It is important to note that the sera of infected mice or humans recognized only the full-length polypeptides of AC-Hly, r-AC-Hly, ΔCla, and ΔH and not their proteolytic fragments. The failure of the sera to recognize these breakdown products, which contain the AC domain and are C-terminally cleaved (see above) (Fig. 2C), further indicates that the modification-and-repeat region of AC-Hly (last 800 residues) needs to be intact to be recognized by these sera.

Inhibition of AC, hemolytic, and cytotoxic activities by sera raised against the truncated AC-Hly derivatives. It was of interest to analyze whether sera of *B. pertussis*-infected mice and/or humans or polyclonal sera raised against the different truncated r-AC-Hly derivatives by immunization with purified proteins could specifically inhibit one of the activities of the toxin. As a necessary control prior to such experiments, the ELISA titers of the various mouse sera were determined by use of full-length r-AC-Hly as the coating antigen for the assay. These titers were found to be similar, with the only exception being that of the anti-ΔC1307 serum, which failed to recognize AC-Hly in the ELISA, although it recognized AC-Hly in the Western blot as well as the other sera did (data not shown).

As shown in Fig. 3A, the AC activity was inhibited by all sera obtained after immunization of mice with purified r-AC-Hly and derived truncated proteins. However, neither the sera from infected mice nor those from infected humans inhibited the AC activity of AC-Hly under identical conditions. This agrees with the results of the Western blot analysis (see above) showing that these sera contain antibodies predominantly directed against the C-terminal portion of AC-Hly (last 800 residues) and not against the AC domain. Indeed, the hemolytic activity of AC-Hly was inhibited by the sera of infected children and less strongly by the sera of infected mice. However, strong inhibition of hemolytic activity was observed with antisera raised by immunization with *B. pertussis* AC-Hly, r-AC-Hly, ΔH, ΔCla, and ΔHR2 (Fig. 3B). This inhibition was due presumably to the presence of specific antibodies against the C-terminal portion of AC-Hly. In fact, the anti-ΔC1307 serum directed against the AC domain of AC-Hly, which lacks such antibodies, did not inhibit the hemolytic activity of AC-

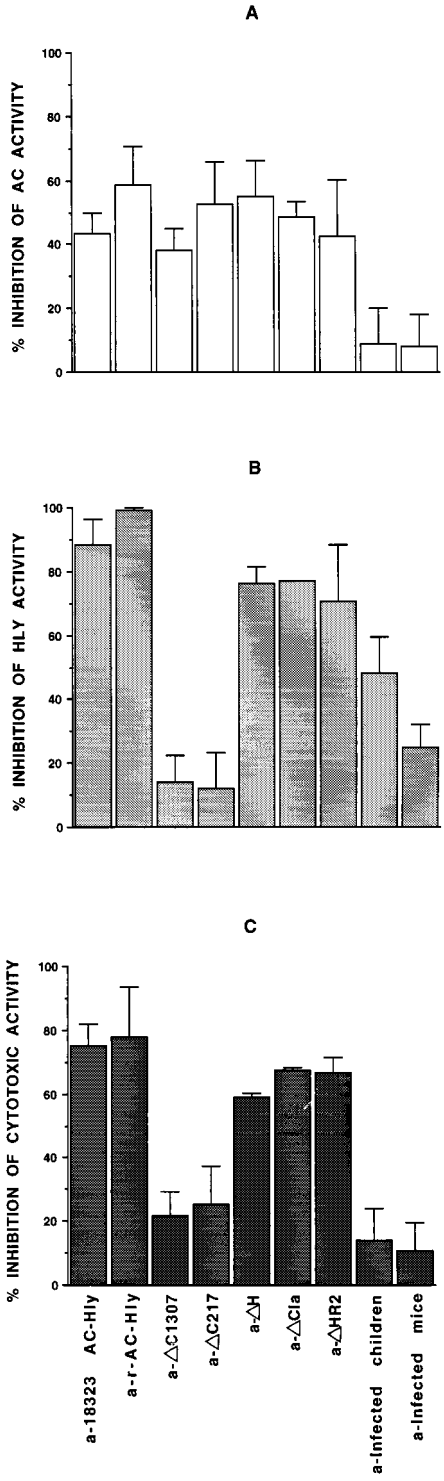

FIG. 3. Inhibition of AC (A), hemolytic (B), and cytotoxic (C) activities of *B. pertussis* AC-Hly by different mouse anti-r-AC-Hly fragment antisera (a) and by sera of *B. pertussis*-infected mice and children. AC-Hly was incubated with various sera as described in Materials and Methods. Error bars indicate standard deviations ($n = 4$).

