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Biochemical characterization of monoclonal antibodies to the Bordetella pertussis Filamentous hemagglutinin (FHA) and Pertussis toxin (PTx): Implications for improved acellular pertussis vaccine design

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by

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Dedication

To Him who gives us strength.

I dedicate this dissertation to my dear husband Dan, and our boys, David and Isaac. To my parents, Daniel and Lilian Acquaye, and my sister Ellen Acquaye. Also, to my late sweet grandma, Lydia Adams.

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To my dear husband, Dan, and our kids David and Isaac, thank you!

To God be the Glory.

Biochemical characterization of monoclonal antibodies to the Bordetella pertussis Filamentous hemagglutinin (FHA) and Pertussis toxin (PTx): Implications for improved acellular pertussis vaccine design

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Incidence rates of whooping cough were dramatically decreased by immunization with whole cell pertussis (wP) vaccines in the 1940s. However, concerns about the safety of the wP vaccine resulted in development of new pertussis vaccines using acellular components of *B. pertussis*. However, *B. pertussis* continues to circulate and cause whooping cough disease, even with the safer acellular pertussis (aP) vaccines. Over the last decade, in spite of the high vaccination coverage in many countries, there has been a significant rise in whooping cough infection in industrialized countries. The filamentous hemagglutinin (FHA) and pertussis toxin (PTx) are two antigens included in all currently licensed aP vaccines. Both FHA and PTx induce an immune response sufficient for protection, although anti-PTx antibodies correlate more with protection.

Most studies assessing the efficacy of aP vaccines have used serum titers as the primary means to measure an antibody response to a given antigen. Single cell techniques however offer an opportunity to better understand the biochemical attributes of individual

antibodies induced upon aP vaccination. In this study, we characterized the antibody responses to FHA and PTx after adult aP booster vaccination. Monoclonal antibodies derived from single B cells which responded to FHA and PTx were purified, the binding affinities to the antigens evaluated, mechanisms of neutralization of FHA and PTx toxicity evaluated, and the binding epitopes of the antibodies analyzed. This study is the first to provide insight into the anti-FHA and PTx antibody repertoire after pertussis vaccination, and also identifies useful antibodies for further elucidation of the structure and function of these antigens. In addition, the mechanisms of neutralization of two potently neutralizing anti-PTx monoclonal antibodies, 1B7 and 11E6 are presented. Antibody binding to live *B. pertussis*, inhibition of PTx binding to a model receptor fetuin and effects on PTx trafficking in CHO-K1 cells were evaluated. Further understanding of the molecular mechanisms of 1B7 and 11E6 neutralization provides a platform for engineering or isolating such unique anti-PTx antibodies for passive immunization therapies.

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Chapter 1: Background and significance

1.1 WHOOPING COUGH INFECTION

Whooping cough is a contagious upper respiratory infection caused by the Gram-negative, aerobic coccobaccillus, *Bordetella pertussis* (Mattoo and Cherry 2005). Other members of the *Bordetella* genus include *B. parapertussis* which also infects humans, and *B. bronchiseptica*, which has a broad mammalian host range including humans. *B. pertussis* infection typically results in a prolonged cough lasting over two weeks (Trollfors and Rabo 1981, Postels-Multani, Schmitt et al. 1995, Birkebaek, Kristiansen et al. 1999, Senzilet, Halperin et al. 2001, Strebel, Nordin et al. 2001, Gilberg, Njamkepo et al. 2002). The coughing is paroxysmal and can severely disturb sleep. Choking and vomiting may also occur (von Konig, Halperin et al. 2002). Other complications, although infrequent have also been recorded, such as syncope (Jenkins and Clarke 1981) and encephalopathy (Halperin and Marrie 1991). People are susceptible to whooping cough infection throughout their lifetime. Severe infection is however more often observed in infants and young children, and the infection may be fatal in infants less than 6 months. Antibiotics such as erythromycin may be used to treat infection early in disease development (Bass 1986). Antibiotics are however usually ineffective past the catarrhal stage of infection when the symptoms are mediated by toxins expressed by the bacteria (Pittman 1984).

Incidence rates of whooping cough were dramatically decreased by immunization with whole cell pertussis (wP) vaccines in the 1940s (Cherry 2012). However, concerns about the safety of the wP vaccine resulted in development of new pertussis vaccines using acellular components of *B. pertussis* (Sato and Sato 1999). Even with the safer

acellular pertussis (aP) vaccines, *B. pertussis* continues to circulate and cause whooping cough disease, in spite of the high vaccination coverage in many countries, (Celentano, Massari et al. 2005, Quinn and McIntyre 2007, Hozbor, Mooi et al. 2009, (CDC) 2012), and over the last decade, there has been a significant rise in whooping cough infection in industrialized countries (Figure 1.1).

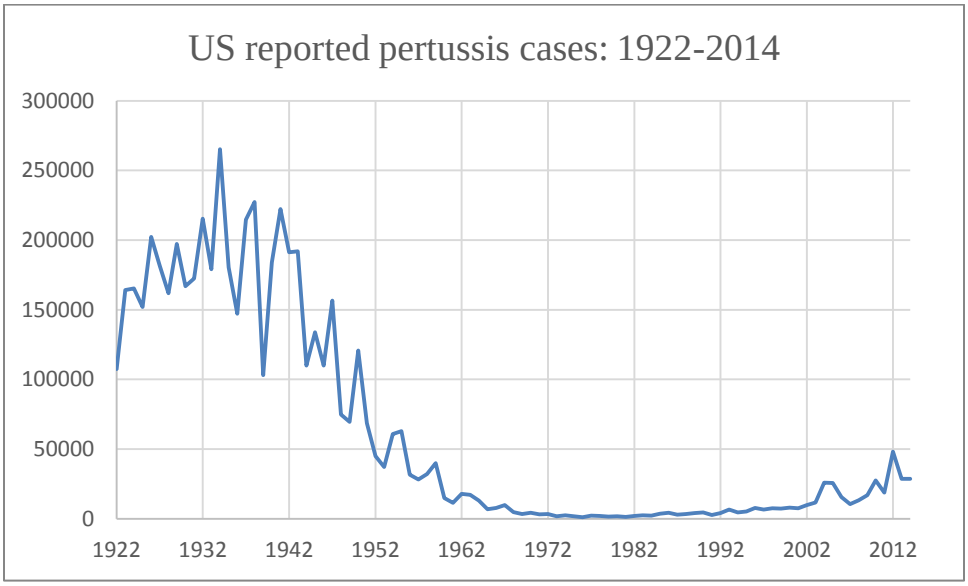


Figure 1.1: Reported incidence of pertussis cases in the US from 1922-2014.
*2014 numbers are provisional (CDC.gov).

More troubling, disease rates have been rising steadily over the past few decades, to a 50-year high of approximately 42,000 cases in 2012 (Tanaka, Vitek et al. 2003, Lavine, King et al. 2013). aP vaccination prevents the severe manifestations of disease, but has been unable to eliminate subclinical infection (Lavine, King et al. 2011). In fact, the temporal decline in post-vaccination immunity has altered disease demographics such

that adults and adolescents now constitute the dominant reservoir (Senzilet, Halperin et al. 2001).

Various reasons have been attributed to the current rise in observed clinical pertussis cases, including increased awareness (Jackson and Rohani 2014), and antigenic divergence between vaccine and circulating clinical strains (Octavia, Sintchenko et al. 2012, Lam, Octavia et al. 2014). However, the pertussis resurgence, closely beginning around the introduction of acellular pertussis (aP) vaccines, raised concerns that the immunity from aP vaccines may be incomplete or wanes more quickly than protection after natural infection (Cherry 2010, Clark, Messonnier et al. 2012, Klein, Bartlett et al. 2012, Sheridan, Ware et al. 2012, Witt, Katz et al. 2012). After infant immunizations at 2, 4 and 6 months, immunity wanes over time, resulting in adults and adolescents reservoirs of the disease (von Konig, Halperin et al. 2002). Although adolescents and adults are often the largest group infected, the burden of disease falls on unimmunized infants, for whom morbidity and mortality from infection is highest (Vitek, Pascual et al. 2003, Elliott, McIntyre et al. 2004, Theilen, Johnston et al. 2008). For this reason, strategies have been put in place to protect infants through booster immunizations for adolescents and adults who serve as reservoirs (Hethcote 1999, Mooi and de Greeff 2007). Significant advances in our understanding of the complex molecular mechanisms of *B. pertussis* pathogenesis, and have led to ongoing research for the development of improved pertussis vaccines.

1.2 *B. PERTUSSIS* VIRULENCE FACTORS

B. pertussis expresses over 20 virulence factors which aid in bacterial colonization and evasion of host immunity (Mooi 1988). Expression of the virulence

genes is modulated by a genetic locus which consists of two closely linked genes *bvgA* and *bvgS* (Beier, Fuchs et al. 1996). The BvgAS component system activates the virulence genes in response to environmental signals such as temperature and nutrients (Konig, Bock et al. 2002, Nakamura, Liew et al. 2006), and also controls phase variation to a non-virulent form with the loss of expression of the virulence factors (Deora, Bootsma et al. 2001). The expressed virulence factors fall into two main groups: adhesins and toxins. The adhesins are mostly found on the surface of the bacteria and are responsible for mediating bacterial attachment to the host. *B. pertussis* adhesins studied in detail include the filamentous hemagglutinin (FHA), fimbrae (FIM), pertactin (PRN) and the serum resistance protein (BrkA). The toxins on the other hand, have immunosuppressive properties to enhance bacterial colonization and include the pertussis toxin (PTx), the adenylate cylase toxin (ACT), tracheal cytotoxin (TCT) and the dermonecrotic toxin (DNT) (Locht 1999). Table 1 shows the virulence factors expressed by the three major *Bordetella* species.

1.3 ACELLULAR PERTUSSIS (AP) VACCINES

The whole cell pertussis (wP) vaccines which preceded the current acellular pertussis (aP) vaccines were made from killed whole cell suspensions of one or more strains of *B. pertussis*, and subsequently treated to reduce toxicity but maintain effectiveness (Organization 2007). However, immunization with wP vaccines raised safety concerns, due to local and systemic reactions such as convulsions and infrequently, neurological reactions (Trollfors 1984). A challenge in aP vaccine development was that the bacterium produces multiple virulence factors, none of which provided a clear correlate of protection (Robbins, Schneerson et al. 1998, Robbins, Schneerson et al.

2009). However, increased knowledge of the molecular mechanisms of *Bordetella* pathogenesis and immunity led to the development of aP vaccine (Sato, Kimura et al. 1984), containing up to five of the virulence factors including PTx, FHA, PRN and fimbriae types 2 and 3 (FIM 2/3).

Table 1.1: Virulence factors expressed by the three major *Bordetella* species.

	B. pertussis	*B. parapertussis*	*B. bronchiseptica*
Adhesins			
FHA	+	+	+
PRN	+	+	+
FIM	+	+	+
Brk	+	Gene present[a]	Gene present[a]
Toxins			
PTx	+	Gene present[b]	Gene present[b]
ACT	+	+	+
TCT	+	+	+
DNT	+	+	+

[a] – The gene for the serum resistance protein (Brk) is present in *B. parapertussis* and *B. bronchiseptica*; however it is not known whether they are expressed.
[b] – The PTx gene is present in *B. parapertussis* and *B. bronchiseptica*, but transcriptionally silent due to the presence of mutations mostly in the promoter regions (Aricò and Rappuoli 1987, Locht 1999). Table adapted from (Locht 1999).

1.4 ANTIBODIES AGAINST aP VIRULENCE FACTORS

The mechanisms underlying protection conferred by pertussis vaccines or natural infection is still an open issue due to the complexity of the range of pharmacological effects from the numerous toxins and adhesins produced by *B. pertussis* (Mills 2001). It is however known that both B and T cells provide protective immunity (Mills and Redhead 1993, Mills, Ryan et al. 1998, Leef, Elkins et al. 2000). Early studies in mice did not provide a clear role for antibodies in protection against *B. pertussis*. Antibody responses to *B. pertussis* in mice after respiratory challenge develop slowly and are detected at low levels, even after the bacteria are cleared (Mills, Barnard et al. 1993). However, results of passive immunization studies indicated antibodies against various *B. pertussis* virulence factors play a protective role against *B. pertussis* infection. Polyclonal pertussis immunoglobulin (P-IVIG), prepared from the sera of immunized human volunteers, passively protected mice against an aerosol challenge and also reversed disease symptoms (Bruss and Siber 1999). Phase I clinical trials with infants treated with P-IVIG also showed a significant reduction in the number and duration of coughing (Granstrom, Olinder-Nielsen et al. 1991). A larger clinical trial however showed no difference in the reduction of symptoms compared to a placebo, and was terminated early due to unavailability of the antibody product (Halperin, Vaudry et al. 2007), and was therefore inconclusive. House hold exposure studies have however also indicated that individuals with high serum antibody levels against PTx, PRN and FIM had lower incidence of severe pertussis (Cherry, Gornbein et al. 1998, Storsaeter, Hallander et al. 1998). Children immunized with a one-component aP vaccine, with higher anti-PTx antibodies, have also been shown to be protected from development of severe pertussis (Taranger, Trollfors et al. 2000). Anti-FHA antibodies are also thought to contribute to protection (He, Viljanen et al. 1994), although the relative value in the absence of

antibodies against PRN and FIM is not clear (Cherry, Gornbein et al. 1998). These studies show a correlation between the levels of serum antibodies to individual virulence factors and protection against severe whooping cough infection.

