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Characterization of Antibody Inhibiting Adherence of *Bordetella pertussis* to Human Respiratory Epithelial Cells

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We have recently established the topographic specificity of the adherence of *Bordetella pertussis* to human ciliated respiratory epithelial cells. For this study, we employed the same quantitative, immunofluorescent adherence assay to test the possibility that sera of patients recovering from naturally acquired whooping cough or immunized with pertussis vaccine may contain activity capable of interfering with this specific adherence. Evaluation of paired sera from six children with culture-proven pertussis demonstrated that antiadherence activity appeared in serum during convalescence from disease. Nine children immunized with diptheria-pertussin-tetanus vaccine also showed activity against adherence, although it was significantly less than in those with clinical disease. Naturally acquired serum antiadherence activity was identified in both immunoglobulin G (IgG) and IgA antibody classes, whereas, as expected, only IgG antibody was present in children receiving the parenteral vaccine. The findings suggest that natural infection or vaccination are associated with the acquisition of serum activity inhibiting the adherence of *B. pertussis* to ciliated cells. Immunization may fail to elicit IgA antiadherence activity.

Bordetella pertussis, the causative agent of whooping cough, adheres specifically to human ciliated respiratory epithelial cells (12). It is likely that this adherence is essential in the development of infection because it permits multiplication of the organism, despite nonspecific host defenses such as mucociliary clearance. Although the mechanism of immunity to infection is unknown, it can be hypothesized that host factors directed against the adherence of *B. pertussis* could be important in recovery from disease and protection from subsequent mucosal infection. We wanted to test this hypothesis, despite the fact that the complicated assay precluded widespread screening of sera. Therefore, we elected to study sera from two specific groups of children: (i) unimmunized children exposed to an outbreak of disease and (ii) newborn infants followed through serial pertussis immunizations. It is difficult to prospectively identify unimmunized children at risk for clinical pertussis in the United States. Thus, to fulfill the criteria of group (i), advantage was taken of an outbreak of disease in an isolated community in Mexico, which permitted the collection of sera from children in the acute and convalescent stages of disease (hereafter referred to as acute and convalescent sera, respectively). Although the number of children in such a group is necessarily small, the results gain importance in view of the stringent selection criterion, i.e., patient sera were immunologically "unfamiliar" with pertussis. Sera fulfilling the criteria of group (ii), i.e., sera from patients receiving carefully supervised serial pertussis immunization, were available through the Pertussis Branch, Bureau of Biologics, Bethesda, Md., from a recently published trial of various pertussis immunization schedules (1). By using an in vitro assay, in which the adherence of *B. pertussis* to human ciliated epithelial cells can be quantitated, we wished to determine whether serum activity interfering with bacterial adherence appeared in these children and compared the activity with that in newborn infants and healthy adults. The implications of differences in levels of antiadherence activity for host susceptibility to the disease are discussed.

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MATERIALS AND METHODS

Adherence assay. Phase I *B. pertussis* Br, a clinical isolate from the University of Virginia, was used for all studies. The strain was stored in the second passage at −70°C in sterilized skim milk. The in vitro adherence assay was performed as previously described (12). In brief, the organism was cultured on Bordet-Gengou medium containing 15% sheep blood for 48 h at 35.5°C and transferred into medium 199S (M. A. Bioproducts, Walkersville, Md.) with a loop (J. F. Morgan, H. J. Morton, and R. C. Parker, Proc. Soc. Exp. Biol. Med. **73**:1, 1950). After the suspension was passed through a 21-gauge needle, a suspension of dispersed aggregates of bacteria was adjusted to 5×10^9 organisms per ml. Human ciliated cells were obtained by brushing trachea that appeared to be normal during bronchoscopy. Cells were suspended in medium 199S to a concentration of 1×10^5 to 5×10^5 cells per ml and used in the assay procedure within 18 h of collection. It is important to note that this relative concentration of organisms and cells was specifically chosen because these conditions yield a maximum number of organisms per cell which is reproducibly countable (i.e., 5), and if adherence is completely blocked, these conditions yield a minimum number of 0 (12).

