MINIREVIEW

Contribution of pertussis toxin to the pathogenesis of pertussis disease

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One sentence summary: This minireview summarizes the evidence that one of the proteins produced by the whooping cough bacterium contributes significantly to the disease that it causes.

ABSTRACT

Pertussis toxin (PT) is a multisubunit protein toxin secreted by Bordetella pertussis, the bacterial agent of the disease pertussis or whooping cough. PT in detoxified form is a component of all licensed acellular pertussis vaccines, since it is considered to be an important virulence factor for this pathogen. PT inhibits G protein-coupled receptor signaling through Gi proteins in mammalian cells, an activity that has led to its widespread use as a cell biology tool. But how does this activity of PT contribute to pertussis, including the severe respiratory symptoms of this disease? In this minireview, the contribution of PT to the pathogenesis of pertussis disease will be considered based on evidence from both human infections and animal model studies. Although definitive proof of the role of PT in humans is lacking, substantial evidence supports the idea that PT is a major contributor to pertussis pathology, including the severe respiratory symptoms associated with this disease.

Keywords: Bordetella; whooping cough; respiratory infection; airway pathology

INTRODUCTION

Pertussis toxin (PT) is produced (at detectable levels) uniquely by Bordetella pertussis. PT is a multisubunit (AB5) protein toxin consisting of an enzymatically active A subunit (S1) non-covalently associated with a pentamer of binding (B) subunits (S2, S3, 2 copies of S4 and S5) (Tamura et al. 1982; Stein et al. 1994). PT is secreted by B. pertussis via a type IV secretion system (Weiss, Johnson and Burns 1993; Shrivastava and Miller 2009) and binds glycoconjugate molecules on mammalian cell surfaces (Witvliet et al. 1989). However, specific receptors for PT have not been identified, and PT appears to bind mammalian cells in a non-saturable and non-specific manner (Finck-Barbancon and Barbieri 1996). After internalization by endocytosis, PT is transported via the retrograde pathway to the endoplasmic reticulum (el Baya et al. 1997; Plaut and Carbonetti 2008), where S1 putatively translocates across the membrane to access the cytosol (Hazes et al. 1996; Pande et al. 2006; Worthington and Carbonetti 2007). The S1 subunit of PT is a member of the family of ADP-ribosylating toxins (Simon, Aktories and Barbieri 2014). It modifies a cysteine residue near the C terminus of the alpha subunit of heterotrimeric G proteins of the G_{i/o} class in mammalian cells, thus inhibiting activation of these G proteins by ligand-bound G protein-coupled receptors (GPCR) (Katada 2012). Since PT intoxicates virtually all mammalian cell types and many GPCRs couple to G, proteins, PT has a broad array of effects on host cell activities. An engineered form of PT with two amino acid changes in the catalytic site of the S1 subunit (9K/129G) has virtually undetectable enzymatic and toxic activity (Pizza et al. 1988; Seubert et al. 2014), demonstrating the essential function of ADP-ribosylation in PT toxicity. This genetically detoxified version of PT has been used as a pertussis vaccine component and retains superior immunogenicity to chemically detoxified PT (Seubert et al. 2014).
et al. 2014). Binding of PT to the cell surface independently of its ADP-ribosylation activity elicits various signaling effects that are relatively transient and concentration dependent (Wong and Rosoff 1996; Zocchi et al. 2005; Schneider, Weiss and Miller 2009; Carbonetti 2010; Mangmool and Kurose 2011). However, there is no evidence that this binding and signaling activity of PT occurs during B. pertussis infection or contributes to pertussis disease.

Several recent reviews on PT cover aspects of its cell biology, role as a virulence factor and involvement in pertussis vaccination (Carbonetti 2010; Locht, Coutte and Mielcarek 2011; Mangmool and Kurose 2011; Melvin et al. 2014; Seubert et al. 2014; Coutte and Locht 2015). This review will focus on the role of PT in the pathogenesis of pertussis infection and disease. As an indication of its toxic effects, PT was originally described as histamine-sensitizing factor (Parfentjev, Goodline and Virion 1947), lymphocytosis/leukocytosis promoting factor (Morse 1965) or islet-activating protein (Yajima et al. 1978) or as an inducer of experimental autoimmune encephalomyelitis (EAE) (Levine et al. 1966), all of which describe its systemic effects on mammalian physiology. But what about its effects on the more obvious respiratory manifestations of pertussis? While there is widespread agreement in the field that PT is an important virulence factor for B. pertussis and an essential component of acellular pertussis vaccines, the role of PT in pertussis respiratory infection and disease is less widely accepted and still somewhat controversial. However, several lines of indirect evidence from human and experimental data from animal studies support the idea that PT contributes importantly to pertussis disease. The first section of this review will consider evidence from human cases and volunteer studies, while the second section will cover evidence from animal model experiments.

