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FEMS Immunology and Medical Microbiology 23 (1999) 273–282

FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY

Adjuvant and protective properties of native and recombinant *Bordetella pertussis* adenylate cyclase toxin preparations in mice

Kalantar Hormozi, Roger Parton, John Coote *

Division of Infection and Immunity, Institute of Biomedical and Life Sciences, Glasgow University, Joseph Black Building, Glasgow G12 8QQ, UK

Received 13 August 1998; received in revised form 26 November 1998; accepted 30 November 1998

Abstract

Bordetella pertussis produces a cell-invasive adenylate cyclase toxin which is synthesised from the *cyaA* gene as an inactive protoxin that is post-translationally activated by the product of the *cyaC* gene. Purified active and inactive CyaA proteins were prepared from *B. pertussis* or from recombinant *Escherichia coli* expressing both *cyaA* and *cyaC* genes or the *cyaA* gene alone, respectively. In addition, a hybrid toxin (Hyb2) in which an internal region of CyaA had been replaced with the analogous region from the leukotoxin (LktA) of *Pasteurella haemolytica*, and which had low cell-invasive activity, was also prepared from *E. coli* expressing the *cyaC* gene. The CyaA preparations showed no evidence of toxicity in a mouse weight-gain test. Active toxin preparations were protective in mice against intranasal challenge with wild-type *B. pertussis*, as evidenced by lung:body weight ratios and bacterial numbers in the lungs, which were comparable to those in mice given whole-cell DPT vaccine. Hyb2 was not as protective as active CyaA and inactive CyaA preparations were not protective. Active CyaA, when co-administered with ovalbumin (OA), had a marked adjuvant effect on the anti-OA IgG antibody response which was not as apparent with inactive CyaA preparations. Similarly, active CyaA stimulated a greater anti-CyaA response than the inactive form.

Keywords: Adenylate cyclase toxin; *Bordetella pertussis*; Adjuvant

1. Introduction

The recent development of acellular pertussis vaccines has emphasised the inclusion of detoxified pertussis toxin (PT) as an essential component, alone or in combination with the adhesin filamentous haemagglutinin (FHA) or other adhesins such as pertactin or fimbriae. Other possible protective antigens have thus far not been considered. Results of recent vaccine efficacy trials indicate that multicomponent acellular vaccines (ACVs) are highly efficacious, but that mono- and two-component ACVs may be less effective [1].

Bordetella pertussis adenylate cyclase toxin (ACT) plays an important role in lung colonisation and establishment of infection, at least in the mouse model. *B. pertussis* strains defective in ACT production are avirulent in mice, show little proliferation and are rapidly cleared from the lungs [2–4]. The N-terminal 400 amino acids of ACT have adenylate cyclase en-

* Corresponding author. Tel.: +44 (141) 330 5845; Fax: +44 (141) 330 4600; E-mail: j.coote@bio.gla.ac.uk

0928-8244/99/$20.00
PII: S0928-8244(98)00146-1

zymic activity which is stimulated up to 1000-fold by host calmodulin. The remainder of the molecule has membrane-targeting and pore-forming activity. ACT invades target cells, whereupon the N-terminal adenylate cyclase enzymic moiety is activated by host calmodulin to produce high levels of cyclic AMP (cAMP) which impairs the functions of immune effector cells and is assumed to assist survival of the bacterium in the initial stages of respiratory tract colonisation [5]. Recent work has indicated that ACT is responsible for apoptosis of mouse alveolar macrophages in vivo [6]. An immune response to this toxin may therefore be an important anti-colonisation factor.

The immune response to ACT has not been extensively investigated. The immunogenic properties of ACT were indicated by reports of high-titre anti-adenylate cyclase (AC) antibodies in pertussis patients and in vaccinated infants and adults [7–9]. Active ACT purified from *B. pertussis* protected against lethal challenge and shortened the period of lung colonisation of infant mice challenged 14 days after vaccination [10,11]. Later work, which expressed in *Escherichia coli* the structural gene, *cyaA*, for ACT and the gene *cyaC* whose product is required for activation of the toxin to an active form, showed that the active form was protective against *B. pertussis* infection of the mouse lung, whereas the inactive toxin was not [9]. Protective anti-CyaA antibodies were directed at the last 800 residues of the protein and were assumed to recognise conformational epitopes created at the C-terminal end following activation by the CyaC protein [12]. In this report we have compared the mouse protective properties of native ACT purified from *B. pertussis* with those of recombinant ACT preparations purified from *E. coli* and examined the adjuvant effect of ACT on the immune response to a co-administered antigen.

