Enzyme-Linked Immunosorbent Assay for Detection of Pertussis Immunoglobulin A in Nasopharyngeal Secretions as an Indicator of Recent Infection

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An enzyme-linked immunosorbent assay was developed for detection of immunoglobulin A (IgA) antibody to Bordetella pertussis (PsIgA) in nasopharyngeal secretions as an indicator of recent infection. Secretion specimens submitted for pertussis culture were examined for PsIgA by this technique. Of 348 specimens tested, B. pertussis was cultured from 57, and PsIgA was detected in 8 culture-positive and 40 culture-negative specimens. The average time between onset of symptoms and specimen collection for the culture-positive, PsIgA-negative specimens was 10 days; for the culture-positive, PsIgA-positive specimens, 15 days; and for the culture-negative, PsIgA-positive specimens, 36 days. Examination of paired samples available from several culture-proven cases demonstrated conversion from a negative PsIgA in the early sample to a positive PsIgA in the follow-up sample. Our results indicate that PsIgA is produced during natural human infection and does not arise as a result of parenteral vaccination. PsIgA usually appears in the nasopharyngeal secretions during the second or third week of illness and persists for at least 3 months. The detection of PsIgA in secretions may be a valuable diagnostic aid in culture-negative patients with pertussis.

The widespread use of pertussis vaccine has been followed by a decrease in severity of the disease, but infection is still common, and instances of pertussis are not infrequently seen in clinical practice (18). Diagnosis on the basis of clinical criteria alone is unreliable, as the presentation may be atypical (9) and other infections sometimes produce signs and symptoms suggestive of pertussis (13).

Definitive diagnosis is dependent on isolation of Bordetella pertussis, but unless good specimens can be collected early in the disease, the culture may be unsuccessful, even with the improved media (2, 14) now available. Direct fluorescent-antibody staining of nasopharyngeal secretions is a useful diagnostic aid, but lack of sensitivity and problems of interpretation limit its value (3, 10). Several different serological methods for detection of serum antibody to B. pertussis have been described, but none has proven reliable for diagnosis of active infection (2, 13). There is still a need for a method to confirm the diagnosis in culture-negative patients.

In pertussis, the bacteria adhere to the ciliated respiratory epithelium, and the infection is essentially superficial (12), yet there is little available information on the local immune response in the course of the disease (9). The purpose of the present study was to develop an enzyme-linked immunosorbent assay (ELISA) for detection of pertussis immunoglobulin A (IgA) (PsIgA) in nasopharyngeal secretions which would permit assessment of the possible significance of IgA as an indicator of recent infection.

MATERIALS AND METHODS

Antigen. The antigen for the ELISA test was prepared from B. pertussis strain 165 obtained from C. R. Manclark, Bureau of Biologics, Food and Drug Administration, Bethesda, Md. This strain contains serofactors 1, 2, 3, 5, and 6. The 48-h growth from 10 charcoal-horse blood agar plates (Oxoid charcoal agar with a final concentration of 10% horse blood and 1% Difco proteose peptone no. 3) was suspended in 0.1 M carbonate buffer (pH 9.6), washed once, and resuspended to a volume of 10 ml in buffer. The suspension was placed in an ice bath and sonicated in a Biosonik III (Bronwill Scientific Inc., Rochester, N.Y.) with a 3/8-in. (ca. 31.5-mm) probe at 180 W output in 30-s pulses until obvious lysis of the cells had occurred. The suspension was then centrifuged at 15,000 rpm for 30 min, and the clear, slightly yellow supernatant was collected. The protein content was determined, and the antigen was dispensed in 0.5-ml amounts and stored at −20°C until use. The antigen remained stable and satisfactory for use throughout the 9-month period of this study.

An antigen solution containing 5 μg of protein per ml was found by checkerboard titration to be optimal
for the ELISA tests.

Conjugate. Peroxidase-labeled anti-human IgA was obtained from Miles Laboratories, Elkhart, Ind. Tests by ELISA for class specificity of the first lot of conjugate used (S345) showed cross-reactivity with IgG and IgM amounting to 10 to 20% of that with IgA. Antibody in secretions is predominantly IgA, and this degree of cross-reactivity is unlikely to be significant in an assay for antibody response in secretions. A second lot of conjugate (S514) gave no cross-reaction with IgG or IgM but contained antibodies directly cross-reactive with the B. pertussis antigen. The cross-reacting antibodies were eliminated by absorption with a suspension of heat-killed B. pertussis cells (strain 165).