Evidence from human pertussis cases, vaccinees and volunteers

Human volunteer experiments

The most convincing evidence that PT contributes to pertussis disease would theoretically be derived from human volunteer experiments involving direct respiratory administration of purified PT or comparison of infection and disease caused by isogenic strains differing only in PT production. However, neither of these studies has been undertaken (or reported at least). The closest is the experimental intranasal infection of adult human volunteers with the candidate pertussis vaccine strain BPZE1 (Mielcarek et al. 2006). This engineered strain expresses the enzymatically inactive 9K/129G version of PT. Volunteers infected with this strain had minor respiratory symptoms that were not significantly greater than those of individuals in the placebo group (Thorstensson et al. 2014), consistent with the idea that PT enzymatic activity contributes to pertussis respiratory disease. However, BPZE1 has two additional genetic alterations—deletion of the dermonecrotic toxin genes and addition of the Escherichia coli ampG gene to reduce the level of tracheal cytotoxin released—that may contribute to any observed phenotypes, so definitive conclusions on the role of PT cannot be made from studies with this strain. There are no reports of experimental respiratory administration of PT to human volunteers. However, one report described intravenous injection of purified PT at a dose of either 0.5 or 1.0 µg/kg (Toyota et al. 1980). These volunteers showed increased insulinemia in a glucose tolerance test, an effect known to be associated with B. pertussis (Parfentjev and Schleyer 1949) and PT (Toyota et al. 1978) in animal studies. However, they did not develop any respiratory symptoms.

Naturally occurring PT-deficient B. pertussis strains

The occurrence of naturally PT-deficient B. pertussis strains isolated from human pertussis cases is extremely rare, a finding that strongly indicates the importance of PT in B. pertussis infection and disease in humans. This is especially so in light of the recent widespread emergence of B. pertussis strains lacking expression of pertactin (Pawloski et al. 2014; Zeddeman et al. 2014) (another component of most acellular pertussis vaccines), likely as vaccine escape mutants (Hegerle, Dore and Guiso 2014; Martin et al. 2015). PT is a component in all acellular pertussis vaccines, yet the same emergence of PT-deficient strains has not occurred. In the single published report of a PT-deficient strain from a case of pertussis in France (Bouchez et al. 2009), the strain was isolated from a 3-month-old unvaccinated infant who was hospitalized with suspicion of pertussis. Although clinical details were scant in this report, the infant apparently suffered a relatively short time course of disease with no leukocytosis. The lack of severe and prolonged respiratory disease in this infant is certainly suggestive of an important role for PT in human pertussis disease, although with just a single case, firm conclusions cannot be made. In such a case, it is also impossible to know when the mutation (in this case deletion of the ptx genes) occurred. It is possible that it occurred during infection or subsequent isolation and passage of the strain—if this were the case, serology might have detected anti-PT antibodies, but no serology was reported for this infant. However, this also highlights a potential problem in identification of such PT-deficient B. pertussis strains, namely that several countries now use serology, detecting anti-PT antibodies, as a diagnostic tool for pertussis, which would negate the ability to detect these strains. With the emergence of technology for relatively easy and inexpensive whole genome sequencing of bacterial strains, identification of PT-deficient B. pertussis strains should become more common if they are indeed circulating and causing disease.