Serum (50 μl) or isolated immunoglobulin fraction was added to 0.5 ml of *B. pertussis* suspension, again passed three times through a 21-gauge needle, and then incubated at 37°C for 30 min. The serum-*B. pertussis* mixture was then added to a suspension of ciliated cells in 0.5 ml of medium 199S and incubated for 3 h at 37°C on a shaker. Cells were rinsed free of nonadherent bacteria over a 3-μm polycarbonate membrane filter (Nucleopore Corp., Pleasanton, Calif.), spread on a slide, and dried. Adherent *B. pertussis* were

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stained with a 1:40 dilution of *B. pertussis* fluorescent antibody (Difco Laboratories, Detroit, Mich.) by a direct antibody technique. Slides were coded and read in a blind fashion by one of us (E.I.T.). By using a phase-contrast microscope equipped with epifluorescence, 25 ciliated cells were located by phase-contrast microscopy, and adherent *B. pertussis* were counted by fluorescence microscopy. Use of this number of cells has been shown to yield reproducible, statistically significant results, while it accommodates the difficulty in obtaining large numbers of human ciliated cells (12). The mean number (± standard error of the mean) of bacteria attached per cell was calculated, and statistical significance of differences was determined by the goodness-of-fit test.

Groups of sera tested. Sera from five groups of patients were examined (informed consent was obtained from the patients, and the study was approved by the Human Investigations Committee, University of Virginia).

(i) Patients with clinical pertussis. Paired sera were drawn 4 weeks apart from six children in Chilapa, Guerrero, Mexico, with culture-positive pertussis. Serum from one adult convalescent from culture-proven pertussis was also available courtesy of R. Pye, Montreal Children's Hospital, Montreal, Canada.

(ii) Vaccinees. (i) Sera (a total of 16 samples) were obtained from four children at birth and 4, 6, and 9 months of age (courtesy of Pertussis Branch, Bureau of Biologics, and L. Baraff, Los Angeles, Calif.) (hereafter referred to as serial sera). All four children were immunized at 2, 4, and 6 months of age; two of the four children also received diptheria-pertussin-tetanus (DPT) vaccine at birth. Other characteristics of these sera have been published previously (1). (ii) Serum was drawn from five healthy children (ages, 2 to 6 years) within 18 months of their third or fourth DPT immunization (supplied by G. Hayden, University of Virginia).

(iii) Maternal-infant pairs. Sera were obtained during delivery of 11 healthy newborn infants from the umbilical cord (hereafter referred to as cord sera). In 8 of the 11 cases, serum from the mother was obtained simultaneously.

(iv) Adults. Serum was drawn from 12 randomly selected healthy adults.

(v) Patients with antibody deficiency. Sera from two patients with agammaglobulinemia (congenital X-linked and acquired common variable agammaglobulinemia with no measurable functional antibody) and from one patient with acquired immunoglobulin A (IgA) deficiency (normal IgG and IgM concentrations with normal tetanus and diptheria antibody titers after DPT) were supplied by E. Pearl, University of Virginia.

Absorption of serum with *B. pertussis*. Representative sera from each group were absorbed against whole *B. pertussis* organisms. Live organisms (10^9) were incubated with 1 ml of serum for 30 min at 37°C and for 2 h at 4°C. Bacteria were removed by centrifugation at 1,500 × *g* for 10 min. Absorption was repeated at least twice on each serum sample.

Selective absorption of immunoglobulin classes. Preliminary investigation of the class of antibody involved in inhibition of adherence was carried out in 15 serum samples from six adults, three children convalescing from pertussis, three newborn infants (cord sera), and three sera from children after they had received the vaccine. IgG was removed by treatment of 1:10 dilutions of sera with the pellet from 4 ml of a 10% suspension of Cowan I staphylococci containing protein A (IgGsorb; The Enzyme Center, Boston, Mass.) (14). IgA and IgM were removed by treatment of 1:10 dilutions of serum with 0.15 and 0.1 ml, respectively, of a 1:2 suspension of Sepharose beads conjugated to anti-human IgA or IgM antibody (Cappel Laboratories, Cochranville, Pa.) (8). Immunoglobulin concentrations were determined before and after treatment by radial immunodiffusion (9). By using protein A, ≥90% of the IgG, ≤20% of the IgM, and ≤40% of the IgA were removed. By using the anti-IgA or anti-IgM Sepharose beads, ≥90% of the IgA or IgM, respectively, but ≤10% of nontarget classes, were removed.