Hly. Interestingly, the serum directed against the ΔC217 protein, which lacks only the last 217 residues, also failed to inhibit the hemolytic activity of AC-Hly at any appreciable level. This indicated that the last 217 residues are either the target of the neutralizing antibodies or are involved in the formation of a structure that elicits synthesis of neutralizing antibodies directed against other portions of AC-Hly. Collectively, these results further indicate that antibodies synthesized after infection are directed mainly against the C-terminal hemolysin portion of AC-Hly and are able to neutralize its hemolytic activity but not the enzymatic activity of its N-terminal AC domain. As shown in Fig. 3C, significant inhibition of the cytotoxic activity of AC-Hly was observed with sera raised against intact AC-Hly and against the truncated proteins ΔH, ΔCla, and ΔHR2, which all contain both the AC domain and the last 217 residues. These antisera also neutralized the AC and hemolytic activities. In contrast, both of the sera which inhibited either hemolytic activity alone, e.g., the sera of infected mice and humans, or the AC activity alone, e.g., the anti-ΔC1306 and anti-ΔC217 sera, failed to inhibit the cytotoxic activity of AC-Hly. Therefore, it appears that the presence of antibodies against both the AC domain and the last 217 residues of AC-Hly may be required to neutralize its cytotoxic activity.

Protective activity of the different AC-Hly recombinant constructs. To localize the AC-Hly epitopes required for its protective activity, we have analyzed the protective activity of the different purified truncated proteins by use of the murine respiratory model. Groups of mice were immunized twice with aluminum hydroxide alone (control) or with purified aluminum hydroxide-adsorbed truncated proteins and then challenged intranasally with a sublethal dose of virulent *B. pertussis* 18323. This model assesses the capacity of the bacteria to adhere, colonize, survive, and multiply in the respiratory tract of the mice. As shown in Fig. 4, bacteria multiplied rapidly in the lungs of control mice for 6 days after infection and then started to be cleared from the lungs. No multiplication of the bacteria was observed in the lungs of mice immunized with *B. pertussis* AC-Hly or r-AC-Hly, and after 3 days, the number of bacteria decreased (Fig. 4). In agreement with our previous observations (5), the protective efficacy of *B. pertussis* AC-Hly was higher than that of r-AC-Hly. A protection similar to that induced by r-AC-Hly was also obtained with the ΔCla protein, suggesting that the deleted portion of ΔCla, between residues 827 and 887, is not required for the induction of protective immunity. The ΔH protein, lacking residues 385 to 828, exhibited a lower protective activity than ΔCla did. No protection was induced by the ΔC1307 protein, lacking the entire hemolysin portion of AC-Hly. Most interestingly, the ΔC217 protein, lacking the last 217 residues, and the ΔHR2 protein, containing the last 217 residues of AC-Hly, both failed to induce protection. It is important to note that the pattern of protective activity correlated with the pattern of recognition of the individual constructs by sera from infected humans and mice.

