EXPERT REVIEWS

Adenylate cyclase toxin-hemolysin relevance for pertussis vaccines

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The adenylate cyclase toxin-hemolysin (ACT, AC-Hly or CyaA) is a key virulence factor of *Bordetella pertussis*. It targets bactericidal activities of phagocytes, such as oxidative burst and complement- or antibody-mediated opsonophagocytic killing of bacteria. Through cAMP signaling, CyaA also skews TLR-triggered maturation of dendritic cells, inhibiting proinflammatory IL-12 and TNF-α secretion and enhancing IL-10 production and Treg expansion, likely hampering induction of adaptive immune responses to *Bordetella* infections. Non-enzymatic CyaA toxoid is a potent protective antigen and adjuvant that boosts immunogenicity of co-administered *B. pertussis* antigens and improves potency of acellular pertussis (aP) vaccines in mice. This makes CyaA a prime antigen candidate for inclusion into a next generation of aP vaccines. Moreover, recombinant CyaA toxoids were recently shown to be safe in humans in frame of Phase I clinical evaluation of a CyaA-based immunotherapeutic vaccine that induces Th1-polarized $CD8^+$ cytotoxic T-lymphocyte responses targeting cervical tumors.

Keywords: adenylate cyclase toxin • antigen delivery • *Bordetella pertussis* • cancer immunotherapy • complement receptor 3 • dendritic cell • pertussis • pertussis vaccine • T-cell epitope • T-cell vaccine • whooping cough

Discovery of adenylate cyclase toxin-hemolysin & its potential as a protective antigen in pertussis vaccines

In 1976, Hewlett and colleagues found that liquid culture supernatants of exponentially growing *Bordetella pertussis* bacteria contain a soluble adenylyl cyclase (AC) enzyme (EC 4.6.1.1) converting ATP to cAMP, a 'second messenger' and key intracellular signaling molecule [1]. The AC was detected also in several commercial whole-cell pertussis (wP) vaccine preparations [2]. Intriguingly, its specific activity was increased over 1000-fold by binding of calmodulin, which is absent in bacteria and occurs in the cytosol of eukaryotic host cells [3]. Confer and Eaton then showed in 1982 that the extracellular bacterial AC enzyme invades phagocytic cells and by elevating their cytosolic concentrations of cAMP it ablates opsonin-induced production of superoxide and opsonophagocytic killing of bacteria [4]. This established the bacterial-surface-associated and -secreted AC of *B. pertussis* as a *bona fide* adenylate cyclase toxin (ACT) that targets sentinel cells of innate immune defense.

In 1983, Weiss *et al.* isolated a Tn5 transposon mutant of *B. pertussis* (BP348) that lacked production of both the AC and the hemolytic activity and was strongly impaired in virulence toward infant mice [5,6]. Further use of the Tn5 mutants revealed that while pertussis toxin (PT) was critical for lethal infection, the newly discovered ACT played a critical role in the capacity of *B. pertussis* to colonize infant mice [7] and its AC activity was particularly important for initiation of bacterial infection [8,9].

At the time of acellular pertussis (aP) vaccine development efforts in the early 80s of the past century, the evidence that ACT was a potent immunosubversive toxin did not receive adequate attention. Despite the seminal observation of Confer and Eaton that ACT subverted a central innate defense mechanism of naive hosts, namely the complement-dependent opsonophagocytic killing of bacteria by neutrophils and alveolar macrophages [4], the AC toxoid of ACT was not considered for inclusion into the aP vaccine. This 'omission' was partly due to the general acceptance of the

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concept of pertussis (whooping cough) being a PT-mediated disease [10]. Attention was, hence, channeled to PT as the prime target for neutralization by aP vaccine-induced antibodies, which by preventing PT-mediated hyperleukocytosis account for the life-saving capacity of the aP vaccine in infants.

An additional factor that contributed to skipping on the potent immunosubversive role played by ACT in pertussis pathogenesis was the lack of knowledge on molecular characteristics of ACT. Compared with PT, the yields of the AC toxin purified from *B. pertussis* cultures were very low and little was known on its immunogenicity and stability. Before 1988, several teams reported and biochemically characterized cell-invasive *B. pertussis* AC toxins of rather varying size, activity and degrees of purity.

The molecular nature of ACT became truly known only in 1988 when the *cyaABDE* operon, encoding the calmodulin-activated AC toxin-hemolysin (CyaA), was cloned and sequenced by Glaser at Institut Pasteur in Paris [11,12]. Sequencing of the operon revealed that CyaA was a 1706 residue-long AC-hemolysin fusion protein. Its AC enzyme domain of 400 residues was found to be fused to an α-hemolysin-resembling repeat-in-toxin (RTX) moiety of 1306 residues, which mediates toxin secretion through the type I secretion system [12–14]. The recombinant toxin expressed from the *cyaA* gene in *Escherichia coli*, however, failed to penetrate lymphocytes [15], indicating that activation by a post-translational modification was required for biological activity of ACT. Indeed, a fifth *cya* gene, *cyaC*, transcribed divergently to *cyaABDE* genes, was soon identified and shown to be essential for production of a hemolytically active and cell-invasive AC toxin [16]. One of the authors of this article (PS) could then design in 1990 a first system for high level production of recombinant and CyaC-activated CyaA in *E. coli* cells [17]. This opened for a wealth of studies on ACT, as recombinant CyaA could be easily purified in sufficient quantities by calmodulin affinity chromatography from urea extracts of inclusion bodies formed in *E. coli*. It could, hence, be conclusively demonstrated in 1993 that CyaA used as an experimental vaccine conferred on mice a high level of protection against a respiratory challenge by *B. pertussis* [18,19]. At that time, the CyaA preparations were still contaminated by uncontrolled amounts of *E. coli* LPS, which may have potentiated the protective capacity of CyaA through unspecific activation of innate immunity. However, the induced protection strongly depended on the level of post-translational modification of proCyaA to CyaA by a covalent amide-linked palmitoylation of the lysine 983 residue by the co-expressed acyl transferase CyaC, thus linking protection to a biologically active conformation of CyaA [20,21]. Indeed, the protective immunogenicity particularly required the structural integrity of the last approximately 900 residues of CyaA that comprised the RTX repeats domain and the palmitoylated lysine 983 residue site [22].