Bordetella parapertussis infection

One school of thought in the pertussis field is that since the closely related human pathogen B. parapertussis can also cause respiratory disease with whooping cough-like symptoms (Hoppe 1999; Watanabe and Nagai 2004) but does not produce PT, then PT therefore cannot be a major contributor to respiratory disease caused by B. pertussis. However, this is a flawed argument because it assumes that the two pathogens differ only by the presence or absence of PT, which is clearly not the case. As well as many differences in gene content revealed by whole genome sequencing (Parkhill et al. 2003; Park et al. 2012), these two pathogens differ in important biological and antigenic properties. For example, B. parapertussis expresses a protective O antigen on its LPS, whereas B. pertussis does not (Wolfe et al. 2007; Goebel et al. 2008). Therefore, PT may contribute to respiratory disease caused by B. pertussis, while distinct properties and mechanisms associated with B. parapertussis infection may contribute to similar respiratory pathogenesis in disease caused by this pathogen. In fact, Koch’s postulates have not been fulfilled for B. parapertussis as a cause of pertussis disease, since disease has not yet been reproduced in an animal model infected with this pathogen (unlike B. pertussis, a strain of B. parapertussis did not induce cough in rats; Parton, Hall and Wardlaw 1994). If serology to detect elevated levels of anti-PT antibodies is not performed on cases of parapertussis infection, then it remains possible that recent pertussis infection and the long-lived effects of PT may have contributed to the disease caused by B. parapertussis infection.
Monocomponent PT acellular pertussis vaccine

A detoxified form of PT is a component of all licensed acellular pertussis vaccines, which generally include one or more additional components that are surface antigens of *B. pertussis* (Klein 2014). Protection against the toxic activities of PT is thought to be especially important to prevent severe disease in infants (see the next subsection). Denmark has used a monocomponent vaccine consisting of hydrogen peroxide-detoxified PT since the 1990s (Thierry-Carstensen et al. 2013) (PT is detoxified by formaldehyde and/or glutaraldehyde in other acellular pertussis vaccines). Clinical vaccine trials revealed an efficacy for this vaccine in the same range as 3- and 5-component acellular pertussis vaccines (around 70-80% against WHO-defined pertussis) (Thierry-Carstensen et al. 2013; Zhang et al. 2014). Importantly, pertussis has remained under control in Denmark, with levels below 10 cases per 100,000 population since introduction of a preschool booster in 2003 (Thierry-Carstensen et al. 2013). Unlike the situation in several other countries, there has been no recent increase in pertussis cases in Denmark (Thierry-Carstensen et al. 2013; Ronn et al. 2014) and no appearance of pertactin-deficient strains (Zeddeman et al. 2014). The infection rate may be significantly higher than the case rate (von Linstow et al. 2010; Ronn et al. 2014), but this is also true in other countries where multicomponent acellular pertussis vaccines are used (Cherry 2005; Pebody et al. 2005). Together, this argues that PT is a crucial factor for pertussis disease pathogenesis, since immune responses to this single component can protect a population from pertussis disease effectively. Once again, the lack of appearance of PT-deficient ‘vaccine escape’ mutants in this population supports the idea that PT is an essential factor for promoting infection and/or transmission of pertussis, as well as its contribution to disease.

Leukocytosis and severe disease in infants

An association between pertussis infection in human infants and leukocytosis (increase in number of circulating white blood cells) has long been recognized (Seitz 1925). From animal studies, we know that PT is a direct cause of leukocytosis (Morse and Morse 1976; Munoz et al. 1981; Nogimori et al. 1984; Hinds et al. 1996). In addition, the infant in France infected with the PT-deficient strain referred to above did not have leukocytosis (Bouchez et al. 2009). However, the adult volunteers injected with purified PT showed no signs of leukocytosis (Toyota et al. 1980), although the dose of PT may have been too low or the volunteers may have had pre-existing anti-PT serum antibodies that neutralized the toxin’s effects. If we assume that PT causes leukocytosis in infants suffering from pertussis, then PT almost certainly contributes to severe and fatal pertussis, since there is a correlation between high levels of leukocytosis and poor outcome in these infants (Pierce, Klein and Peters 2000; Surridge, Segedin and Grant 2007; Rowlands et al. 2010). Blocked pulmonary capillaries as a result of leukocytosis may contribute to the pulmonary hypertension commonly seen in infants hospitalized with severe pertussis (Pierce et al. 2000; Donoso et al. 2005). The mechanism by which PT induces leukocytosis has not been established, although in one study, lymphocytes from infected infants were found to be deficient in expression of the lymph node homing marker L-selectin (Hudnall and Molina 2000), which may contribute to the lack of migration of these cells away from the circulation. If *B. pertussis* is restricted to the respiratory tract during infection, then sufficient toxin would have to access the circulation from this site to produce the effect, assuming a direct action of PT on leukocytes as the cause. An alternative possibility is that unrecognized disseminated infection occurs in infants with severe disease and high levels of leukocytosis, and that PT released from these disseminated bacteria contributes to the leukocytosis. Although there are no reports of disseminated pertussis infection in infants, it is not clear whether appropriate culture of blood samples for growth of *B. pertussis* is routinely performed on these infants before antibiotics have been administered, and so disseminated infection may have been missed.