DISCUSSION

Antibodies against AC-Hly are common in sera of infected children (1, 19), and we have shown previously that immunization with AC-Hly protects mice against bacterial colonization by *Bordetella* spp. (5, 20, 21, 31). The availability of a set of truncated forms of the recombinant AC-Hly has allowed us to study the importance of the different domains of the protein in the induction of protective immunity upon vaccination with AC-Hly. We presently show that the anti-AC-Hly antibodies synthesized upon infection by *B. pertussis* are directed predominantly against the modification-and-repeat domain of AC-Hly (last 800 residues) or against a conformational epitope(s) which is present only when the carboxy-terminal and modification regions are intact. Indeed, only truncated AC-Hlys hav-

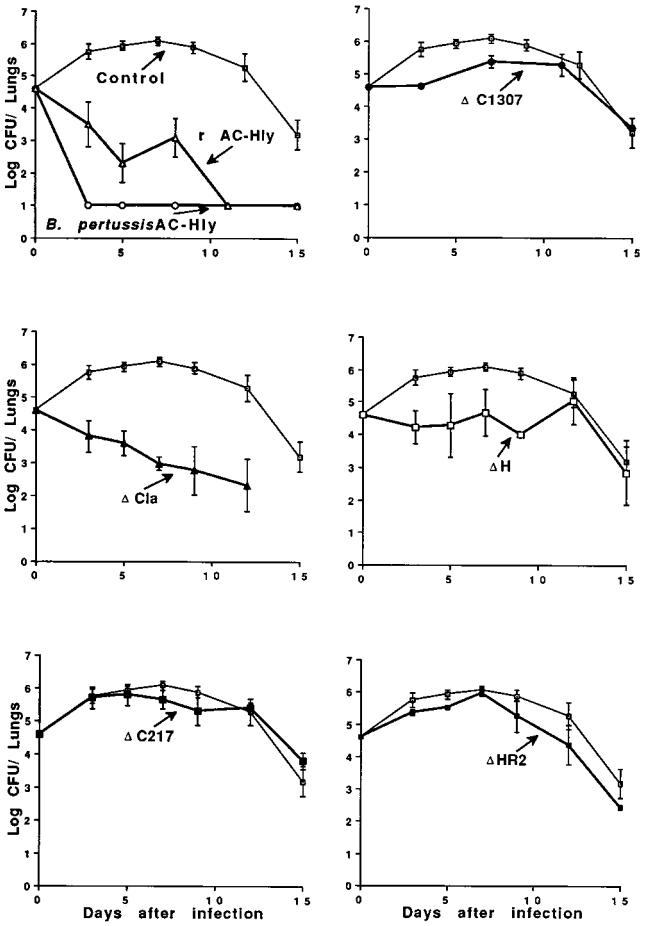

FIG. 4. Protective activities of r-AC-Hly and r-AC-Hly truncated proteins. Mice 3 to 4 weeks old were immunized twice, at a 2-week interval, with 15 μg of r-AC-Hly (△), or ΔCla (▲), or ΔC1307 (●), or ΔHR2 (■), or ΔC217 (■), or ΔH (□), or *B. pertussis* AC-Hly (○) adsorbed on aluminum hydroxide, or with buffer containing aluminum hydroxide alone as a control (⊡). They were infected intranasally 2 weeks later with 10^5 CFU of *B. pertussis* 18323. The plots show the geometric standard deviation (error bars) for six mice per time point.