Isolation of the IgG and IgA classes. Serum (5 ml) from five adults (high antiadherence activity) and 5 ml of pooled cord serum were dialyzed overnight at 4°C against 1 liter of 0.1 M potassium phosphate buffer (pH 8.0) and applied to a column (2 by 18 cm) of DEAE cellulose equilibrated with the same buffer (3). Upon washing the column with 500 ml of buffer, IgG appeared in the void volume. The IgA-rich fraction which bound to the column was then eluted with 500 ml of 1 M NaCl (pH 6.5) in 0.1 M KH_2PO_4 buffer. Both the IgG- and IgA-rich fractions were then concentrated to 10 ml of vaccuum dialysis and then dialyzed overnight at 4°C against medium 199S without albumin. Residual IgG in the IgA-rich fraction was removed with protein A as described above. Immunoelectrophoresis revealed no contamination of IgG with IgA and vice versa. The adult IgG fraction contained 568 mg of IgG per dl (whole serum pretreatment, 1,112 mg/dl) and the IgA-rich fraction contained 65 mg of IgA per dl (whole serum pretreatment, 85 mg/dl); the cord IgG fraction contained 230 mg of IgG per dl (whole serum pretreatment, 760 mg/dl), and the IgA-rich fraction contained <1 mg of IgA per dl (whole serum pretreatment, 2 mg/dl).

RESULTS

In the presence of serum devoid of antibody (agammaglobulinemic sera), the maximum number of *B. pertussis* organisms adherent per cell was 4.8 ± 0.2 (mean, 4.4 ± 0.4) (Table 1). Five of six acute sera drawn from children during the culture-positive, paroxysmal phase of clinical pertussis had no antiadherence activity (4.3 ± 0.2 organisms per cell). In contrast, convalescent sera drawn 4 weeks later demonstrated activity which virtually completely prevented adherence (0.6 ± 0.03) in all six children ($P < 0.01$). These data are consistent with the hypothesis that clinical whooping cough is, indeed, associated with the acquisition of adherence-inhibiting activity in serum.

Sera from neonatal infants, who are known to be unusually susceptible to disease, demonstrated activity inhibiting adherence to 2.1 ± 0.2, which is a level intermediate

TABLE 1. Effect of selective removal of immunoglobulin classes from serum and nasal secretions on adherence of *B. pertussis* to human ciliated cells

Serum (no. of patients)	Mean no. of adherent bacteria per cell			
	Unabsorbed	Absorbed[a]		
		−IgG	−IgA	−IgM
Agammaglobulinemic (control)	4.4	4.7	4.5	4.5
Convalescent from pertussis (6)	0.4	3.5[b]	2.4[b]	0.9
Adult, high level (9)	0.4	3.4[b]	2.2[b]	0.7
Cord (11)	2.1	5.4[b]	2.3	2.4
Vaccinee (5)	3.7	5.4[b]	3.6	3.6
Adult, low level (3)	3.4	4.7[b]	3.6	3.6

[a] Immunoglobulin class denotes the class removed from serum; values denote residual serum antiadherence activity. Maximum variation for each value was ±0.4 organisms per cell.

[b] For these values there was a significant difference between the absorbed and unabsorbed values ($P < 0.01$).

between acute and convalescent sera from children with clinical disease ($P < 0.01$). All 11 of the cord sera examined exhibited the same intermediate level of antiadherence activity, regardless of the corresponding maternal sera activity. This suggests that transplacentally acquired antiadherence activity provides only partial protection from adherence either because of quantitative or qualitative differences in serum antibodies.

