Antibody-mediated inhibition of *Bordetella pertussis* adenylate cyclase–haemolysin-induced macrophage cytotoxicity is influenced by variations in the bacterial population

N. Hegerle$^{1,2}$ and N. Guiso$^{1,2}$

$^1$Prevention and Molecular Therapies of Human Diseases Unit, Institut Pasteur, Paris, France
$^2$Institut Pasteur, CNRS-URA3012, Paris, France

Whooping cough is a vaccine-preventable disease presenting with epidemic cycles linked to natural and/or vaccine-driven evolution of the aetiological agent of the disease, *Bordetella pertussis*. Adenylate cyclase–haemolysin (AC-Hly) is a major toxin produced by this pathogen, which mediates macrophage apoptosis *in vitro* and *in vivo*. While current acellular pertussis vaccine (APV) formulations do not include AC-Hly, they all contain pertussis toxin and can comprise filamentous haemagglutinin (FHA), which interacts with AC-Hly, and pertactin (PRN), which has been hypothesized also to interact with AC-Hly. We aimed to study the capacity of specific antibodies to inhibit the *in vitro* *B. pertussis* AC-Hly-mediated cytotoxicity of J774A.1 murine macrophages in a background of a changing bacterial population. We demonstrate that: (i) clinical isolates of different types or PRN phenotype are all cytotoxic and lethal in the mouse model of respiratory infection; (ii) lack of PRN production does not impact AC-Hly-related phenotypes; (iii) anti-AC-Hly antibodies inhibit cell lysis whatever the phenotype of the isolate, while anti-PRN antibodies significantly inhibit cell lysis provided the isolate produces this antigen, which might be relevant *in vivo* for APV-induced immunity; and (iv) anti-FHA antibodies only inhibit lysis induced by isolates collected in 2012, maybe indicating specific characteristics of epidemic lineages of *B. pertussis*.

INTRODUCTION

*Bordetella pertussis* is the Gram-negative bacterium responsible for whooping cough, a severe acute respiratory disease life-threatening for partially or unvaccinated newborns. The colonization of the human respiratory tract by *B. pertussis* is achieved through expression of an array of virulence factors that promote adhesion to the respiratory epithelium and subversion of the immune system to avoid rapid clearance of the pathogen (Mattoo & Cherry, 2005). While adenylate cyclase–haemolysin (AC-Hly) and pertussis toxin (PT) are well-known toxins involved in immune modulation (Carbonetti, 2010; Fedele *et al.*, 2013), filamentous haemagglutinin (FHA), pertactin (PRN), fimbriae 2 (FIM2) and FIM3 are adhesins involved in the mechanical adherence of *B. pertussis* to the respiratory epithelium (Jacob-Dubuisson & Locht, 2007). Colonization of the respiratory tract of partially or unvaccinated newborns is accompanied by characteristic clinical manifestations and can be fatal (Cherry *et al.*, 2012; Mattoo & Cherry, 2005). To protect this population from the disease, whole-cell pertussis vaccine (WPV) was introduced in 1959 in France for primary and booster immunization of children. This strategy managed to control circulating isolates similar to WPV strains, and dramatically decreased the morbidity and mortality of the disease in this age group (Baron *et al.*, 1998; Bonmarin *et al.*, 2007; Bouchez *et al.*, 2008; Caro *et al.*, 2005, 2006; Hegerle & Guiso, 2013; Weber *et al.*, 2001). In 1998, acellular pertussis vaccines (APVs) were introduced for adolescent booster vaccination (Institut de Veille Sanitaire, 1998) and afterwards replaced WPV for child immunization as well. Adult APV booster vaccination was later introduced (Institut de Veille Sanitaire, 2004, 2008), with the aim of protecting newborn children from pertussis by increasing the herd immunity. But despite vaccination, whooping cough still remains a major issue and resurgence of this disease has occurred in several countries (He & Mertsola, 2008; Mooi *et al.*, 2014; Plotkin, 2014).