2. Materials and methods

2.1. Bacterial strains and plasmids

B. pertussis 18-323 is a reference strain (NCTC 10739) and the standard strain for mouse intracerebral challenge in vaccine potency testing. It was used in this study for mouse intranasal challenge in protection tests. BPDE386 (kindly supplied by Alison Weiss, University of Cincinnati, OH, USA) is a derivative of *B. pertussis* strain Tohama. It contains an oligonucleotide insertion which prevents expression of the *cyaC* gene encoding CyaC required for the post-translational activation of CyaA, encoded by the *cyaA* gene [13,14]. This strain produces CyaA which has AC enzymic activity, but no invasive or cytolytic activity. BP348 (pRMB1) is a Tn*5* insertion *cyaA* gene mutant of *B. pertussis* Tohama [15] containing a recombinant cosmid (pRMB1) encoding the entire *cya* locus [16]. This strain produces large amounts of active CyaA which has both AC enzymic and cytotoxic activities [17].

E. coli BL21/DE3 (F^- *ompT* r_B^- m_B^-) was used as host strain for production of recombinant proteins. The CyaA and CyaC proteins were expressed from separate compatible plasmids which allowed inactive CyaA to be prepared in the absence of CyaC and active CyaA to be made in its presence [18,19]. The regions containing the initiating ATG codons of *cyaA* and *cyaC* of *B. pertussis* strain Taberman were converted to *Nde*I (CATATG) sites by PCR. The *Nde*I site introduced by PCR allowed direct linkage of the PCR-amplified coding sequence of each gene to the T7 gene 10 leader sequence of the T7 RNA polymerase vector pET11a (Novagen). The T7 and *cyaC* sequences were subsequently subcloned into pACYC184 for co-expression of CyaC with CyaA. Recombinant proteins, either CyaA alone to produce inactive toxin or in combination with CyaC to produce active toxin, were expressed in *E. coli* from the authentic ATG codons and contained no foreign amino acids. A hybrid CyaA toxin (Hyb2), in which an internal domain of CyaA (amino acids 688–918) has been replaced with a 238-amino acid sequence from the analogous region of LktA of *Pasteurella haemolytica* (residues 379–616) [19], was also expressed, alone or in combination with CyaC, in *E. coli* and purified. This hybrid protein has full AC enzymic activity, but low invasive and cytolytic activities.

2.2. Preparation and purification of CyaA for immunisation

B. pertussis strains were grown on Bordet-Gengou (BG) medium (Gibco) containing 20% (v/v) defibri-

nated horse blood for 72 h at 37°C in a humid atmosphere. Bacteria harvested from lawn plates were cultured in cyclodextrin liquid medium [20] containing 10% (w/v) bovine serum albumin (Sigma) for 48 h at 37°C. Cells were harvested by centrifugation and extracts prepared in 8 M urea as described previously [21]. Recombinant CyaA and Hyb2 proteins were expressed as inclusion bodies in *E. coli* and extracts of these were prepared in 8 M urea as described previously [18,19]. All CyaA and Hyb2 preparations were purified by DEAE-Sepharose column chromatography [22] followed by calmodulin-agarose chromatography [17].

2.3. Immunisation and intranasal challenge

Male, 3–4 week old HAM ICR (CD-1) mice randomised in groups of 10 were injected subcutaneously (s.c.) with 250 μl containing 15 μg per mouse of purified CyaA or hybrid CyaA preparation and alhydrogel (250 μg ml^{-1}) in phosphate-buffered saline (PBS) or 1/5 human dose of adsorbed DPT vaccine (Wellcome Trivax-AD) using two doses at a 2-week interval. One group of mice was vaccinated with alhydrogel in PBS as a control.