Almost four decades from the discovery of ACT, there is now a substantial body of knowledge available on the mechanism of action of CyaA on host cells and on its multiple roles played in subversion of both innate immunity and in hijacking of adaptive host immune responses [23–25]. This constitutes solid grounds for considering inclusion of the AC toxoid into the next generation of aP vaccines. It is plausible to assume that a capacity to induce ACT-neutralizing antibody responses may improve the recently questioned capacity of aP vaccines to prevent *B. pertussis* colonization and transmission in fully aP-vaccinated children or adults on longer term and under outbreak settings [26–30]. There are good reasons to believe that absence of neutralization of the immunosuppressive action of ACT may facilitate the escape of bacteria from surveillance of host immune system and allow *B. pertussis* to colonize respiratory epithelia of aP-vaccinated subjects. The subversive effects on immune defense of the host elicited by cAMP signaling of ACT would range from early paralysis of bactericidal activities of phagocytes, to the capacity of ACT to fool and suppress induction of adaptive T-cell immune responses, up to a modulatory action of ACT on antigen-presenting intraepithelial dendritic cells (DCs) and expansion of suppressive Treg cells. As outlined below, this would be complemented by manipulation of physiology of respiratory epithelial cells, such as enhanced chloride and mucosal fluids secretion providing nutrients for *B. pertussis* growth on epithelial surfaces.

What makes CyaA rather unique among toxins and antigens produced by *Bordetellae* pathogenic to mammals is the conserved expression of CyaA by all three species pathogenic to mammals, *B. bronchiseptica, B. parapertussis* and *B. pertussis*, respectively. The amino acid sequences of CyaA produced by the three species show some residue divergence, particularly in the C-terminal repeat domains, potentially reflecting adaptation for binding to complement receptors of diverse hosts [31,32]. This may account for lack of cross-protective immunogenicity of CyaA between these *Bordetellae* [33]. Within the *B. pertussis* species, the CyaA sequence is extremely conserved and one of the least variable antigens, since the recent genomic sequencing of a worldwide collection of 343 *B. pertussis* strains isolated between 1920 and 2010 revealed a single nucleotide polymorphism in the *cyaA* gene that leads to a residue substitution in the produced protein, namely a rather conservative substitution of the valine residue 892 by a methionine [34]. This highlights that by difference to major aP antigens like pertactin, filamentous hemagglutinin (FHA), PT and fimbriae, the CyaA may be under strong structure–function optimization pressure, albeit it is hard to predict if antigenic variants of it will not be selected once the CyaA would make part of the aP vaccine.

Mechanism of biological action of CyaA in *Bordetella* infections

The CyaA toxin is a multifunctional protein of 1706 residues in length (Figure 1). It consists of an N-terminal enzymatic AC domain of about 400 residues that is fused to a pore-forming RTX family hemolysin/cytolysin (Hly) moiety of about 1300 residues [25]. The latter itself consists of a hydrophobic pore-forming domain (residues 500–700), a fatty acyl-modified domain (residues 800–1000), of a vast calcium-binding domain

characteristic of RTX proteins (residues 1000–1600), and a C-terminal secretion signal [14]. The Hly part of CyaA is involved in cell binding and enables delivery of the enzymatic AC domain into the cytosol of host cells to convert ATP to cAMP [3,35]. In parallel, CyaA is able to form small cation-selective pores in the cytoplasmic membrane of target cells, which account for the moderate hemolytic activity toward erythrocytes [36,37]. Both toxin activities then depend on the post-translational activation of proCyaA to CyaA, which consists in covalent palmitoylation of the ε-amino groups of lysine residues 860 and 983 by a co-expressed protein acyl transferase, CyaC [16,17,21,38,39]. Finally, the CyaA toxin structure and activity depends on calcium ion loading of the numerous (~40) binding sites in the RTX domain [40–42].

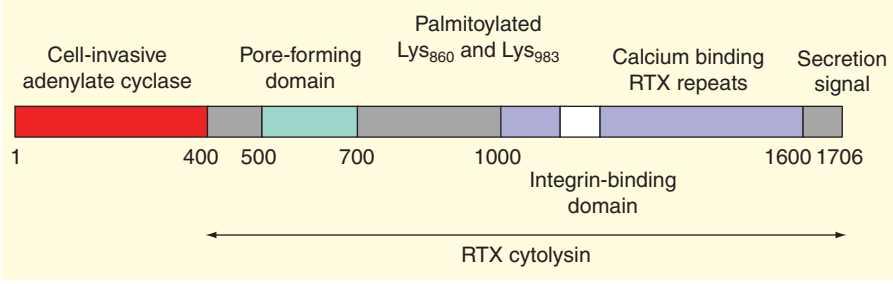

Figure 1. Schematic representation of adenylate cyclase toxin-hemolysin structure. CyaA consists of N-terminal cell-invasive adenylate cyclase domain and RTX cytolysin moiety. RTX cytolysin contains several subdomains: pore-forming domain, activation domain with two sites of post-translational palmitoylation, calcium-binding nonapeptide repeats and C-terminal secretion signal.
CyaA: Adenylate cyclase toxin-hemolysin; RTX: Repeat-in-toxin.

CyaA was shown to exert a complex array of cytotoxic and immunosubversive activities on host phagocytes, to which the toxin specifically binds with high affinity through the complement receptor 3 (CR3), also known as the $\alpha_M\beta_2$ integrin, CD11b/CD18, or Mac-1 [32]. The toxin initially interacts with N-linked oligosaccharides [43], recognizes CR3 through its CD11b subunit [32] and delivers the AC enzyme into phagocytes in two steps [44]. As schematically depicted in Figure 2, a membrane-inserted translocation precursor of CyaA generates a calcium-conducting path across cell membrane and mediates influx of extracellular calcium ions into cells [45]. This activates calpain-mediated processing of the talin tether of CD11b/CD18 and triggers relocation of the toxin-receptor complex into lipid rafts. There, translocation of the AC domain across the cytoplasmic membrane into cytosol of phagocytes is completed [44]. Indirect evidence suggests that the cell-invasive AC and the pore-forming activities of CyaA are accomplished by distinct subpopulations of CyaA conformers that employ the same transmembrane CyaA segments within target cell membrane in an alternative way [25]. The balance between the two activities can, indeed, be shifted in either direction by specific residue substitutions [46–49].