WPV is composed of killed bacteria and induces a broad-range immune response against *B. pertussis*, while all licensed APVs include PT in addition to FHA, or FHA and PRN, or FHA, PRN, FIM2 and FIM3. APV-induced immunity is thus narrower, but targets every circulating isolate producing the

Abbreviations: AC, adenylate cyclase; APV, acellular pertussis vaccine; FHA, filamentous haemagglutinin; Hly, haemolysin; PRN, pertactin; PT, pertussis toxin; WPV, whole-cell pertussis vaccine.
antigens included in the vaccine. Although demonstrated to be a major toxin in the murine model of respiratory infection (Bouchez et al., 2009; Fedele et al., 2013; Khelef et al., 1994), AC-Hly has not been included in any APV formulation so far. This toxin drives CD11b/CD18-expressing eukaryotic cells (Osickova et al., 2010) to an apoptotic state in vivo (Gueirard et al., 1998) and in vitro (Khelef et al., 1993; Osickova et al., 2010), and can thus directly target airway macrophages in its human host.

Although capable of inducing apoptosis on its own (Khelef & Guiso, 1995), AC-Hly has been demonstrated to interact with FHA in vitro in a non-covalent manner (Zaretzky et al., 2002), the lack of this adhesin causing an increased secretion of AC-Hly in the culture supernatant (Zaretzky et al., 2002). Interactions between PRN and AC-Hly have only been hypothesized so far (Khelef & Guiso, 1995), but could draw attention in a background of an increasing prevalence of natural PRN-deficient isolates in countries vaccinating with APVs (Barkoff et al., 2012; Hegerle & Guiso, 2013; Miyaji et al., 2013; Otsuka et al., 2012; Pawloski et al., 2014; Queenan et al., 2013; Quinlan et al., 2013). The loss of this adhesin does not impact the virulence or transmission of the bacterium (Bodilis & Guiso, 2013) and, despite the effectiveness of current APVs in preventing severe pertussis in children less than 6 months of age (Bodilis & Guiso, 2013), great concerns have arisen regarding the duration of APV-induced immunity if the prevalence of PRN-deficient isolates increases.

We thus sought to analyse the possible interaction existing between PRN and AC-Hly, and the changes that can occur in the B. pertussis AC-Hly-related phenotype by means of enzymatic activity determination and AC-Hly-mediated macrophage cytotoxicity. To study possible variations in antibody recognition of B. pertussis isolates from different vaccine era or pertussis cycles, we performed inhibition assays with specific antisera targeting AC-Hly, PT, PRN and FHA. The murine model of respiratory infection was also used to assess differences in the in vivo virulence of the isolates.

**METHODS**

**Isolates and growth conditions.** B. pertussis reference strain Tohama and WPV strains Bp1414 and Bp1416 were part of the collection of the Institut Pasteur (Njamkepo et al., 2002). PRN-deficient and PRN-producing clinical isolates belonged to the collection of the National Reference Centre held in our laboratory since 1993. Strain and isolate characteristics are summarized in Table 1.

Bacteria were grown on Bordet–Gengou agar (BGA; Difco) supplemented with 15% defibrinated sheep blood at 36°C for 72 h and plated again for 24 h before each cytotoxicity assay. When necessary, isolates and reference strains were cultured in modified Stainer–Scholte medium until OD₅₅₀ 1, starting from OD₅₅₀ 0.2. Characterization of clinical isolates was performed by PFGE, genotyping and serotyping as described elsewhere (Mooi et al., 2000, 2009).

