Antibodies to *Bordetella pertussis* adenylate cyclase are produced in man during pertussis infection and after vaccination

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Summary. *Bordetella pertussis* produces several potential virulence factors. One of these is an adenylate cyclase which penetrates eukaryotic cells, is activated by calmodulin and generates high levels of intracellular cAMP. We have found that pertussis infection in man leads to production of high titres (2000–8000) of anti-*B. pertussis* adenylate cyclase antibodies. Such antibodies also are produced after pertussis vaccination. They persist into adulthood, cross the placenta and disappear a few months after birth. The anti-adenylate cyclase antibodies found in human serum during pertussis infection do not neutralise the catalytic and penetrative activities of the enzyme.

Introduction

Bordetella pertussis, the causative agent of whooping cough, produces several potential virulence factors that may play a role in the pathogenesis of the disease.^{1,2} The most studied and best characterised virulence factor is pertussis toxin (PT). PT causes many biological responses including lymphocytosis, histamine sensitisation and stimulation of insulin secretion.^{1,2} This toxin has recently been purified and shown to ADP-ribosylate several guanine-nucleotide binding proteins.³ ADP-ribosylation of G_i, the inhibitory guanine-nucleotide binding protein of the hormone-sensitive host adenylate cyclase (AC) system, blocks receptor-mediated inhibition of AC, thereby leading to increased intracellular cAMP levels.³ A role for PT in immunity to *B. pertussis* infection has been demonstrated both in animal models and in human disease. In mice, passive or active immunisation with PT induces protection from the disease produced by both intracerebral and aerosol challenges.^{4,5} Granström *et al.*⁶ demonstrated a rise in neutralising antibodies to PT in human sera after infection.

Another toxin that dramatically affects host cAMP metabolism is *B. pertussis* AC. The location of the enzyme in the bacterial cell is mainly extracytoplasmic^{7,8} and its activity strongly depends on the eukaryotic calcium-binding protein calmodulin (CaM).⁹ The enzyme penetrates eukaryotic cells and, upon activation by CaM, produces high levels of intracellular cAMP.^{10,11} As a result, bactericidal functions of polymorphonuclear leucocytes and macrophages are impaired.^{1,10} Thus, the enzyme may play an important role in the pathogenesis of the disease by inhibiting host defences. Weiss *et al.*¹² showed, by transposon Tn5-induced mutagenesis, that a *B. pertussis* strain deficient in AC was avirulent in an animal model of pertussis infection. Moreover, virulence for mice was restored when the mutant harboured a recombinant plasmid expressing AC activity.¹³ Recently, *B. pertussis* AC was purified, cloned and the genetic determinant sequenced.^{13–16} The availability of a purified preparation of *B. pertussis* AC has enabled us to test for the presence of anti-AC antibodies in patients with pertussis and in subjects after vaccination.

Materials and methods

Patients and subjects

Fifteen patients aged 6–61 (mean 23 SD 18) years with

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pertussis were included in the study. Although pertussis is mainly considered to be a disease of the very young, it has recently been observed more frequently in older children and adults.¹⁷ Two of the patients had not been vaccinated and in four patients the vaccination status was unknown. Serum was obtained by two of us (E.W. and R.K.) 15–100 (mean 37 SD 21) days after symptoms started. Diagnosis of pertussis in suspected cases was based on a high titre of IgG against PT.¹⁸ Additionally all these subjects had a high titre of IgA antibodies against a whole-cell lysate of *B. pertussis* in comparison with a standard reference serum.¹⁹ These patients also showed PT-neutralising antibodies as determined by the Chinese hamster ovary (CHO) neutralisation test.⁶ Serum samples were also obtained from four neonates (cord blood), four unvaccinated infants 4–5 months old, four vaccinated children 2–3 years old, and four vaccinated adults 25–40 years old. IgG against PT and PT-neutralising antibodies (CHO assay) were determined. The relevant patients and other subjects, including mothers of neonates, had been vaccinated with the whole-cell vaccine in childhood. The four vaccinated children were immunised three times during their first year of life, and given a booster-dose a year later. None of the control subjects had had clinical pertussis.

Purification of *B. pertussis* AC

Purified preparations of *B. pertussis* AC were obtained from a wild type *B. pertussis* strain and from a cloned *Escherichia coli* strain expressing the enzyme. *B. pertussis* strain 165 was grown and harvested as described previously.¹¹ The 200-Kda form of the enzyme was purified according to the procedure of Rogel *et al.*¹⁵ This form of the enzyme was chosen because it represents the product of the entire *B. pertussis* AC gene.^{16,20}

A gene library of *B. pertussis* was constructed from chromosomal DNA of strain Tabl by Brownlie *et al.*¹³ A 10-kb DNA fragment, containing the structural gene of *B. pertussis* AC and two out of three genes required for AC secretion,²¹ was subcloned into the vector pIC20H to give the plasmid pRMB3. This plasmid was transformed into *E. coli* H1469 Lof strain and the enzyme produced was purified as described previously.²²