The toxin does not depend on receptor-mediated endocytosis and delivers its cell-invasive AC domain directly across the plasma membrane into cytosol of cells expressing the CR3 receptor ($CD11b^+$ cells), where the calmodulin-activated AC enzyme converts cytosolic ATP to cAMP. Supraphysiological levels of cAMP are generated in phagocytes within minutes of cell exposure to picomolar toxin concentrations and near-instantly ablate the bactericidal oxidative burst and opsonophagocytic killing capacities of neutrophils and macrophages [4,24,25,50,51]. At about two orders of magnitude lower efficacy, CyaA can also penetrate cells lacking the CR3 receptor ($CD11b^-$ cells) and thanks to its extremely high specific AC enzyme activity, it still can increase cAMP concentrations to easily detectable levels also in a variety of epithelial and other host cell types [25,52]. Recently, higher than anticipated levels of CyaA were detected in nasopharyngeal fluids and washes from diseased infants and experimentally infected olive baboons [53]. This suggests that CyaA may be playing a rather prominent role in modulation of respiratory epithelia function and damage during natural infections. CyaA-bearing outer membrane vesicles released by *B. pertussis* were reported to deliver CyaA also into polarized airway epithelial cells that lack CR3 and appear to be quite resilient to direct CyaA translocation across the membrane of apical cell surface [54,55]. cAMP signaling of CyaA delivered by outer membrane vesicles can, hence, be expected to importantly compromise the barrier function of airway epithelial layers. This may enable also free secreted toxin to access the basolateral side of polarized epithelia, from where CyaA was shown to efficiently invade epithelial cells and deregulate chloride secretion and potentially enhance also mucus production [55].

The recent development of an olive baboon weanling challenge model, in which pathophysiology of human pertussis can be truly reproduced, will enable use of AC-deficient *B. pertussis* strains for addressing of the question whether CyaA action dampens or promotes infected tissue inflammation. Induction of IL-6 production by CyaA in human tracheal epithelial cells was previously observed *in vitro* [56] and *in vivo* this would likely recruit neutrophils to the site of infection, at least as observed in the mouse respiratory challenge model [57,58]. Neutrophils would also be recruited by two parallel outcomes of CyaA action, the first depending on cAMP signaling of CyaA and induction of COX-2 expression in macrophages, yielding release of chemoattractive prostaglandins [59]. The other mechanism would involve permeabilization of intraepithelial ($CD11b^+$) DCs, where K^+ efflux through CyaA pores in the membrane of LPS-primed DCs was shown to contribute to NALP3 inflammasome complex formation, caspase-1 activation and release of proinflammatory IL-1β [60], which would also recruit neutrophils to the site of infection. In the mouse model, indeed, neutrophils would be the prime target of CyaA [61]. However, what role, if any, do neutrophils and airway tissue inflammation play in human infections by *B. pertussis* remains

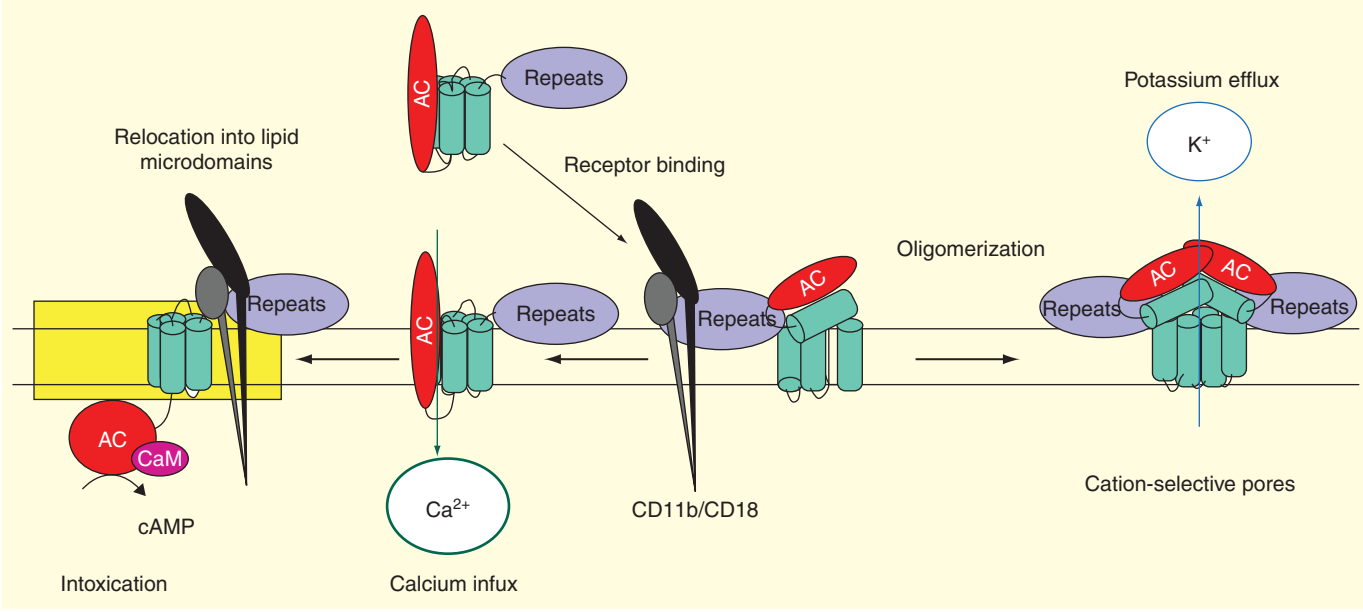

Figure 2. Schematic representation of adenylate cyclase toxin-hemolysin action. *Bordetella* adenylate cyclase toxin-hemolysin binds to the complement receptor 3 (known as CR3, $\alpha_M\beta_2$ integrin, CD11b/CD18 or Mac-1, respectively), penetrates the cytoplasmic membrane of phagocytes and employs two distinct conformers to exert its multiple activities. One conformer forms cation-selective pores that permeabilize phagocyte membrane for efflux of cytosolic potassium. The other conformer conducts extracellular calcium ions across cytoplasmic membrane of cells, relocates into lipid rafts, translocates the adenylate cyclase enzyme domain into cells and converts cytosolic ATP to cAMP.