**Determination of AC activity.** For AC activity measurement, isolates were grown as previously described (Hegerle et al., 2013) in enriched Stainer–Scholte medium and harvested at mid-exponential phase at OD₅₅₀ 1 ± 0.2. For secreted AC activity, the bacterial suspension was centrifuged at 6000 g for 10 min in a bench centrifuge and the resulting supernatant tested for AC activity by radioactive assay as previously described (Ladant et al., 1986). The proportion of membrane-bound AC activity was estimated as the ratio between the total AC activity (total ACact) minus the supernatant AC activity (supernatant ACact) and the total AC activity.

\[
\text{Membrane-bound activity}\% = \frac{\text{Total AC}_{\text{act}} - \text{Supernatant AC}_{\text{act}}}{\text{Total AC}_{\text{act}}} \times 100
\]

**Production of murine polyclonal antisera.** Specific polyclonal mouse antisera directed against AC-Hly, PT, FHA and PRN were obtained as described by Weber et al. (2001). Briefly, 4-week-old BALB/c mice were immunized subcutaneously four times at 4 week intervals with 10 μg of each purified antigen adsorbed onto aluminium hydroxide and were bled 7 days after the last injection. PT (PtxA2), FHA and PRN (PRN1) were purified from B. pertussis in their native form, while AC-Hly was produced as a recombinant protein (Betsoû et al., 1993). PtxA2- and PRN1 type antigens were used since these are the vaccine type antigens of currently licensed APVs. The specificity of each polyclonal antiserum was verified by Western blotting against the purified antigens and whole B. pertussis bacterial extracts. All murine immune and naive sera were heat inactivated for 30 min at 56 °C prior to use in the inhibition assays.

**Western blot analysis of virulence factor production.** Clinical isolates were tested for virulence factor production by Western blot using the mouse polyclonal antisera obtained after immunizing mice with purified antigens as described above and by Weber et al. (2001).

**J774A.1 cell culture and cytotoxic/inhibition assays.** J774A.1 (ATCC TIB-67) murine monocyte/macrophage cells were cultured and cytotoxic assays were performed as previously described (Bouchez et al., 2009). Briefly, the J774A.1 murine macrophages were cultured in T150 filter-capped flasks at 37 °C with 5% CO₂ in RPMI 1640 + Glutamax 1 (Invitrogen) complemented with 1% sodium pyruvate 100 mM solution, 1% HEPES 1 M solution, 1% antibiotic–antimycotic solution (Invitrogen) and 10% PBS (BioWest). Cells were transferred into 96-well plates 24 h before lysis experiments were conducted at a final concentration of 5 × 10⁴ cells per well. On the day of infection, adherent cells were washed three times with the same culture medium lacking antibiotic–antimycotic solution, and the number of cells was determined by counting after Trypan blue staining.

B. pertussis isolates were suspended in cell culture medium and were added to the cells at a final m.o.i. of 100:1 in a final volume of 100 μl per well of the 96-well culture plate. Bacteria were deposited by light centrifugation at 600 g for 5 min at room temperature prior to incubation. Bacteria and cells were left in contact for 8 h and cell lysis was assessed with the CytoTox 96 non-radioactive assay kit (Promega). Cytotoxicity was determined as a percentage of cell lysis determined by OD₄₉₅ measurement as presented in the formula below, where ‘OD₄₉₅ isolate’ is the measured optical density of the well containing the B. pertussis isolate, ‘OD₄₉₅ control’ is the optical density of the control well containing only cells, and ‘OD₄₉₅ total lysis’ is the optical density measured in the well where all cells are lysed by adding the Promega lysis buffer.

\[
\text{Cell lysis }\% = \frac{\text{OD}_{495\text{ total lysis}} - \text{OD}_{495\text{ control}}}{\text{OD}_{495\text{ total lysis}}} \times 100
\]

For inhibition assays, the bacterial suspensions were incubated for 30 min at 37 °C with 5% CO₂ and with the appropriate polyclonal immune serum at a final dilution of 1:500. This serum dilution was chosen as it causes 50% lysis inhibition of the strain Tohama.
after 8 h of incubation. The specific polyclonal immune sera used for inhibition were those used for Western blotting and thus directed against AC-Hly, PT, FHA and PRN. As FHA and PT were unable to inhibit Tohama-induced cell lysis, they were used at an arbitrary concentration. Inhibition was calculated as the ratio between lysis without serum and the lysis without serum minus the lysis in the presence of serum and the lysis without serum, as shown below.