For challenge, *B. pertussis* 18-323 was grown as a lawn on BG agar for 24 h at 37°C and bacteria were suspended in a casamino acids solution [16]. A sublethal dose (10^6 cfu per mouse in 50 μl) was instilled intranasally into five mice of each group 7 days after the second vaccination. The weight of the mice before and at intervals up to 7 days after challenge were recorded. Mice were killed 7 days after challenge and the lungs were removed aseptically. Lung pathology and lung weight as per cent of body weight were determined and they were then homogenised in casamino acids solution. Dilutions of homogenate were cultured on BG agar at 37°C in a humid atmosphere and colonies counted after 72 h.

2.4. Adjuvanticity of CyaA

Groups of 20 mice were injected s.c. with 250 μl PBS containing ovalbumin (Sigma) 20 μg per mouse alone or mixed with 15 μg per mouse of purified active or inactive recombinant CyaA. Two weeks after the first vaccination, 10 mice from each group were given a second injection. Sera were taken from five mice of each group at 7-day intervals after the first injection.

2.5. Toxin assays

AC enzymic activity was determined in the presence of 1 μM calmodulin (Sigma) as described previously [23]. One unit of AC enzymic activity produces 1 μmol of cAMP in 1 min at 30°C and pH 8.0. Protein concentrations were determined by the method of Bradford [24]. Invasive AC activity was determined with BHK21 cells using a shape change assay described previously [21]. BHK21 cells were mixed with serial dilutions of toxin preparations containing 3 mM $CaCl_2$ and allowed to spread in the absence of serum on fibronectin-coated multiwell plates. After 90 min at 37°C, the cells were fixed and stained with Coomassie blue and examined microscopically for the stellated morphology which results from cAMP elevation. Protein concentration required for 50% stellation was estimated from a dose-response curve of mean percentage stellation as a function of protein concentration. Haemolytic activity was measured by mixing an aliquot of the reaction mix with sheep erythrocytes, followed by incubation at 37°C for 18 h as described previously [19]. Cytotoxicity against mouse macrophage-derived (J774.2) cells was measured as described previously [19]. Assays were performed in 96-well tissue culture plates. Toxin dilutions (50 μl) were mixed with 50 μl of cell suspension. After incubation for 2 h at 37°C in a humid atmosphere, cell viability was determined using the CellTiter96™ kit (Promega) according to the manufacturer's instructions. This assay is based on the inhibition of the ability of active mitochondria in living cells to reduce tetrazolium dye.

2.6. SDS-PAGE and immunoblotting

Proteins were separated on SDS-polyacrylamide 7.5% or 10% gels and visualised by staining with Coomassie blue or transferred electrophoretically to Hybond C membranes (Amersham). Membranes were blotted with rabbit anti-CyaA serum and bound antibody was detected with alkaline phosphatase-labelled anti-rabbit immunoglobulin (Scottish Antibody Production Unit). For rabbit antibody production, CyaA was resolved by SDS-PAGE, the

respective 200-kDa protein band excised, washed in sterile PBS, crushed, suspended in sterile PBS and dialysed against saline at 4°C for 18 h. The supernate from the dialysis sac was emulsified in an equal volume of Freund's incomplete adjuvant (Sigma), injected intramuscularly in a rabbit and the final antiserum collected 1 week after the rabbit had received four booster injections at monthly intervals.

