Peter Sebo
Institute of Microbiology of the CAS, Prague
thanks to
‘democracy’
and
acellular vaccines
whooping cough is back
(to the wealthiest countries...)
We've got a problem again...

Confirmed clinical pertussis in 1990-2013 in CR (10 million people)
We had 2,518 cases in 2014 (like in 1961...)

The famous Czech wP was “improved”...
aP introduced
**B. pertussis** is armed with **numerous parallel and redundant virulence systems** (cytotoxins, complement resistance factors, immunomodulators and adhesins).
aP vaccines can be improved, but...

only a next generation of non-reactogenic wP vaccine will be able to stop the spread of the bug in the wealthiest populations be it as a booster in older kids and adults – inducing sterilizing immunity or as priming in a pediatric hexavaccine
ACT relevance for pertussis vaccines


1980  Wolff *et al.*: *Calmodulin activates prokaryotic adenylate cyclase*. PNAS 77: 3841


...*For unknown reasons, humans infected with the bacterium *Bordetella pertussis* are exceptionally vulnerable to secondary infections.* Bordetella species elaborate a soluble, heat-stable, and *highly active adenylate cyclase*. This enzyme is internalized by phagocytic cells and catalyzes the unregulated formation of adenosine 3',5'-monophosphate (cyclic AMP), thereby *disrupting normal cellular function*. This unusual phenomenon *may explain Bordetella-induced aphylaxis*...

**aphylaxis** = absence of phylaxis or immunity

Obsolete term meaning lack of protection against disease

Lack of protection against disease. Also called *nonimmunity*. 
Phagocyte impotence caused by an invasive bacterial adenylate cyclase.

Culture supernatants contain very little of active ACT = huge potency!

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Cyclic AMP (pmole/10^7 PMN)</th>
<th>Adenylate cyclase (pmole/10^7 PMN-min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>Incubated for 20 minutes at 37°C, washed, trypsinized, washed, homogenized</td>
<td>4.9</td>
<td>0, 0*</td>
</tr>
<tr>
<td>Neutrophils plus B. pertussis extract (540 μg/10^7 PMN)</td>
<td>Incubated for 20 minutes at 37°C, washed, trypsinized, washed, homogenized</td>
<td>1296</td>
<td>41.9, 28.0, 45.1</td>
</tr>
<tr>
<td>Neutrophils plus B. pertussis extract (540 μg/10^7 PMN)</td>
<td>Incubated for 20 minutes at 0°C, washed, trypsinized, washed, homogenized</td>
<td>6.7</td>
<td>4.2, 4.3, 4.8</td>
</tr>
</tbody>
</table>

*Limit of detection, < 1 pmole per 10^7 PMN per minute.
Confer DL and Eaton JW, Science 217:948

Phagocyte impotence caused by an invasive bacterial adenylate cyclase.

Fig. 2. Neutrophil killing defect induced by *Bordetella* extract. Human neutrophils (2 × 10⁷ per milliliter) suspended in Hanks balanced salt solution were incubated for 5 minutes at 37°C with an equal volume of dialyzed *Bordetella* extract or dialysate control. The killing of *Staphylococcus aureus* 502A was assessed as described (12) by admixing 5 × 10⁶ neutrophils, 2 × 10⁸ bacteria, and 0.1 ml of pooled human serum in a total volume of 1 ml. Numbers of viable bacteria remaining were determined by plating dilutions of the incubation suspension removed at 0, 30, and 60 minutes. Each point represents the mean of quadruplicate determinations. Control tubes containing no neutrophils showed no change in bacterial count. Symbols: ○, *Bordetella*-treated neutrophils; ●, control neutrophils.

Fig. 3. Accumulation of cyclic AMP in human neutrophils (PMN) incubated with dialyzed *Bordetella* extract. Neutrophils, 10⁷ per milliliter in Hanks balanced salt solution, were incubated at 37°C with equal volumes of dialyzed *Bordetella* extract (protein content, 520 μg/ml) for the times shown. Total cyclic AMP was determined as described (16). Values shown represent the means and standard error of seven separate (duplicate) determinations with neutrophils from four different donors. Normal neutrophils contain 2 to 5 pmole of cyclic AMP per 10⁷ cells, and these amounts do not change during control incubations. Separate experiments (not shown), in which neutrophil pellets were obtained by brief centrifugation after incubation, indicated that > 90 percent of the total recoverable cyclic AMP is associated with the cell pellet.
Nobody doubts that antibodies against PT save infant`s lifes

= PT needs to be in the aP vaccine

but:

*B. parapertussis* does not need PT to cause whooping cough

and

Adenylate cyclase toxin is critical for colonization by *Bordetella pertussis*


The bug really needs ACT for knocking down innate immunity:

- All *Bordetellae* pathogenic to mammals produce adenylate cyclase toxin-hemolysin (Except for certain *B. bronchispetica* lineages)

- ACT sequences are highly conserved in *B. pertussis* isolates

- Strains not producing ACT have not been isolated from patients, so far (in contrast to PT, FHA or pertactin, which all are dispensable)

- ACT is an extremely potent toxin that knocks-down phagocytes in 30-60 seconds (PT needs 30 min to get internalized into cells and effects manifest in 8-12 h)

- ACT instantaneously blocks oxidative burst of neutrophils at pM conc. in 30 seconds

- ACT blocks uptake of complement-opsonized particles at pM conc.
Not surprisingly, hence, ACT is a protective antigen

(at that time ACT samples contained LPS)
Highly purified CyaA-AC\(^{-}\) protects on its own


The *Bordetella pertussis* Type III Secretion System Tip Complex Protein Bsp22 Is Not a Protective Antigen and Fails To Elicit Serum Antibody Responses during Infection of Humans and Mice

Rodrigo Villarino Romero, Ilona Bibova, Ondrej Cerny, Branislav Vecerek, Tomas Wald, Oldrich Benada, Jana Zavadilova, Radim Ousicka, Peter Sebo

Institute of Microbiology of the ASCR, Prague, Czech Republic; National Institute of Public Health, Prague, Czech Republic

Poster: Villarino, Bibova et al.
Evidence from other labs


Addition of CyaA-AC\(^-\) improves performance of the aP vaccine

Effect of Different Forms of Adenylate Cyclase Toxin of *Bordetella pertussis* on Protection Afforded by an Acellular Pertussis Vaccine in a Murine Model\(^7\)

Gordon Y. C. Cheung,\(^1\) Dorothy Xing,\(^2\) Sandra Prior,\(^2\) Michael J. Corbel,\(^2\) Roger Parton,\(^1\) and John G. Coote\(^1\)

Division of Infection and Immunity, Glasgow Biomedical Research Centre, University of Glasgow, Glasgow,\(^1\) and Division of Bacteriology, National Institute of Biological Standards and Control, South Mimms, Hertfordshire,\(^2\) United Kingdom

Received 14 July 2006/Accepted 12 September 2006

Four recombinant forms of the cell-invasive adenylate cyclase toxin (CyaA) of *Bordetella pertussis* were compared for the ability to enhance protection against *B. pertussis* in mice when coadministered with an acellular pertussis vaccine (ACV). The four forms were as follows: fully functional CyaA, a CyaA form lacking adenylate cyclase enzymatic activity (CyaA\(^e\)), and the nonacylated forms of these toxins, i.e., proCyaA and proCyaA\(^e\), respectively. None of these forms alone conferred significant (\(P > 0.05\)) protection against *B. pertussis* in a murine intranasal challenge model. Mice immunized with ACV alone showed significant (\(P < 0.05\)) reductions in bacterial numbers in the lungs after intranasal challenge compared with those for control mice. When administered with ACV, both CyaA and CyaA\(^e\) further reduced bacterial numbers in the lungs of mice after intranasal challenge compared with those for ACV-immunized mice, but the enhanced protection was only significant (\(P < 0.05\)) with CyaA\(^e\). Coadministration of CyaA\(^e\) with ACV caused a significant (\(P < 0.05\)) increase in immunoglobulin G2a antibody levels against pertactin compared with those in mice immunized with ACV alone. Spleen cells from mice immunized with ACV plus CyaA\(^e\) secreted larger amounts of interleukin-5 (IL-5), IL-6, gamma interferon (IFN-\(\gamma\)), and granulocyte-macrophage colony-stimulating factor (GM-CSF) than did cells from mice immunized with ACV plus CyaA or ACV alone after stimulation in vitro with a mixture of *B. pertussis* antigens. Spleen cells from mice immunized with ACV plus CyaA\(^e\) also secreted larger amounts of IFN-\(\gamma\) and GM-CSF than did cells from mice immunized with CyaA\(^e\) alone after stimulation in vitro with CyaA\(^e\). Macrophages from mice immunized with ACV plus CyaA\(^e\) produced significantly (\(P < 0.05\)) higher levels of nitric oxide than did macrophages from mice immunized with CyaA\(^e\) alone, ACV alone, or ACV plus CyaA after stimulation in vitro with a mixture of *B. pertussis* antigens or heat-killed *B. pertussis* cells. These data suggest that the enhancement of protection provided by CyaA\(^e\) was due to an augmentation of both Th1 and Th2 immune responses to *B. pertussis* antigens.
ACT relevance for pertussis vaccines

- **ACT is a protective antigen** in the i.n. or aerosol challenge model
- **anti-ACT antibodies are common and abundant in convalescent patients**

- Documented adjuvant effect of the nonenzymatic AC toxoid on co-administered B. pertussis antigens

- **ACT polarizes T cell responses towards Th1** even when administered with alum!