\[
\text{Lysis inhibition (\%) } = \frac{\text{Isolate lysis} - \text{Isolate lysis with serum}}{\text{Isolate lysis}} \times 100
\]

**RESULTS**

**AC activity and secretion**

All isolates produced comparable amounts of AC activity in vitro regardless of their PRN phenotype (Table 1) and were haemolytic on BGA plates. AC-Hly toxin thus conserved both its pore-forming haemolytic activity and its AC activity whether it was produced in a PRN-deficient or in a PRN-producing isolate. The absence of PRN production does not impact the distribution of AC activity as over 90% remained surface bound whatever the phenotype of the isolate (Table 1), which is consistent with published observations (Zaretzky et al., 2002).

**B. pertussis-induced macrophage lysis**

All isolates were cytotoxic for J774A.1 murine macrophages in vitro whatever their phenotype (Fig. 1). Although variable, no significant difference or trend was found in cell cytotoxicity. The loss of PRN production thus does not impair the toxicity of B. pertussis isolates to macrophages.

**Inhibition of B. pertussis-induced macrophage lysis**

Serum obtained from unvaccinated naive mice was heat inactivated and used as a control and reference for the inhibition achieved by naive serum was performed using GraphPad Prism version 5.0b for Mac (GraphPad Software). A normality test was performed for Tohama and confirmed the Gaussian distribution of the results.