2.7. Enzyme-linked immunosorbent assay (ELISA)

For detection of anti-CyaA antibodies, ELISA plates (Immulon-2, Dynatech) were coated overnight at 4°C with 100 μl 50 mM sodium carbonate buffer (pH 9.8) containing 50 μg ml^{-1} purified active recombinant CyaA or 1 mg ml^{-1} ovalbumin (Sigma). Plates were washed with PBS (pH 7.4) containing 0.05% (v/v) Tween 20 (Sigma) and blocked by incubation with PTB buffer (PBS (pH 7.4) containing 0.05% (v/v) Tween 20 and 2% (w/v) bovine serum albumin (Sigma)) for 1 h at 37°C. After washing the plates as above, 100 μl of two-fold serial dilutions of test sera in washing buffer were dispensed into the ELISA plate wells and incubated for 1 h at 37°C. After washing, the plates were incubated with sheep anti-mouse-peroxidase conjugate (SAPU) diluted 1:2000 in wash buffer (100 μl per well). Following incubation at 37°C for 1 h, a peroxidase substrate solution (0.034% (w/v) *ortho*-phenylene diamine (Sigma), 0.002% (v/v) H_2O_2 in citrate-phosphate buffer (pH 5)) was added and the ELISA plate incubated in the dark for 20 min. The reaction was stopped by addition of 50 μl per well of 12% (v/v) H_2SO_4 and the absorbance values were measured at OD_{492nm} in an Anthos ELISA reader. Antibody titres were determined as ELISA U ml^{-1} of neat mouse serum by assigning an arbitrary unitage of 100 ELISA U ml^{-1} to pooled reference antisera raised against either purified active recombinant CyaA or ovalbumin according to the immunisation schedule given above. By interpolating at OD_{492nm} 0.5, the antibody titre in ELISA U ml^{-1} was calculated from the ratio of test serum titre to reference serum titre $\times 100$. With sera from non-vaccinated control mice the anti-OA and anti-CyaA antibody levels were below the limit of detection, with absorbance values at background levels ($OD_{492nm} < 0.2$), even in undiluted sera.

3. Results

3.1. Production and properties of CyaA proteins

Active and inactive CyaA, either the native forms from *B. pertussis* or the recombinant forms produced in *E. coli*, were purified by affinity chromatography. All preparations contained a major polypeptide of approximately 200 kDa M_r which reacted with anti-CyaA polyclonal antiserum in Western blots (data not shown). Hyb2 was also purified as a ~200-kDa protein which reacted with anti-CyaA polyclonal antiserum (data not shown). The purified proteins produced some faint lower-M_r bands which probably represented breakdown products of the full-length protein. The specific AC activities of all

Table 1
Properties of CyaA toxins from *B. pertussis* and recombinant *E. coli* strains[a]

CyaA preparation	Enzymic AC activity (U mg $protein^{-1}$)	Invasive activity (μg protein ml^{-1} for 50% stellation of BHK cells)	Haemolytic activity (μg protein ml^{-1} for 20% haemolysis)	Cytotoxic activity (μg protein ml^{-1} for 50% killing)	
				J774.2 cells	BL3 cells
Active nCyaA[b]	619 ± 41	2.3 ± 0.6	5.9 ± 1.2	2.7 ± 0.3	2.6 ± 0.5
Inactive nCyaA[b]	603 ± 27	> 58	> 63	> 60	> 51
Active rCyaA[c]	626 ± 37	2.9 ± 0.4	9.4 ± 1.5	3.3 ± 0.4	3.3 ± 1.1
Inactive rCyaA[c]	674 ± 73	> 72	> 89	> 72	> 57
Active Hyb2	291 ± 53	> 46	24.4 ± 2.0	15.6 ± 1.7	19.1 ± 2.6
Inactive Hyb2	408 ± 28	> 72	> 64	> 83	> 117

[a]Results are the mean values ± S.E.M. of four separate determinations.
[b]nCyaA, native CyaA prepared from *B. pertussis*.
[c]rCyaA, recombinant CyaA.