Significant differences have been observed in both the T and B cell immune responses generated after aP vaccination compared with wP vaccines or natural infection (Podda, Bona et al. 1995). Analysis of T cells in infants primed with aP vaccines, showed a Th2 skewed or mixed Th2/Th1 response, whereas wP vaccines or natural infection induced predominantly Th1/17 responses (Ross, Sutton et al. 2013). In addition, individuals immunized with aP vaccines are observed to have a higher magnitude of T cell and antibody responses with a slower rate of decay, compared to individuals with natural infection or primed with wP vaccines (Dalby, Petersen et al. 2010). Antibody responses to FHA, FIM and PRN in aP vaccines are higher in magnitude compared to responses to the wP vaccines or natural infection (Edwards, Meade et al. 1995, Berbers, van de Wetering et al. 2013). The anti-FHA and PRN antibodies however decline more quickly (Giuliano, Mastrantonio et al. 1998, Berbers, van de Wetering et al. 2013). Anti-PTx antibody levels are variable depending on the aP vaccine (Edwards, Meade et al. 1995), but may be lower in magnitude compared to natural infection (Berbers, van de Wetering et al. 2013). The decay kinetics of anti-PTx antibodies induced to aP vaccines also appear to be variable in comparison to natural infection. One study, suggests anti-PTx levels may decline faster after aP vaccination (Berbers, van de Wetering et al. 2013), while another study suggests the contrary (Dalby, Petersen et al. 2010). Serum antibody levels and the kinetics of antibody decay after aP vaccination therefore do not provide a complete picture of the protection from aP vaccine immunization still protects from severe disease. In addition, some aP vaccines with lower efficacy may also induce high antibody levels (Greco, Salmaso et al. 1996, Gustafsson, Hallander et al. 1996, Olin,

Rasmussen et al. 1997), further suggesting not all the virulence factors in the aP vaccines contribute to protection against severe infection.

1.5 OBJECTIVES OF THIS DISSERTATION

FHA and PTx are antigens included in almost all current aP vaccines. Both FHA and PTx induce an immune response sufficient for protection, however anti-PTx antibodies correlate more with protection. Most studies assessing the efficacy of aP vaccines have used however serum titers as the primary means to measure an antibody response to a given antigen, while only a few have used ELISpot analysis to monitor antigen specific B cell populations (Stenger, Smits et al. 2010, Hendrikx, de Rond et al. 2011, Hendrikx, Ozturk et al. 2011). While overall serum antibody titers are high in aP vaccinated individuals, serum ELISAs do not distinguish between high concentrations of weakly binding antibodies and low concentrations of tightly binding antibodies, or easily identify the spectrum of epitopes recognized by the polyclonal serum. Serum antibody studies have however provided a broad understanding of the quality of antibodies induced by infections versus acellular vaccines.

Recent developments in isolation and analysis of monoclonal antibodies from single B cells (Meijer, Andersen et al. 2006, Tiller, Meffre et al. 2008) have provided great insights into antigen-specific antibody repertoires, facilitating identification of rare, neutralizing antibodies (Yu, Tsibane et al. 2008) and correspondingly, the epitopes recognized (Krause, Tsibane et al. 2011). For instance, the techniques of isolating monoclonal antibodies from polyclonal serum (Cheung, Beausoleil et al. 2012, Wine, Boutz et al. 2013) have been used to identify neutralizing antibodies against pathogens such as HIV (Zhu, O'Dell et al. 2012, Zhu, Ofek et al. 2013), 1918 influenza virus

(Wrammert, Smith et al. 2008, Yu, Tsibane et al. 2008, Krause, Tsibane et al. 2011). Single cell techniques therefore offer an opportunity to better understand the biochemical attributes of individual antibodies induced upon aP vaccination, and determination of the number of antibody sequence families responding to a given antigen. In addition, the ligand binding affinity and neutralization capacity of each antibody can be determined. Identified neutralizing antibodies also have the potential to be used for passive immunotherapy.

In the next two chapters of this study, antibody responses to FHA and PTx after aP booster vaccination are characterized. Monoclonal antibodies derived from single B cells which responded to FHA and PTx were purified from transiently expressing CHO-K1 cells. The binding affinities to the antigens were characterized, the mechanisms of FHA and PTx neutralization evaluated, and the binding epitopes of the antibodies analyzed. This study, the first to provide insight into the anti-FHA and PTx antibody repertoire after pertussis vaccination, also identifies useful antibodies for further elucidation of the structure and function of these antigens. In the third chapter of this thesis, the molecular mechanisms of neutralization of two potently neutralizing anti-PTx monoclonal antibodies, 1B7 and 11E6 are presented. Antibody binding to live *B. pertussis*, inhibition of PTx binding to a model receptor fetuin and effects on PTx trafficking in CHO-K1 cells were evaluated. Further understanding of the molecular mechanisms of 1B7 and 11E6 neutralization provides a platform for engineering or isolating such unique anti-PTx antibodies for passive immunization therapies.

Chapter 2: Characterization of antibodies from single B cells induced to the filamentous hemagglutinin (FHA) after acellular pertussis booster immunization.

2.1 INTRODUCTION

FHA, one of the first major *B. pertussis* virulence factors to be discovered (Arai and Sato 1976, Sato, Izumiya et al. 1981) is a large immunogenic antigen found both surface associated and secreted. FHA is first synthesized as a large ~370 kDa precursor from the fhaB gene (Delisse-Gathoye, Locht et al. 1990, Domenighini, Relman et al. 1990), after which it undergoes extensive N-terminal (Jacob-Dubuisson, Buisine et al. 1996, Lambert-Buisine, Willery et al. 1998) and C-terminal proteolytic processing to yield the ~220 kDa mature protein (Delisse-Gathoye, Locht et al. 1990, Domenighini, Relman et al. 1990), which is the major secreted product. Structurally, FHA has been shown to be a monomeric filamentous rod, about 50 nm in length with a globular end (Makhov, Hannah et al. 1994, Kajava, Cheng et al. 2001).

Mature FHA is thought to have four binding domains responsible for *B. pertussis* adherence to receptors on host cells (Brennan, Hannah et al. 1991, Prasad, Yin et al. 1993, Hannah, Menozzi et al. 1994). A heparin binding domain (HBD), located at the N-terminal end of FHA (Hannah, Menozzi et al. 1994, Colombi, Horton et al. 2004), is involved in the carbohydrate dependent hemagglutination of red blood cells (Sandros and Tuomanen 1993, Menozzi, Mutombo et al. 1994), and binds receptors with sulfated glycoconjugates such as heparin which may be present on mucus secreting epithelial cells (Menozzi, Mutombo et al. 1994). The relevance of the HBD in the pathogenesis of *B. pertussis* infection is however unclear as it is not sufficient for adherence to epithelial

cells and macrophages, but appears to contain neutralizing epitopes sufficient for protection (Alonso, Reveneau et al. 2002).

A second region comprising the tri-peptide residues 1097-1099, contains the amino acid sequence Arg-Gly-Asp (RGD) is involved in binding to integrins on macrophages and ciliated cells (Ishibashi, Claus et al. 1994, Ishibashi, Relman et al. 2001). The RGD domain on FHA is involved in binding to the leukocyte response integrin/integrin-associated protein (LRI/IAP) complex and the integrin $\alpha_M\beta_2$/complement receptor 3 (CR3)/macrophage receptor 1 (Mac1) on macrophages and possibly other leukocytes, and enhances the attachment and invasion of *B. pertussis* (Saukkonen, Cabellos et al. 1991, Ishibashi, Claus et al. 1994). The RGD domain on FHA also appears to be involved in binding to the epithelial integrin, $\alpha_5\beta_1$, and enhancing bacterial invasion of human epithelial cells (Ishibashi, Relman et al. 2001). The RGD domain however does not appear to be crucial for colonization, as *B. pertussis* strains with an RAD mutation still maintain virulence similar to parental strains (Ishibashi, Relman et al. 2001, Julio, Inatsuka et al. 2009).

A third region close to the RGD sequence, the carbohydrate binding domain (CBD), is found within residues 1141-1279. The CBD binds to lactosylceramides, and *B. pertussis* strains with in-frame deletions of the CBD fail to adhere to macrophages and ciliated cells *in-vitro* (Prasad, Yin et al. 1993). Recently, a region within the C-terminus of the mature FHA (MCD), located ~500 amino acids at the end of the mature FHA has been implicated in *B. pertussis* adherence to epithelial cells and macrophages (Julio, Inatsuka et al. 2009). The MCD also contains neutralizing epitopes sufficient for protection (Knight, Huang et al. 2006).

FHA mediates *B. pertussis* adherence to epithelial cells and macrophages *in-vitro* (Urisu, Cowell et al. 1986, Ishibashi, Relman et al. 2001) and is also required for

colonization of the trachea *in-vivo* (Jacob-Dubuisson, Buisine et al. 1996, Julio, Inatsuka et al. 2009). In addition to mediating adherence, FHA also has important immunomodulatory functions. FHA binding to the LRI/IAP complex on macrophages via its RGD domain upregulates CR3 binding activity (Ishibashi, Claus et al. 1994), while the interaction of the FHA RGD with the VLA-5 integrin on epithelial cells, upregulates ICAM-1 expression (Ishibashi and Nishikawa 2002, Ishibashi and Nishikawa 2003). FHA has also been shown to inhibit $CD4^+$ T cell proliferation (Boschwitz, Batanghari et al. 1997) and induce apoptosis (Abramson, Kedem et al. 2001) or immunosuppressive effects (McGuirk and Mills 2000, McGuirk, McCann et al. 2002) on macrophages and dendritic cells. Due to its importance as an adhesin and immune-modulator, FHA is included in almost all currently licensed aP vaccines.

Anti-FHA antibodies are thought to be protective (He, Viljanen et al. 1994) by blocking bacterial adhesion and promoting bacterial clearance (Sato, Izumiya et al. 1981). To better understand protection and the rapid waning of immunity induced by aP vaccines, we evaluated the quality of individual antibody responses to FHA after an adult booster immunization. The DNA from single B cells responding to FHA isolated from the adult volunteers cloned into expression vectors were kindly provided by Excelimmune Inc. The single B cells were isolated from adult volunteers immunized with a booster aP vaccine and DNA sequences corresponding to antibodies responding to each antigen extracted. The antibody sequences were cloned into mammalian expression vectors, and the fully human antibodies subsequently purified from CHO-K1 cell lines. Of the greater than 50 unique anti-FHA antibodies identified, six were selected for further characterization. The selected purified antibodies bound FHA with high affinity, were diverse in the germline sequence families, but appeared to recognize an immunodominant epitope. We identified two neutralizing, antibodies, B6, and H6 in an *in-vitro*

hemagglutination assay. B6 also inhibited *B. pertussis* binding to CHO-K1 epithelial cells, while H6 potently inhibited FHA binding to the macrophage CR3 receptor. All the tested antibodies bound whole *B. pertussis* cells suggesting they may be substrates for complement mediated killing of *B. pertussis*. This study, the first to provide insight into the anti-FHA antibody repertoire after pertussis vaccination, also identifies useful antibodies for further elucidation of the FHA structure and function and provides promising candidates for passive immunotherapy for whooping cough infection.

2.2 MATERIALS AND METHODS

2.2.1 Antibody plasmid DNA

DNA plasmid vectors encoding antibody sequences corresponding to single B cells responding to FHA were kindly provided by Excelimmune, Inc. The B cells were isolated from two healthy adult volunteers on day 6 after receiving a booster vaccination with the acellular pertussis vaccine, either Adacel, (Sanofi Pasteur, Lyon, France), or Boostrix (GlaxoSmithKline Biologicals, Rixensart, Belgium and Novartis Vaccines and Diagnostics, GmbH & Co. KG, Marburg, Germany. Subjects were recruited as part of their routine medical care. All donors completed a uniform blood donor history questionnaire. An IRB approved consent form was obtained from each donor giving permission to collect their blood for research purposes.

The single B cells responding to native FHA were then isolated using established methodologies as described below (Coronella, Telleman et al. 2000). The heavy and light chain antibody sequences provided were in either a one or two plasmid vector system (Table 2.1). The one plasmid system contained both the heavy or light chain in a single

vector with a neomycin/kanamycin resistance cassette and other functional elements such as a ColE1 origin of replication, and a CMV promoter for high level protein expression in mammalian cells. The dual plasmid system was made up of two plasmids vectors, each containing either the heavy or light chain sequences with a neomycin/kanamycin resistance cassette and other functional elements such as a ColE1 origin of replication, and a CMV promoter.