an unresolved question. Pertussis is unique among infectious diseases by the absence of accompanying fever and other evidence of an inflammatory illness, even if severe and fatal [62,63]. Indeed, the limited histologic examination of human biopsies from pertussis patients that were free of obvious secondary infections did not reveal any massive neutrophil infiltration into *B. pertussis*-infected airway epithelia [64].

On the other hand, CyaA action was found to promote apoptosis of lung alveolar macrophages of experimentally infected mice and to subvert *in vitro* the profiles of secreted cytokines through biasing of TLR ligand-induced maturation of professional antigen-presenting cells, such as DCs, toward a 'tolerogenic' phenotype [65–71]. In particular, CyaA action was found to suppress TLR-induced secretion of proinflammatory IL-12 and TNF-α cytokines and to promote enhanced release of the immunosuppressive IL-10 cytokine from mouse and human DCs. We could recently confirm these effects also with TLR-activated DCs that were incubated with low and physiologically relevant CyaA amounts (10 ng/ml), or with live bacteria [72]. Such subversively matured mouse and human DCs expressed reduced surface amounts of the CD40 co-stimulatory molecule (involved in antigen-specific stimulation of T cells), exhibited enhanced TLR-stimulated chemotactic migration and a reduced capacity to stimulate antigen-specific T-cell immune responses *in vitro*. Moreover, the CyaA and TLR-treated DCs stimulated expansion of antigen-specific $FoxP3^+CD25^+CD4^+$ Treg lymphocytes *in vitro*. This suggests that CyaA may be contributing to the previously observed expansion of Treg cells in *B. pertussis*-infected mouse tissues [23]. This raises an intriguing hypothesis that outmigration of CyaA-subverted ('tolerogenic') DCs from airway epithelia into lymph nodes might be interfering with development of adaptive B- and T-cell responses to *B. pertussis* infection, thereby delaying clearance of bacteria and impacting on development of immune memory.

Immunogenicity in *Bordetella* infections & protective antigen activity of CyaA

Due to unavailability of sufficient amounts of sufficiently pure CyaA at the time of intense pertussis research in the era of aP vaccine development, and because of absence of ACT from the aP vaccine, only limited data on immunogenicity of CyaA in human infections by *B. pertussis* are available. Arciniega and colleagues noted in 1991 that antibodies to CyaA were common in sera from individuals diagnosed with pertussis [73]. They also found that for various *B. pertussis* antigens, the antibodies reactive with CyaA, presumably of maternal origin, were the most prevalent in cord-blood sera of newborns [74]. However, it remains unclear if these were due vaccination or previous infections of mothers. Some level of cross-reactive antibodies recognizing ACT may also be induced by RTX proteins produced by human microbiota, including the α-hemolysin HlyA produced by uropathogenic as well as many commensal *E. coli* strains, the FrpA/C proteins produced by the *Neisseria meningitidis* in nasopharynx, or LtxA produced by the oral cavity colonizer *Aggregatibacter actinomycetemcomitans* [14]. Presence of specific antibodies recognizing purified CyaA in immunoblots was, however, clearly observed in sera of infants diagnosed with whooping cough that were selected so as to be older than 8 months, in

order to exclude the presence of maternal antibodies, and younger than 2 years of age, so that their full clinical history could be known [22,75]. A post-vaccination study then revealed that antibodies to CyaA were induced by whole-cell pertussis vaccination series and increased steadily for up to 158 months after vaccination. This was potentially due in part to confounding contribution of cross-reacting antibodies induced against other RTX proteins of infecting bacteria other than *B. pertussis*, as increase of CyaA-recognizing antibodies preceded the increase of anti-PT antibodies resulting from a boosting exposure of the vaccinated subjects to unrecognized *B. pertussis* infections [76]. This diagnostic complication was overcome by the use of only the AC domain as antigen, as this is unique to CyaA. The presence of specific anti-AC antibodies in sera of 51 patients with culture-confirmed pertussis could, hence, be unambiguously demonstrated [77].

The largest in kind and best controlled study of induction of anti-CyaA antibodies upon natural infection in vaccinated and unvaccinated children was published in 2004 [78]. Using appropriate controls to exclude confounding recognition of unrelated bacterial components in the used CyaA preparations, Cherry and colleagues used a collection of sera samples from four vaccine efficacy studies and from German children and adults with pertussis. Using 79 post-vaccination sera from recipients of various whole-cell (DTP) and acellular (DTaP) vaccines from various producers, and a 13 DT only-vaccinated sera set as negative control, the anti-CyaA antibody levels induced by DTP or DTaP vaccination were found to be rather low and only slightly higher in some DTP recipients over recipients of the DTaP vaccine that contained only traces of ACT, if any. A vigorous antibody response to CyaA was, however, detected in the 20 sera of unvaccinated children with confirmed *B. pertussis* infection and 4 sera of unvaccinated children with *B. parapertussis* infection, yielding 48- and 28-fold increase of geometric mean antibody titers over the geometric mean antibody titers found in sera of unvaccinated non-infected children, respectively. The anti-CyaA responses were strikingly lower, though present, in vaccinated children that experienced infection due to failure of DTP (5 children) or DTaP (10 children) vaccine [78]. Together with the work of Grimprel and colleagues, cited above, this conclusively demonstrated that ACT was well immunogenic during natural *Bordetella* infections [76].