Sera from four infants were studied serially to determine the changes in antiadherence activity during the first year of life and the effect of vaccination on the level of this activity. Sera from vaccinees of all ages showed less activity against adherence (mean, 3.5 ± 0.2) than serum from patients convalescent from pertussis ($P < 0.01$) (Fig. 1). The little serum activity detected at birth (mean, 2.4 ± 0.4) was consistently lost by 4 months of age (mean, 4.3 ± 0.3) ($P < 0.01$) if children were unimmunized at birth. Despite receiving at least four doses of vaccine, all five older children still had levels of antiadherence activity less than that from convalescent sera (mean, 3.7 ± 0.3) ($P < 0.01$). It appears, therefore, that antiadherence activity is low, but detectable, in cord serum, but without immunization disappears within several months of birth. Although vaccination results in a variable appearance of adherence-inhibiting activity, the level remains significantly lower than that after natural infection.

Serum from adults segregated into two levels of antiadherence activity when tested in the assay. Of 12 sera, 9 had high-level activity which inhibited adherence as effectively as sera from patients convalescing from disease (0.4 ± 0.2) ($P > 0.5$). In contrast, 3 of 12 sera had low-level activity and resembled that in vaccinees (3.4 ± 0.3) ($P > 0.5$). Although documentation of immunization histories were not available, all subjects denied immunization, and most subjects gave a history of clinical whooping cough during childhood.

In summary, testing of the five groups of sera revealed reproducible and statistically different levels of antiadherence activity: high (0.4 ± 0.2) as in convalescent serum and low (3.5 ± 0.3 to 4.3 ± 0.2) as in acute and vaccinee serum ($P < 0.001$ by goodness-of-fit test). Low-level activities were not a reflection of person-to-person variation in high antiadherence antibody titers since dilution to 1:100 did not alter the level of antiadherence activity in a given serum sample, and dilution to 1:1,000 resulted in either complete loss of activity or persistence of activity at the original level. These data raised the possibility that antiadherence activity may represent high-titer antibody and that different levels of activity do not represent variations in amount of antibody but rather differences in immunoglobulin class or target antigen of the antibody(ies).

Serum antiadherence activity is antibody. Absorption of sera from each study group against whole *B. pertussis* cells completely removed antiadherence activity at all levels. To examine the possibility that antiadherence activity specific to *B. pertussis* may in large part represent specific antibody, preliminary experiments were carried out in which classes of immunoglobulin were absorbed from sera (Table 1). In only two groups of sera did removal of imunoglobulins other than IgG alter the antiadherence activity. Although no serum contained IgM with antiadherence activity, the IgG and IgA fractions from both convalescent and high-level adult sera contained antiadherence activity. In contrast to high-level adult serum, activity in cord serum was entirely within the IgG fraction and was presumably acquired transplacentally. Similarly, vaccination produced serum antiadherence activity in the IgG class alone. Sequential removal of IgG, IgA, and IgM classes from adult serum completely abolished antiadherence activity. To confirm these observations derived from removal of immunoglobulin classes from serum, the classes were isolated from pooled adult ($n = 5$) and cord ($n = 5$) serum and tested in the assay. The IgG class was found to contain the expected intermediate level of antiadherence activity in both adult and cord sera (2.4 ± 0.3 and 2.0 ± 0.2, respectively). Antiadherence activity was found in the IgA fraction of adult serum (3.4 ± 0.3), whereas no activity was found in the cord serum IgA fraction.