Immunoblotting

Purified 200-Kda form of the enzyme at a concentration of 0.5–1 µg/lane was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) and then electrophoretically transferred to nitrocellulose in a buffer containing 15.6 mM Tris-HCl and 120 mM glycine at pH 8.3. The transfer was conducted for 2 h at 190 mA. The nitrocellulose was cut into individual strips, blocked for 30 min with phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) 2% w/v and then incubated overnight at 4°C with dilutions of various sera, in a final volume of 10 ml. The incubation solution contained PBS supplemented with BSA 2% w/v. The strips were then washed four times,

each for 10 min, with the following solutions: (1) PBS, (2 and 3) PBS containing Tween 20 0.2% w/v, and (4) PBS. The nitrocellulose strips were then incubated for 2 h at room temperature in PBS containing BSA 2% w/v which was supplemented with ¹²⁵I-labelled protein A 10⁵ cpm/ml. Unbound protein A was removed by repeated washing with PBS containing Tween 20 0.2% w/v. The strips were dried and autoradiographed at –70°C for 14 h.

Inhibition of AC activity

AC activity was determined in a total volume of 50 µl as described previously.¹¹ The ability of the various sera to inhibit AC activity was assessed as follows: 20-µl volumes of undiluted sera, and of sera diluted 1 in 10 and 1 in 100 in PBS supplemented with BSA 1% w/v, were incubated for 4 h at 4°C with 20 µl of the 200-Kda form of the enzyme purified from *B. pertussis*, at an activity of 8 nmol of cAMP/min/ml. At the end of the incubation period, three samples, each of 10 µl, were withdrawn and assayed for AC activity. As a control we used neutralising anti-*B. pertussis* AC serum.¹⁵ This serum was obtained from only one guinea-pig out of six immunised with purified *B. pertussis* AC; the rest of the animals produced non-neutralising anti-*B. pertussis* AC antibodies (E. Hanski, unpublished observation). This neutralising serum completely inhibited the AC activity of the 200-Kda form of the enzyme at dilutions up to 1 in 200.¹⁵

Inhibition of penetrative activity

Penetrative activity was determined by measurement of intracellular cAMP accumulation as described previously.¹¹ Human lymphocytes were used as target cells for the enzyme. The invasive form of *B. pertussis* AC was isolated by gel filtration of a crude bacterial extract.¹⁵ The specific activity of this preparation was 3.1 µmol of cAMP/min/mg protein and its capacity for penetration was 0.35 µmol of cAMP/mg of lymphocyte protein/mg of bacterial protein. The invasive enzyme (40 µl) was incubated with the various sera (40 µl of undiluted serum or serum diluted 1 in 10 or 1 in 100 in PBS supplemented with BSA 0.1% w/v) for 5 h at 4°C before testing. Then, three 20-µl samples were withdrawn and the penetrative activity was determined. In control experiments the neutralising anti-*B. pertussis* AC serum completely blocked the entry of the invasive form of enzyme at dilutions of up to 1 in 200.

Results

We tested human sera for the presence of anti-*B. pertussis* AC antibodies. High amounts of anti-AC antibodies were detected in all patients with pertussis (table I). In contrast, anti-AC antibodies could not be detected in sera from healthy non-vaccinated infants as shown for one of them in fig. 1. The patients' sera used in this study contained IgA antibodies against a whole-cell lysate of *B.*

Table I. Anti-*B. pertussis* AC antibody titres in various groups

Group	Number of subjects	Titre*
Pertussis patients	15	2000–8000
Neonates	4	100–300
Non-vaccinated infants	4	Not detected
Vaccinated children	4	200–800
Vaccinated adults	4	200–600

*The presence of anti-*B. pertussis* AC antibodies was assessed by immunoblotting. The titre indicates the maximum serum dilution which could specifically detect the 200-Kda form of *B. pertussis* AC according to the conditions described in *Materials and methods*.

pertussis at titres with a relative potency index of > 5, as determined by the method of Nagel and Poot-Scholten.¹⁹ The anti-PT IgG and CHO-neutralising antibody titres of different donor groups are shown in table II. There was no difference between the titres of vaccinated and non-vaccinated patients. In non-vaccinated infants, anti-PT antibodies were hardly detected, whereas in neonates CHO-neutralising antibodies were detected in higher titres. In vaccinated children, CHO-neutral-

ising titres were even higher, approaching those detected during pertussis infection. Table I shows the titres of anti-*B. pertussis* AC antibodies in the various groups studied. Very high titres (2000–8000) were found in patients with pertussis, irrespective of their vaccination status. In contrast, in non-vaccinated infants 4–5 months old, no such antibodies could be detected even at a dilution of 1 in 20 (table I; fig. 1). However, anti-*B. pertussis* AC antibodies could be detected in cord blood and in vaccinated children and adults.

To exclude the possibility that a minor contaminant of the AC preparation, of similar molecular weight, was responsible for the interactions on the Western blots, the control experiment shown in fig. 2 was performed. *B. pertussis* AC was purified from an *E. coli* strain harbouring a plasmid that encodes for the structural gene of *B. pertussis* AC.^{13, 21} All patients' sera interacted with the enzyme produced in *E. coli*, as shown for one patient in fig. 2. This interaction was specific since recognition of the enzyme was not detected with serum from a non-vaccinated infant.