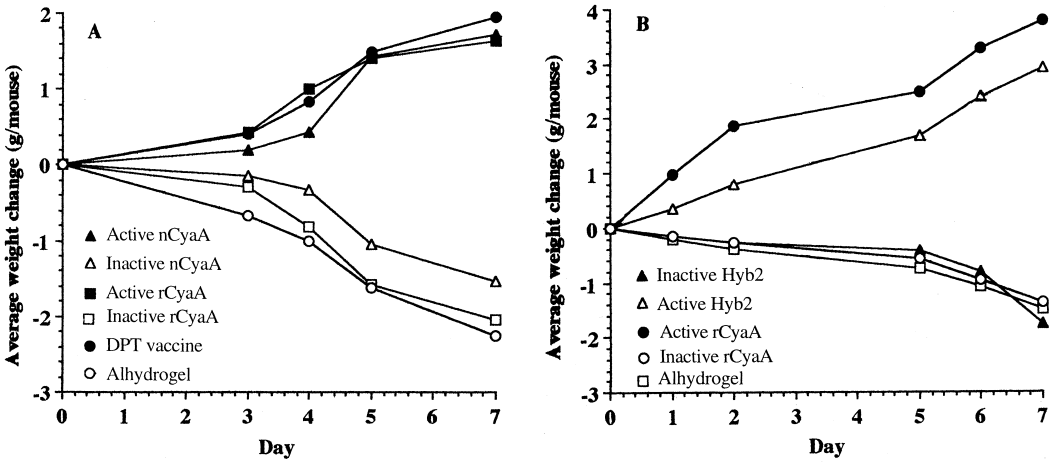

Fig. 1. Weight change of mice after challenge. Mice, randomised in groups of five, were challenged intranasally with *B. pertussis* 18-323 1 week after the second vaccination with purified CyaA and hybrid CyaA preparations or, in control mice, with DPT vaccine or alhydrogel alone. The experiment reported in panel A was repeated with similar results. nCyaA, native CyaA prepared from *B. pertussis*; rCyaA, recombinant CyaA.

the toxins were similar (Table 1). The toxins produced in the absence of CyaC activity had no cell-invasive, haemolytic or cytotoxic activity. The cell-invasive and cytotoxic activities of the native CyaA and recombinant CyaA produced in the presence of CyaC were similar, but the haemolytic activity of the recombinant toxin was lower than that of the native protein, a feature of the toxin produced in *E. coli* which has been reported previously [25]. The toxic activities of Hyb2 were all noticeably lower than the CyaA proteins, in agreement with previous work [19].

3.2. Mouse weight-gain test for toxicity

The effect of vaccination of mice with purified CyaA or hybrid CyaA preparations from *B. pertussis* strains and recombinant *E. coli* strains was studied over 21 days post vaccination. All mice in each group gained weight in a similar and uniform manner over the 21-day period and no deaths were recorded (data not shown).

3.3. Mouse protection

Mice immunised with two doses of vaccine preparation were challenged intranasally with 10^6 cfu per mouse of *B. pertussis* 18-323 1 week after the second vaccination. After challenge, mice vaccinated with active CyaA or DPT vaccine continued to gain weight in a similar manner to those that were left as non-vaccinated, non-challenged controls (Fig. 1A). Mice receiving active Hyb2 gained weight, but not as well as those receiving active CyaA or DPT vaccine (Fig. 1B). In contrast, mice injected with inactive CyaA, inactive Hyb2 or alhydrogel alone showed a marked weight loss over the same period after challenge (Fig. 1). Two deaths were recorded 2 days after challenge in the group given alhydrogel alone, but no deaths were observed in groups receiving inactive toxin preparations.

There were clear differences in the lung weight as a percentage of body weight (up to two-fold) of mice vaccinated with any of the inactive toxins and alhydrogel compared to mice vaccinated with any of the active toxins and DPT vaccine as well as non-vaccinated, non-challenged control mice (Table 2). The lung counts of *B. pertussis* from mice vaccinated with any of the inactive toxins and alhydrogel showed up to 3 $\log_{10}$ differences compared to mice vaccinated with active toxins or DPT vaccine. Although the active Hyb2 preparation was clearly protective, in the one experiment performed, it did not appear to be as effective as the active CyaA preparations. In two experiments, the active native CyaA was as protective as the whole cell DPT vac-

cine and the active recombinant CyaA only slightly less efficient (Table 2).

3.4. Mouse IgG antibody responses to CyaA antigens

All CyaA preparations used with alhydrogel were highly antigenic in stimulating anti-CyaA IgG antibody responses in mice. Serum IgG titres ranged between 67.4 ± 1.7 and 83.1 ± 3.4 ELISA U ml^{-1} (mean values of 10 individual mouse sera ± S.E.M.) taken 7 days after the second vaccination. Inactive CyaA preparations with alhydrogel were as effective as active CyaA preparations in raising antibodies which cross-reacted with active CyaA as coating antigen in the ELISA assay. This meant that, although inactive CyaA was not protective, it nevertheless raised antibody to CyaA to a level equivalent to that of the protective, active form of the toxin. Active and inactive Hyb2 preparations also raised anti-CyaA antibodies to a similar level to those raised by CyaA (data not shown). Western blot analysis showed that sera raised against either the active or non-active recombinant CyaA recognised both active and inactive CyaA preparations prepared from *E. coli* or *B. pertussis* as well as the Hyb2 toxin, again indicating that the toxin preparations had certain common epitopes (data not shown).