Protective antigenicity of partially purified CyaA was demonstrated as early as in 1989 using the mouse challenge model by Guiso and colleagues [18]. Since then, both passive immunization with anti-CyaA antibodies, as well as active immunization with CyaA-derived material purified from *B. pertussis,* and in particular with the more homogeneous and LPS-free AC^- toxoid preparations purified from recombinant *E. coli,* were shown to confer on mice a high level of protection against a respiratory challenge by *B. pertussis* [18,20,22,79–82].

Only deletion constructs of CyaA having the structure of the last approximately 900 residues of ACT intact and bearing the palmitoylated lysine 983 residue were recognized in immunoblots by sera of *B. pertussis*-infected mice and humans [22]. Deletion constructs having this C-terminal moiety of CyaA intact, while lacking residues 385–828, still induced toxin-neutralizing antibodies in mice [22]. This suggests that under *in vivo* conditions, a small number of conformational epitopes in the structure of the receptor-binding domain of CyaA are recognized and induce toxin-neutralizing antibodies. A construct lacking only the last 217 out of 1706 residues of CyaA was not conferring any protective immunity, nor induced any toxin-neutralizing antibodies. This highlighted the importance of the C-terminal region for AC toxin structure and activity, as revealed by other studies as well [83,84]. Indeed, a protein lacking residues 385–1489, but bearing the last 217 residues could still induce antibodies neutralizing toxin activity *in vitro*, but failed to confer protective immunity *in vivo* [22]. This was most likely due to the absence of the key protective epitope of CyaA that is located at the junction of the AC domain to the hemolysin moiety, between residues 373–400, to which a monoclonal antibody (3D1) binds and subsequently blocks AC domain translocation into target cells [85,86].

Adjuvant activity of CyaA on co-administered antigens

Of particular interest in the light of potential use of CyaA in a next generation of combination acellular pertussis vaccines are the reports documenting the adjuvant effect of the non-enzymatic AC toxoid on co-administered *B. pertussis* antigens [80,81,87,88]. Intraperitoneal co-administration of the CyaA* ($CyaA\text{-}AC^-$) toxoid enhanced serum IgG antibody responses to pertussis toxin or pertussis toxoid, FHA and pertactin antigens, respectively, as well as the T-cell immunity-mediated IFN-γ-dependent macrophage activation [80]. Intranasal co-administration of CyaA* with ovalbumin enhanced significantly the IgG and IgA antibody responses against ovalbumin in the serum and in lung and nasal secretions of immunized mice. It further boosted the induced ovalbumin-specific proliferative T-cell responses in spleens and it strongly enhanced the antibody response to pertactin, thus potentiating significantly its protective efficacy against intranasal challenge of mice with *B. pertussis* [81]. Finally, co-administration of the CyaA* toxoid together with a diluted commercial aP vaccine importantly improved the resulting protection against intranasal challenge by *B. pertussis* in a manner depending on the amount of CyaA* added, presumably through a mechanism of augmentation of Th1 and Th2 immune responses to *B. pertussis* antigens present in the aP vaccine [88]. This was accompanied by increased IgG2a antibody levels to pertactin contained in the aP vaccine and enhanced IL-5, IL-6 and IFN-γ and GM-CSF secretion by splenocytes and enhanced NO production by macrophages restimulated by heat-killed *B. pertussis* cells, as compared with immunization with CyaA* alone or aP alone [88]. Hence, co-administration of LPS-free CyaA* contributed to enhanced protection beyond the induction of anti-CyaA antibodies and could partly shift the polarization of the immune response from a typical Th2 type, seen with the aP vaccine in mice, to a mixed Th1/Th2 type of response. This would go well also with the large body of evidence on the Th1-polarizing capacities of

the AC toxoid vaccines used as tools for delivery of T-cell antigens for presentation on DCs and induction of tumor and virus antigen-specific $CD8^+$ cytotoxic lymphocyte responses, which is discussed at length below [89–102].

The molecular nature of the Th1-polarizing adjuvant capacity of AC toxoids is currently subject to intense examination. Results of the Leclerc team at Institut Pasteur in Paris would suggest a role of the CD11b/TLR signaling axis [103], while our own results would point toward the importance of pore-forming activity of the AC toxoid in permeabilization of CD11b-expressing antigen-presenting cells and their MAPK activation-dependent maturation upon interaction with TLR ligand-free toxoid [Kosova et al., Manuscript in preparation].

The Th1-polarizing capacity together with importance of ACT as a virulence factor in early phases of bacterial colonization and its protective immunogenicity, make AC toxoid to a prime candidate for examination as an additional antigen for improvement of aP vaccine formulations, particularly in light of the accepted opinion that a Th1/Th17 response correlates with efficient protection against *B. pertussis* infection [23].

Production possibilities, process & formulation issues

It is not surprising that purification of useful quantities of CyaA from wild-type *B. pertussis* culture supernatants containing 10–20 ng of CyaA per ml of the typically used Stainer-Scholte liquid medium turned out to be difficult [36,53]. Larger quantities of cell-associated CyaA can be extracted from bacterial cell surface with 4 M urea solution but are still not sufficient for obtaining enough of purified material for formulation into a commercial vaccine at an economical scale. Moreover, the protein is partially denatured during extraction and purification procedure.

Using genetically modified *B. pertussis* carrying plasmids with the entire *cya* locus the yields of purified *Bp*-CyaA from 4 M urea extracts of bacterial cells could be augmented at a laboratory scale to the level of 1–5 mg/l of culture without any optimization by the authors of this review [Sebo P, Unpublished Data]. This would approach levels of the other produced *B. pertussis* antigens that are currently formulated into the aP vaccine. Recently, the authors of this review developed procedures allowing obtaining such amounts of soluble native CyaA from *B. pertussis* culture supernatants, eliminating the need for antigen extraction with 4 M urea. If performed on the same run with purification of the other antigens formulated into the aP vaccine, such option might potentially be economically viable. Given that at 10–20 ng of ACT produced *in vivo* per milliliter of serous fluid of mucosal secretions does still allow induction of IgG and IgA antibody responses against conformational epitopes, it appears likely that the soluble folded native CyaA toxoid would be quite immunogenic in a vaccine preparation, as well.

