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Minireview

MINIREVIEW

Pertussis leukocytosis: mechanisms, clinical relevance and treatment

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One sentence summary: Pertussis infection in young infants is characterized by high white blood cell counts (leukocytosis) and this minireview covers the causes, clinical findings and possible treatments for pertussis leukocytosis.

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ABSTRACT

The significant and sometimes dramatic rise in the number of circulating white blood cells (leukocytosis) in infants suffering from pertussis (whooping cough) has been recognized for over a century. Although pertussis is a disease that afflicts people of all ages, it can be particularly severe in young infants, and these are the individuals in whom leukocytosis is most pronounced. Very high levels of leukocytosis are associated with poor outcome in infants hospitalized with pertussis and modern treatments are often aimed at reducing the number of leukocytes. Pertussis leukocytosis is caused by pertussis toxin, a soluble protein toxin released by *Bordetella pertussis* during infection, but the exact mechanisms by which this occurs are still unclear. In this minireview, I discuss the history of clinical and experimental findings on pertussis leukocytosis, possible contributing mechanisms causing this condition and treatments aimed at reducing leukocytosis in hospitalized infants. Since recent studies have detailed significant associations between specific levels of pertussis leukocytosis and fatal outcome, this is a timely review that may stimulate new thinking on how to understand and combat this problem.

Keywords: leukocytosis; lymphocytosis; pertussis toxin; *Bordetella pertussis*

INTRODUCTION

Pertussis is a severe and prolonged coughing disease caused by infection of the respiratory tract with *Bordetella pertussis*, a Gram-negative bacterial pathogen (Kilgore *et al.* 2016). The disease affects individuals of all ages, but can be particularly severe in young infants, with frequent hospitalizations and occasional deaths (Berger *et al.* 2013). In addition to the hallmark paroxysmal coughing, pertussis in infants and young children is frequently characterized by a significant rise in the number of circulating white blood cells or leukocytes (leukocytosis) (Heininger *et al.* 1997). This is typically especially pronounced for lymphocytes (Hodge *et al.* 2003), and the term lymphocytosis is often used instead. However, leukocytosis is rarely seen in adolescents (Heininger *et al.* 1997) and adults (Aoyama *et al.* 1992) with pertussis. The normal number of circulating leukocytes in the bloodstream of humans is 4500–11 000 per μl. Newborns have a higher count but this falls to adult levels within the first few years of life (Schelonka *et al.* 1994). Various medical conditions, such as leukemia, use of certain drugs or other infections, can also cause leukocytosis (Abramson and Melton 2000), but the more pronounced lymphocytosis is not typically observed in these cases as it is in pertussis. In infants suffering from pertussis, hyperleukocytosis (>100 000/μl) can occur, and although this is a prognosticator of poor outcome (Pierce, Klein and Peters 2000), it is still unclear whether it is a

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contributor to fatality or just an associated marker of severe disease. The following sections discuss the history of clinical and experimental findings on pertussis leukocytosis, the discovery of pertussis toxin (PT) as the bacterial factor promoting leukocytosis, possible mechanisms by which the toxin mediates this effect and treatments aimed at reducing leukocytosis in hospitalized infants. With increasing levels of pertussis in many countries around the world and the failure of acellular vaccines to control this infection and disease (Mooi, Van Der Maas and De Melker 2014), increased understanding of the pathogenesis of pertussis disease is timely and important.