3.5. Adjuvanticity of CyaA on the antibody response to ovalbumin

Mice were vaccinated twice, with a 2-week interval, with purified active or inactive CyaA with or without ovalbumin (OA) as the test antigen. Sera were taken over a period of 4 weeks at 1-week intervals starting from day 7 and titrated by ELISA. Two identical experiments produced similar results and the data from one are shown in Fig. 2. The primary anti-OA antibody response was enhanced 3–4-fold in mice vaccinated with OA plus active CyaA compared with the mice injected with OA alone (Fig. 2A). A slight adjuvant effect of inactive CyaA was apparent, but it was much weaker than that of the active CyaA. The secondary IgG anti-OA antibody

Table 2
Active protection against intranasal challenge with *B. pertussis* by vaccination of mice with CyaA preparations

Vaccine	Experiment No.	Mean lung weight (g) as % body weight ± S.E.M.[a]	Mean *B. pertussis* cfu per lung $\times 10^{-3}$ ± S.E.M.[a]
Active nCyaA[b]	1	0.82 ± 0.05	0.68 ± 0.18
	2	0.73 ± 0.02	2.6 ± 0.11
Inactive nCyaA[b]	1	1.42 ± 0.02	4220 ± 380
	2	1.29 ± 0.04	1700 (median)
Active rCyaA[c]	1	1.01 ± 0.04	0.91 ± 0.28
	2	0.89 ± 0.02	3.52 ± 0.33
	3	0.86 ± 0.03	5.9 ± 0.16
Inactive rCyaA[c]	1	1.45 ± 0.06	> 5000
	2	1.37 ± 0.03	4500 (median)
	3	1.39 ± 0.06	> 25000
Active Hyb2	1	1.0 ± 0.01	15.7 ± 0.13
Inactive Hyb2	1	1.47 ± 0.07	> 25000
Alhydrogel only	1	1.45 ± 0.07	5730 ± 420
	2	1.45 ± 0.02	4940 ± 640
	3	1.55 ± 0.09	> 25000
DPT vaccine	1	0.84 ± 0.01	< 0.5
	2	0.93 ± 0.02	4.78 ± 1.17
	3	0.87 ± 0.02	3.6 ± 0.05
Control[d]	1	0.71 ± 0.01	< 25

[a]Results are the mean values from five individual mice ± S.E.M. or median values for lung counts where counting was recorded from confluent culture plates.
[b]nCyaA, native CyaA prepared from *B. pertussis*.
[c]rCyaA, recombinant CyaA.
[d]Control, non-vaccinated and non-challenged mice.