Technically easier and very cost–effective option, however, is the production of CyaA using highly optimized expression plasmids in *E. coli* cells into inclusion bodies. These can be easily separated, washed to high purity and solubilized in 8 M urea, so as to yield >90% homogeneous extract. CyaA purification is performed under denaturing conditions in 6–8 M urea solutions by combination of ion-exchange and hydrophobic or affinity (on immobilized recombinant calmodulin) chromatographies. Complete endotoxin removal is then achieved by column washes with 60% isopropanol solutions or by inclusion of a hydroxylapatite chromatography step [51,104–106]. Yields largely exceeding 100 mg of purified CyaA per liter of medium density bacterial culture are easily obtained under laboratory conditions and can be substantially increased by proper optimization of culture density and purification protocols in an industrial scale process. Among the advantages of this approach is the long-term stability of the purified denatured CyaA in 8 M urea solutions that can be frozen or lyophilized for long-term storage. Highly biologically active and/or antigenic CyaA is obtained by simple dilution of concentrated CyaA stocks over 100-fold into calcium-containing buffer lacking urea, or even with physiological saline [18,20,22,79–82].

The technology of CyaA production into inclusion bodies in *E. coli* has already been successfully used for three different cGMP batches of immunotherapeutic vaccines used in Phase I clinical trials of CyaA-based immunotherapeutic vaccines, as discussed below. It has, hence, reached maturity of an optimized biopharmaceutical scale technology. Currently, development of procedures for refolding of the recombinant denatured protein into monomeric soluble CyaA antigen suitable for formulation into combination vaccines is successfully underway.

For the future use in pertussis vaccines of the *E. coli*-produced recombinant CyaA (*rEc-CyaA*), it remains to be explored to what extent, if at all, its mixed acylation status impact on its protective immunogenicity. By difference to the *B. pertussis*-produced native *Bp-CyaA* protein, which has the lysine 860 and 983 residues post-translationally modified by C16:0 palmityl residues, an approximately 1:1 mixture of saturated C16:0 palmityl 500 and unsaturated palmitoleil (*cis*-Δ9 C16:1) fatty acyl residues was found to be used for activation of *rEc-CyaA* in *E. coli* [107,108]. This heterogeneity of post-translational modification is, however, unlikely to affect the specific immunogenicity of *rEc-CyaA*. The native *Bp-CyaA* and the recombinant *rEc-CyaA* proteins exhibit, indeed, an identical capacity to bind and penetrate target cells and differ only in the specific propensity to form the oligomeric CyaA pores in target cell membranes [38,108,109]. A rigorous antigenic potency comparison of the native and recombinant CyaA has not yet been possible, because sufficiently homogeneous CyaA preparations isolated from *B. pertussis* were not available.

Application of CyaA for antigen delivery in therapeutic anti-cancer vaccines

The most advanced application of non-enzymatic (genetically detoxified) CyaA-AC^- toxoids is their use as T-cell antigen delivery tool in anti-cancer immunotherapeutic T-cell vaccines for induction of potent antigen-specific cytotoxic $CD8^+$ T-lymphocyte (CTL) immune responses [89,91,101].

The principle of the technology is schematically depicted in Figure 3. It was discovered in the 90s at the Institut Pasteur in Paris [93,98,99]. Over the past 20 years of collaborative efforts of