Table 2.1: Plasmid vectors for expression of anti-FHA antibodies

Heavy chain antibody	**Light chain antibody**	**Plasmid Vector**	**Antibody ID used in this study**
42.11.D4.22G2.VH	42.11.D4.23G2.K	Single	D4
42.11.G2.22E4.VH	42.11.G2.23E4.K	Single	G2
42.12.A9.22G6.VH	42.12.A9.23G6.K	Single	A9
55.12.G10.34E2.VH	55.12.G10.32E4.K	Single	G10
55.12.H2.34A3.VH	55.12.H2.32A5.K	Single	H2
55.15.C12.34A5.VH	55.15.C12.32B7.K	Dual	C12
55.15.E10.34E5.VH	55.15.E10.32G7.K	Single	E10
55.16.H6.34D7.VH	55.16.H6.32A9.K	Dual	H6
55.18.D12.34F8.VH	55.18.D12.32G10.K	Single	D12
55.19.B6.34A9.VH	55.19.B6.32A11.K	Dual	B6
55.19.C2.34G9.VH	55.19.C2.32E11.K	Dual	C2
55.22.E7.34C11.VH	55.22.E7.33A1.K	Single	E7
57.52.B5.1A1.VH	57.52.B5.1A1.K	Single	B5

2.2.2 Antibody screening

2.2.2.1 Isolation of plasma cells, antibody cloning

Isolation of plasma cells and antibody cloning were perfomed by excelimmune inc as described. Plasma B cells were enriched from PBMCs using a plasma cell isolation kit (Miltenyi-Biotech), labeled with antibodies (BioLegend) to CD19, CD38 and Kappa or Lambda light chains and single-cell sorted by FACS (BD FACSJazz). The single sorted B cells were then distributed into 96-well PCR plates containing reverse transcriptase buffer, RNase inhibitor (NEB) and primers for the heavy chain J region and both light chains (Kappa and Lambda). Reverse transcription was performed using the Superscript III reverse transcriptase (Invitrogen). The cDNA was divided and separate semi-nested PCRs were performed to produce VH (VDJ) and light chain (V_LC_L) antibody amplicons. For antibody expression, an IgG1 constant region was grafted onto the cloned VDJ sequences. Cognate pairing of antibodies was maintained and antibodies were transiently expressed under EF1alpha promoters in HEK293 mammalian cells (ATCC) in a 96 well format. Transfection was achieved by polyethylenimine (Polysciences) using a standard transfection protocol. From the microplates, supernatants were removed and screened for antigen-antibody interactions in custom Luminex™ assays.

2.2.2.2 Antibody-antigen screening

Luminex™ MagPlex® microspheres were coated with antigens using an xMAP Antibody Coupling Kit according to the manufacturer's instructions (Luminex Corporation). In brief, the desired number of microspheres were placed in a microtube, washed and activated for coupling using the activation buffer. Sulfo-NHS was added, followed by EDC. The microspheres were incubated for 20 minutes at room temperature

on a rotator in the dark. The microspheres were washed before coating conditions were added and microspheres were then incubated for 2 hours at room temperature while rotating. The microspheres were then washed and placed in storage buffer (1% PBS, 1% BSA and 0.05% sodium azide). For all washing steps, a DynaMag™ Magnet (Invitrogen) was used. Each Luminex™ MagPlex® microsphere received a single antigen for coupling. For multiplexing, antigens were coupled to Luminex™ MagPlex® microspheres with unique spectral signatures. Antigens were purchased from List Labs and included Fimbriae 2/3, Pertactin, Pertussis Toxin (whole), Pertussis Toxin Protomer A and Pertussis Toxin Protomer B. Coupled beads were then used to perform the Antigen-Antibody assays. The assay was run according to manufacturer's instructions (Luminex). Briefly, coupled microspheres were placed in wells of 96 well microplates and incubated with supernatants derived from transfected HEK293 cells for 30 minutes. The microplates were then washed with assay buffer (1X PBS pH 7.4 with 1% BSA) and a PE conjugated goat anti-FC antibody (Jackson ImmunoResearch) was added and incubated for another 30 minutes. After incubation, the microplates were washed and resuspended in assay buffer. Microplates were then read in a Luminex FlexMap 3D. Reactivities were scored based on values of fold over background.

2.2.3 Bacterial strains and growth conditions

The *Bordetella* strains used were *B. pertussis* Tahoma I (ATCC# BAA-587), *B. pertussis* ΔFHA, a streptomycin resistant *B. pertussis* Tahoma I derivative lacking the structural gene for FHA, D420, a recent clinical isolate of *B. pertussis pertussis* (from Eric Harvill, Penn State University), *B. bronchiseptica* strain RB50 (ATCC# BAA-588), and *B. parapertussis* strain 12822 (ATCC # BAA-589). The *Bordetella* strains were

grown on Bordet-Gengou agar supplemented with 15% sheep blood (BD Biosciences). Liquid cultures were grown from colonies on the plates which showed hemolysis due to production of the adenylate cyclase toxin (ACT), and indicative of the expression of the virulence genes. Cultures were grown in Stainer Scholte medium (Stainer and Scholte 1970) with 1g/L of hepatikis (2,6-O-dimethyl-β-cyclodextrin) added for the production of FHA. Plasmid vectors for transient expression of antibodies in CHO-K1 cells were purified from the *E. coli* strain DH5α. Transformation of the antibody plasmid vectors into *E. coli* was done by electroporation. Cells successfully transformed were selected on 2XYT plates containing ampicillin.

2.2.4 Antigens and antibodies

2.2.4.1 FHA

Mature FHA produced by *B. pertussis* strain 165 was purchased from List Biological Laboratories, Inc. (Campbell, CA), or obtained through BEI Resources, NIAID, NIH. FHA from *B. pertussis* TahomaI strain, D420 strain (recent clinical isolate) or from *B. bronchiseptica* were purified from the culture supernatant using heparin affinity chromatography as described (Menozzi, Gantiez et al. 1991). FHA concentrations were determined using a BCA assay using BSA as a standard. Purified FHA was stored in 50 mM Tris, pH 8, 500mM NaCl at -20^{o}C. The FHA fragments 861-1655 (pMAL 83) and 1655 (pMAL 85) were generously provided by Dr. Camille Locht. Construction and expression of pMAL 83 and 85 have been previously described (Leininger, Bowen et al. 1997). FHA fragments 1-435 and 1156-1295 the corresponding coding regions were amplified from *B. pertussis* TahomaI cells and cloned into the pMALc-5x vector (NEB) between the NdeI and HindIII restriction sites. The FHA 1-435 peptide fragment was

amplified using the forward primer 5'- aggaaccacatatgaacacgaacctgtacaggctggtctt - 3' and reverse primer 5'- aagaactacagcaagcttcgacagcttgtccgccttgagg - 3'. The 1156-1295 fragment was amplified using the forward primer 5'- aatcggacatatggctgccaaaggggacaagggcaagcc and reverse primer 5' - gctaactaagcttgccgtccgagccgaccaggaa - 3'. Restriction sites are underlined. The pcr reactions contained 5% DMSO to enhance fragment amplification from the GC-rich *B. pertussis* genomic DNA. The amplified fragments were pcr purified (Qiagen purification kit), digested with the NdeI and HindIII restriction enzymes and ligated to the pMALc-5x vector, similarly digested and gel purified (Qiagen purification kit).

2.2.4.2 Antibodies

The antibodies were transiently expressed on a laboratory scale in adherent CHO-K1 cells from mammalian plasmid DNA containing cDNA sequences of the antibody genes. The plasmid vectors were purified using a midiprep plasmid preparation kit (Qiagen). Concentration and quality of plasmid DNA were assessed by A_{260} measurements on a nanodrop 2000 (ThermoScientific). Three confluent T-150 flasks containing adherent CHO-K1 cells were transfected with 150 µg of the plasmid DNA using Lipofectamine 2000 reagent (Life Technologies). After a total of three changes of complete antibody media media (DMEM media (Life Technologies) with 10% Ultra Low IgG FBS (Life Technologies) and 1X Penicillin/Streptomycin (Life Technologies)), the saved culture supernatants were pooled and concentrated by overnight precipitation with 50% saturated ammonium sulfate. Antibodies were purified by protein A affinity chromatography as described after dialysis into sodium phosphate buffer pH 7.2, 150mM NaCl (Sutherland and Maynard 2009). Protein concentrations were determined by

absorbance at 280 nm on a nanodrop 2000 (Thermo Scientific) using an extinction coefficient of 1.4. Antibody purity was verified with 12% SDS-PAGE gels stained with GelCode Blue (Pierce). Human intravenous pertussis immunoglobulin (P-IGIV) (Bruss, Malley et al. 1999) was obtained from the Massachusetts Public Health Biologic Laboratory. Polyclonal mouse anti-FHA antibody was obtained from NIBSC. HRP and fluorescent conjugated secondary antibodies were obtained from Southern Biotech and Thermoscientific respectively. The IgG3 anti-*B. pertussis* lipo-oligosaccharide (LOS) antibody (D26E) was obtained from Thermoscientific.

2.2.5 Phylogenetic analyses

The V_H and V_K sequences of the isolated antibodies were mapped to the closest human germline VDJand VJ sequences respectively using NCBI/IgBLAST (Ye, Ma et al. 2013). The human germline sequences from NCBI/IgBLAST are obtained from the IMGT database (Lefranc, Giudicelli et al. 2005). To obtain the closest human germline sequence, we adapted the methodology of (Jayaram, Bhowmick et al. 2012) by selecting the sequence hit with the highest score. The score is determined after calculating the final points after adding points for each match and subtracting points for each mismatch (Ye, Ma et al. 2013). The germline sequences are presented in the form IGHV1-46*03. The first two variables IG represent an immunoglobulin. The next variable, H represents the genetic locus, (H, K or L). The next, V represents the type of gene segment (V D or J), the number after the gene segment, 1, represents the gene family. The subsequent number, 46, represents the individual gene, and the last two numbers, 03, correspond to the allele. The gene segment pairings (VDJ or VJ) for each genetic locus (H or K) were compared and the frequencies of pairing for the putative VDJ or VJ presented in a heat

map. The individual gene families and alleles were also analyzed by indicating the frequencies in a pie chart. Since the antibody isolation maintained the original pairing of the heavy and light chains, the full V_H or Vκ sequences were then aligned using Phylogeny.fr (Dereeper, Guignon et al. 2008) to generate phylogenetic trees.

2.2.6 Thermal stability analysis

Thermal stability of the purified antibodies was analyzed in an applied Biosystems ViiA 7s instrument. Three replicates of 100 µg/ml solutions of the antibodies were prepared in PBS using the protein thermal shift dye (Life Technologies) according to the manufacturer's protocol. The antibodies were heated from 25°C to 90°C with a scanning rate of 1°C/min increments. To determine the melting temperature (Tm), the data was fit to a sigmoidal four parameter (4PL) non-linear regression model using Graph pad prism 5.

2.2.7 Antigen specific responses

2.2.7.1 Indirect ELISA assays

High binding ELISA plates (Costar) were incubated overnight at 4°C with FHA at a concentration of 1 µg/ml in PBS, pH 7.4. All volumes were 50 µl/well unless otherwise stated. The plates were washed after each incubation step with wash buffer (PBS, pH 7.4 + 0.05% Tween-20) to remove any unbound proteins. Serially diluted antibodies prepared in blocking buffer, were added to the plates after blocking with 5% milk in wash buffer. Subsequently, HRP-conjugated anti-human Fc antibodies (Southern Biotech) diluted 1:1000 were used to detect bound antibodies. The plates were developed with TMB

substrate (ThermoScientific) according to the manufacturer's instructions and quenched with 1N HCl. Absorbances were read at 450 nm in a SpectraMax M5 instrument (Molecular Devices) using the SoftMax Pro v5 software. The relative binding affinities of antibodies to respective coated antigens were determined by estimation of the concentration of each antibody required to achieve 50% maximal binding at saturation (EC_{50}). The EC_{50} values were determined from fitting the binding curves to the 4 parameter logistic (4PL) non-linear curve model using the GraphPad Prism 5 software.

2.2.7.2 Competition ELISA assays

The ability of the antibodies to compete with a biotinylated competitor antibody for binding to overlapping was evaluated by a competition ELISA. The competitor antibody was biotinylated with 20 molar excess EZ-link Sulfo-NHS-LC-Biotin following the manufacturer's instructions. Excess biotin was removed by dialysis against PBS overnight at 4^{o}C. FHA was coated at 1 µg/ml overnight at 4^{o}C. Serially diluted antibodies incubated with a constant concentration of a biotinylated competitor antibody were added to the coat of FHA. The biotinylated antibody was detected with streptavidin-HRP, and the reaction developed using TMB substrate as above. The concentration of antibody required to achieve 50% inhibition (IC_{50}) was determined from fitting the binding curves to the 4 parameter logistic (4PL) curve model.

2.2.7.3 Western blot

To detect antibody epitopes on FHA, 5 µg of full length FHA from *B. pertussis* TahomaI was electrophoresed in a 7.5% gel and then transferred to a PVDF membrane at 20V, 4^{o}C overnight. The membranes were blocked with PBS + 5 % milk, washed with

PBS-tween, incubated with 25 µg of antibody, and subsequently with HRP-conjugated secondary antibodies. Binding to FHA was detected using a colorimetric TMB substrate kit (Fisher scientific).