**Table 1. Characteristics of selected B. pertussis strains and isolates**

Isolates shown in bold were collected during the last pertussis cycle in France (2012). ND, Not determined; NL, Not lethal.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Year</th>
<th>WB*</th>
<th>FIM†</th>
<th>Type (PFGE/ptxP/ptaX/PRN) ‡</th>
<th>ACact/ACbound (U ml⁻¹/% total) §</th>
<th>LD₅₀ (c.f.u. per mouse)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tohama</td>
<td>&lt;1954</td>
<td>+</td>
<td>2</td>
<td>II/1/1/1</td>
<td>43.1 ± 3.2/89.1 ± 1.3</td>
<td>8 × 10⁷</td>
<td>Bouchez et al. (2009)</td>
</tr>
<tr>
<td>Bp1414</td>
<td>&lt;1954</td>
<td>+</td>
<td>2/3</td>
<td>II/1/4/1</td>
<td>44.2 ± 1.5/88.8 ± 3.8</td>
<td>8 × 10⁷</td>
<td>Hegerle et al. (2012)</td>
</tr>
<tr>
<td>Bp1416</td>
<td>&lt;1954</td>
<td>+</td>
<td>3</td>
<td>III/1/2/1</td>
<td>41.0 ± 4.5/88.2 ± 5.8</td>
<td>3 × 10⁷</td>
<td>Hegerle et al. (2012)</td>
</tr>
<tr>
<td>FR3713</td>
<td>2007</td>
<td>+</td>
<td>3</td>
<td>IV/3/1/2</td>
<td>54.0 ± 8.2/98.8 ± 1.0</td>
<td>2 × 10⁷</td>
<td>Hegerle et al. (2012)</td>
</tr>
<tr>
<td>FR3749</td>
<td>2007</td>
<td>PT</td>
<td>3</td>
<td>IV/Δ/Δ/2</td>
<td>53.6 ± 5.6/96.9 ± 0.4</td>
<td>ND/ND</td>
<td>This study</td>
</tr>
<tr>
<td>FR4929</td>
<td>2011</td>
<td>+</td>
<td>3</td>
<td>IV/3/1/2</td>
<td>49.1 ± 7.4/100 ± 0.0</td>
<td>6 × 10⁷</td>
<td>This study</td>
</tr>
<tr>
<td>FR5133</td>
<td>2012</td>
<td>+</td>
<td>3</td>
<td>IV/3/1/2</td>
<td>58.8 ± 9.9/94.0 ± 5.1</td>
<td>3 × 10⁷</td>
<td>This study</td>
</tr>
<tr>
<td>FR5392</td>
<td>2012</td>
<td>+</td>
<td>3</td>
<td>IV/3/1/2</td>
<td>46.8 ± 7.8/97.3 ± 0.3</td>
<td>6 × 10⁷</td>
<td>Bouchez et al. (2009)</td>
</tr>
<tr>
<td>FR3693</td>
<td>2007</td>
<td>PRN− (IS)</td>
<td>3</td>
<td>IVs/3/1/2</td>
<td>55.9 ± 1.8/98.6 ± 0.4</td>
<td>2 × 10⁴</td>
<td>Bouchez et al. (2009)</td>
</tr>
<tr>
<td>FR3793</td>
<td>2007</td>
<td>PRN− (Δ)</td>
<td>3</td>
<td>IVs/3/1/2</td>
<td>56.7 ± 9.4/93.9 ± 0.8</td>
<td>3 × 10⁷</td>
<td>Hegerle et al. (2012)</td>
</tr>
<tr>
<td>FR4596</td>
<td>2009</td>
<td>PRN− (IS)</td>
<td>3</td>
<td>IVs/3/1/2</td>
<td>51.7 ± 7.4/94.8 ± 3.6</td>
<td>1 × 10⁷</td>
<td>This study</td>
</tr>
<tr>
<td>FR4684</td>
<td>2010</td>
<td>PRN− (Δ84)</td>
<td>3</td>
<td>III/1/2/1</td>
<td>56.2 ± 6.2/100 ± 0.0</td>
<td>2 × 10⁷</td>
<td>This study</td>
</tr>
<tr>
<td>FR4953</td>
<td>2011</td>
<td>PRN− (stop)</td>
<td>3</td>
<td>IV/3/1/2</td>
<td>50.3 ± 9.9/89.5 ± 8.1</td>
<td>4 × 10⁷</td>
<td>This study</td>
</tr>
<tr>
<td>FR5187</td>
<td>2012</td>
<td>PRN− (Δ)</td>
<td>3</td>
<td>IVa3/1/2</td>
<td>67.2 ± 6.2/100 ± 0.0</td>
<td>2 × 10⁷</td>
<td>This study</td>
</tr>
<tr>
<td>FR5388</td>
<td>2012</td>
<td>PRN− (IS)</td>
<td>3</td>
<td>IVa3/1/2</td>
<td>79.4 ± 6.2/100 ± 0.0</td>
<td>3 × 10⁷</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Western blot (WB) analyses were conducted using specific anti-FHA, anti-PRN, anti-PT and anti-AC-Hly antisera. If not specified, the isolate expressed all tested virulence factors and is designated by +. Δ, Deletion of gene; Δ84, deletion of 84 bp at the beginning of the gene; IS, insertion sequence inside the gene of interest; stop, SNP causing a stop codon at position 1479.

†Isolate type is given as the type of fimbriae (FIM) produced.

‡Isolate type is given by its PFGE group, PT operon promoter (ptxP) allele type, PT subunit A (ptaX) allele type and prn allele type as follows.

§Total AC activity (ACact) of the bacterial suspension is given as the amount (nmol) of cAMP formed min⁻¹ ml⁻¹ (U ml⁻¹), while the ‘bound’ activity corresponds to the proportion of ACact that remains bound to the cell surface. Values are given as the mean ± SD for at least three independent measurements.
calculation of cell lysis inhibition. Co-incubating bacteria with the naive serum did not impact their cytotoxicity (Fig. 2a); neither did the serum impact J774A.1 cell viability throughout the incubation time (not shown).

As expected, immune serum containing polyclonal antibodies directed against AC-Hly significantly inhibited cell lysis in every case (Fig. 2a), with a mean inhibition of 67.0 ± 3.2 % for the tested isolates. In contrast, anti-PT antibodies never inhibited cell lysis (not shown), which was in accordance with our expectations, as it does not play a role in macrophage lysis.