EARLY STUDIES

Leukocytosis in pertussis patients has been described since the late 1800s, when Froehlich (Froehlich 1897), Meunier (Meunier 1898) and others (De Amici GaP 1898) found leukocytosis to be present in the large majority of pertussis cases they examined and considered it to be of diagnostic value. Churchill noted that leukocytosis was more pronounced at younger ages and at early stages of the disease (Churchill 1906). In addition, he found that lymphocytosis (greater than normal percentage of lymphocytes among circulating leukocytes) is also frequently present and characterizes pertussis but not other diseases that could be confused for whooping cough (Churchill 1906). In these early studies, there was considerable debate about the time of appearance of leukocytosis and lymphocytosis relative to disease symptoms, the specific cell types showing increased numbers and percentages and the diagnostic value of these blood cell counts (reviewed in 1933 by Dolgopol (Dolgopol 1933)). Sauer and Hambrecht cautioned that the absence of leukocytosis early during cough disease should not discount pertussis as the diagnosis, and they described an early leukopenia in children with pertussis before peak leukocytosis during the paroxysmal phase (Sauer and Hambrecht 1931). Kolmer also found that leukocytosis and lymphocytosis were highest at the paroxysmal stage of disease, and that the highest levels of leukocytosis (up to 65 000/μl) were observed in children with fatal complications (Kolmer 1909). In a 1925 review of the early literature on pertussis leukocytosis (Seitz 1925), Seitz noted several pertussis cases where hyperleukocytosis had been observed. In three cases, the counts exceeded 200 000—all of these children and most of the others with counts above 100 000 died from the disease.

Early experimental studies in non-human primates not only confirmed that *Bordetella pertussis* infection was the cause of whooping cough, but also confirmed it as the cause of the associated leukocytosis. In a study published in 1929, Sauer and Hambrecht experimentally infected macaques with *B. pertussis* and saw significant leukocytosis in animals that became sick with pertussis-like symptoms (as high as 97 000 in one animal) (Sauer and Hambrecht 1929). Inaba and Inamori observed leukocytosis and lymphocytosis in experimentally infected macaques and young dogs, with counts as high as 40 000 (Inaba and Inamori 1934). Rich and colleagues also saw leukocytosis in experimentally infected chimpanzees (Rich *et al.* 1936). Animal models also provided the opportunity to test various extracts from *B. pertussis* cultures for their capacity to promote leukocytosis in the absence of live bacteria. Ehrich and colleagues found that an ultrasonicated extract of a *B. pertussis* culture produced leukocytosis and lymphocytosis when injected into rabbits, whereas a heated extract produced only granulocytosis (Ehrich *et al.* 1942). This indicated the involvement of a heat-labile component in pertussis leukocytosis, whereas activity of the heated extract was likely due to endotoxin. Bradford *et al.* also found evidence of the involvement of a heat-labile factor in leukocytosis induced by injection of *B. pertussis* extracts into mice (Bradford, Scherp and Tinker 1956). These and other studies demonstrated that active disease was not a prerequisite for leukocytosis and that an unknown heat-labile 'toxin' was likely the factor inducing this effect.

STUDIES OF STEPHEN MORSE

The studies of Morse in the 1960s and 70s contributed significantly to our understanding of experimental pertussis leukocytosis. Using adult mice injected intravenously with suspensions of killed *Bordetella pertussis* ('pertussis vaccine'), he observed a dose-dependent leukocytosis peaking 3–5 days post-injection and consisting primarily of small (naïve) lymphocytes, but with rises also in large lymphocytes, monocytes and granulocytes (Morse 1965). This property of the pertussis vaccine was cell associated and heat labile, and the effect was abrogated by prior immunization of mice with the same material, indicating that the leukocytosis-inducing factor is antigenic (Morse 1965). Histological studies suggested that lymphocytosis resulted from the release of cells from tissues and lymphoid organs in treated animals, rather than the production of new cells (Morse 1965). This was confirmed by studying leukocytosis in mice injected with tritiated thymidine to label newly produced cells, with the finding that pertussis vaccine induced no greater labeling of lymphocytes than in control mice (Morse and Riester 1967).