- **AC toxoid** drives maturation of DC and when mixed with aP vaccine antigens and alum AC toxoid 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....

We shall know in a few years...

PRESS RELEASE
Paris and Toulouse, February 2nd, 2015

GENTICEL LICENSES VAXICLASE PLATFORM TO SERUM INSTITUTE OF INDIA FOR USE IN PERTUSSIS VACCINES

- Serum Institute of India, world's largest producer of vaccines, to evaluate Vaxiclase for use in multivalent vaccines containing pertussis antigens
- Preclinical stage license agreement entitles Gentcel to up to $57 million in upfront & milestones payments plus single digit royalties on net sales

GENTICEL (Euronext Paris and Brussels: FR0011790542 - GTCL), a French biotechnology company and leading developer of therapeutic vaccines, today announces that it has licensed its Vaxiclase technology to Serum Institute of India Ltd. (SIIL), for use as a component in acellular multivalent combination vaccines including pertussis antigens.

The license granted by Gentcel to SIIL provides the Vaxiclase platform for inclusion in multivalent vaccines that also protect against Bordetella pertussis, the causative agent of whooping cough. The license covers all countries of the world except major pharmaceutical markets, in particular the USA, Canada, New Zealand, Australia, Japan, Israel, Turkey and greater Europe.
Vaccines that are built with the CyaA vector are chimeric recombinant proteins consisting of the CyaA protein and the antigen of choice.

September 2012 - Genticel S.A. completed Phase I clinical trial for HPV16/18-induced cervical carcinoma

Using a cGMP batch of the adenylate cyclase (CyaA-AC') toxoid for delivery of HPV E7 antigen as immunotherapeutic vaccine

safe, immunogenic, inducing CD8+ CTLs and HPV 16/18 virus load reduction demonstrated

Entered phase II trial = will be of interest to see pertussis incidence in CyaA-E7 toxoid treated woman…

IPO on April 4, 2014 at Euronext Paris and Brussels - 34 millions Euro
Adenylate cyclase toxin hijacks the β₂ integrin receptor into lipid rafts to accomplish membrane translocation in two steps.

CyaA-AC- higher concentrations induces maturation of DC through LPS-independent TLR4 and TRIF signaling.

Antigen Targeting to CD11b+ Dendritic Cells in Association with TLR4/TRIF Signaling Promotes Strong CD8+ T Cell Responses

• CyaA binds CD11b+ dendritic cells (DCs) and induces their maturation...
• DCs sense CyaA through the TLR4/Toll/IL-1R domain–containing adapter-inducing IFN-β pathway, independent of the presence of LPS
• leading to the induction of strong immune responses.

The three cytotoxic activities of ACT adenylate cyclase toxin & pore-forming hemolysin/Cytolysin

Sebo et al. (1991) Gene 104:19
Sakamoto et al. (1992) J. Biol. Chem. 267, 13598
Benz et al. (1994) J. Biol. Chem. 269, 27231
Hackett et al. (1995) J. Biol. Chem. 270, 20250
Gray et al. (1998) J. Biol. Chem. 273, 18260
Osickova et al. (1999) J. Biol. Chem. 274, 37644
Basler et al. (2007) J. Biol. Chem. 282, 12419
However, already cell permeabilization by the Pore-forming activity by low amounts of CyaA-AC-induces maturation of DC (Svedova et al. 2015 Immun Cell Biol, in press)
Toxoid activates DC in function of Pore-forming activity (K⁺ efflux)

<table>
<thead>
<tr>
<th>CyaA mutants</th>
<th>Ca²⁺ influx</th>
<th>Specific cell-permeabilizing activity (K⁺ efflux)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyaA-AC</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>CyaA-E570Q-K860R-AC</td>
<td>+++</td>
<td>-/+</td>
</tr>
<tr>
<td>CyaA-E509K-E516K-AC</td>
<td>-/+</td>
<td>+++</td>
</tr>
</tbody>
</table>

BMDC
BMDC
CyaA-AC

Toxoid activates DC in function of Pore-forming activity (K⁺ efflux)
The CyaA-AC\textsuperscript{-} Toxoid primes activation of DC by cell-permeabilities, causing K\textsuperscript{+} efflux and p38 MAPK activation. At higher toxoid concentrations, CyaA-mediated clustering of CD11b/CD18 with TLR4 and TRIF signaling occurs.

