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Immunoglobulin A Antibodies to Pertussis Toxin and Filamentous Hemagglutinin in Saliva from Patients with Pertussis

G. ZACKRISSON,[1]* T. LAGERGÅRD,[2] B. TROLLFORS,[3] AND I. KRANTZ[4]

Department of Clinical Bacteriology,[1] Department of Medical Microbiology,[2] Department of Pediatrics,[3] and Department of Infectious Diseases,[4] University of Göteborg, S-413 46 Göteborg, Sweden

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Immunoglobulin A (IgA) antibodies against pertussis toxin (PT) and filamentous hemagglutinin (FHA) in 181 saliva samples obtained during various stages of pertussis from 112 patients were determined. Saliva samples obtained within 5 days after the onset of symptoms did not have detectable IgA antibodies against either of the two antigens. Of the samples obtained between 6 and 50 days after the onset of symptoms, 72% had antibodies against FHA but only 40% had antibodies against PT. With few exceptions, saliva samples obtained more than 50 days after the onset of symptoms contained antibodies against both antigens. In the 59 patients from whom paired saliva samples were obtained at intervals of 2 to 5 weeks, a significant increase in the geometric mean FHA antibody titers but not PT antibody titers occurred. However, increases that were fourfold or greater were observed against FHA in only 19 patients and against PT in 14 patients. Thus, IgA antibodies against FHA and PT in saliva develop during pertussis, and the importance of secretory IgA antibodies for protection against infection and disease should be investigated. Determination of these antibodies in paired saliva samples is, however, of little value for the laboratory diagnosis of pertussis.

Serum antibodies against two protein antigens of *Bordetella pertussis*, pertussis toxin (PT) and filamentous hemagglutinin (FHA), develop in the great majority of patients with pertussis. Determination of antibodies against these proteins in paired serum samples markedly improves the possibilities of diagnosing the disease (7, 8, 10, 20, 21). Acellular pertussis vaccines currently undergoing clinical trials contain detoxified PT alone or in combination with FHA, and the serum antibody response after the injection of the two antigens has been studied (2, 3, 9, 11–13, 17). Detoxified PT induces protection against disease in humans (1, 4), but it is not yet known if this protection is mediated through a serum antibody response. Animal experiments indicate that FHA is also a protective antigen in animals (15), but the role of serum antibodies against FHA for protection in humans is not clear. In contrast to this interest in serum antibodies against *B. pertussis* antigens, little attention has been focused on the development of mucosal antibodies against PT and FHA after disease and vaccination. *B. pertussis* attaches to the cilia of the human respiratory epithelium but does not invade tissues (14, 19). It is therefore possible that secretory immunoglobulin A (IgA) is important in protection against infection by blocking attachment. Since infection with pertussis results in long-lasting immunity against reinfection, the first step in evaluating the role of IgA antibodies in protection would be to study the development of secretory PT and FHA antibodies in patients with pertussis. In the present study, we have assayed IgA antibodies against PT and FHA in saliva from patients with pertussis. The possibility of using the determination of antibodies in saliva for the diagnosis of pertussis has also been evaluated.

MATERIALS AND METHODS

Patients. The patients participated in a study of the serum antibody response to PT and FHA (20, 21) which was approved by the Ethical Committees of the Universities of Umeå and Göteborg. After informed consent from the patients or their parents had been obtained, nasopharyngeal, serum, and saliva samples were obtained. From the patients who returned 2 to 5 weeks later, second serum and saliva samples were obtained. From 112 of the originally included 172 patients, one or more saliva samples were obtained.