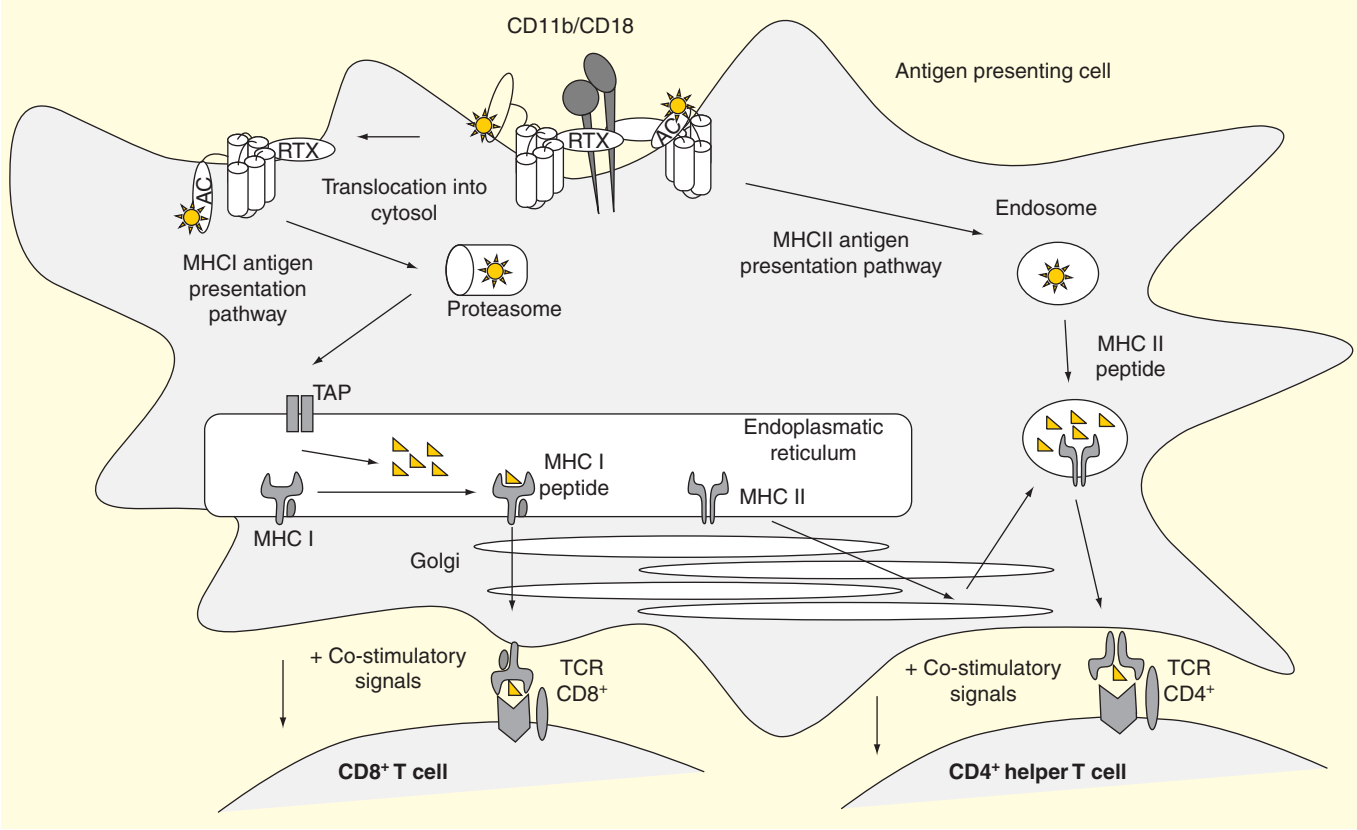

Figure 3. Use of adenylate cyclase toxin-hemolysin as an antigen delivery tool for induction of specific CD8+ cytotoxic lymphocyte responses such as in vaccines for immunotherapy of cancer. Heterologous T-cell antigens (star with yellow nucleus) from intracellular bacteria, parasites, viruses or tumors are genetically engineered as inserts into the catalytically inactive AC domain of CyaA. The purified CyaA toxoid carrying passenger antigens binds complement receptor 3 (the CD11b/CD18 heterodimer) on the surface of antigen-presenting cells, in particular of dendritic cells. The toxoid penetrates the cellular membrane in two different conformations that give rise to molecules with the AC domain translocated into cell cytosol, directly across the cytoplasmic membrane, and to molecules forming oligomeric membrane channels permeabilizing cells and with the AC domain stuck at the external face of cellular membrane. The translocated AC domain bearing the inserted antigen is processed inside the cytosol of antigen-presenting cells by proteasome. The peptide epitopes (yellow triangles) are next transported by TAP1 into the endoplasmic reticulum and bind to newly synthesized MHC I glycoprotein molecules. The MHC I – peptide complexes are transported to the cell surface and presented to CD8+ T lymphocytes, thereby promoting their antigen-specific activation. Cell permeabilization by oligomeric pores induces potassium leakage form cells and activation of MAPK driving maturation of dendritic cells. Upon CD11b/CD18 receptor-mediated endocytosis, the membrane-associated molecules with passenger antigens are processed to antigenic peptides in endosomes as well and bind to MHC II molecules. The MHC II complexes are next exposed on cell surface and presented to CD4+ T cells, inducing strong Th1-polarized Th responses.
AC: Adenylate cyclase enzyme; CyaA: Adenylate cyclase toxin-hemolysin.

three teams, including the one of the coauthor of this review (PS), the initial research tool has now been developed into an established experimental therapy tested in humans. A vast body of scientific literature on the use of the CyaA vector for delivery of a broad spectrum of T-cell antigens is now available and the technology is protected by a patent portfolio owned and managed by Institut Pasteur. In 2004, the company BT Pharma, now Genticel S. A., took a license for the use of the CyaA vector in immunotherapy of cervical cancer in HPV-infected women [110]. After a successful Phase I trial, proving safety, immunogenicity and yielding a first indication of clinical efficacy of the therapeutic vaccine upon intradermal or subcutaneous delivery of the E7 oncoantigen from human papilloma viruses 16 and 18 [97,111], a Phase II clinical trial has recently been initiated. In parallel, a CyaA-based immunotherapeutic vaccine is under Phase I/II study by the academic Theravac consortium in patients with advanced metastatic melanoma disease [112]. In this case, the CyaA is used to deliver an HLA*0201-restricted tyrosinase epitope [89].

The use of CyaA for delivery of immunotherapeutic T-cell vaccines is based on the capacity of CyaA to accommodate within the permissive sites of the AC domain large antigenic polypeptide inserts without losing the capacity to target and penetrate the CR3-expressing myeloid DCs [90,92–94,96,98,99,113–118]. Upon delivery into the cytosol of DC, the antigens are processed by proteasome and the generated peptides are transported into the endoplasmic reticulum via TAP1 transporter to be loaded onto neosynthesized MHC class I molecules for presentation to CD8+ T lymphocyte on DC surface [100,119,120]. As a result, potent prophylactic and immunotherapeutic CD8+ CTL

immune responses can be induced *in vivo* against a very broad array of T-cell antigens originating from viruses, intracellular bacteria, parasites and tumors [89,91–99,102,117,121,122].

Among the particularly promising features of this T-cell vaccine delivery technology is the strong Th1 polarization of the induced immune responses, the proved efficacy in animal models and robustness of the production system of the recombinant vaccine that consists of a chemically defined purified recombinant protein molecule that can be manufactured on industrial scale. Recently, the entire AC domain of the toxoid could be replaced by a synthetic polyepitope construct that was still delivered into DC *in vitro* and stimulated antigen-specific and potent T-cell responses in mice and capable of *ex vivo* expansion of CMV-specific human CTLs, thus opening for design of polyvalent therapeutic T-cell vaccines [118,123].