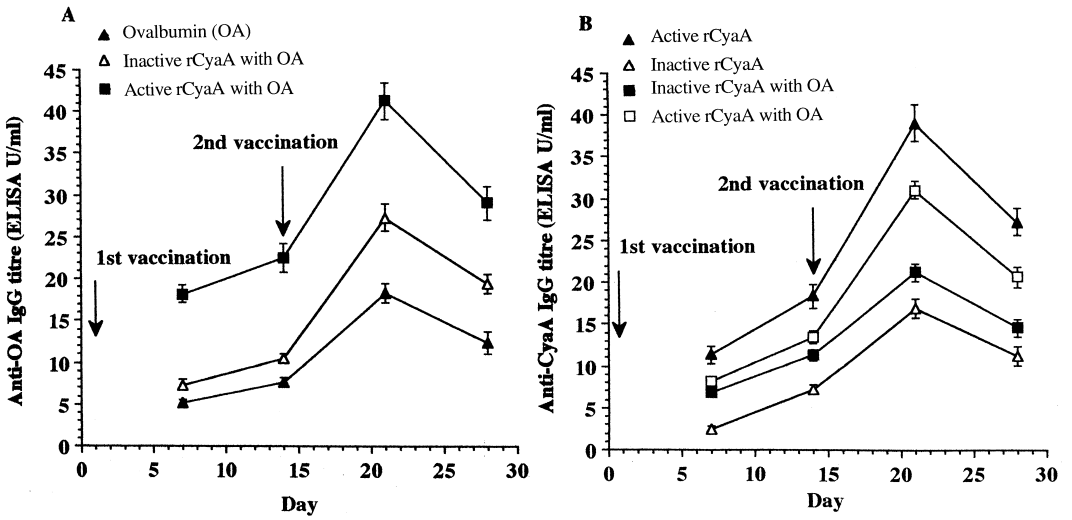

Fig. 2. Effect of purified recombinant CyaA preparations on primary and secondary anti-OA or anti-CyaA antibody responses. Mice, randomised in groups of 20, were vaccinated subcutaneously at a 2-week interval with 15 μg CyaA per mouse with or without 20 μg ovalbumin, or with 20 μg ovalbumin alone. Anti-OA or anti-CyaA IgG antibodies were measured by ELISA in sera from five mice per group at each sampling time. OA was used as coating antigen for the results shown in panel A, whereas purified active recombinant CyaA (rCyaA) was used as coating antigen in panel B. The experiment was repeated with similar results.

response was also increased in mice injected with OA plus active CyaA compared with the mice receiving OA alone. Again, the effect with inactive CyaA was not as marked.

The primary and secondary IgG anti-CyaA antibody responses were also evaluated (Fig. 2B). The primary response was enhanced 2–3-fold in mice primed with active CyaA compared with mice vaccinated with inactive CyaA. The secondary response was also increased 2–3-fold. OA had no adjuvant effect on the response to active CyaA and, in the two experiments preformed, appeared to reduce both the primary and secondary responses, whereas the responses to the inactive form of CyaA were apparently slightly increased in the presence of OA.

4. Discussion

All purified CyaA and hybrid toxin preparations were produced as full-length proteins which ran as 200-kDa proteins on SDS-PAGE gels, although some evidence of lower-M_r fragments was evident in the preparations. The full-length CyaA protein, in either the native or recombinant form, is susceptible to proteolytic degradation [9,17,25,26]. Although the native and recombinant CyaA preparations had similar AC-specific activities and cell-invasive and cytotoxic activities, the CyaA produced in *E. coli* had a lower haemolytic activity, a feature noted previously [25]. This is probably due to differences in the way in which the modifying C protein behaves in the different bacterial host backgrounds. Biochemical characterisation of the CyaA produced in *E. coli* has revealed that, whereas CyaA from *B. pertussis* is exclusively palmitoylated at Lys-983, the recombinant CyaA represented a heterogeneous population of CyaA proteins with 13% myristoylated and 67% palmitoylated at Lys-983 and an additional 67% of the Lys-860 residues palmitoylated [27]. The hybrid CyaA toxin (Hyb2), where an internal domain of CyaA close to the CyaC modification site at Lys-983 is replaced with a 238-amino acid sequence from the homologous region of LktA of *P. haemolytica* [19], when expressed in combination with CyaC, had full AC enzymic activity, but low invasive and cytolytic activities. This hybrid protein possesses a putative acylation site for LktA at Lys-544 and is more efficiently activated by LktC than by CyaC in vivo in *E. coli* [19]. However, Hyb2 is clearly activated by CyaC which may mean that, although it has low toxic activity, there may never-

theless be a conformational change in the protein through the action of CyaC which creates immunogenic and protective properties and this was investigated.

The data from the mouse weight-gain test (toxicity test) following immunisation with active and inactive toxin preparations showed no clear toxic effect on the mice. Indeed, there were no obvious differences between mice vaccinated with the active invasive toxins and those given the inactive non-invasive forms. This indicated that, at least at the dosage used here, active CyaA was not creating a toxic physiological response, and no acute toxic effects of active CyaA on the whole animal have been reported. This contrasts with the effects on the whole animal of pertussis toxin which has a lethal dose in the mouse of 0.5 μg [28]. Both of these toxins act by raising the intracellular cAMP concentration in mammalian target cells. The action of CyaA is very rapid with cAMP accumulating immediately after cell exposure and AC activity reaching a peak within 10–40 min. The high AC activity is maintained only for as long as the target cells are exposed to the toxin as removal of exogenous toxin promotes a rapid decline in intracellular AC activity due to proteolytic destruction of the internalised CyaA [29]. PT irreversibly inactivates the inhibitory subunit of the eukaryotic AC [30], but although stimulation of AC activity has been reported, PT is a slower acting toxin than CyaA and the accumulation of cAMP in target cells has not been demonstrated as it has with CyaA. The functional differences between the toxins are such that CyaA must be continuously present to promote cAMP accumulation whereas the consequences of PT action will be prolonged even in the absence of the toxin.