In total, 181 saliva samples from the 112 patients were available for analysis of IgA antibodies against PT, and 177 of them were also available for analysis of IgA antibodies against FHA. IgG, IgM, and IgA antibodies against PT were determined in 163 simultaneously obtained serum samples, and in 149 of these samples IgG, IgM, and IgA antibodies against FHA were also determined. All 112 patients had by the first or second visit developed clinical symptoms of pertussis, with a paroxysmal cough for at least 6 weeks, whooping attacks, and often vomiting. Virtually all of them had been in contact with other patients with typical symptoms at day-care centers or within the family. Laboratory verification of the pertussis diagnosis revealed that 71 patients had organisms isolated from the nasopharynx, 20 had a significant increase in serum PT and FHA antibodies, 7 had a significant increase in serum PT antibodies, 6 had a significant increase in serum FHA antibodies, 1 had lymphocytosis, 4 had exposure to a culture-verified case, and 3 had clinical symptoms alone. Table 1 shows the age distribution of the patients. Thirty-six patients had been vaccinated with a whole-cell vaccine. From 48 patients only one saliva sample was available, from 56 patients two were available, from 3 patients three were available, and from 3 patients four were available. At the first visit the duration of symptoms was estimated. In most cases this estimate could be made with an accuracy of a few days but, because of the insidious onset of symptoms and the possibility that some children may have had another concurrent respiratory tract infection, the estimated date of the onset of symptoms was uncertain in some cases.

Controls. Saliva samples were collected from 30 healthy adults, aged 21 to 60 years. Ten of them had a history of

* Corresponding author.

TABLE 1. Age distribution of 112 patients with pertussis

Culture result	No. of patients aged:					
	≤6 mo	7–11 mo	1–5 yr	6–10 yr	11–20 yr	>20 yr
Positive	6	4	29	16	1	15
Negative	1	0	13	15	1	11

whooping cough in childhood, 8 had been vaccinated with pertussis whole-cell vaccines, and 11 had neither been vaccinated nor had had pertussis. No information was available for one of them.

Saliva. From older children and adults saliva samples were obtained by letting the patients spit in a plastic cup. From younger children saliva was aspirated with a pipette. The saliva and serum samples were stored frozen at −26°C until analyzed.

ELISA. (i) PT antibodies in saliva. In the PT enzyme-linked immunosorbent assay (ELISA), flat-bottom polystyrene microdilution plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated overnight with 50 μg of fetuin (GIBCO Laboratories, Grand Island, N.Y.) per ml at room temperature (22). After the plates were washed with phosphate-buffered saline (PBS) three times, PT at a concentration of 4 μg/ml was added to each well and incubated for 2 h at room temperature, and the plates were washed again. After being thawed, the saliva samples were centrifuged at 4,800 rpm (Wifug Lab Centrifuges, Bradford, England), and the supernatants were diluted twofold in 0.05% PBS–Tween 20 (Kebo, Stockholm, Sweden). Dilutions from 1:4 to 1:64 were tested in duplicate. After 4 h of incubation at room temperature, the plates were washed with PBS-Tween three times, a 1:400 dilution of swine anti-human IgA conjugated to alkaline phosphatase (Orion Diagnostica, Helsinki, Finland) was added, and the plates were incubated at room temperature overnight. The concentrations of antigen and conjugate were determined by checkerboard titration. The plates were washed with PBS-Tween, and *p*-nitrophenyl phosphate (disodium) diluted in 1 M diethanolamine buffer (pH 9.8) was added at a concentration of 1 mg/ml. The enzyme reaction time was correlated to the absorbancy values of three reference sera, two positive and one negative, used as controls. A_{405}s were determined. Coating with fetuin alone was also performed as a control. The antibody content in saliva was expressed as the highest saliva dilution showing an extinction value of 0.2 above the background. When geometric mean antibody levels in saliva were determined, samples with a titer of <1/4 were arbitrarily assigned a value of 1/2 and samples with a titer of >1/64 were arbitrarily assigned a value of 1/128.

(ii) FHA antibodies in saliva. In the FHA ELISA, the microdilution plates were coated overnight with FHA diluted in 50 mM Tris hydrochloride buffer (pH 8.0) at room temperature. The concentration of FHA was 2 μg/ml, as determined by titration curves. After the plates were washed with PBS-Tween, saliva diluted in PBS-Tween (as described above for the PT ELISA) was added and incubated at room temperature for 4 h. The remaining steps were identical to those in the PT ELISA.