In attempts to identify and purify the leukocytosis-inducing factor, he found that it was released into the supernatant of liquid *B. pertussis* cultures, that the activity of this factor was equivalent to that induced by injection of pertussis vaccine and that it was destroyed by heat and reduced by treatment with proteolytic enzymes (Morse and Bray 1969). For subsequent studies he used culture supernatant or partially purified fractions instead of pertussis vaccine to induce leukocytosis. To determine whether the cells or the tissues were affected by pertussis treatment to induce leukocytosis, he isolated peripheral blood lymphocytes (PBL) from mice with pertussis-induced leukocytosis or from control mice (Morse and Barron 1970). After radioactive labeling *in vitro*, these cells were transferred to mice and the tissue distribution of labeled PBL was followed. He found that PBL isolated from pertussis-treated mice migrated to tissues and lymph nodes at much lower levels than PBL isolated from control mice, and concluded that the inhibition of lymphocyte emigration from the circulation in pertussis-treated mice is due to an effect on the lymphocytes themselves and not on the tissues (Morse and Barron 1970). In a follow-up study using a similar approach, he found that labeled lymphocytes had reduced homing to lymphoid tissue whether the lymphocytes or the recipient mice were treated with pertussis lymphocytosis-promoting factor (LPF) (Taub *et al.* 1972). He also found that LPF could bind to erythrocytes and lymphocytes and then spontaneously elute from these cells to affect other cells, and proposed this as a possible explanation for the inhibitory effect when LPF was injected into recipient mice (Taub *et al.* 1972; Adler and Morse 1973). Using histopathology analysis, he found early massive depletion of lymphocytes from the spleen, and to a lesser extent from the thymus, in LPF-treated mice and subsequent depletion of lymphocytes from lymph nodes (Athanassiades and Morse 1973). He also found evidence of the failure of circulating lymphocytes to emigrate back from the blood through lymph node postcapillary venules (Athanassiades and Morse 1973).

Morse was also able to purify LPF from *B. pertussis* culture supernatant and showed that this single factor was also responsible for the histamine sensitization and hypoglycemia-inducing effects associated with culture supernatant, but was distinct from the hemagglutinating factor (Morse and Morse 1976). As little as 20 ng of purified LPF when injected intravenously into mice caused significant leukocytosis, with increases in both T and B lymphocytes (Morse and Morse 1976). Although he did not elucidate the molecular mechanism of LPF activity (he did show inhibition of lymphocyte responses to adrenergic stimuli by culture fractions in an earlier study (Parker and Morse 1973)), Morse's studies collectively represented a major advance in our understanding of pertussis leukocytosis and lymphocytosis, and helped lay the foundation for molecular analysis of LPF (subsequently known as PT) activity.

MECHANISMS CAUSING LEUKOCYTOSIS IN PERTUSSIS

Many studies have firmly established that PT is the leukocytosis-promoting factor produced by *Bordetella pertussis*. In addition to mice, other experimental animals, including rats (Samore and Siber 1992), pigs (Elahi *et al*. 2005) and macaques (Hinds *et al*. 1996; Pauza *et al*. 1997), develop leukocytosis when treated with purified PT. Mice and baboons infected with *B. pertussis* have reduced levels of leukocytosis when treated with PT-specific monoclonal antibodies (Nguyen *et al*. 2015). In addition, humans infected with the related pathogen *B. parapertussis*, which can cause respiratory disease but does not produce PT, do not develop leukocytosis (Heininger *et al*. 1994), and an infant from which a PT-deficient *B. pertussis* strain was isolated also did not have leukocytosis (Bouchez *et al*. 2009). Furthermore, lymphocytes are not the only leukocyte subset contributing to leukocytosis, since monocytes and neutrophils are similarly affected by PT treatment (Meade, Kind and Manclark 1984; Im *et al*. 1989). However, the specific mechanisms by which PT induces leukocytosis are still not completely elucidated.

A major advance was the discovery of the molecular mechanism of action of PT by Ui and colleagues in the 1970s and 80s. They referred to PT as 'islet-activating protein' since they were studying its capacity to stimulate insulin secretion in rodents, as well as its stimulation of increased cAMP levels in treated cells. They discovered that PT modified a mammalian cell membrane-associated component of the adenylate cyclase system by ADP-ribosylation (Katada and Ui 1982) and that the target protein modified by PT was a previously undescribed guanine nucleotide regulatory protein (G_i protein) that inhibited adenylate cyclase (Murayama and Ui 1983). Subsequently, it was revealed that many G protein-coupled receptors (GPCRs) exert signaling effects through this PT-sensitive G_i protein, explaining the multitude of activities ascribed to PT (Reisine 1990). Genetically inactivated versions of PT that retained the capacity to bind to cells were then used to distinguish between effects of PT that are dependent or independent of its enzymatic activity, and using this approach, ADP-ribosylation activity of PT was found to be necessary for induction of leukocytosis (Black *et al*. 1988; Nencioni *et al*. 1990).