*Bordetella pertussis* ptxP3 strains

The PT subunits are encoded by an operon of ptx genes (Locht and Keith 1986; Nicosia et al. 1986). Recent years have seen the widespread emergence of *B. pertussis* strains with the ptx operon promoter allele, ptxP3 (Rart et al. 2010; Kallonen et al. 2012; Lam et al. 2012; Schmidtké et al. 2012; Bowden et al. 2014). There is speculation that this polymorphism is associated with increased *B. pertussis* fitness in the face of acellular pertussis vaccine-elicited immunity in humans (Mooi et al. 2009). When grown in vitro, these strains apparently have marginally increased transcription of ptx genes (King et al. 2013; de Gouw et al. 2014) and production of PT (Mooi et al. 2009; de Gouw et al. 2014) compared to ptxP1 strains (which predominated previously). Therefore, it is tempting to conclude from this that increased PT production correlates with increased pathogenicity of *B. pertussis* strains in vaccinated human populations. However, there are many other genetic differences between ptxP3 strains and ptxP1 strains (Bart et al. 2010; King et al. 2013; de Gouw et al. 2014). Indeed, one study showed that a ptxP3 strain with its ptx promoter replaced by the ptxP1 sequence colonized mouse airways as efficiently as the ptxP3 parent strain (King et al. 2013), suggesting that the genetic background of these strains plays an important role in virulence. Therefore, any conclusions on the role of PT in the pathogenicity of these strains are tentative at best. Furthermore, definitive conclusions on the differences between ptxP3 and ptxP1 strains will have to be derived from a comparison of many more strains of each type than the very small numbers of strains investigated in these preliminary reports.

Evidence from animal models of pertussis

Effects of administration of purified PT

When administered systemically to experimental animals, PT recapitulates the leukocytosis seen in human infants with pertussis disease (Morse and Morse 1976; Munoz et al. 1981; Nogimori et al. 1984; Hinds et al. 1996). The mechanism by which PT induces leukocytosis is not clear, but a study in macaques showed that PT treatment reduces surface expression of the integrin LFA-1 on lymphocytes (Schenkel and Pauza 1999), which may affect the migration properties of these cells. PT inhibition of chemokine receptor signaling also likely contributes to leukocytosis, since much of leukocyte migration between the vasculature, tissues and lymphatics is controlled by chemokines and their receptors, which are GPCRs coupled to G proteins and therefore inhibited by PT (Cyster 1999; Campbell and Butler 2000). PT may also have deleterious effects on heart function independently of leukocytosis, since PT treatment of experimental animals demonstrates that its target G proteins are involved in control of heart rate and other cardiac functions (Adamson et al. 1993; Grimm et al. 1998; Zheng et al. 2005; Wainford, Kurtz and Kapusta 2008). Systemic administration of PT also induces vasodepressor sensitization to histamine (Munoz et al. 1981), although whether this is a component of pertussis disease in humans is not clear. Again, the mechanism underlying this effect has not been elucidated, although the H1 histamine receptor is involved.
Histamine sensitivity may involve PT inhibition of signaling by sphingosine-1-phosphate (S1P) receptors, another G<sub>i</sub>-linked GPCR, since mice lacking S1P in plasma were sensitized to histamine in a manner similar to PT treatment (Camerer et al. 2009). PT inhibition of S1P receptor signaling also reduces pulmonary vascular barrier integrity (Dudek et al. 2007), and therefore may contribute to edema in the lungs, which is seen by autopsy examination in at least some infants who died from pertussis infection (Sawal et al. 2009). Reduced vascular barrier integrity may also contribute to the pathogenesis of EAE in animal models (Bennett et al. 2010), many of which require PT to stimulate disease (Munoz, Bernard and Mackay 2003, 2007). Therefore, even though PT itself is apparently not a direct cause of cough, it may contribute to the paroxysmal nature of pertussis cough by preventing desensitization of receptors stimulated by tussive agents (Maher, Dubuis and Belvisi 2011), thereby preventing the cessation of the coughing response. Since PT effects are long-lived in experimental animals (Carbonetti et al. 2003, 2007), PT may also contribute to the longevity of pertussis cough after infection has been cleared.