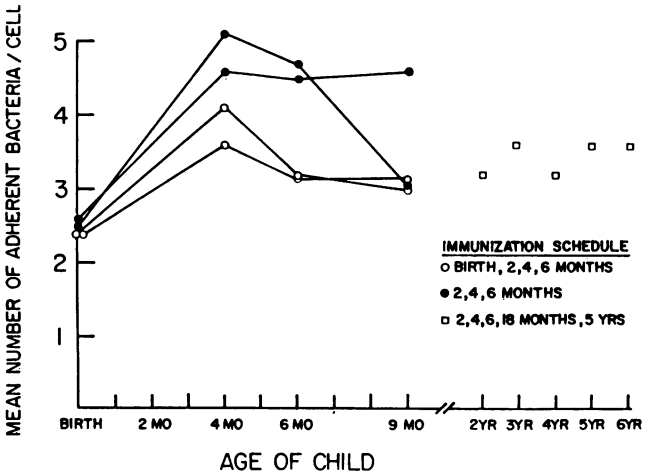

FIG. 1. Effect of immunization with DPT on serum antiadherence activity. Serum antiadherence activity is compared in two groups of children: four children followed serially from birth to 9 months of age after receiving DPT by the schedule depicted in the figure (solid lines connect serial values for each child) and five children vaccinated appropriately for their age from whom a single serum sample was available (□). Activity present in cord serum is seen to be lost by 4 months of age in children unimmunized at birth and appears in three of four cases after ≥3 DPT doses. All five older children demonstrated an intermediate level of antiadherence activity similar to the 9-month-old infants.

DISCUSSION

B. pertussis is a prototype mucosal bacterial pathogen of the respiratory tract. This organism not only adheres specifically to ciliated cells but also multiplies noninvasively to the respiratory epithelium. The importance of adherence in the pathogenesis of pertussis is suggested by the high degree of specificity of the bacterial-ciliary interaction (13). We hypothesized that activity directed against this essential adherence process may be important in recovery from clinical disease and immunity to reinfection. This concept has precedence in infections of other mucosa (5, 11, 15). Circulating antibody plays a role in defense of the respiratory mucosa against viral pathogens, although its role in bacterial respiratory disease is as yet uncertain (7). It is not unreasonable, however, to expect that circulating and locally produced antiadherence antibody may play a role in immunity to clinical pertussis. Although our adherence assay is highly specific and quantitative, the expansion of the microscopic evaluation of human cells to large numbers of tests is not feasible. Because no alternative assay has been developed as yet, we felt that important information could be gained from the application of this technique, despite the limitation of numbers if the sera tested were strictly chosen, i.e., documented pertussis in a previously unexposed, unimmunized population and supervised serial vaccination of newborn infants.

Our results indicate that serum activity directed against the specific adherence of *B. pertussis* to human ciliated cells clearly exists. It appears that this activity involves specific antibody. Activity appears after infection and, to a lesser extent, after immunization. In both cases, activity can be abolished by absorption of immunoglobulin classes from serum. Both the IgG and IgA fractions isolated from convalescent serum have antiadherence activity. This evidence suggests that for *B. pertussis* and perhaps other bacterial pathogens, antibody that prevents bacterial adherence may contribute to lower respiratory tract immunocompetence. The relative role of antiadherence antibody in relation to other mucosal defense mechanisms in recovery from disease remains to be established.

Low serum antiadherence activity was associated with the lack of IgA antiadherence antibody. *B. pertussis*-specific IgA, as measured by the enzyme-linked immunosorbent assay, appears in serum and mucosal secretions after respiratory challenge with the organism but not with parenteral challenge with prepared antigen (4, 10). We also noted the lack of sIgA antiadherence activity in extracts of nasal secretions of three vaccinees (data not shown). These findings suggest that *B. pertussis* may have to be presented by the mucosal route to elicit the IgA response critical to high-level antiadherence activity. This may contribute to the lack of efficacy of the current pertussis vaccine.

Variation in the immunoglobulin class of antiadherence activity may have implications for understanding the epidemiology of pertussis. The susceptibility of newborn infants to infection may be a result of the lack of IgA antiadherence antibody, which is presumably absent by virtue of exclusion of this antibody class from transplacental transport. It appears that many adults have high levels of protection against adherence in serum. This could arise from either disease-induced immunity which is long lived or immunity anamnestically recalled by subclinical infection. Support for the role of subclinical infection in maintaining immunity is twofold. Vaccinees and a subset of adults studied here clearly have lowered protection against adherence in vitro and may be susceptible to either colonization or disease. These adults and vaccinees may represent the two groups recently observed to harbor the organism in the nasopharynx (asymptomatically) (2). They may also represent a natural source of *B. pertussis* spread in the community (6).

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