Extending the findings of Morse described above, Sugimoto *et al*. used an *in vitro* mouse thymus model to show that PT intoxication of lymphocytes, but not of epithelium, inhibited migration of the lymphocytes across the epithelial layer (Sugimoto *et al*. 1983), consistent with the idea that PT acts on the circulating cells rather than the tissues to induce lymphocytosis. Several studies found that an important aspect of PT induction of leukocytosis is its inhibition of lymphocyte extravasation. The lymph node homing marker L-selectin (CD62L) on lymphocytes mediates their initial attachment and rolling on lymph node high endothelial venules, and upregulation of the secondary adhesion molecule LFA-1 (CD11a/CD18) on lymphocytes mediates arrest for subsequent extravasation (Warnock *et al*. 1998) (Fig. 1). Spangrude *et al*. found that PT-treated lymphocytes lost their ability to extravasate to lymph nodes *in vivo* but retained the ability to bind to post-capillary high endothelial cells *in vitro* (Spangrude, Braaten and Daynes 1984), indicating that PT specifically inhibited the extravasation step. Bargatze and Butcher dissected this further, finding that PT treatment of lymphocytes specifically inhibits their activation-dependent arrest on lymph node high endothelial venules and that this activity was dependent on ADP-ribosylation activity (Bargatze and Butcher 1993). Warnock *et al*. found that LFA-1-dependent arrest of lymphocytes was abolished by treatment with PT but not with genetically inactivated PT, leading to the conclusion that PT inhibits G_i protein-linked signaling necessary for the sticking of lymphocytes prior to extravasation at high endothelial venules in lymph nodes (Warnock *et al*. 1998).

Phenotypic analysis of the lymphocyte subsets from mice (Mu, Cooley and Sewell 1994) or children (Kubic, Kubic and Brunning 1991) undergoing pertussis leukocytosis suggested an expansion of a normal naïve cell population and not proliferation of activated cells. Further phenotypic analysis of leukocytes from infants with pertussis revealed a dramatic reduction in L-selectin expression on all leukocyte subsets (Hudnall and Molina 2000; Hodge *et al*. 2003), possibly contributing to the lack of extravasation of these cells (Fig. 1). PT treatment of mice also reduced expression of L-selectin on effector T cells in a study of experimental autoimmune encephalitis (Amend *et al*. 2006). In macaques with PT-induced lymphocytosis, circulating lymphocytes had reduced surface expression of LFA-1 (Schenkel and Pauza 1999) (although this was not seen in the studies on human infants). How PT might reduce expression of these adhesion markers is not clear, but one study found that IL-6 reduced L-selectin levels on neutrophils in rabbits (Suwa *et al*. 2002), and increased IL-6 has been associated with pertussis (Torre *et al*. 1993) and with PT activity (Torre *et al*. 1993; Andreasen, Powell and Carbonetti 2009; Richard *et al*. 2011). In contrast, extravasation of T cells into non-lymphoid tissue is not inhibited by PT treatment (Cose *et al*. 2006; Caucheteux, Torabi-Parizi and Paul 2013).