Anti-FHA antibodies presented variable results depending more on the isolate's era of collection than its PRN phenotype (Fig. 2b). It thus appeared that the anti-FHA antiserum significantly inhibited cell lysis induced by FR5133 (51.5 ± 8.2 %), FR5187 (PRN⁻; 59.2 ± 4.1 %), FR5388 (PRN⁻; 56.3 ± 8.9 %) and FR5392 (63.4 ± 10.1 %), collected during the last French epidemic of pertussis in 2012, while the other isolates remained unaffected by the addition of anti-FHA antibodies.

The results obtained with anti-PRN antiserum are more surprising as this serum was able to significantly inhibit B. pertussis-induced cell lysis with a mean inhibition of 72.3 ± 4.1 % for isolates producing PRN. The inhibitory capacity of anti-PRN antiserum was abolished if the isolates did not produce PRN and dropped to a mean of 5.0 ± 1.0 % inhibition for PRN-deficient isolates (Fig. 2c).

**Murine model of intra-nasal infection**

As previously reported (Bouchez et al., 2009; Hegerle et al., 2012), apart from the PT-deficient isolate FR3749, all isolates remained capable of establishing a lethal colonization in vivo in the mouse model of respiratory infection. Despite inter-isolate differences such as observed between Bp1414 and FR3793 (PRN-deficient), intra-species variability was low and LD₅₀ values were quite homogeneous whatever the phenotype of the tested B. pertussis isolate (Table 1). The PRN deficiency of FR3793 cannot alone explain the increased LD₅₀, which is probably linked to other unknown genetic and/or proteomic differences.

**DISCUSSION**

AC-Hly is the secreted B. pertussis toxin responsible for macrophage apoptosis (Gueirard et al., 1998; Khelef et al., 1993; Osickova et al., 2010) and is mandatory for establishment of successful colonization in the mouse model of respiratory infection (Carbonetti et al., 2005; Khelef et al., 1992). Its importance for the pathogenesis of pertussis is further underlined by the rarity of isolates not producing...
Fig. 2. Inhibition of J774A.1 cell lysis by (a) naive serum [black circles], and polyclonal immune sera (grey bars) targeting (a) AC-Hly, (b) FHA and (c) PRN. Results are the mean ± SE for at least three independent experiments. Inhibition was calculated against the control condition without serum as presented in Methods. Statistical significance was determined between the inhibition of the naive serum and the inhibition of the polyclonal immune serum being considered; ***P<0.05.
this toxin (C.-H. Wirsing von Koenig, Direktor des Instituts für Hygiene und Labormedizin, HELIOS-Klinikum Krefeld, Krefeld, Germany, personal communication), as well as the genetic invariability of cyaA, the gene encoding this virulence factor (Chenal-Francisque et al., 2009; unpublished data).

The determination of AC enzymic activity of clinical PRN-deficient and PRN-producing isolates demonstrates that this particular adhesin was not involved in the distribution or amount of AC activity produced (Table 1). All isolates were also cytotoxic for J774A.1 murine macrophages in vitro and no statistically significant difference was found whatever the phenotype of the isolate (Fig. 1). Thus, there is no evidence of any PRN–AC-Hly interaction being involved in the process of macrophage cytotoxicity in vitro. These results are consistent with the in vivo murine model of respiratory infection since all tested isolates are evenly lethal, apart from FR3793, for which there is no obvious reason explaining this phenotype (Table 1).

Nonetheless, while the expected inhibition of cell lysis with specific anti-AC-Hly antiserum occurred whatever the isolate’s phenotype (Fig. 2a), specific anti-PRN antiserum was capable of inhibiting macrophage lysis induced by B. pertussis isolates that still produced PRN, the lack of which impaired this inhibitory capacity (Fig. 2c). As these two virulence factors did not seem to directly interact according to previous observations, this could be linked to anti-PRN antibodies preventing the correct secretion and presentation of AC-Hly to the target cell, but it remains that these antibodies are thus capable of inhibiting macrophage cytotoxicity in vitro and therefore might also act in vivo.