For both lots of conjugate, a titer of 1:400 was found by titration with known positive and negative controls to be optimal.

ELISA procedure. The procedure followed was essentially that of Voller et al. (19). The wells of polystyrene flat-bottom microtiter plates (Dynatech Laboratories Inc., Alexandria, Va.) were sensitized by adding 200 µl of antigen diluted in carbonate buffer (pH 9.6) and holding at 4°C overnight. The plates were then emptied and washed by carefully flooding with phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20, agitating for 3 min on a mechanical rotator (circular path, 180 rpm), and repeating this process for a total of three washes. Secretion samples diluted 1:2 in phosphate-buffered saline-0.05% Tween 20 containing 1% bovine serum albumin were added to the wells in 200-µl amounts, and the plates were held at room temperature for 2 h. The plates were washed as before, and 200 µl of a 1:400 dilution of peroxidase-labeled anti-human IgA was added to each well. After a 2.5-h period at room temperature, the plates were again washed, and 200 µl of freshly prepared enzyme substrate was added (5-aminosalicylic acid [Aldrich Chemical Co., Inc., Milwaukee, Wis.] plus 0.05% H2O2) (20). The plates were left at room temperature for 1 h, and the enzyme reaction was then terminated by addition of 50 µl of 3 M NaOH.

The results were read visually and by spectrophotometer. Once the optimal parameters for the ELISA procedure were established, the tests gave results that for the majority of specimens were clear-cut and easy to read visually. Tests scored as positive gave a distinct color change (some degree of brown being evident) from the pale yellow of the negative samples and controls. A positive result corresponded to an absorbance of 0.155 or greater when a 1:4 dilution of the well contents was read in a Bausch & Lomb Spectronic 20 at 450 nm. Some specimens gave results between a clear-cut positive and negative. For the purpose of this study, these "doubtful" results were considered negative. For diagnostic purposes, a follow-up specimen from the patient would be requested for testing.

A rough quantitation of PsIGA content in positive samples was obtained by retesting serial dilutions of the secretions.

Specimens. The specimens examined were nasopharyngeal secretions submitted for pertussis investigation to the diagnostic microbiology laboratory at the Isaak Walton Killam Hospital for Children, Halifax. They were collected by suction with a fine, flexible plastic catheter (no. 5, French) and syringe. In the laboratory, the specimens were cultured for B. pertussis on charcoal-horse blood agar with and without added cephalxin (40 µg/ml) (15), smears were prepared for fluorescent-antibody staining for B. pertussis, and the remainder of the specimen was rinsed out of the catheter with 0.5 ml of phosphate-buffered saline and stored at −70°C until the ELISA tests were set up.

The majority of specimens were collected from patients presenting to the emergency or outpatient departments of the hospital and represented sporadic cases of respiratory illness. There was no major outbreak of pertussis or other respiratory illness during this time.

Controls. As a positive control, human pertussis immune globulin (Cutter Laboratories, Berkeley, Calif.) was used. Pooled negative secretions served as a negative control.

Absorption tests. To test for specificity, selected positive secretions were absorbed for 2 h at 37°C with dense, washed, heat-killed suspensions of various organisms. For each absorption test, a duplicate sample of the secretions was diluted in parallel with the absorbed secretion and also held for 2 h at 37°C. The absorbed and unabsorbed secretions were then tested in parallel by the ELISA test. The reactions were read for absorbance at 450 nm by an 8-channel photometer (Titerhek Multiaskan; Flow Laboratories, Inc., McLean, Va.).

Organisms used for absorption tests were: B. pertussis 165, B. pertussis 1494 (vaccine strain from D. W. Stainer, Connaught Laboratories, Toronto), Bordetella parapertussis 45170 (Provincial Laboratory of Public Health, Edmonton), Bordetella bronchiseptica R 1714-68 (A. J. Wort, Halifax), Escherichia coli ATCC 29522, and clinical isolates of Haemophilus influenzae type b, Pasteurella multocida, and Streptococcus pyogenes from the University of Alberta Hospital, Edmonton.