Other related mechanisms may also contribute to PT-mediated leukocytosis. Chemokine receptors are PT-sensitive GPCRs that control much of cellular migration (Luther and Cyster 2001; Zabel, Rott and Butcher 2015), and therefore PT inactivation of chemokine signaling through these receptors very likely also contributes to leukocytosis. For example, PT promotes lymphocyte egress from the spleen back into the circulation by inhibiting chemokine receptor CCR7-mediated retention in the tissue (Pham *et al*. 2008). PT also promotes B cell egress from the bone marrow by inhibiting CXCR4-mediated retention (Beck *et al*. 2014) (Fig. 1). PT accelerated the maturation and release of thymocytes into the circulation as naïve T cells, although a chemokine receptor dependence was not shown in that study (Person, Korngold and Teuscher 1992). In addition, PT intoxication of endothelial cells may contribute to leukocytosis, since one study showed that endothelium-specific G_i deficiency caused reduced leukocyte extravasation (Pero *et al*. 2007), and G_i is the molecular target of PT activity.

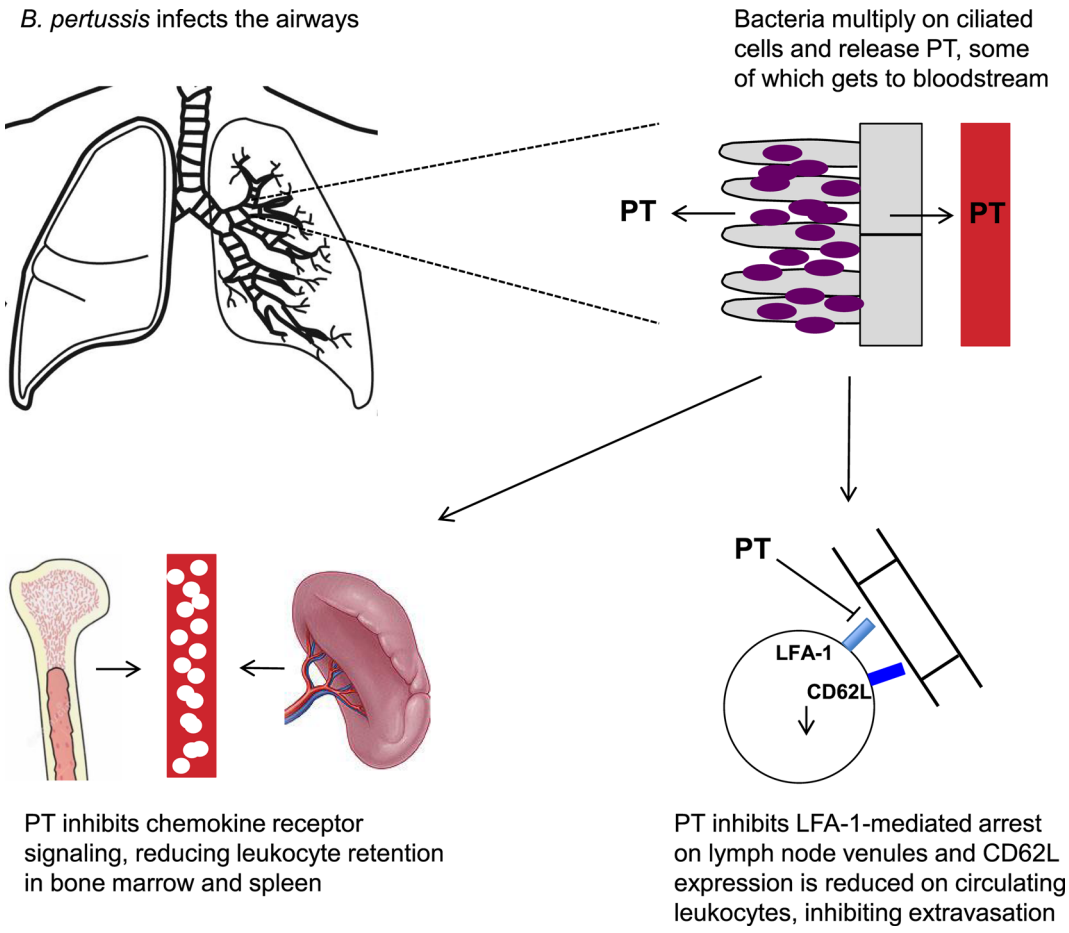

Figure 1. Possible mechanisms of PT-mediated leukocytosis in pertussis.