**Infection with PT-deficient B. pertussis strains**

Substantial information on the role of PT in B. pertussis infection and disease has been derived from animal model studies comparing infection with isogenic strains differing only in PT production. We and others have used this approach to demonstrate that PT modulates a number of immune and inflammatory responses to B. pertussis infection in mice. For example, PT suppresses early recruitment of neutrophils to infected lung tissue and airways (Carbonetti et al. 2003, 2005; Kirimanjeswara et al. 2005), by inhibiting the production of neutrophil-recruiting chemokines by resident cells in the lung (Andreasen and Carbonetti 2008). PT also inhibits anti-bacterial activity of resident airway macrophages (Carbonetti et al. 2007), which scavange inhaled particles, including bacteria, and maintain relative sterility of the lower airways. The specific recognition molecules and signaling pathways that are inhibited by PT to alter macrophage function have not yet been elucidated, though there are a number of PT-sensitive GPCRs expressed by macrophages (Fpr, leukotriene, prostat glandin and complement receptors for example) that may be targets (Lattin et al. 2007). PT also suppresses serum antibody levels to B. pertussis during infection (Mielcarek et al. 1998; Carbonetti et al. 2004), an effect that may reduce natural immunity to reinfection.

The general inhibitory effect of PT on innate immunity and early inflammatory events promotes B. pertussis growth in the airways (Carbonetti et al. 2003, 2007). However, at the peak of infection, PT production by B. pertussis correlates with increased inflammation and pathology in the airways of mice (Khelef et al. 1994; Connelly, Sun and Carbonetti 2012). This enhanced airway pathology is not simply due to higher bacterial loads achieved by PT-producing versus PT-deficient strains, since mice with equivalent bacterial loads of the two strains demonstrated PT-dependent pathology (Connelly et al. 2012). How does the role of PT switch from inhibiting inflammatory events early after inoculation to promoting inflammatory pathology at peak infection? One potential mechanism involves the PT-dependent upregulation of pendrin in the airways. Pendrin is an epithelial anion exchanger (Nofziger et al. 2011), whose activity appears to promote inflammatory pathology in the airways during B. pertussis infection (Scanlon et al. 2014) (see our other review in this issue for more on this finding). Another possibility is that PT inhibits host mechanisms of resolving inflammation after the initial proinflammatory phase. Consistent with this idea, mice infected with high doses of a PT-deficient strain resolved inflammatory airway pathology relatively rapidly, whereas infection with an isogenic PT-producing strain that achieved equivalent bacterial loads significantly prolonged inflammatory events and airway pathology (Connelly et al. 2012). At least one class of inflammation resolving molecules, a group of endogenous lipids termed resolvin and lipoxins (Levy and Serhan 2014), signal through PT-sensitive GPCRs (Maddox et al. 1997; Krishnamoorthy et al. 2010). Therefore, PT may exacerbate and prolong acute inflammatory pathology by inhibiting the ability of resolvents to suppress and resolve inflammation at peak infection. Whether this inflammatory pathology correlates with the respiratory symptoms of human pertussis, including the severe cough, is unclear. Unfortunately, mice do not cough (to any stimulus), so it is not possible to test these correlations definitively in mice. However, in similar assays to those mentioned above, we have found that mice infected with a PT-producing B. pertussis strain have significantly exacerbated respiratory reflex responses to intratracheal administration of bradykinin compared to mice infected with a PT-deficient strain (Canning and Carbonetti, unpublished data). In addition, rats experimentally infected with B. pertussis develop paroxysmal coughing lasting several days (Hall, Parforn and Wardlaw 1994), whereas rats infected with a PT-deficient strain (or with B. parapertussis) did not cough (Parforn et al. 1994), indicating an association of PT with pertussis cough pathology. Recently, a new animal model of pertussis has been developed using young baboons inoculated with B. pertussis. These infected animals cough paroxysmally and develop other pertussis symptoms characteristic of pertussis disease in young children (Warfel et al. 2012; Warfel and Merkel 2014). However, baboons inoculated with a naturally occurring PT-deficient B. pertussis isolate did not develop cough or other signs of pertussis disease, even though they were colonized to the same extent and duration as animals inoculated with a PT-producing strain (T. Merkel, pers. comm.). Together these findings indicate that even though PT may not be the direct cause of pertussis cough, there is a strong association between PT production and cough responses in B. pertussis-infected animals, and this may be due to PT promotion of inflammatory responses at the peak of infection.