Despite the known interaction between FHA and AC-Hly, anti-PRN antiserum had no effect on cell lysis induced by the majority of tested isolates (Fig. 2b). FR5133, FR5187 (PRN-deficient), FR5388 (PRN-deficient) and FR5392, collected during the last French cycle of pertussis in 2012, were the only isolates sensitive to anti-AC-Hly antiserum. No mutation was found in fhaB, the gene encoding FHA, in FR5133 or in FR5187 as compared with the reference strain Tohama, whereas sphpB1, the subtilisin-like protease involved in FHA processing and maturation (Coutte et al., 2001), presented a non-synonymous SNP. This mutation was also found in the isolate FR4929 (unpublished data), which was not inhibited by anti-AC-Hly antiserum, thus ruling out possible involvement of this SNP in the observed anti-FHA-mediated inhibition of cell lysis.

Besides vaccine-driven evolution of B. pertussis populations (Octavia et al., 2011), epidemic lineages circulate (Advani et al., 2009; Weber et al., 2001) and need to be considered as well since they are possibly responsible for the characteristic cycles of pertussis, as they might present a particular phenotype and/or fitness. The result obtained with anti-FHA antiserum might reflect such a population of B. pertussis, as FR5133 and FR5187 were collected during the same pertussis cycle and are closely related, as evidenced by their segregation in a neighbour-joining tree built from the concatenated sequences of their SNPs obtained against B. pertussis reference strain Tohama (unpublished data). These two isolates, as well as FR5388 and FR5392, may thus share specific characteristics, yet to be determined, that impact the anti-FHA-mediated inhibition of cell cytotoxicity.

While PT, FHA and PRN can be targeted by APV-induced immunity, AC-Hly cannot, and any related phenotype should, therefore, remain unaffected during infection in an APV-immunized host. In vitro studies, however, demonstrated that AC-Hly interacts with FHA (Zaretzky et al., 2002) and it has been hypothesized that PRN might interact with this toxin as well (Khelef & Guiso, 1995). These interactions may participate in pertussis pathogenesis since a higher concentration of AC-Hly is localized at the site of adhesion through non-covalent interaction with FHA (Zaretzky et al., 2002), while AC-Hly seems mandatory for optimal FHA binding activity to epithelial cells (Perez Vidakovics et al., 2006). We here demonstrate that loss of PRN production does not impact AC-Hly-related phenotypes, whereas the presence of specific anti-FHA or anti-PRN antibodies does. Although these inhibitory capacities only occur if the isolates were collected during the last cycle of pertussis in 2012 or produce PRN, active immunization with APVs may impact AC-Hly-related phenotypes in vivo despite the lack of this toxin in all currently licensed APVs. Loss of PRN production might thus be an advantage for this pathogen in an APV-immunized population, while the sudden inhibition obtained with anti-FHA antiserum for isolates collected in 2012 remains puzzling.

Further in vivo and field studies are needed to explore the impact of PRN deficiency on the effectiveness of APV-induced immunity, but all licensed APVs include PT and monovalent PT APV is capable of inducing a protective immunity in the population (Petersen et al., 2012). Most APVs also induce anti-FHA antibodies, which seem to impair in vitro macrophage cytotoxicity induced by currently circulating isolates, which may also be relevant in vivo. Although development of new vaccines and evolution of vaccination strategies will probably occur in the next decade, high vaccine coverage with effective APVs will increase herd immunity and should enable control of the disease and protect children in the meanwhile.

ACKNOWLEDGEMENTS

We thank GlaxoSmithKline laboratories for the gift of purified antigens. This work was supported by the Institut Pasteur Foundation (URA CNRS3012) and GlaxoSmithKline Biologicals, Rixensart, Belgium.

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Edited by: P. Langford