2.2.7.4 Surface Plasmon resonance

A BIAcore 3000 instrument (GE Healthcare) was used to quantitatively measure the equilibrium binding affinity of selected antibodies from the anti-FHA repertoires. The antibodies were covalently coupled to CM5 chips (GE healthcare) using standard amine coupling with a 50/50 solution of 1-ethyl-3-[3-(dimethylamino) propyl] carbodiimide hydrochloride and N-hydroxysuccinimide, in 100 mM sodium acetate buffer (pH 5.0) to achieve ~1000 - 1600 response units. The reaction was then quenched with 1.0 M ethanolamine-HCl, pH 8.5. Samples of antigen at different concentrations in running buffer (HBS, pH 7.4 0.005% Tween) were run in duplicate over the chip at 30 µl/min, with injection and dissociation times of 3 and 5 min respectively. A 30 s injection of 3.6 M magnesium chloride 1 mM Glycine, pH 2 at 30 µL/min was used to regenerate the surface between antigen injections. The baseline was corrected by subtracting simultaneous runs over a second flow cell which had been coupled with an irrelevant human antibody. The on (k_{on}) and off (k_{off}) rates were estimated by globally fitting five different concentrations of antigen to the 1:1 Langmuir binding equation in the BIAevaluation v3.0 (Pharmacia Biosensor AB) software. The dissociation constant, K_D, was determined from the ratio of the k_{on} and k_{off}.

2.2.7.5 Expression of antibody epitopes on whole B. pertussis

The expression of anti-FHA epitopes on live *B. pertussis* were assessed as follows. Bacteria were harvested from cultures grown on Bordet Gengou agar, resuspended in PBS with 5% glycerol (PBSG), and the OD_{600}/mL adjusted to 1. The cells were washed with PBS and then incubated with 1 µg of each antibody in 100 µL at room temperature. Bound antibodies were detected after washing with a 1:500 dilution of goat anti human Fc Alexa Fluor 647 conjugate or $F(ab')_2$ goat anti mouse Fc Alexa Fluor 488 (Jackson Immunoresearch). 10 000 events per treatment were collected on a BD SLRII Fortessa flow cytometer. The cell associated fluorescence for a gated population of the cells was determined using the FlowJo_V10 software. An anti-*Bordetella* LOS mouse IgG3 antibody (D26E) (Thermoscientific) was used as a positive control. To detect D26E binding to *B. pertussis*, the antibody was incubated with the *B. pertussis* cells in PBS on ice. Bound antibody was detected after washing with a 1:50 dilution of goat anti mouse Fc Alexa Fluor 488 conjugate (Jackson Immunoresearch).

2.2.8 *In-vitro* neutralization of FHA binding activities

2.2.8.1 Inhibition of FHA mediated hemagglutination

The ability of the anti-FHA antibodies to inhibit the FHA meidated hemagglutination of red blood cells (RBC) was analyzed as follows. Serially diluted antibodies were first prepared in a round bottom micro-titer plate. An amount of 0.5 µg of FHA was then added to each well and the mixture incubated at 25°C for 30 minutes. Sheep RBC was added to a final concentration of 0.5% and the total volume of the reaction topped up to 100 µl with PBS. The preparation was incubated at 25°C for approximately 3 hours. The lowest amount of antibody that could inhibit

hemagglutination caused by FHA was taken as the neutralizing dose. Up to 30 molar excess antibodies were tested for FHA neutralization.

2.2.8.2 Inhibition of B. pertussis binding to CHO-K1 cells

FHA binds epithelial cells or macrophages to mediate *B. pertussis* adherence to the host. We tested the ability of isolated monoclonal antibodies to inhibit B. *pertussis* binding to CHO-K1 cells using a modified form of the adherence assay (Hazenbos, van den Berg et al. 1994, Cotter, Yuk et al. 1998). *B. pertussis* cells were labeled with FITC (Thermoscientific) as previously described (Hazenbos, van den Berg et al. 1994) with minor modifications. 10^8 cfu/mL of bacteria were incubated in a 100 µL solution of 1 mg/ml of FITC in 100 mM carbonate buffer, pH 9.2. The labeling was carried out at room temperature for 30 minutes, after which the cells were washed three times with PBS to remove excess FITC. To assay for inhibition of adherence of Bp-FITC to CHO-K1 cells, 1 x 10^6 CHO-K1 cells were first allowed to adhere in 12-well plates for 1 hour at 37^oC, 5% CO_2. The CHO cells were maintained in media without antibiotics (DMEM buffer with 10% FBS). The CHO cells were then incubated with the Bp-FITC in the presence of 25 µg/ml antibodies. The plates were spun down to promote contact between the bacteria and CHO cells, and incubated for another hour at 37^oC. Unbound bacteria were washed with PBS, after which the adherent cells were scraped off and resuspended in 500 µL FACS buffer (PBS + 2% FBS). Bound Bp-FITC cells were analyzed by flow cytometry using a BD SLRII Fortessa instrument. The analysis did not differentiate between bound and internalized *B. pertussis*.

2.2.8.3 Inhibition of FHA binding to the macrophage receptor CR3

The ability of the antibodies to inhibit FHA binding to the macrophage complement receptor 3 (CR3, CD11b/CD18, $\alpha_M\beta_2$ integrin or Mac1) was evaluated in a competition ELISA. FHA binding to CR3 was first confirmed using an indirect capture ELISA. To assess inhibition of FHA binding to CR3, high binding ELISA plates (Costar) were coated overnight with CR3 at 1 μg/ml in PBS. The plates were then blocked with blocking buffer (2% PBS-BSA). The antibodies were then serially diluted, and incubated for 1 hour at room temperature in the presence of a constant concentration of 247 nM FHA. The antibody-FHA mixtures were then added to the CR3 coat. Bound FHA was detected with a polyclonal mouse anti-FHA mixture and a 1:1000 dilution of rabbit α mouse HRP-conjugated antibodies (Invitrogen). The plates were developed with TMB substrate and the absorbance at 450nm recorded. The absorbance were normalized to the highest in each data set and plotted using the GraphPad Prism 5 software.

2.2.8.4 Complement mediated bactericidal activity

The ability of the anti-FHA antibodies to induce complement mediated bactericidal activity was assessed. *B. pertussis* were grown on Bordet Gengou plates for 3 days at 37^{o}C. 3 x 10^5 bacteria were then incubated with antibody in 20 μl for 30 minutes at 37^{o}C in a 96-well round bottom plate. Antibody depleted complement was then added and the mixture incubated for another hour 37^{o}C. PBS with 10 mM EDTA was added to stop the complement reaction, and serial dilutions plated on Bordet Gengou plates. The plates were incubated at 37^{o}C and colony forming units determined after 3 days of growth to assess bactericidal activity.

2.3 RESULTS

2.3.1 Vaccination with aP induces high numbers of B cells responding to native FHA

From the FHA specific responses of the individual B cells isolated, greater than 50 unique anti-FHA B cells were identified, compared to only 5 unique anti-PTx B cells. The higher number of B cells responding to FHA is consistent with previous reports of a higher magnitude of serum responses to FHA in comparison with variability in the anti-PTx response (Berbers, van de Wetering et al. 2013). FHA is also normally secreted at much higher levels than PTx by *B. pertussis*. The high levels of secreted FHA may be important for promoting adherence of phase variants which may arise *in vivo*, and for neutralizing bactericidal antibodies when attached to the bacterial cell surface (Mooi 1988). High FHA secretion by *B. pertussis* may also allow FHA to act as a decoy antigen by presenting epitopes which allow binding by anti-FHA antibodies without effectively neutralizing FHA activities.

2.3.2 B cell responses to FHA after aP booster vaccination arise from discrete germline families

There was significant similarity in the sequences forming the framework regions of the 13 antibody sequences analyzed. The sequences of the antibodies, aligned using ClustalOmega (Sievers, Wilm et al. 2011), indicated the framework regions of the antibody sequences exhibited the most similarity, with the biggest differences in the CDRs (not shown). From an NCBI/IgBLAST search of the isolated sequences of the variable heavy and light chains; potential germline sequences of the isolated antibodies were identified by selecting germline sequences in the IMGT database with the highest score. From the identified parental germline sequences, the anti-FHA antibodies had discrete parental clones, with a few antibodies sharing the same parental germline

sequences. Within both the variable light and heavy chain regions, the IgH and IgK V3 gene segments were predominant, while the D3 gene segment of the heavy chain was the most utilized. The J5 gene segment occurred more in the heavy chain, while J1 and J2 were used equally in the light chain (Figure 2.1). Initial studies of VDJ gene segment usage revealed pairing preferences exist among gene segments (Reth, Jackson et al. 1986). Subsequently, Volpe and Kepler (Volpe and Kepler 2008) provided statistical analysis of preferred D-J gene segment pair preferences after analyzing almost 6500 human immunoglobulin heavy chain genes. They showed that 5'D segments showed increased frequency of pairing with the 3'- most J segments. Furthermore, some 3' D segments paired more frequently with the closest 5' J segments (1 to 4). From their observations, they concluded multiple D-J rearrangements occur prior to the formation of a complete VDJ gene. Although we analyzed only 13 antibodies, we observed a few antibodies also showed pairing between the 5' D segment and the 3' J segment or vice versa. The small data set of antibodies characterized however limits the extent of analysis of pairing frequencies.

We also analyzed the sequences at the level of the genes and alleles (Figure 2.2). Within the heavy chain, the 43D*03 gene and allele combination in the V segment was the most represented. Also, the 19*01 and 26*01 in the D segment, and the 5*02 in the J segment were most utilized. Within the light chain, the 20*01 and 1*01 combinations were most utilized in the V and J gene segments respectively. The number of mutations in the amino acids of the isolated sequences compared to the putative parental germline sequences ranged from 5 to 34 within the V regions. The D and J regions on the other hand, had few changes with 0 to 4 mutations compared to the putative germline sequences (Tables 2.2 and 2.3). Since the antibody isolation maintained the original pairing of the heavy and light chains, the full VH or Vκ sequences were then aligned

using Phylogeny.fr. The phylogenetic tree generated after combining the full V_H and V_L sequences also suggested the antibodies descended from discrete germline families (Figure 2.3). This study is the first to provide insight into the anti-FHA antibody repertoire after pertussis vaccination. The sequence information obtained during the formation of anti-FHA antibodies in the immune repertoire can potentially be used to guide efforts to generate stable and/or humanized antibodies for *in-vivo* therapeutic use.

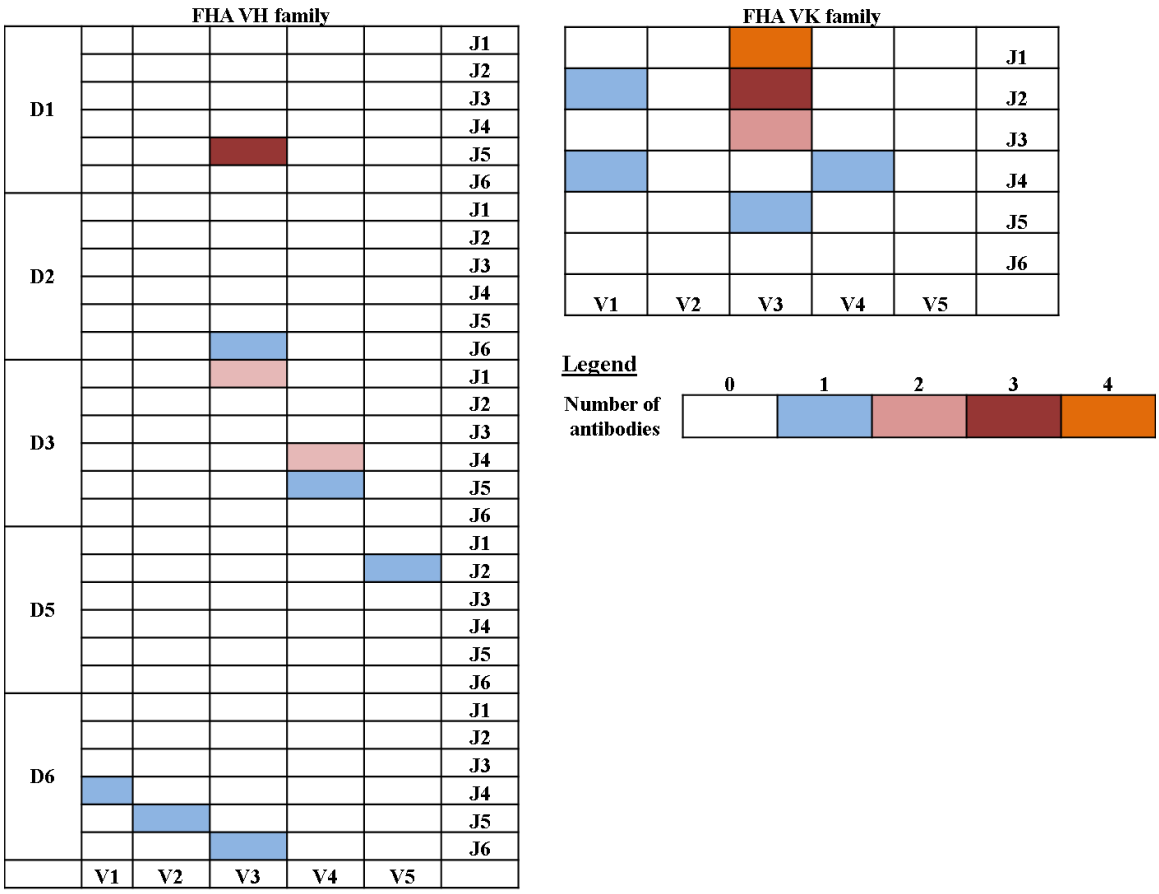


Figure 2.1: VDJ gene family usage of the anti-FHA heavy and light chains.

Each panel represents the number of unique VDJ patterns existing in the heavy and light chains of the thirteen isolated anti-FHA antibody sequences. The color legend shows the number of antibodies with the corresponding VDJ genes. The white space represents absent VDJ recombination types in the repertoire.