How does PT gain access to circulating cells to induce leukocytosis during pertussis infections and why does this occur only in young children and not in adolescents and adults? One possibility is that due to the endemic nature of pertussis in human populations, adolescents and adults have sufficient levels of PT-neutralizing antibodies, or generate them rapidly in a memory immune response, from prior infections or vaccinations. Therefore, PT is neutralized before it can cause leukocytosis in these individuals, whereas naïve infants have no such protection. However, leukocytosis is not seen in naïve adult mice infected with *B. pertussis*, whereas it is present in infected neonatal mice (our unpublished data). Based on our unpublished results using neonatal mice infected with *B. pertussis*, we speculate that bacteria multiply to greater numbers in the respiratory tract of young children than in adults and therefore sufficient PT may be produced and released into the bloodstream to affect circulating leukocytes.

CLINICAL FINDINGS AND RELATIONSHIP TO FATAL PERTUSSIS

As described above, early studies of pertussis leukocytosis detailed some of the clinical findings, including the kinetics of the response, the predominant lymphocytosis and the presence of hyperleukocytosis in extreme cases. More recently, in a large study of pertussis patients, Heininger *et al.* found leukocytosis in 72% of unvaccinated individuals with pertussis (at the time of their visit to a doctor) versus 38% of controls (individuals with cough disease not due to pertussis), and these numbers were 76% and 37% when looking at lymphocytosis (Heininger *et al.* 1997). Leukocyte counts were generally higher in younger children than in adolescents. They concluded that leukocytosis and lymphocytosis are strong and specific indicators of pertussis infection, but that sensitivity of this finding is limited. Shojaei *et al.* compared confirmed versus suspected cases of pertussis and found that leukocytosis and lymphocytosis were present significantly more frequently in confirmed cases (Shojaei *et al.* 2014). However, in a review of the literature on pertussis lymphocytosis, Levene and Wacogne cautioned that the presence of lymphocytosis was not very specific to pertussis and that ~50% of children with suspected pertussis and raised lymphocyte counts will not have pertussis (Levene and Wacogne 2011).

Several studies have proposed a correlation between high levels of leukocytosis and fatal outcome in pertussis. Hyperleukocytosis was an independent predictor of death among 13 critically ill infants with pertussis in the UK (Pierce, Klein and Peters 2000). Other studies showed that high leukocytosis was similarly predictive of death among pertussis infants in Canada (Mikelova *et al.* 2003), New Zealand (Surridge, Segedin and Grant 2007), Argentina (Gentile *et al.* 2014), Tunisia (Borgi *et al.* 2014) and the USA (Murray *et al.* 2013). Winter *et al.* analyzed this association more closely in a larger study of fatal pertussis cases in the USA, finding that leukocytosis above 70 400 was particularly predictive of death, especially if combined with low birth weight (Winter *et al.* 2015). But is hyperleukocytosis a direct contributor to fatal pertussis disease or is it simply a correlate?

Pulmonary hypertension is a common complication among critically ill infants with pertussis (Goulin, Kaya and Bradley 1993; Donoso *et al.* 2005) and histopathological autopsy findings of leukocyte thrombi in pulmonary vessels have led some to speculate that leukocytosis may contribute to pulmonary hypertension by blocking pulmonary capillaries and restricting blood flow (Pierce, Klein and Peters 2000; Paddock *et al.* 2008; Sawal *et al.* 2009). However, Winter *et al.* note that PT may cause more widespread and damaging effects on cardiac and respiratory function through inhibition of key G_i protein signaling in heart and lungs, and that leukocytosis may just be a PT-associated marker of severe disease rather than a proximate cause of death (Winter *et al.* 2015).