Serum IgG, IgM, and IgA antibodies against PT and FHA were determined as described previously (20, 21). A titer of ≥1/100 was considered positive.

Antigens used in the ELISA. PT was prepared by R. Sekura, National Institute of Child Health and Human Development, Bethesda, Md. (16). The purity was verified by the *Limulus* amoebocyte lysate assay and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining. FHA was a gift from F. Arminjon, Institute Mérieux, Marcy l'Etoile, France. The lipopolysaccharide content was less than 10 ng/mg, as measured by the *Limulus* assay, pyrogen tests, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining.

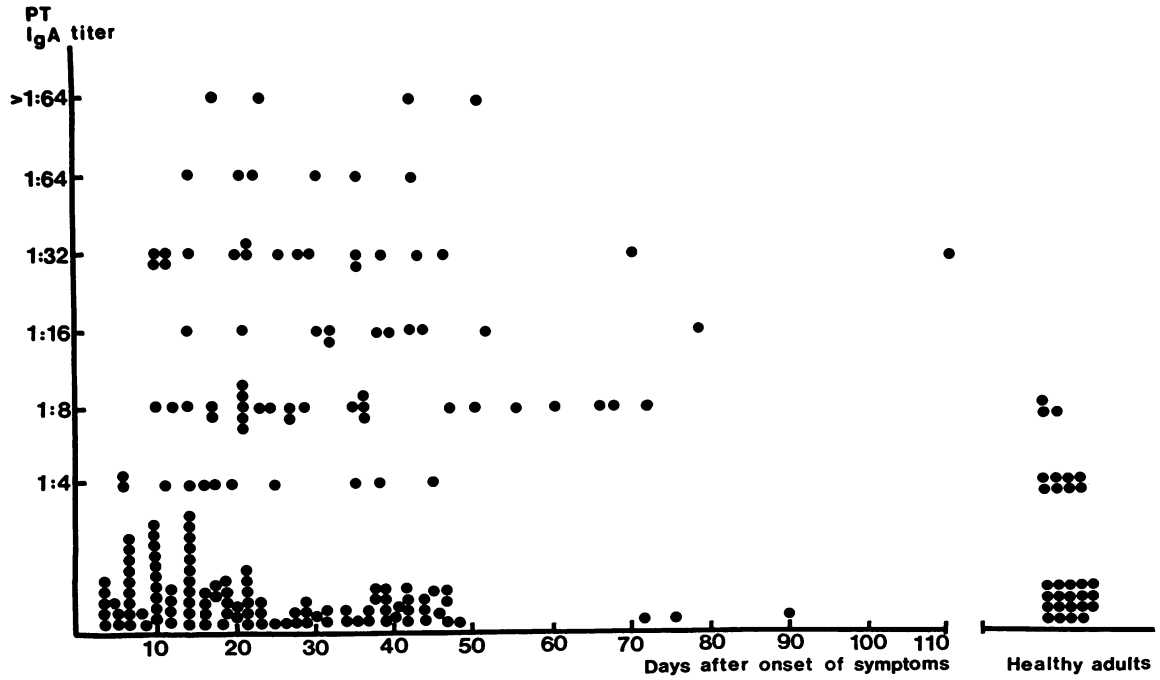

FIG. 1. IgA antibody levels against PT in 181 saliva samples from patients with whooping cough and 30 healthy adults.