RESULTS

A total of 348 routinely collected specimens of nasopharyngeal secretions submitted to the microbiology laboratory of the Isaak Walton Killam Hospital for pertussis investigation were examined for the presence of pertussis IgA by the ELISA technique. The results of cultures for B. pertussis and the ELISA tests are shown in Table 1. B. pertussis was cultured from 57 specimens (16.4%). PsIGA was detected in 8 of the culture-positive and 40 of the culture-negative specimens for a total of 13.8% of specimens.

| TABLE 1. Results of culture and ELISA tests for PsIGA on 348 nasopharyngeal secretions |
|---------------------------------|-----|-----|
| Result                        | n   | % of |
| Culture positive, PsIGA negative | 49  | 14.1 |
| Culture positive, PsIGA positive   | 8   | 2.3  |
| Culture negative, PsIGA positive   | 40  | 11.5 |
To correlate these results with the stage of disease, the case records of all patients with specimens giving a positive culture or ELISA test were reviewed for information regarding the time of specimen collection in relation to onset of symptoms. Records of 39 patients contained this information, and the results of 46 specimens submitted from these 39 patients are plotted in Fig. 1. Specimens producing positive cultures were collected 3 to 25 days after onset of symptoms, those positive for PsIgA were collected 6 days to 12 weeks after onset.

The average time between onset of symptoms and specimen collection for the culture-positive, PsIgA-negative specimens was 10 days; for the culture-positive, PsIgA-positive specimens, 15 days; and for the culture-negative, PsIgA-positive specimens, 36 days (Fig. 1).

In Table 2, the PsIgA result of these 46 specimens is related to the week of illness. Of 13 specimens collected in the first week of illness, 1 was positive (7.7%) for PsIgA. The positive specimens increased to 41.7% in week 2, 54.6% in week 3, and 100% by weeks 4 to 12.

To determine the specificity of an immunological procedure, the test is usually applied to a series of specimens from known positive and negative patients. In pertussis this is complicated by the problems of diagnosis, both clinical and bacteriological, and the absence of a recognized and reliable alternative serological procedure for comparison. To provide evidence for specificity, therefore, absorption tests were carried out on selected secretion specimens positive for PsIgA (Table 3). Absorption with E. coli, H. influenzae type b, P. multocida, or S. pyogenes (tested because it may cross-react in the direct immunofluorescence test for B. pertussis) had no significant effect on the PsIgA result. Absorption with B. pertussis reduced the level of PsIgA to the negative range, whereas absorption with B. parapertussis resulted in a 45% reduction in the ELISA readings. Of interest is the high degree of cross-reactivity shown between B. bronchiseptica and B. pertussis in these tests. Absorption with B. bronchiseptica R1714-68 removed substantially all PsIgA.

Other evidence for the specificity of the test comes from a review of the case records of patients with a positive PsIgA result. Information was available on 24 such patients (Table 4). Of these, 15 were proven instances of pertussis by positive culture or positive direct fluorescent-antibody test, on the same specimen that was PsIgA positive or on an earlier specimen. The other nine patients had symptoms suggestive of pertussis, a persistent, spasmodic or paroxysmal cough together with (in seven patients) whoop, cyanosis, or vomiting.

ELISA tests carried out on paired early and follow-up secretion samples on seven patients are shown in Fig. 2. The results demonstrate the conversion from a negative PsIgA result in the early specimen to a positive result in the follow-up specimen. In six of these patients, the early specimen was culture positive; in the seventh, a 5-month-old child with croupy cough with chok-
ing spells, cyanosis, and vomiting, erythromycin was administered before the first specimen was taken, and cultures were negative.

We have insufficient data to make an accurate assessment of the persistence pattern of PsIgA. Samples collected from four patients 22 to 26 weeks after onset of pertussis had titers that were low (1:2 or 1:4) or negative, suggesting that by 6 months PsIgA production had declined.

Our results indicate that immunization with pertussis vaccine does not give rise to PsIgA in nasopharyngeal secretions. Over 85% of the secretion specimens tested in this study were negative for PsIgA, and many of these specimens came from immunized children. In Table 5 the immunization status of patients in whom PsIgA was detected is compared with that of patients who were PsIgA negative and culture positive.

In both groups, a majority of patients had completed the primary series of three diphtheria-pertussis-tetanus immunizations. Similarly, the children whose results are shown in Fig. 2 had received at least two shots of diphtheria-pertussis-tetanus but their nasopharyngeal secretions were negative for PsIgA when first examined for suspected pertussis infection, further evidence that immunization does not stimulate PsIgA.

**DISCUSSION**

The superficial localization of *B. pertussis* to the ciliated epithelium of the respiratory tract suggests that local immunity may be significant in host response to infection. We have demonstrated that the ELISA technique can be used for detection of pertussis IgA and that the presence of IgA in nasopharyngeal secretions corre-
lapses well with recent infection. Holt (7) demonstrated the presence of “antiadhesion” antibody in the saliva of an experimentally infected Taiwan monkey, and Geller and Pittman (5) detected pertussis-specific IgA and IgG by immunofluorescence in tracheal washings 15 days after intranasal infection of mice. Nasally administered pertussis vaccine was shown by Thomas (16) to provoke a secretory IgA response in human volunteers, but no studies of local response during human disease have been published.