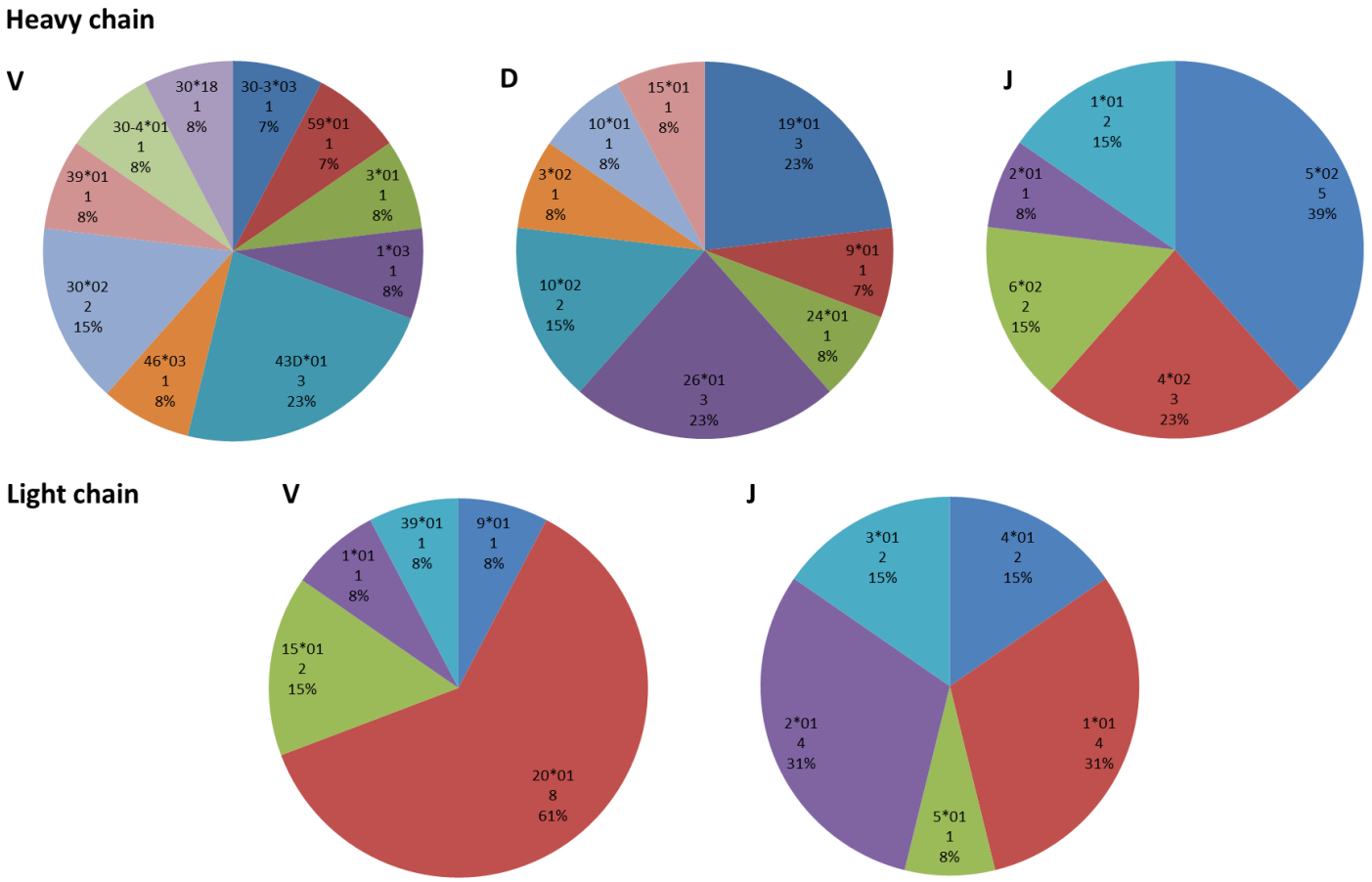


Figure 2.2: Gene and allele family usage of the isolated anti-FHA antibodies.

Genes and alleles are shown in the pie chart in the a*b format. The number and percentage of antibodies represented is indicated below each gene and allele used.

Table 2.2: VDJ germline family usage of the isolated anti-FHA antibody light chains.

Antibody	Chain	Germline V	Germline D	Germline J	Mismatches
D4.K	Light	IGKV1-9*01	-	IGKJ4*01	12,0
A9.K	Light	IGKV3-20*01	-	IGKJ1*01	11,1
G10.K	Light	IGKV3-20*01	-	IGKJ1*01	9,0
H2.K	Light	IGKV3-20*01	-	IGKJ5*01	9,1
C12.K	Light	IGKV3-20*01	-	IGKJ2*01	9,0
E10.K	Light	IGKV3-20*01	-	IGKJ1*01	12,1
H6.K	Light	IGKV3-15*01	-	IGKJ3*01	21,1
D12.K	Light	IGKV3-20*01	-	IGKJ2*01	9,0
B6.K	Light	IGKV3-20*01	-	IGKJ2*01	12,0
C2.K	Light	IGKV4-1*01	-	IGKJ4*01	13,0
E7.K	Light	IGKV3-15*01	-	IGKJ3*01	23,1
B5.K	Light	IGKV1-39*01,IGKV1D-39*01	-	IGKJ2*02	34,2
G2.K	Light	IGKV3-20*01	-	IGKJ1*01	5,0

Table 2.3: VDJ germline family usage of the isolated anti-FHA antibody heavy chains.

Antibody	Chain	Germline V	Germline D	Germline J	Mismatches
D4.VH	Heavy	IGHV3-30-3*03,IGHV3-30*04	IGHD6-19*01	IGHJ5*02	18,1,1
A9.VH	Heavy	IGHV4-59*01	IGHD3-9*01	IGHJ4*02	20,0,0
G10.VH	Heavy	IGHV3-30-3*01	IGHD6-19*01	IGHJ6*02	18,0,1
H2.VH	Heavy	IGHV5-10-1*03	IGHD5-24*01	IGHJ2*01	18,0,1
C12.VH	Heavy	IGHV3-43D*01	IGHD1-26*01	IGHJ5*02	23,0,0
E10.VH	Heavy	IGHV1-46*03	IGHD6-19*01	IGHJ4*02	7,0,0
H6.VH	Heavy	IGHV3-30*02	IGHD3-10*02	IGHJ1*01	25,0,0
D12.VH	Heavy	IGHV3-43D*01	IGHD1-26*01	IGHJ5*02	23,0,0
B6.VH	Heavy	IGHV3-43D*01	IGHD1-26*01	IGHJ5*02	24,0,0
C2.VH	Heavy	IGHV4-39*01	IGHD3-3*02	IGHJ5*02	21,0,0
E7.VH	Heavy	IGHV3-30*02	IGHD3-10*02	IGHJ1*01	25,0,0
B5.VH	Heavy	IGHV4-30-4*01	IGHD3-10*01	IGHJ4*02	24,0,1
G2.VH	Heavy	IGHV3-30*18	IGHD2-15*01	IGHJ6*02	21,0,4

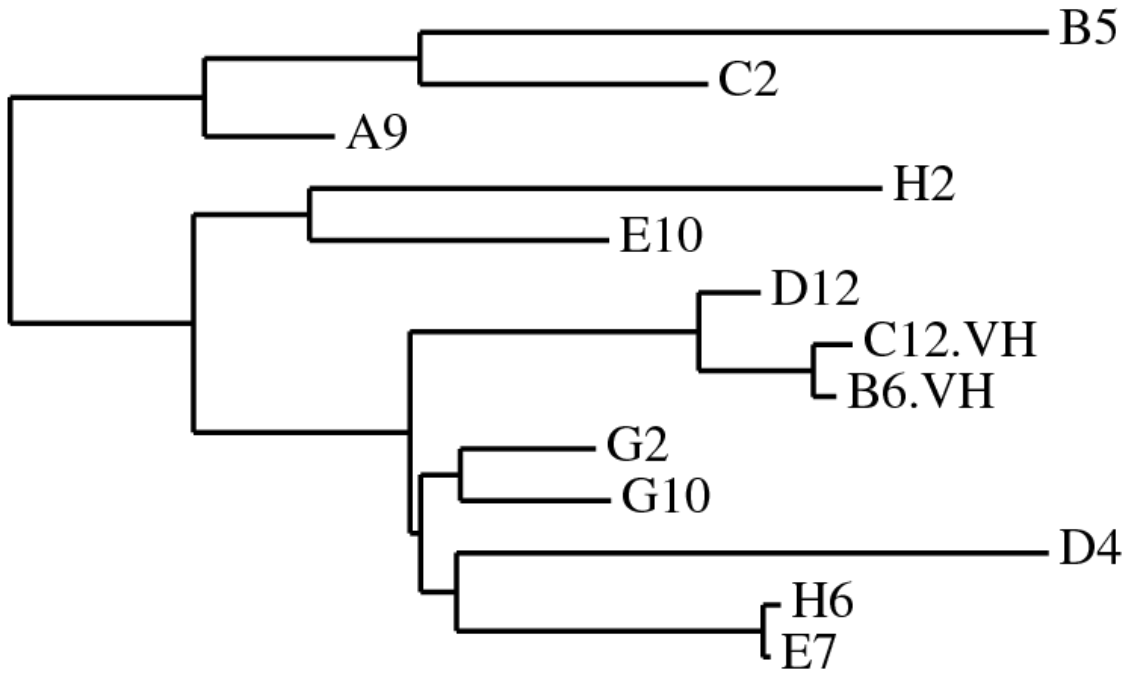


Figure 2.3: Phylogenetic tree of isolated anti-FHA antibodies.

The phylogenetic tree was constructed from the VH and Vk gene sequences of the isolated anti-FHA antibodies using phylogeny.fr. The tree shows the diversity of germline families of the antibodies. The length of a branch is proportional to the number of mutations in the antibody compared to a putative germline.

2.3.3 Antibody expression and stability

To select anti-FHA antibodies for further characterization, the thirteen anti-FHA antibodies were screened for specific activity, estimated as the ratio of FHA binding to expression levels (Fig 2.4 A). Six isolates with varying FHA specific activity, which are representative of different phylogenetic families were subsequently purified for further characterization (Fig 2.4 B). The different antibodies exhibited low expression levels in 6 well culture plates, ranging from 0.01 to 0.37 μg/mL (Table 2.4, column 2). Typical yields for therapeutic antibodies are in the range of g/L after optimization (Wurm 2004). The low yields of these antibodies on the laboratory scale may be due to low transfection efficiency in the 6-well plate format, or to inherently poor expressing strains due to the DNA sequence. Although D12, G10, H2 and H6 exhibited the lowest expression levels from the 6-well plate format, their expression levels did not correlate with expression levels from T-150 flasks. Yields obtained for these antibodies from T-150 flasks were approximately 2 μg/mL (not shown). From a therapeutic standpoint, relevant low expressing antibodies may need further engineering for increased expression levels. Further studies on improving yields can be done by testing different transfection conditions and/or expressing the antibodies in suspension cultures in bioreactors. Antibodies selected by phage display appear to express better, and may indicate a methodological bias for selection of inherently better folding antibodies. Yields of relevant antibodies from this study could therefore be improved significantly through *in-vitro* sequence engineering to select for better expressing variants (Mason, Sweeney et al. 2012).

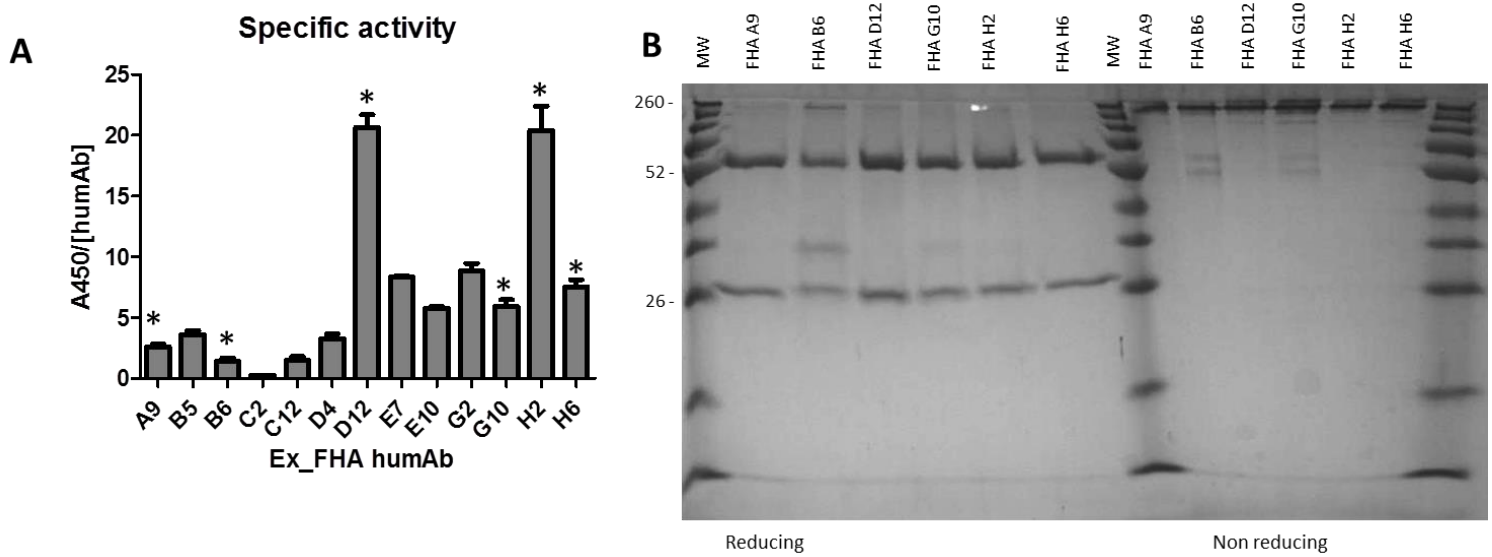


Figure 2.4: Selection and purification of anti-FHA antibodies.