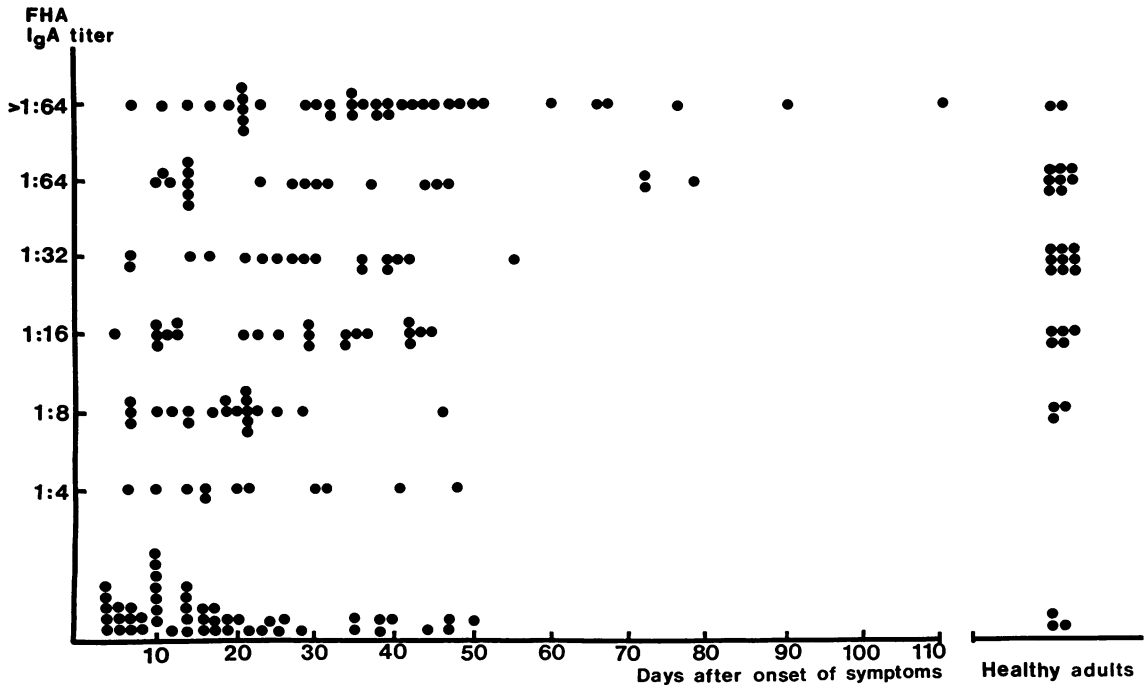

FIG. 2. IgA antibody levels against FHA in 177 saliva samples from patients with whooping cough and 30 healthy adults.

RESULTS

Figures 1 and 2 show the PT and FHA antibody levels in saliva in the 30 healthy adults and, related to the duration of symptoms, in the saliva samples from the 112 patients. Among the healthy adults, more had detectable antibodies against FHA than against PT (27 of 30 versus 11 of 30; $P < 0.001$; Z test for comparison of two proportions). Two healthy adults had anti-FHA titers of $\geq$1/64, and the median anti-FHA titer was 1/32. All healthy adults with detectable anti-PT antibodies in saliva had low titers, 1/4 to 1/8.

There was a wide variation in both anti-FHA and anti-PT titers in saliva from the patients, but some relationship to the duration of symptoms could be discerned. All samples obtained within 5 days after the onset of symptoms lacked detectable antibodies against both PT and FHA. Of the samples obtained 6 to 49 days after the onset of symptoms, 72% (114 of 158) had detectable FHA antibodies, while fewer, only 40% (65 of 161), had detectable PT antibodies ($P < 0.001$; Z test for comparison of two proportions). Of the samples obtained $\geq$50 days after the onset of symptoms, only 1 of 13 lacked detectable antibodies against FHA and 3 of 14 lacked those against PT.

In 62 patients, antibody titers in paired saliva samples obtained at intervals of 2 to 5 weeks could be compared. The geometric mean FHA antibody titers increased from 1/12 to 1/23 ($P < 0.001$; paired t test). Increases that were fourfold or greater were, however, seen in only 19 patients. The geometric mean PT antibody titers were 1/4.2 for the first saliva sample and 1/5.6 for the second saliva sample (nonsignificant; paired t test). Increases that were fourfold or greater were seen in only 14 patients.