PT is also associated with lethality of B. pertussis infection in neonatal mice. Alison Weiss’ group showed that 6-day-old Balb/c mice succumbed to infection at doses that are sublethal for adult mice.
mice (Weiss and Goodwin 1989). However, neonatal mice inoculated with strains carrying a transposon insertion causing decreased secretion of PT showed significantly reduced lethality (Weiss and Goodwin 1989). We have recently found that PT production is required for lethality of *B. pertussis* in neonatal C57BL/6 mice (our unpublished data). In addition to lethality, we have found that other characteristics of PT-associated pertussis disease seen in adult mice are quite different in infected neonatal mice, and these may account for the lethality of disease in the young animals. Since lethality only in the very young is the same scenario as in human pertussis, neonatal mouse models of pertussis infection and disease may reveal novel characteristics of disease pathogenesis that can then be targeted for development of improved therapeutic approaches for treatment of severe pertussis in young infants.

CONCLUSION/OUTLOOK

In this minireview, I have attempted to review the evidence, from both humans and animal models, that PT contributes to pertussis disease. Recent reviews on PT have either been broad with only a subsection on this topic (Carbonetti 2010; Locht et al. 2011; Coutte and Locht 2015) or have been focused on vaccine-related (Seubert et al. 2014) or cell biology (Mangmool and Kurose 2011) aspects of the toxin. The contribution of PT to pertussis respiratory disease is somewhat controversial, with at least some investigators in the pertussis field skeptical of its involvement. PT is clearly an important virulence factor for *B. pertussis* infection and for at least some aspects of pertussis disease, such as leukocytosis, for which there is substantial evidence of the involvement of PT. It is unlikely that PT alone can cause respiratory symptoms associated with pertussis disease, since complex host responses to the bacterial infection in the respiratory tract are almost certainly involved in this pathology. Definitive evidence supporting a role for PT in respiratory pertussis disease will have to come from human volunteer studies with isogenic PT-producing and PT-deficient strains, but it is not clear if such studies will be approved by regulatory agencies. In the absence of such studies, the relatively new baboon model of pertussis may be a valuable tool for investigating both basic mechanisms in pertussis disease as well as novel therapeutic and vaccine approaches.

As described above, some lines of evidence from both human and animal studies already indicate that PT contributes to the severe respiratory symptoms of pertussis, including the paroxysmal cough, even if it is not the direct initial stimulus. Therefore, therapies directed at the effects of PT activity on the host may prove beneficial in the treatment of pertussis. Since PT-mediated modification of target G proteins is a stable and long-lived effect, targeting modified G proteins for rapid turnover may be a therapeutic approach. However, it is unclear which specific host cells are targeted by PT to produce these effects. In addition, our current understanding of G protein turnover in mammalian cells is not sufficient to design any therapeutics aimed at removing this G protein modification. Instead, therapeutics targeting PT-mediated effects may have to be aimed at downstream host responses that lead to disease pathogenesis. In our accompanying review in this issue, we describe two host-targeted therapeutic approaches that we are pursuing that emerged from our studies on the role of PT in the mouse model. If either of these approaches proves beneficial, it will not only provide much needed novel therapeutics for pertussis treatment, but will also validate the mouse model as an important tool in the investigation of pertussis disease pathogenesis and the discovery of potential therapeutic approaches.

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