Our findings indicate that PsIgA is produced during natural human infection and usually appears in nasopharyngeal secretions during week 2 or 3 of illness. Eight of the specimens examined were positive by culture and for PsIgA, but in the group as a whole, PsIgA was more commonly found in patients from whom the organism could no longer be recovered. The evidence suggests that parenteral vaccination does not provoke a secretory IgA response. We have insufficient data to estimate long-term persistence of PsIgA, but our observations suggest that it may persist for 3 months after the onset of symptoms and decline to a low level by about 6 months.

Early development of the secretory IgA system in the human infant is illustrated by our results. A child 5 months old at the time of sampling (49 days after clinical onset) had a titer of 1:256. In a child who had developed pertussis at 4 weeks of age, a suction sample collected 5 months later showed a 1:4 titer of PsIgA. Tomasi (17) has stated that secretory IgA approaches adult levels by the age of 6 months but that serum IgA does not reach adult levels until 5 to 15 years.

Evidence for specificity of the test comes from correlation with culture results and clinical data and from cross absorption experiments. Hoiby et al. (6) showed by immunoelectrophoretic methods that 2 of 44 antigens detectable in B. pertussis sonicates were present in many other gram-negative bacteria and that P. multocida had 4 cross-reactive antigens. We found that preabsorption of PsIgA-positive secretions with three cross-reactive species (including P. multocida) did not affect the PsIgA result, whereas absorption with either of two strains of B. pertussis rendered the test negative. The antigenic interrelationships of Bordetella species found in our tests are similar to those reported by other investigators (1, 4, 6, 8).

The likelihood of a positive PsIgA test occurring in infections with B. parapertussis or B.
bronchiseptica could not be assessed, as no such infections were encountered. The high degree of cross-reactivity between B. pertussis and B. bronchiseptica may constitute one possibility of nonspecificity, but human infections with B. bronchiseptica are believed to be uncommon (13). B. parapertussis has been found to cause 5 to 20% of human Bordetella infections (11), but its degree of cross-reactivity with B. pertussis (<50%) is less than that of B. bronchiseptica. Both B. parapertussis and B. bronchiseptica are less exacting than B. pertussis in growth requirements and should be more easily recovered in culture if present. Cultures should be specifically examined for these organisms as well as for B. pertussis.

We believe that the detection of PsIgA may be a useful procedure for retrospective diagnosis of pertussis after the disease has progressed to a stage at which cultures are unlikely to be positive. It may also be of value in assessing the significance of morphologically abnormal bacteria sometimes seen in direct immunofluorescence tests. In clinical practice, it is necessary to differentiate between true pertussis and somewhat similar syndromes associated with viral infection (2, 9, 18), and the ELISA PsIgA test should help in this and in investigations of the possible concomitant occurrence of bacterial and viral infection (2, 9).

Broome et al. (3) have emphasized the importance of accurate diagnosis for meaningful study of the incidence and spread of pertussis and have pointed out the shortcomings of clinical diagnosis. The reliability of culture is not known with certainty because of lack of any independent method of diagnosis. It is known that the organisms are recovered with decreasing frequency as the disease progresses (9) and that highest recovery rates are found early in the disease if carefully collected specimens are planted without delay on freshly prepared selective media. Our study indicates the probability of recovering B. pertussis declines significantly by the end of week 3 of symptoms. The problems of interpretation of direct immunofluorescence preparation have been commented on by Broome et al. (3). The results of tests for serum antibody in pertussis have been variable and not reliable for diagnosis of recent infection (2, 13), although IgM determinations may be useful in immunized patients (Goodman, unpublished data). We believe that detection of secretory PsIgA is a potentially useful adjunct or alternative to isolation of the organism for diagnosis of pertussis, particularly if symptoms have been present for more than 2 weeks.

We recognize that unanswered questions remain concerning sensitivity and specificity of the test in clinical and epidemiological applications. There is a need to extend the search for PsIgA to children and adults without a history of recent upper respiratory tract infection as well as to investigate the reaction in larger numbers of persons with infection proven by culture. We feel that the results we have obtained justify publication at this stage to stimulate interest in the possibilities of the PsIgA test and thus to broaden the base for long-term assessment of its place in the diagnosis of pertussis.

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LITERATURE CITED