So, why is ACT so much relevant for pertussis vaccines?

What does it do and how does it work?
Adenylate cyclase toxin - cytolysin

AC domain

RTX hemolysin moiety

CR3 binding segment

1

400

500

700

1000

1706

Hydrophobic segments

Palmitoylation on K860 and K983

Secretion signal

T25

T18

N

C

I

II

CBS

III

42 calcium binding repeats

X-(L/I/F)-X-G-X-G-X-(D/N)-D

Guo Q. et al. (20005) EMBO J. 24, 3190–3201
ACT is an RTX protein secreted by a type I system.

Need to unfold and refold on the way to target...

>100 μM Ca\(^{2+}\)

<100 nM Ca\(^{2+}\)
Calcium-driven formation of an intramolecular Brownian ratchet directs movement of large RTX proteins through type I secretion system conduits
ACT/cAMP signaling breaks the hell loose... and supresses TLR signaling of the bug...

**signal transduction events:**
- NF-κB: ↓
- MAPK – p38, ERK, JNK: ↓

**expression and upregulation of TLR:**
- TLR1-6, 9, TLR4, TLR2: up

**mucin:** MUC2, MUC5AC: up

**other soluble factors:**
- $O_2^-$, NO: ↓
- PGE2: ↑

**defensins and other antimicrobial peptides:**
- hβdefensin2: ↓
- βdefensin1: ↓
- cathelicidin: ↓

**AEC**

**other cells**

**cytokine and chemokines:**
- IL-1α, ↑
- IL-1β, ↑
- IL-6, ↑
- IL-8, ↑
- IL-10, ↑
- TNFα: ↓
- IFNβ: ↓
- TGF-β: ↓
- GM-CSF, MCP-1: ↓
- MIP-1α: ↓
- RANTES:...

**expression of costimulatory x inhibitory molecules:**
- CD80, CD86, ↑
- CD40, ↓
- CD54, B7-H2, B7-H3: x: ↑
- FasL: ↑
- PD-L1, PD-L2: ↓
ACT targets myeloid phagocytes bearing \( \alpha_M \beta_2 \) integrin CD11b/CD18

- \( \beta_2 \) subfamily
- **complement receptor 3 (CR3), Mac-1, Mo-1, \( \alpha_M \beta_2 \)**
- monocytes, granulocytes, macrophages, NK cells, neutrophils and *dendritic cells*, certain B cell subtypes

ACT first recognizes N-linked glycans of CD11b/CD18

Morova et al. (2008) PNAS 105, 5355

**The Yin: ACT as a SWIFT SABOTEUR**

Low ACT (CyaA) concentrations make a difference on respiratory mucosa...

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**Respiratory Epithelium:**
- Ciliated cells
- Nonciliated cells (Goblet cells, Absorptive cells)
- Mucus layer

**B. pertussis:**
- Pertactin
- FHA
- fimbriae
- Pertinax

**Pathophysiology:**
- ACT (CyaA) secretion
  - ATP intoxication
  - CAMP formation
  - CD11b/CD18
- Channel formation
- Phagocytic functions
- Phagocytosis
- Superoxid production

**Immunological Response:**
- IL-1β
- IL-10, IL-6, IL-1β, IL-17
  (Semi-mature state of DC?)
- IL-12, TNFα, CCL3
- Cell lysis
- Apoptosis
- T regulatory response

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Vojtová et al., 2006, Curr. Op.. Microbiol. 9, 69-75
Signaling disarming phagocytes
Proteinaceous segments specifically involved in CyaA binding:

CD11b – residues 614-682
CD11b - residues 342-424
cAMP-elevating AC activity of ACT (CyaA) enables B. pertussis growth in the presence of neutrophils

Cerny et al., unpublished
AC activity of *B. pertussis* ACT blocks ROS production by neutrophils and NET formation

*Cerny et al. unpublished*
• cAMP-mediated suppression of neutrophil extracellular trap formation and apoptosis by the *B. pertussis* adenylate cyclase toxin.
• Convalescent-phase antisera from patients recovering from pertussis block ACT-mediated inhibition of the oxidative burst and NET formation
CyaA-induced morphological rearrangements

Mouse macrophage-like cell line J774 A.1:

Kamanova et al. (2008)
*J. Immunol.* 181, 5587-97

JanaK
ACT at low doses ablates complement-mediated opsonophagocytosis (through RhoA inactivation)

A

![Graph showing cAMP levels over time](image)

B

![Graph showing phagocytosis](image)
Non-opsonized *B. pertussis* can persist within macrophages due to cAMP elevating activity of ACT.
ACT-produced cAMP signaling through PKA activates SHP-1 tyrosine phosphatase that blocks iNOS gene transcription.