Expert commentary

The major weakness of current aP vaccines consists in relying on only a few selected antigens (PT, FHA, PRN, fimbriae 2/3) that are adjuvanted by the Th2-polarizing alum. These are used to induce immunity against a pathogen like *B. pertussis* that has numerous already known, and possibly a few as yet unknown, redundant and parallel immunosuppressive and adhesive mechanisms. Of them at least the action of ACT, playing a critical role in the early phases of bacterial colonization, appears to be of crucial importance for suppression of host innate immunity mechanisms, for modulation of adaptive T- and B-cell responses and for evasion of host immunity. The absence of ACT, the major antigen lipooligosaccharide and of the complement resistance factors and siderophore or lactoferrin acquisition receptors in the current aP vaccine formulation, likely accounts for the increasingly apparent failure of the aP vaccine to confer a long-term protection against whooping cough in highly vaccinated populations. Duration of protective immune memory in fully vaccinated older children and teenagers remains a matter of controversy and some recent reports would indicate that it might range between 3 and 5 years post-vaccination only. Furthermore, over 50% of *B. pertussis* strains that are currently isolated from fully vaccinated children do not produce PRN, a key protective antigen of the aP vaccine [26,28,124]. This suggests that immunity induced by aP does protect against critical pulmonary forms of pertussis in infants, preventing PT-caused hyperleukocytosis and fatal outcomes, but does not confer a sufficiently potent or long-lasting protective immunity against infection and transmission of *B. pertussis* bacteria. This would allow for selection of vaccine escape mutants in highly vaccinated populations. Such hypothesis would also be supported by the observations reached in the recently developed baboon model that reproduces well the course of pertussis illness in humans [125,126]. In contrast to wP vaccine-protected animals, in which bacterial proliferation was restricted and clearance of a heavy experimental infection occurred within 2 weeks, the challenged aP-vaccinated baboon weanlings developed a higher level of colonization that persisted for 5 weeks. Moreover, aP-vaccinated animals were not protected against proliferation of *B. pertussis* upon natural infection by cage mates and could transmit the disease to naïve cage mates [127]. Given that reactogenicity of wP vaccines appears as unacceptable in the most developed countries, where pertussis is on a steep rise again [26,28,124], there is an urgent public health need to improve the composition of the aP vaccine so as to enhance protection of aP-vaccinated subjects against colonization by the pathogen and block its transmission. Given the protective immunogenicity of CyaA and the major immunosuppressive role played by the AC toxin in *Bordetella* infections, it appears logical that CyaA toxoids would be considered as a first choice antigen for inclusion in the next generation of aP vaccines. The way towards this has recently been paved by construction of a doubly detoxified CyaA antigen that besides lacking the AC enzyme activity is also devoid of most of its pore-forming activity accounting for the hemolytic properties of CyaA [48]. The CyaA-AC$^-$Hly$^-$ 'phenotype' of the toxoid, however, does not impact on its immunogenicity in mice [Svedova *et al.*, in preparation] and protection of the construct by a recent US patent offers a commercial advantage for its incorporation into aP vaccines from the perspective of a pharmaceutical company. It hence appears likely that CyaA will be considered as a new antigenic component in future aP vaccines, if its contribution to protective potency of the aP formulations, in terms of Th1 polarization and protection against colonization by the pathogen, can be confirmed in vaccination experiments in baboon weanlings.

Five-year view

Given the steep reappearance of whooping cough and the steadily rising incidence of pertussis disease in most of the countries that shifted to the use of aP vaccines some years ago, pertussis is again among the major public health threats in the most developed countries, including the USA, Australia and the EU countries. The mortality rate in these countries will remain low with implementation of maternal immunization that will protect the youngest infants by maternal antibodies. The aP is also highly effective in preventing severe critical pertussis in toddlers. With the evolving pertussis epidemiology and prevalence of the whooping cough disease in older children and teenager populations, however, the need for development of a vaccine capable of stopping the spread of the upcoming pertussis epidemics is sorely needed. A hectic race for development of next generation of still safe and more efficient pertussis vaccines over the next 5 years can be expected. The range of potential approaches toward tackling of the problem is fairly broad and surely in 5 years' time there will be a whole range of novel pertussis vaccines under clinical development. This will include development of a live attenuated vaccine based on the BPZE1 strain [128] to which the rights have recently been acquired by a US-based private equity [129]. In parallel, chemically and genetically detoxified less reactogenic wP vaccines will be developed by several teams and companies worldwide, while the major vaccine producers will continue working on adding further protective *B. pertussis* antigens into their current aP vaccines. Replacement of the used adjuvant towards a more Th1-polarizing one in the novel aP vaccine formulations will be considered. Perhaps not in 5 years, but

certainly within a decade, a whole range of novel pertussis vaccines will be registered, be it next-generation wP or live attenuated pertussis monovaccines, applied to stop the epidemic spread of *B. pertussis* infections, or improved DTaP and Tdap vaccines with enhanced number of *B. pertussis* antigens and used for booster vaccination of adolescents and adults. Intensive work is likely to be performed also on reformulation and supplementing of the current pediatric hexavaccine aP component with additional antigens and/or aiming to replace it by a fully genetically detoxified, less reactogenic wP component.

Financial & competing interests disclosure

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Key issues

- Adenylate cyclase toxin-hemolysin (CyaA) is a key immunosubversive toxin of pathogenic *Bordetellae* and neutralizes host phagocytes by inhibiting their bactericidal capacities, thereby suppressing host innate immunity in the early phases of bacterial colonization.
- Adenylate cyclase toxin (ACT) subverts also adaptive immune responses through modulatory action on host dendritic cells.
- At the time of current acellular pertussis (aP) vaccine development efforts, ACT was unavailable as antigen that could be produced at an economic scale, little was known of its nature, mechanism of action, structure, protective antigenicity and possible use in aP vaccines.
- Over the past 25 years, much was learned on ACT, its immunogenicity in human infections was demonstrated, its protective vaccine antigen potency was shown in mice respiratory challenge models and mechanism of action was largely deciphered.
- Large-scale production of recombinant adenylate cyclase toxoids has been optimized and clinical cGMP batches have been produced and tested to be safe in Phase I clinical trials of T-cell vaccines developed for cancer immunotherapy.
- CyaA toxoid carrying HPV-16 and 18 oncoantigen E7 is currently under Phase II clinical trial evaluation as immunotherapeutic for papilloma virus-induced cervical cancer in women.
- Genetically fully detoxified CyaA-AC$^-$Hly$^-$ toxoid has been developed, protected by an US patent and awaits evaluation as novel aP vaccine component in baboon weanling immunization and *B. pertussis* challenge studies.
- Current aP vaccines induce short-lasting immune memory and predominantly Th2-polarized immune response against only few *B. pertussis* antigens among which ACT, a key virulence factor is missing. It is expected that addition of CyaA and perhaps of some additional *B. pertussis* antigens into a next generation of aP vaccines, will significantly improve the Th1/Th2 polarity, efficacy and duration of immune memory induced upon vaccination of infants.

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