The relationship between the presence or absence of IgA antibodies in saliva and antibodies of one or more classes in serum against PT and FHA in all cases in which saliva and serum had been obtained simultaneously was as follows. In the 136 cases with detectable serum antibodies against FHA, 78% (106) of the corresponding saliva samples contained detectable IgA. In the 131 cases with detectable serum antibodies against PT, 49% (64) of the corresponding saliva samples contained detectable IgA. In the 107 cases with detectable serum IgA antibodies against FHA, 84% (90) of the corresponding saliva samples contained IgA antibodies. In the 102 cases with measurable serum IgA antibodies against PT, 54% (55) of the corresponding saliva samples contained IgA antibodies.

Of 32 cases with no anti-PT antibodies in serum, 6 had anti-PT IgA in saliva. Of 13 cases with no anti-FHA antibodies in serum, 5 had anti-FHA IgA in saliva. Of 61 cases with no anti-PT IgA in serum, 15 had anti-PT IgA in saliva. Of 41 cases with no anti-FHA IgA in serum, 21 had anti-FHA IgA in saliva.

Of 177 saliva samples tested for the presence of IgA antibodies against both FHA and PT, 63 had antibodies against both antigens and 40 lacked antibodies against both; 64 samples had anti-FHA antibodies but not anti-PT antibodies, and 10 had anti-PT antibodies but not anti-FHA antibodies.

DISCUSSION

Little is known about the importance of mucosal defense factors for protection against *B. pertussis* infections. Since the organism is not invasive but induces the pathogenic process from colonization of ciliated cells in the respiratory tract (14), factors which inhibit colonization should be protective. FHA is considered to be of importance for attachment of the organism to ciliated cells (18). It is therefore important to study secretory IgA antibodies against FHA. In the present study, we have shown that IgA antibodies against FHA develop in the majority of patients with clinical whooping cough. The samples obtained from patients with symptoms of less than 6 days all lacked IgA antibodies against FHA, while all but one sample obtained after 50 days

or more of symptoms had detectable antibodies. Even though there was no close correlation between detectable antibodies and the duration of symptoms in the time interval of 6 to 50 days, our data indicate that FHA antibodies in saliva develop in a large majority of patients with pertussis during late convalescence. These antibodies probably remain detectable for a long time, since 90% of the healthy adults had detectable IgA antibodies against FHA in saliva, most of them in high titers. It should therefore be of value to study the role of secretory IgA antibodies against FHA in protection against pertussis in vaccine studies.

IgA antibodies against PT in saliva also developed in many of the patients with pertussis. Like FHA antibodies, PT antibodies were not detected in any saliva sample from patients with a disease duration of 6 days or less. PT antibodies in saliva were detected less often than FHA antibodies in saliva in patients with a disease duration of more than 6 days and also less often in the 30 healthy adults.

The absence of IgA antibodies against FHA and PT in saliva samples obtained very early in the disease indicates that mucosal antibodies against these antigens may play a role in defense against infection.

It has been suggested that the determination of IgA antibodies against PT and FHA in saliva (6) could be used for the diagnosis of pertussis. Our results do not agree with this, since only about one-third of the patients had fourfold or greater increases in FHA antibodies and about one-fourth had increases in PT antibodies in saliva. A high titer of FHA antibodies in saliva may also be difficult to interpret as a sign of recent infection, since several healthy adults had high titers of FHA antibodies in saliva even though their known contact with *B. pertussis* antigens through natural infection or vaccination with whole-cell vaccines had occurred in early childhood. It must be emphasized that several factors other than antibody production must affect the antibody content in a saliva sample; most important of these is the amount of fluid in the saliva, which is dependent on various stimuli of the salivary glands (5).

In conclusion, IgA antibodies against FHA and PT in saliva develop in the majority of patients with pertussis, but the determination of antibodies in saliva is of less diagnostic value than the determination of antibodies in serum.

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