The ability of sera from pertussis patients to neutralise *B. pertussis* AC activity was examined by two assays: inhibition of enzymic activity and inhibition of penetration of the enzyme into target cells. None of the human sera examined showed such properties. In animals (rabbits and guinea-pigs) immunised with purified *B. pertussis* AC, high titres of anti-*B. pertussis* AC were detected by immunoblotting.^{14, 15} However, neutralisation of enzymic activity and penetrative ability of *B. pertussis* AC was found in the serum of only one guinea-pig out of six immunised. This serum completely inhibited both activities at a dilution of 1 in 200.¹⁵

Fig. 1. Immunoblotting of *B. pertussis* AC, with human sera from: (1) pertussis patient, diluted 1 in 1000; (2) neonate, 1 in 100; (3) non-vaccinated infant, 1 in 50; (4) vaccinated child, 1 in 300; (5) vaccinated adult, 1 in 300.

Discussion

This study demonstrates the capacity for production of antibodies against *B. pertussis* AC in man and is the first direct evidence that AC is produced by *B. pertussis* during infection. The anti-AC antibodies are produced in high titres after infection. These antibodies are also produced after vaccination, persist into adulthood, cross the placenta and disappear a few months after birth. It appears that higher titres of anti-AC antibodies are produced after infection than after vaccination, although this may be due to differences in the timing of the observations. Further studies are required to substantiate this finding. Recently, it has been reported that anti-filamentous haemagglutinin (FHA) antibodies appear in sera of pertussis

Table II. Anti-pertussis toxin antibody titres in various groups

Group	Number of subjects	IgG titre	CHO-neutralising antibody titre
Pertussis patients	15	Range 1600–25600 Median 3200–6400	Range 20–640 Median 160–320
Neonates	4	< 50, < 50, < 50, 100	1, 4, 4, 8
Non-vaccinated infants	3	< 50, < 50, < 50	0, 0, 4
Vaccinated children	3	< 50, < 50, 800	16, 16, 160

*See *Materials and methods* for anti-PT IgG and CHO-neutralising antibody assays.

patients²³. Since FHA has a molecular weight of 210–220 Kda,²³ it could have contaminated the preparations of the 200-Kda form of AC, purified from the wild type *B. pertussis* strain. To exclude this possibility, *B. pertussis* AC cloned in *E. coli* was utilised and showed the same response as observed for the enzyme purified from the native *B. pertussis* cells.

B. pertussis AC antibodies could be detected by immunoblotting with protein A, which detects human IgG. These antibodies did not neutralise the enzymic activity or penetrative ability of the

enzyme but the lack of neutralising capacity does not indicate that these antibodies are insignificant for host immunity. Brezin *et al.*²⁴ showed that both AC enzymic-activity-neutralising and non-neutralising antibodies prevented the lethal effects of the haemorrhagic alveolitis in mice infected with *B. pertussis*. It is possible that, during infection, there is production of different local antibodies that can neutralise the activities of *B. pertussis* AC. Alternatively, locally produced antibodies, which are similar to those detected by us in the serum, may be important for defence against the AC toxin in the respiratory tract. AC toxin, either secreted by the bacteria²⁵ or extracted from them,^{10,11} penetrates various eukaryotic cells. Its mechanism of penetration into respiratory tract cells during infection is not known. Hewlett *et al.*²⁶ suggested that a direct contact between bacteria and target cells is required for intoxication with *B. pertussis* AC. During infection, locally produced antibodies similar to those detected by us in human sera may interfere with the penetration of the invasive enzyme *via* direct contact. Recently, Raptis *et al.*²⁷ reported that hyperimmune human sera could neutralise *B. pertussis* AC activity but details were not provided. The apparent discrepancy between these results and ours is as yet not understood.

In the case of PT, the appearance of neutralising antibodies has been considered to be further evidence for its role in virulence. Granström *et al.*⁶ claimed that PT-neutralising antibodies are important for long term immunity against pertussis. However, some of the patients reported in their study, in spite of having initial high titres of PT-neutralising antibodies, developed pertussis. Furthermore, Trollfors *et al.*²⁸ did not find a correlation between anti-PT IgG and CHO-neutralising antibodies during pertussis infection. In some of their patients, CHO-neutralising antibodies were not detected. However, it was not reported whether or not the course of the disease in these patients was more severe than in the other patients.

Fig. 2. *B. pertussis* AC expressed in *E. coli* is specifically recognised in an immunoblot by serum from a pertussis patient. Lane 1 shows the reaction with serum from a pertussis patient, diluted 1 in 4000; lane 2, reaction with serum from a non-vaccinated infant diluted 1 in 50.

The use of *B. pertussis* AC for diagnosis of pertussis was described by Confer and Eaton.²⁹ Nasopharyngeal swabs, carrying as few as 100 *B. pertussis* organisms, induced detectable levels of cAMP. The observation that anti-AC antibodies are produced during pertussis infection in titres apparently higher than those appearing after

vaccination may form a basis for their use as an additional tool for diagnosis of pertussis.

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