A) Specific activity of anti-FHA antibodies. Binding of antibodies to FHA from crude culture supernatant after transient CHO cell expression was monitored using an FHA capture ELISA. The antibody concentrations in the crude culture supernatant were determined using an Fc capture ELISA with irrelevant monoclonal antibody as a standard to estimate the antibody concentrations. The specific activity was determined from the ratio of FHA binding (A450nm) to the estimated concentration of antibody. Antibodies marked with * were selected for further characterization. **B)** Gel electrophoresis of selected purified antibodies in reduced and non-reduced conditions. Molecular weights (MW) in kDa of the standard are indicated. The antibody heavy and light chains are at the expected sizes of ~50 and 25 kDa respectively in reducing conditions. In non-reducing conditions, the antibody sizes are ~150 kDa.

To examine the thermal stability of the selected anti-FHA antibodies, the antibodies were heated fro-m 25°C to 90°C in the presence of the protein thermal shift dye (Life Technologies). The melting temperature (T_m) was determined from fitting the fluorescence data normalized to the maximum and minimum fluorescence. The six anti-FHA antibodies also exhibited diverse thermal stability profiles (Figure 2.5) with melting temperatures ranging from 64.45 ± 0.04 to 74.38 ± 0.06 (Table 2.4). During development of antibodies for therapeutic use, antibodies with superior biophysical properties such as high thermostability are selected Relevant antibodies identified in this study with low stability can be engineered for increased thermostability.

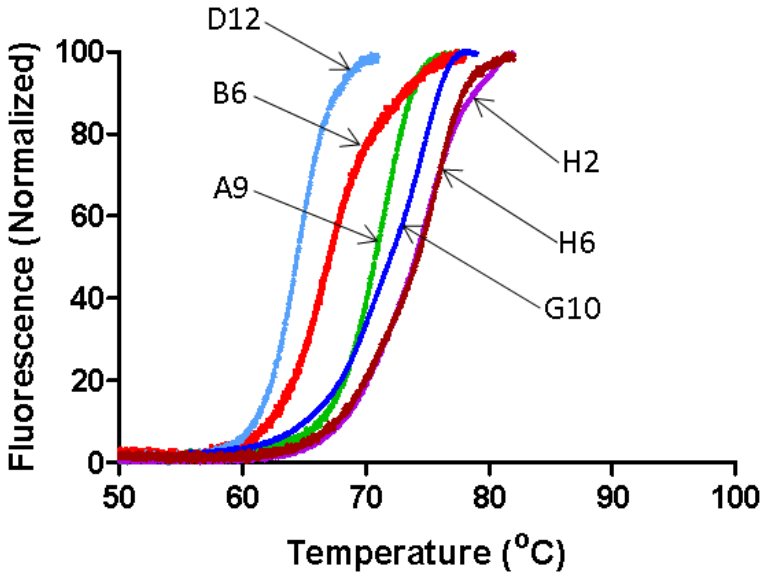


Figure 2.5: Melting temperature profile of selected purified anti-FHA antibodies.

The anti-FHA antibodies, each at a concentration of 100 µg/ml, were heated up to 90°C in PBS with the protein thermal shift dye. The T_m was determined from fitting the data to a sigmoidal four parameter logistic (4PL) non-linear regression model after normalizing the data to the maximum and minimum fluorescence. Data after the maximum fluorescence are not shown.

Table 2.4: Expression levels and stability of anti-FHA antibodies

Antibody	**Expression level (µg/ml)**	**Melting temperature, T_m (^oC)**
A9	0.14 ± 0.005	70.89 ± 0.02
B6	0.37 ± 0.05	67.36 ± 0.03
D12	0.02 ± 0.01	64.45 ± 0.04
G10	0.06 ± 0.01	73.11 ± 0.05
H2	0.03 ± 0.002	74.19 ± 0.04
H6	0.03 ± 0.0004	74.38 ± 0.06

2.3.4 Purification of FHA from *Bordetella sp.*

High yields of up to 80 mg/L have been reported for FHA purified from different strains of *B. pertussis* using various chromatographic techniques (Sato, Cowell et al. 1983, Wong and Skelton 1989, Ozcengiz, Kilinc et al. 2004). The interaction of *B. pertussis* FHA with the glycoconjugate heparin is one of the techniques exploited for its purification. Using heparin affinity chromatography as described (Menozzi, Gantiez et al. 1991), FHA from two strains of *B. pertussis* (the recent clinical isolate D420 and the classical strain TahomaI) and *B. bronchiseptica* strain RB50 were purified. The FHA from *B. pertussis* and *B. bronchiseptica* were electrophoretically similar with *B. bronchiseptica* FHA running slightly larger due to its slightly larger nucleotide sequence

as previously reported (Jacob-Dubuisson, Kehoe et al. 2000, Inatsuka, Julio et al. 2005). The yields of FHA in this study from the heparin affinity were approximately 80-fold lower than reported using ionic or hydrophobic interactions (Sato, Cowell et al. 1983, Wong and Skelton 1989, Ozcengiz, Kilinc et al. 2004) (Table 2.5). FHA from *B. parapertussis* could also not be purified by heparin affinity. The apparent lack of affinity of *B. parapertussis* FHA with heparin is consistent with the inability of heparin to inhibit *B. parapertussis* binding to epithelial cells, in contrast to *B. pertussis* (van den Akker 1998).

Table 2.5: Yields of FHA from *Bordetella sp.* using heparin affinity chromatography

***Bordetella* strain**	**Estimated FHA yields (mg/L)**
B. pertussis strain D420 (recent clinical isolate)	4.32 ± 3.09
B. pertussis strain TahomaI	2.84 ± 1.59
B. bronchiseptica strain RB50	0.73 ± 0.01
B. parapertussis strain 12822	ND

2.3.5 *B. pertussis* anti-FHA antibodies after aP booster immunization bind FHA with high affinity

To assess the binding of the antibodies to the respective antigens, indirect ELISAs were used to assess the apparent binding affinities, based on the concentration of antibody resulting in 50% of the maximum signal also called the 50% effective concentration (EC_{50}). The anti-FHA antibodies exhibited nM binding affinity to purified FHA from different strains of *B. pertussis* (Table 2.6, columns 2-4). There were however differences in the estimated affinities, with H6 showing the lowest affinity for FHA. Surface plasmon resonance (SPR), the industry standard for determination of binding affinities was then used to determine the on (k_{on}) and off (k_{off}) rates of the B6 and H6 anti-FHA antibodies (Figure 2.6). The affinities determined from antibody binding to *B. pertussis* strain D420 FHA, showed B6 had a K_d of 0.19 ± 0.04 nM, approximately six-fold higher affinity than H6, with a K_d of 1.27 ± 0.05 (Table 2.6, column 4). The estimated affinities are both consistent with expected affinities in the range of 10-0.1 nM for antibodies in the circulating immune repertoire (Foote and Eisen 1995).

Other *Bordetella* species such as *B. bronchiseptica* and *B. parapertussis* also cause whooping cough, although *B. parapertussis* is better able to successfully infect and cause disease in immunocompromised humans (Watanabe and Nagai 2004). FHA is one of the common virulence factors produced by the *Bordetella* strains, in addition to pertactin, adenylate cyclase toxin, tracheal cytotoxin and dermonecrotic toxin (Mattoo, Foreman-Wykert et al. 2001). The FHA proteins produced by *B. bronchiseptica* strain RB50 and *B. parapertussis* strain 12822, are very similar to that of *B. pertussis* strain TahomaI, with internal variations in sequence (Kloos, Mohapatra et al. 1981, Parkhill, Sebaihia et al. 2003) and are important for adherence and invasion of a variety of cells

lines *in-vitro* (Ewanowich, Sherburne et al. 1989, van den Akker 1998, Ishibashi, Relman et al. 2001, Julio, Inatsuka et al. 2009).

The apparent binding affinities (EC_{50}) were compared for the six anti-FHA antibodies to purified FHA from two strains of *B. pertussis* (TahomaI – laboratory *B. pertussis* strain; D420 – recent *B. pertussis* clinical isolate), and one strain of *B. bronchiseptica* (strain RB50). The *B. pertussis* anti-FHA antibodies similarly bound FHA from *B. bronchiseptica* with high nM affinity (Figure 2.7, Table 2.6, column 5). This agrees with reports that anti-FHA antibodies in B. *pertussis* induced serum cross react with *B. bronchiseptica* (Goebel, Zhang et al. 2009). The B6 antibody however showed reduced affinity to *B. bronchiseptica* FHA, suggesting its epitope may not be fully conserved in *B. bronchiseptica* FHA. FHA from *B. parapertussis* could however not be purified by heparin affinity. The apparent lack of affinity of *B. parapertussis* FHA with heparin is consistent with the inability of heparin to inhibit *B. parapertussis* binding to epithelial cells, in contrast to *B. pertussis* (van den Akker 1998). Binding of the six *B. pertussis* anti-FHA monoclonal antibodies was therefore assessed to whole cell cultures or cell culture supernatant of *B. parapertussis* using an indirect ELISA. The binding affinities to whole cells were in the range of 0.005 to 0.11 nM, (Table 2.6, column 6). The estimates of the affinities may however not be definite due to the longer time need to generate the signal in the ELISAs. The H2 and H6 antibodies however appeared to show more specific binding compared to the other four antibodies to the *B. parapertussis* culture supernatant (not shown). Polyclonal antibodies to *B. pertussis* FHA and other antigens have also been shown to weakly cross-react with *B. parapertussis* bacterial suspensions although the two FHA proteins may be immunologically distinct (Khelef, Danve et al. 1993). The slightly stronger binding of H2 and H6 to *B. parapertussis* culture supernatant would also be consistent with their major binding epitopes being on

the C-terminus of FHA (discussed below). *B. pertussis* and *B. parapertussis* share 85.7% and 87.5% sequence identity and similarity respectively. The C-terminus of *B. parapertussis* FHA however most closely resembles *B. pertussis* FHA, since the N-terminus of *B. parapertussis* FHA has additional sequences.

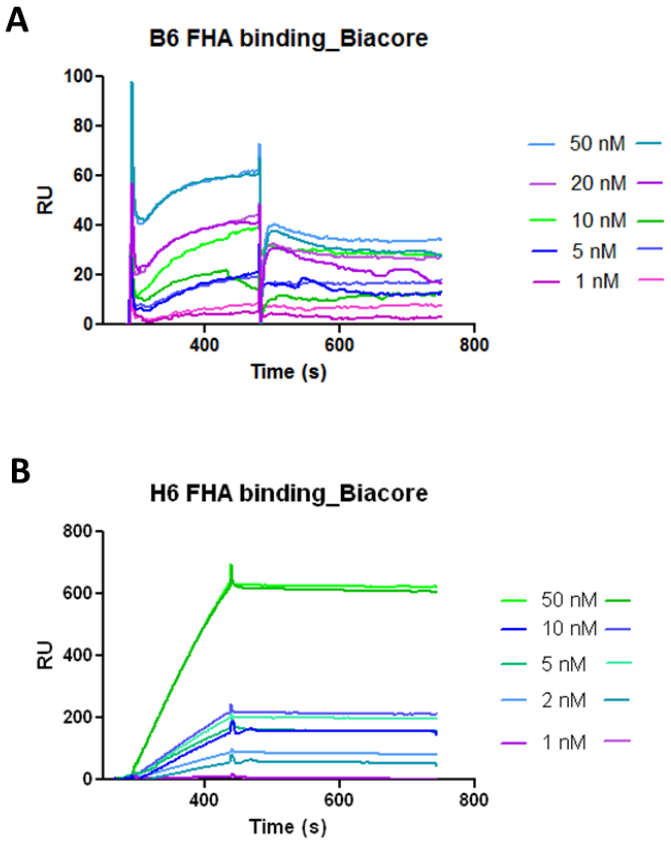


Figure 2.6: Biacore sensograms for B6 and H6 binding to *B. pertussis* D420 FHA.

The **A)** B6 and **B)** H6 antibodies were immobilized on a CM5 chip to a level of ~900 and 1600 RU respectively. *B. pertussis* strain D420 FHA was then injected at the indicated concentrations at a flow rate of 30 µL/min. Baseline correction was performed by subtracting simultaneous runs over a second flow cell with an isotype control protein immobilized. The antibody binding affinity (K_d) was determined from the ratio of the off-rate (k_{off}) to the on-rate (k_{on}), after globally fitting the binding curves to the 1:1 Langmuir model in the BIAevaluation v3.0 software.