Cell-invasive Adenylate cyclase 

Hydrophobic domain 

Palmitoylated Lys860 and Lys983 

RTX-hemolysin repeats 

Translocation 

Calcium influx 

Potassium efflux 

Cation selective pores 

SHP-1 active conformation 

AP1 c-Fos iNOS

Ondřej Černý

CyaA/cAMP-triggered SHP-1 activation extends *B. pertussis* survival inside macrophages

*Ondra*  
CyaA/cAMP-triggered SHP-1 activation causes BimEL stabilization, Bax activation and macrophage apoptosis

Ahmad et al. 2015, Cell. Microbiol, in press
Signalling pathways influenced by CyaA

**CyaA**

- cAMP↑
- PKA↑
- SHP-1↑
- AKT↓
- JNK↓
- AP-1↓
- iNOS↓
- NADPH oxidase↓
- Macrophage apoptosis↓
- RNS↓

- Epac↑
- NF-κB↑
- FoxO3a↑
- BimEL↑

- PLC↓
- JNK, p38↑

- K+ efflux↑

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- Deregulated DC maturation
- ROS↓

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- Block of killing of bacteria
- Adaptive immunity↓

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Černý *et al*, 2015 JI and manuscript in preparation

Adkins 2014 *PLoS One*

Švédová *et al*, 2015 CIB in press

Ahmad *et al*, *Cell. Microbiol* 2015 in press
Suppressing adaptive immunity
Adenylate cyclase toxin hijacks the β₂ integrin receptor into lipid rafts to accomplish membrane translocation in two steps.

ACT (CyaA) skewes TLR-stimulated cytokine production in DC towards tolerance?

\[ n = 4, \, * \, P < 0.05; \, 100 \, \% \, \text{urea} + \text{LPS (buffer)} \]
cAMP signaling of ACT dampens and skews adaptive immunity towards initial $T_{h2}/T_{r1}$-mediated tolerance of colonizing *Bordetellae* followed by delayed $T_{h1}/T_{h17}$ mediated clearance?

Several reports indicated that CyaA subverts adaptive T cell immune responses

*Relman lab:* Boschwitz et al. (1997) *JID* 176:678

Hence:
The cAMP signaling of ACT,
prevails and
knocks down the innate immunity
and
dampens the adaptive immune response
in order
to enable host colonization by Bordetellae
Explaining why is it so important to add the AC toxoid into the aP vaccine if we are serious about breaking the vicious circle of epidemic whooping cough spread in the most developed countries ...
To sum up this talk:

Rise in pertussis incidence in the most developed countries will likely not stop until the adenylate cyclase toxoid has been added to the aP vaccine…

And a less reactogenic nextgen wP vaccine is used as booster…
Sebolab = a PI Confederation...

**Radim Osička** – CR3 interactions and cAMP signaling of ACT

**Jiří Mašín** – membrane penetration and pore-forming activity of ACT

**Láďa Bumba** – structure, folding and secretion of ACT

**Branko Večerek** – sRNA regulation of *Bordetella virulence*

**Irena Adkins** – ACT and fooling od DC maturation and cAMP-mediated inhibition of T cell immune response

**Peter Šebo** – strategic steering, mentoring and paper editing, signaling of cAMP and phagocyte action

**Ondřej Staněk** – novel antigen delivery tools, Veterinary vaccines

**Jana Holubová + Karolína Škopová** – novel pertussis whole cell vaccine
External collaborations:

**Institut Pasteur:**
- teams:
  - Nicole Guiso
  - Claude Leclerc

**University of Virginia**
- Erik L. Hewlett

**Trinity College**
- Kingston Mills
  - Aisling Dunne

**Institute of Microbiology**
- Lidia Tučková
  - Marek Kovář
  - and their teams

**University Wurzburg**
- Roland Benz
  - and his team

**Bernhard Nocht Institut:**
- Thomas Jacobs
  - Susanne Tartz

**MH Hannover**
- Ingo Just
  - Harald Genth

**VLA Surrey**
- Martin Vordemeier