ing this region intact were recognized by sera of infected mice and humans in Western blots and were the only proteins exhibiting the capacity to induce protection in mice. This indicates that at least in the case of mice, a rather limited set of identical or overlapping epitopes may account for both the induction of synthesis of anti-AC-Hly antibodies by infected subjects and for induction of synthesis of protective antibodies upon vaccination by AC-Hly. These results are consistent with our previous observation that the CyaC-mediated posttranslational modification of the toxin is essential for its protective activity (5). Those results had already indicated that the acylation either induces a particular conformation of AC-Hly, required for the display of protective epitope(s), or itself generates a rather limited set of protective epitopes on the toxin molecule. As shown here, removal of the last 217 residues of AC-Hly, which are distal to the modification site at lysine 983, abolished recognition of this protein (ΔC217) by the sera of infected mice and humans as well as its protective activity. However, this was not due to a lack of modification of the ΔC217 protein because this protein is fatty-acylated when produced in the presence of CyaC (29). Moreover, the last 217 residues per se were not recognized by these sera nor did they exhibit protective activity when presented in the ΔHR2 protein lacking the modification site (lysine 983) and a large portion of the repeats. Hence, both the last 217 residues and the modification site are important for protective activity of AC-Hly. The most straightforward interpretation of these results is that interaction of the modification region with the last 217-residue portion of AC-Hly is required for forming or displaying the protective epitopes on AC-Hly. It was surprising to find that neither the ΔC217 protein lacking only the last 217 residues of AC-Hly nor the ΔHR2 protein lacking the modification region and a large part of repeats, but containing the last 217 residues, was recognized by the polyclonal sera of infected humans and/or mice (early sera). These antisera might have a narrow specificity against a unique epitope or a small set of epitope(s) located at the break point of the repeat portions present in the ΔC217 and ΔHR2 proteins (proline 1489). However, this possibility appears rather unlikely with pooled polyclonal antisera. In addition, the sera of infected mice and humans recognized the major full-length polypeptides of the different proteins but failed to recognize the C-terminally-cleaved proteolytic fragments present in the preparations, fragments which were recognized by the anti-AC monoclonal antibody. Collectively, these results suggest that the formation of protective epitopes on AC-Hly and recognition of AC-Hly by the sera of infected subjects require a particular structure formed only by an intact modification-and-repeat region of AC-Hly. It is conceivable that formation of this structure may require the posttranslational fatty-acylation of AC-Hly at lysine 983 and may be abolished by removing the C-terminal portion of AC-Hly. In this respect, it is important to note that both the hemolytic and cytotoxic activities of AC-Hly are lost upon removal of the last 75 residues of AC-Hly, which contain the unprocessed secretion signal and are not directly involved in its pore-forming activity (4, 29). These observations further support the hypothesis that the extreme C-terminal portion of AC-Hly plays an essential role in the overall AC-Hly structure. We have shown previously that AC preparations containing several polypeptides of 40 to 50 kDa, purified from *B. pertussis* culture supernatants, are protective antigens at very low doses (two 4-μg doses per mouse). This suggested that major protective epitopes are localized on the AC portion of AC-Hly (20, 21). Our present results suggest that contamination by AC-Hly fragments spanning the modification-and-repeat region of AC-Hly accounted for the protective activity associated with our earlier preparations of the AC domain. However, these contaminants were minor, and it would be rather surprising if they displayed activity. Alternatively, under certain conditions, the region between amino acids 385 and 450 to 500 may also play a role in AC-Hly protective activity. The structure of this region may be modified in AC-Hly in the absence of the last 217 amino acids or in the absence of acylation. The structure and presentation of the 40- to 50-kDa N-terminal portion to the immune system may induce protection when cleaved from the rest of the AC-Hly molecule in *B. pertussis* culture supernatants. One argument in favor of this hypothesis is that the monoclonal antibody, directed against the part of the molecule located between amino acids 385 and 400, is a protective antibody (20). The capacity of an N-terminal portion of AC-Hly to be a protective antigen still remains an open question. Studies are being undertaken to test this hypothesis.

The result reported here, that the C-terminal modification-and-repeat portion of AC-Hly is crucial for the immunoprotective activity of the entire AC-Hly toxin molecule, is in agreement with our recent observation that the deduced amino acid sequence of AC-Hly from the animal pathogen *Bordetella bronchiseptica*, which does not exhibit cross-protective activity

against infection by *B. pertussis* (18), differs from the *B. pertussis* AC-Hly sequence only in the C-terminal repeat region (6).

We have also examined whether a correlation exists between the protective activity of a given truncated r-AC-Hly in vivo and its capacity to induce the synthesis of antibodies which could neutralize toxin activity in vitro. However, no such correlation was found. While all proteins exhibiting protective activity induced synthesis of neutralizing antibodies, the ΔHR2 protein, which was not protective at all, induced a strong neutralizing antibody response. This suggests that the presence of antibodies neutralizing AC-Hly toxin activity is not a reliable measure of the induced protection against infection by *B. pertussis.*

It is important to note that the ΔCla protein, lacking part of the hydrophobic domain, exhibited protective activity. This recombinant protein is a good candidate for inclusion in future acellular vaccines against pertussis, since it does not exhibit any residual cytotoxic activity (29).

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