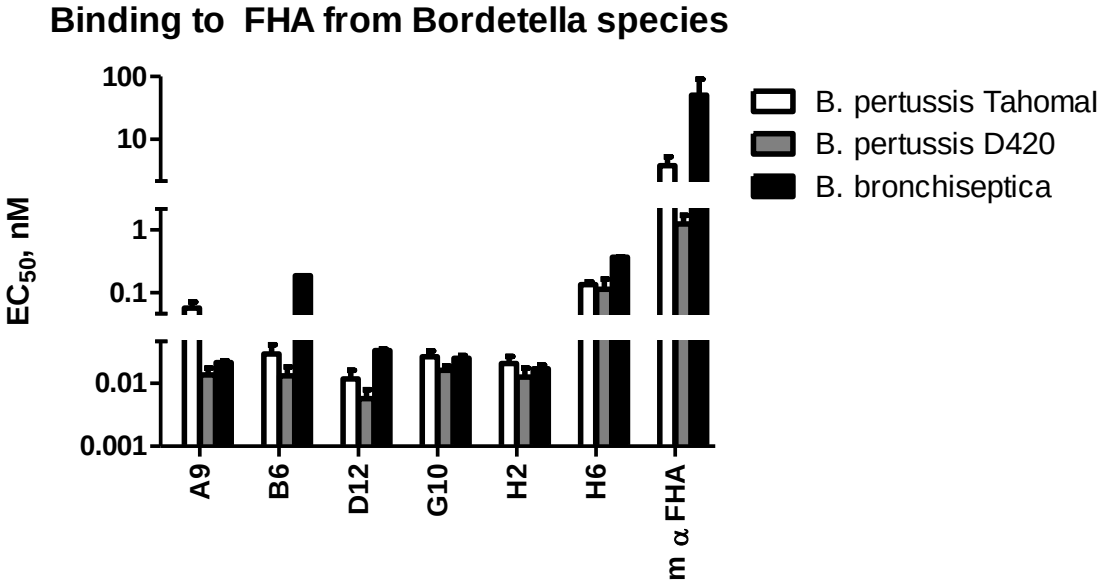


Figure 2.7: Binding affinity of the selected anti-FHA antibodies to purified FHA from *B. pertussis* and *B. bronchiseptica* FHA.

Purified FHA from the *Bordetella sp*. indicated was coated at a concentration of 1 µg/mL. Serially diluted antibodies were added to FHA coat and binding detected with HRP conjugated anti-human Fc antibodies after TMB substrate development. Binding curves were fit to the four parameter logistic (4PL) non-linear equation using GraphPad prism 5 to estimate EC_{50} values. The EC_{50} are shown as $\log_{10}$ of the estimated mean values. The ELISAs were repeated at least twice with similar results. Error bars represent the standard error of the mean from replicate experiments.

Table 2.6: Estimated affinities for anti-FHA antibodies binding to FHA from major to *Bordetella* species.

Antibody	**EC_{50}, nM ± SE** ***B. pertussis*** **strain 165**	**EC_{50}, nM ± SE** ***B. pertussis*** **strain TahomaI**	**EC_{50}, nM ± SE (K_d, nM ± SE)** ***B. pertussis*** **strain D420**	**EC_{50}, nM ± SE** ***B. bronchiseptica*** **strain RB50**	**EC_{50}, nM ± SE** ***B. parapertussis*** **strain 12822^{+}**
A9	0.07 ± 0.02	0.06 ± 0.01	0.01 ± 0.004	0.02 ± 0.002	0.003± 0.001
B6	0.16 ± 0.06	0.03 ±0.01	0.01 ± 0.005 (0.19 ± 0.04)	0.19 ± 0	0.003± 0.002
D12	0.08 ± 0.03	0.01 ± 0.004	0.006 ± 0.002	0.03 ± 0.002	0.003 ± 0.002
G10	0.27 ± 0.05	0.03 ± 0.006	0.02 ± 0.003	0.03 ± 0.003	0.005 ± 0.004
H2	0.05 ± 0.02	0.02 ± 0.006	0.01 ± 0.005	0.02 ± 0.002	0.03 ± 0.03
H6	0.30 ± 0.05	0.13 ± 0.01	0.11 ± 0.05 (1.27 ± 0.05)	0.37 ± 0.002	0.004 ± 0.17*

$^{+}$ - EC_{50} determined from antibodies binding to whole *B. parapertussis* cells.

* - Determined from one ELISA with two replicates. The other EC_{50} are from at least two independent experiments each with two replicates.

2.3.6 Neutralizing anti-FHA antibodies after aP vaccination inhibit *B. pertussis* FHA mediated binding activities

FHA has multiple binding specificities, with at least three binding domains responsible for *B. pertussis* adherence to host cells. FHA interacts with glycoconjugates on the surface of eukaryotic cells (Brennan, Hannah et al. 1991, Prasad, Yin et al. 1993) to mediate attachment. The FHA mediated hemagglutination of red blood cells (RBC) involves FHA binding to surface glycoconjugates and is inhibited by glycoconjugates such as heparin (Menozzi, Mutombo et al. 1994). This suggests inhibition of FHA mediated hemagglutination may directly correlate with inhibition of FHA binding and subsequently *B. pertussis* adhesion to host cells. Of the six selected anti-FHA antibodies, B6 and H6 were able to inhibit FHA mediated hemagglutination (Figure 2.8A). H6 more potently inhibited hemagglutination at an antibody:FHA molar ratio of 0.97 ± 0.31, while B6 inhibition was at a ratio of 20.5 ± 5.08 (Figure 2.8B). The rest of the antibodies tested did not inhibit hemagglutination up to 30 molar excess concentrations. Leininger et al. (Leininger, Bowen et al. 1997) identified two immunodominant domains on FHA recognized by antibodies in sera from infants immunized with either aP or wP vaccines or from patients with pertussis disease. Leininger et al. also observed that some antibodies that bound the C-terminal domain, more potently neutralized the FHA mediated hemagglutination of RBC. On the other hand, some antibodies which recognized the N-terminus of FHA weakly inhibited the hemagglutination. The more potent ability of H6 to inhibit FHA mediated hemagglutination therefore indicated its epitope may be closer to the C-terminal domain of FHA, while the weaker inhibition of hemagglutination by B6 indicated its epitope lies closer to the N-terminus. Elucidation of the B6 and H6 epitopes would therefore supplement knowledge about the relationship of the structure of FHA

and how this relates to hemagglutination. This information would also lead to better understanding of the FHA mediated adherence of *B. pertussis* to cells *in-vivo*.

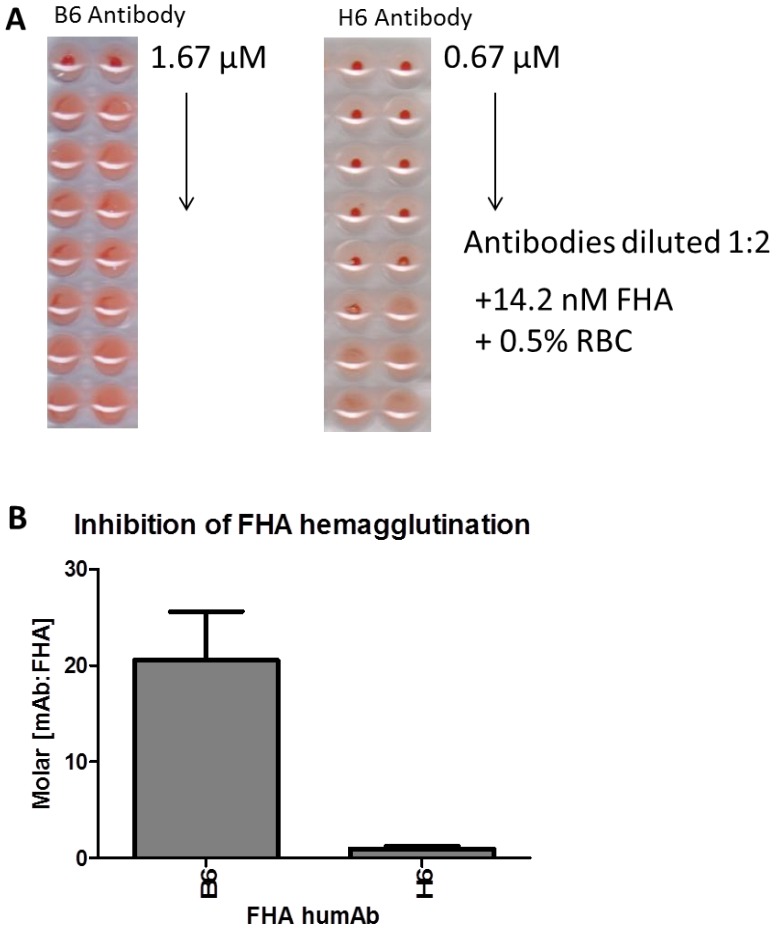


Figure 2.8: Inhibition of FHA mediated hemagglutination of RBC.

A) B6 and H6 anti-FHA antibodies inhibit FHA mediated hemagglutination. FHA was added to the serially diluted antibodies and the mixture incubated at 25°C for 30 minutes. Sheep RBC was added to a final concentration of 0.5% and the total volume of the reaction topped up to 100 µl with PBS. The lowest amount of antibody that could inhibit hemagglutination caused by FHA was taken as the neutralizing dose. The rest of the anti-FHA antibodies tested did not inhibit FHA hemagglutination up to 30 M excess. **B)** Molar neutralization of FHA mediated hemagglutination.

FHA mediates *B. pertussis* adhesion to and invasion of epithelial cells *in-vitro* (Tuomanen and Weiss 1985, Urisu, Cowell et al. 1986, van den Berg, Beekhuizen et al. 1999, Ishibashi, Relman et al. 2001, Ishibashi and Nishikawa 2002). Anti-FHA antibodies have also been shown to be important for inhibiting the attachment of *B. pertussis* to mammalian cells *in-vitro* (Sato, Izumiya et al. 1981, Tuomanen, Zapiain et al. 1984, van den Berg, Beekhuizen et al. 1999). To assess the relevance of the isolated anti-FHA antibodies in inhibiting *B. pertussis* binding to epithelial cells, FHA binding to CHO-K1 epithelial cells was first confirmed in a FACS assay. FHA was found to bind to CHO-K1 cells in a dose dependent manner (Figure 2.9A) and also increased the adherence of *B. pertussis* to the CHO-K1 cells when added exogenously (Not shown). The ability of five of the selected anti-FHA antibodies were then tested for inhibition of *B. pertussis* binding to CHO-K1 epithelial cells. Using *B. pertussis* conjugated to FITC, and an MOI of approximately 100, B6 was found to inhibit binding to the CHO-K1 cells (Figure 2.9B). B6 may therefore bind an epitope sufficient for inhibition of *B. pertussis* binding to CHO-K1 epithelial cells. FHA interaction with glycoconjugates and integrins on the surface on epithelial cells is thought to be important for *B. pertussis* adhesion (Menozzi, Gantiez et al. 1991, Menozzi, Mutombo et al. 1994, Ishibashi, Relman et al. 2001) and B6 may inhibit these interactions. Further characterization of the B6 epitope showed it binds strongly bound an N-terminal fragment of FHA. An N-terminal epitope of B6 is also in agreement with the study by Leininger which identified neutralizing N-terminal antibodies which weakly inhibit FHA mediated hemagglutinatoin (described below). A previous study also identified an antibody to the N-terminus of FHA which inhibited *B. pertussis* binding to epithelial cells (Leininger, Probst et al. 1993). More in depth analysis of the B6 epitopes would therefore contribute to information on the functional binding site of FHA to epithelial cells.

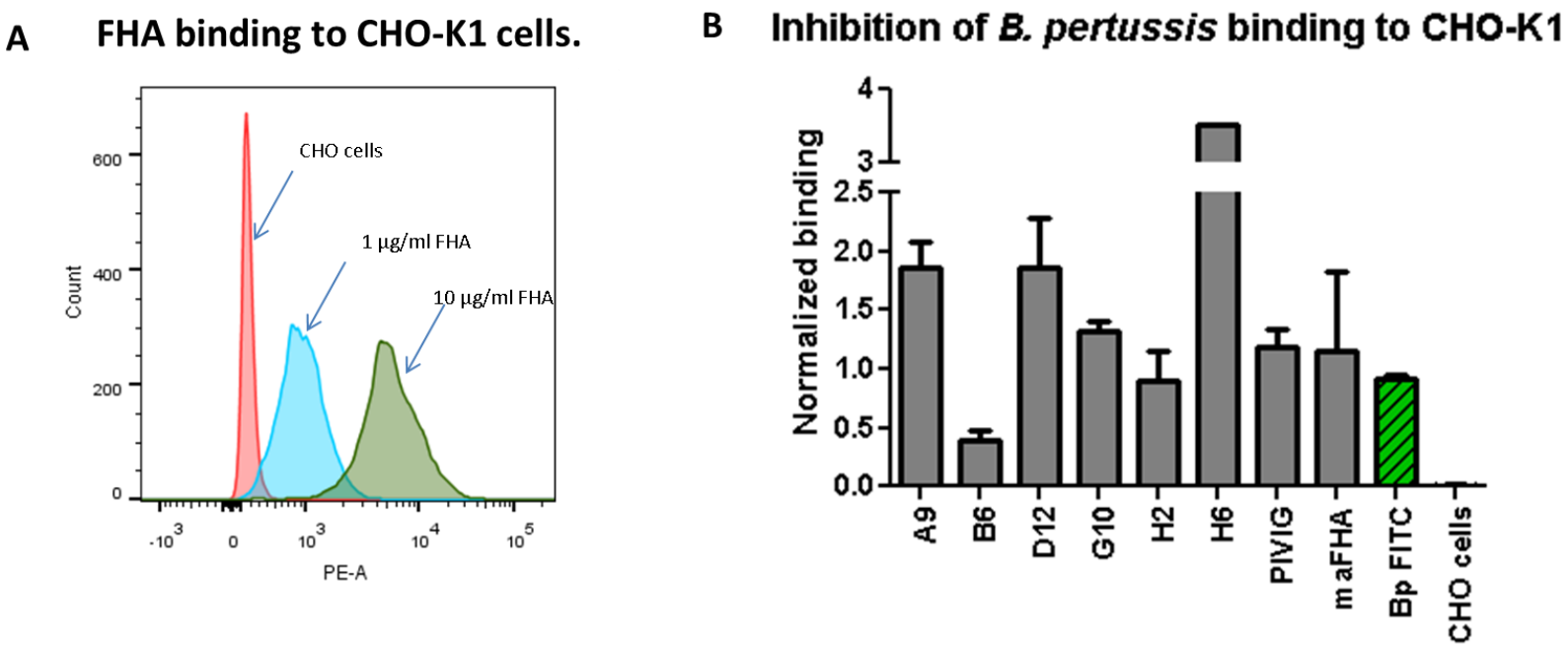


Figure 2.9: Inhibition of *B. pertussis* adhesion to CHO-K1 epithelial cells.