TREATMENTS

One of the conventional emergency treatments for infants with life-threatening pertussis disease is extracorporeal membrane oxygenation (ECMO) to provide cardiac and respiratory support (Williams *et al.* 1998). However, the success rate of this treatment for these infants is relatively low, with ∼70% mortality rate (Halasa *et al.* 2003), possibly because of the leukocytosis. Increasingly, treatment of life-threatening pertussis includes some measure to reduce the number of circulating leukocytes (reviewed in Scanlon, Skerry and Carbonetti 2015). Romano *et al.* reported a case of a 3-month-old infant hospitalized with severe pertussis, including hyperleukocytosis and pulmonary hypertension, who was treated successfully by double volume exchange transfusion to reduce the leukocyte mass (Romano *et al.* 2004). Rowlands *et al.* found that addition of leukodepletion by exchange transfusion in the intensive care of infants with critical pertussis reduced the mortality rate from 44% to 10% (Rowlands *et al.* 2010). Additionally, there have been similar reports of cases where single or double volume exchange transfusion was successful in reducing leukocytosis and improving the outcome for infants with severe pertussis disease (Rowlands *et al.* 2010; Martinez *et al.* 2011; Lashkari, Karuppaswamy and Khalifa 2012; Onoro *et al.* 2012; Berger *et al.* 2013; Kuperman *et al.* 2014). One of these cases also reported the beneficial effects of hyperhydration as a treatment for the leukocytosis (Lashkari, Karuppaswamy and Khalifa 2012), while another report found that leukopheresis as a method of leukodepletion was not effective in preventing death (Onoro *et al.* 2012). Assy *et al.* described the use of leukofiltration using a leukocyte filter during ECMO in the successful treatment of an infant (Assy *et al.* 2015). However, exchange transfusion was not successful in preventing fatal outcome in all cases. As reviewed by Kuperman *et al.* (Kuperman *et al.* 2014), out of 47 reported pertussis cases that were treated by exchange transfusion, only 30 survived. From a study of 10 infants with critical pertussis who received exchange transfusion, Nieves *et al.* concluded that the treatment may be ineffective if organ failure has already occurred (Nieves *et al.* 2013). They also pointed out that even if leukocytosis is not the direct cause of death in these infants, exchange transfusion may still be beneficial by reducing the level of circulating PT, which may have other life-threatening effects as mentioned above.

Another potential treatment to reduce leukocytosis in pertussis patients is administration of PT-specific antibodies to reduce its effects. Bruss *et al.* found that administration of intravenous pertussis immunoglobulin (P-IGIV), with high levels of anti-PT antibodies, to infants with pertussis resulted in significant reduction in leukocytosis by 3 days post-treatment, as well as reductions in paroxysmal coughing and bradycardic episodes (Bruss *et al.* 1999). However, a more recent clinical trial of P-IGIV treatment found no significant benefit in disease symptoms, although leukocytosis was not measured in this study (Halperin *et al.* 2007). Very recently, Nguyen *et al.* administered humanized murine monoclonal antibodies specific for PT to experimentally *Bordetella pertussis*-infected animals (Nguyen *et al.* 2015). They found that this treatment was more effective than P-IGIV in preventing leukocytosis in mice and was effective in reducing leukocytosis when administered therapeutically to infected baboons. This may provide a more specific and effective antibody-based therapy for treatment of leukocytosis and other PT-associated effects in human pertussis.

CONCLUSION

Leukocytosis and lymphocytosis are strongly associated with pertussis infection, particularly in infants, although the timing of measurement versus onset of infection and disease can influence the diagnostic value of this finding. Hyperleukocytosis is associated with fatal outcome in infants hospitalized with pertussis and modern emergency treatments often include a measure to deplete circulating leukocytes. A causal link between leukocytosis and pertussis fatality is unclear and will probably have to be determined in experimentally infected animals. PT is clearly the leukocytosis-promoting factor of *Bordetella pertussis* and probably acts through a combination of effects on leukocyte surface adhesion molecules and chemokine receptor signaling to cause this effect. Further investigation of pertussis leukocytosis and other PT effects will increase our understanding of the pathogenesis of this disease and will inform development of improved treatments for hospitalized infants suffering from critical pertussis.

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