Purified active CyaA, either the native form from *B. pertussis* or the recombinant form, exhibited protective activity in mice against intranasal challenge with *B. pertussis* 18-323 similar to the protective efficacy of the whole-cell DPT vaccine. This was shown by low lung:body weight ratio and low bacterial numbers in the lungs compared to mice vaccinated with purified inactive CyaA of 200 kDa. These data confirmed that CyaC-mediated modification of CyaA toxin is critical for its protective activity. Our data from two separate experiments indicated that the recombinant active CyaA had only slightly less protective efficacy than the native active toxin. Betsou et al. [9] found, using the rate of clearance of *B. pertussis* challenge from the lungs of mice as a measure of protection, that bacterial numbers decreased more rapidly in mice immunised with active CyaA prepared from *B. pertussis* than in the group immunised with active recombinant CyaA. Any differences in the immunogenic behaviour of the CyaA toxins are presumably related to differences in the manner in which the CyaC protein post-translationally modifies the protoxin in the different bacterial host backgrounds, as noted above. The active Hyb2 protein was also protective against *B. pertussis* challenge, albeit at a lower efficacy than active CyaA. Thus this hybrid toxin, which showed $< 20\%$ of the cell invasive and cytotoxic activities of CyaA, acted nevertheless as a protective antigen. This suggested that much of the protective epitope(s) structure determined by the C-terminal portion of CyaA had been retained. Another hybrid toxin (Hyb1), which contained the N-terminal enzymic domain and the pore-forming domain from CyaA (amino acids 1–687) with the remainder of the protein derived from the C-terminal end of LktA (amino acids 379–953) [19], showed no protective activity and exhibited a similar pattern of response in mice to inactive CyaA (data not shown).

Co-administration of active rCyaA with OA enhanced several-fold the specific IgG antibody responses to OA compared with administration of OA alone. The enhancement in antibody response was not observed to the same extent with inactive CyaA. This suggests that the invasive activity of CyaA may be responsible primarily for the enhancement of antibody responses to OA via increases in the level of intracellular cAMP in immune effector cells. In keeping with this, the active rCyaA stimulated a greater IgG response to itself than the inactive rCyaA. It has been reported that cAMP upregulates IL-4 and IL-5 production by activated $CD4^+$ T cells [31]. It is well documented that PT augments IgG and IgE antibody responses to concurrently administered antigens [32–34], but the mechanism(s) by which PT exerts adjuvanticity is not clear.

The nature of the protective immune response generated by CyaA requires further investigation. As an invasive protein which is degraded intracellularly, it may be processed via the MHC class I-restricted

antigen presentation pathway which is important for cell-mediated immunity. Recent work has established that *B. pertussis* can invade and survive within a variety of eukaryotic cells, including macrophages and human respiratory epithelial cells, and this may relate to an intracellular persistent state against which humoral immunity is not effective. Induction of cell-mediated immunity has been shown to be necessary for effective clearance of *B. pertussis* in the mouse [35–37], but the role of ACT in the induction of T-cell responses has not been studied. In addition, it will be of interest to study any enhancing effect of CyaA on the antibody response to antigens currently included in acellular vaccines such as PT, FHA and PRT, and to investigate if the AC activity of CyaA is required for its adjuvant activity. Some doubts have been expressed about the use of CyaA as a vaccine antigen because of a cross-reaction with mammalian brain adenylate cyclase [38], but this cross-reaction may be confined to the catalytic and calmodulin binding sites at the N-terminal end of the toxin and it may be possible to alleviate this problem by deletion or alteration of these regions.

Acknowledgments

We thank Susan Campbell for excellent technical assistance. The work was supported in part by the Wellcome Trust and an Iranian Government scholarship to K.H.

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