A) Biotinylated FHA binds to CHO-K1 epithelial cells in a dose dependent manner. Dilutions of biotinylated FHAwere added to CHO-K1 cells. Bound FHA was detected with PE conjugated streptavidin and assessed by flow cytometry. **B)** Anti-FHA antibodies inhibit *B. pertussis* adhesion to CHO-K1 epithelial cells. CHO-K1 epithelial cells were incubated with *B. pertussis* cells conjugated to FITC (Bp-FITC) at an MOI of 100 in 12-well culture plates. To assess inhibition of binding, 2.5 µg of the antibodies were pre-incubated with Bp-FITC. Bound *B. pertussis* cells after washing were detected by flow cytometry after scraping the cells off. The assays were repeated twice except for H6, and error bars represent the standard error of the mean from replicate experiments. H6 inhibition of Bp-FITC binding was performed once due to limited antibody.

In addition to binding to epithelial cells, FHA also mediates *B. pertussis* adhesion to host macrophages by interacting with integrins such as the macrophage CR3 receptor (Relman, Tuomanen et al. 1990, Ishibashi, Claus et al. 1994). FHA binding to the soluble CR3 receptor was confirmed in a capture ELISA (Figure 2.10A). The ability of the anti-FHA antibodies to inhibit FHA binding to soluble CR3 was then assessed in a competition ELISA assay. H6 more potently inhibited the interaction of FHA with CR3, however, the other antibodies tested also weakly inhibited FHA binding to CR3 (Figure 2.10B). The absorbance at 450nm was normalized to the highest absorbance in each data set to account for the amplified signal observed in the samples with antibody added. The amplified signal in samples with antibody added relative to the no antibody control may have been due to non-specific binding of other antibodies in the secondary GαR HRP antibody to the human antibodies. Other tested secondary antibodies exhibited non-specific inhibition of FHA binding.

Initial studies implicated the FHA RGD motif in mediating the interaction of FHA with CR3 to enhance *B. pertussis* attachment to macrophages (Ishibashi, Claus et al. 1994). Further studies using variants of *B. pertussis* containing an RAD amino acid substitution at the RGD motif, suggests the RGD motif may only play a partial role in mediating *B. pertussis* attachment to macrophages *in-vivo* (Hellwig, Hazenbos et al. 1999). *B. pertussis* attachment to host macrophages via the interaction of FHA with CR3 may however allow internalization of the bacteria which may serve as a reservoir, and prolong clinical manifestations of the disease (Ishibashi, Claus et al. 1994, Hellwig, Hazenbos et al. 1999). Disruption of the FHA-CR3 interaction by neutralizing anti-FHA antibodies may therefore be useful for reducing bacterial persistence. Further *in-vitro* and *in-vivo* studies are therefore needed to assess the role of antibodies like H6 in reducing *B. pertussis* entry and survival in CR3 bearing cells such as macrophages.

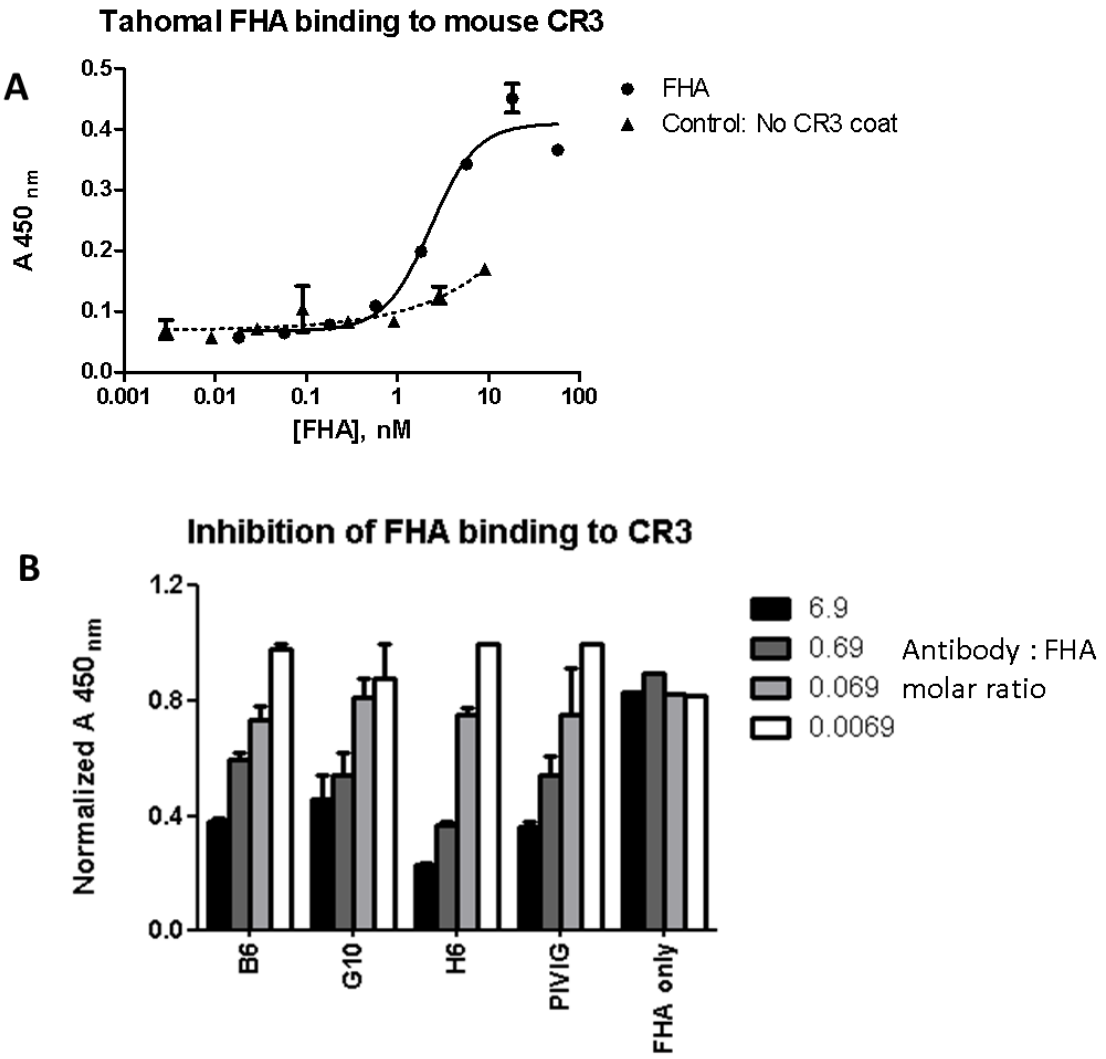


Figure 2.10: Inhibition of FHA binding to the macrophage CR3 receptor

A) FHA binding to the macrophage CR3. FHA was serially diluted and added to a coat of CR3 at 1 µg/ml. Bound FHA was detected using polyclonal mouse anti-FHA antibody and goat-anti-mouse HRP. The control wells had no CR3 coated to detect non-specific binding of FHA to the coat. **B)** Antibody mediated inhibition of FHA binding to CR3. Serially diluted antibodies with a constant concentration of 247 nM FHA were added to a coat of CR3 at 1 µg/ml. Bound FHA was detected using polyclonal mouse anti-FHA antibody and a polyclonal rabbit anti-mouse HRP secondary. The absorbance at 450nm was normalized to the highest absorbance in each data set to account for amplified signal observed in the samples with antibody added compared to the no antibody control.

2.3.7 Anti-FHA antibodies after aP vaccination recognize immunodominant epitopes in the N and C terminus of FHA

The 220kDa active form of FHA is derived from a large 370 kDa precursor, with further proteolytic breakdown products which are thought to play different roles during infection (Domenighini, Relman et al. 1990). Domenighini et al (Domenighini, Relman et al. 1990) have suggested a scheme for the proteolytic digest of FHA from its precursor utilizing immunoblot analysis and sequencing of the FHA fragments. The ~150 doublet, 125, 75 and 58 kDa fragments are attributed to the C-terminus of FHA, while the 98 kDa fragment is attributed to the N-terminus. Studies have subsequently shown that the C-terminal ~500 amino acid end of FHA designated as the type I region, is immunodominant after either immunization or infection with *B. pertussis* (Leininger, Bowen et al. 1997, Piatti 1999). Neutralizing antibodies induced to the type I region FHA strongly inhibit FHA mediated hemagglutination (Leininger, Bowen et al. 1997). A second immunogenic domain in FHA, located in the N-terminus, designated as the type II region, was also identified by Leininger et al. (Leininger, Bowen et al. 1997). Neutralizing antibodies induced to the type II region however exhibited a weaker inhibition of FHA mediated hemagglutination. The more potent inhibition of FHA mediated hemagglutination by H6, and weaker inhibition by B6 gave an indication that H6 binds the C-terminus, while B6 binds the N-terminus.

Using a western blot, we directly assessed binding of the antibodies to the FHA fragments produced after SDS gel electrophoresis. The A9, B6, D12, and G10 anti-FHA antibodies strongly recognized smaller FHA fragments in the blot, as well as larger precursor forms of FHA (Figure 2.11). Recognition of the ~150 kDa doublet attributed to the C terminal portion of FHA would be consistent with results that suggest the C terminal end of FHA is immunodominant after vaccination or disease, and suggests these

antibodies may have linear elements to their epitopes. However, these antibodies also appeared to recognize an ~100 kDa fragment, which could be the designated N-terminal fragment (Domenighini, Relman et al. 1990). The results of the hemagglutination assay putatively assigning B6 to the N-terminus was therefore conflicting with the ability of B6 to recognize fragments attributed to both the N and C-termini.

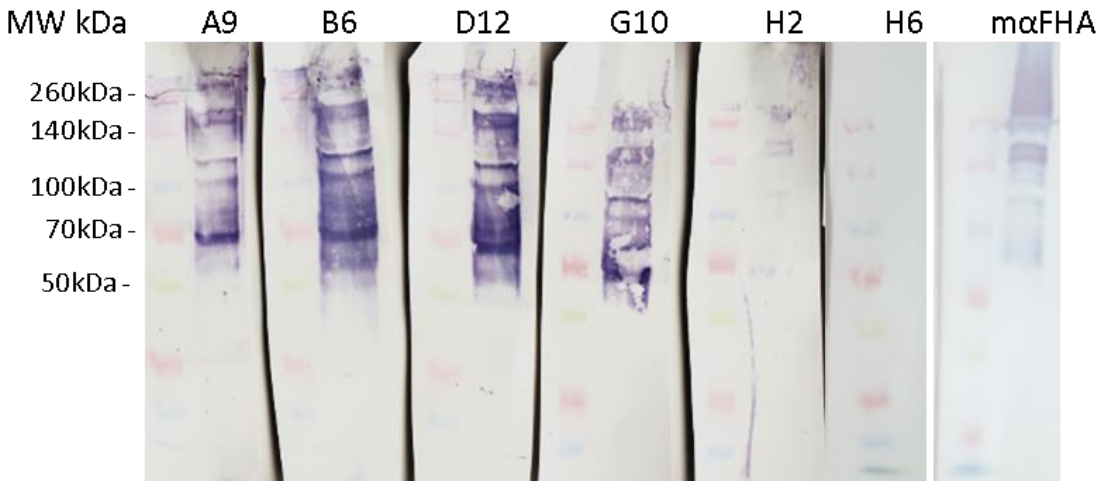


Figure 2.11: Western blot analysis of binding of the isolated anti-FHA antibodies to FHA fragments.

An amount of 5µg of FHA from *B. pertussis* was electrophoresed in a 7.5% gel and transferred to a PVDF membrane under standard conditions. Strips of PVDF containing FHA were incubated with the anti-FHA antibodies and binding to the FHA fragments detected with a secondary HRP conjugated antibody. The blots were developed with a chromogenic substrate

To further clarify the regions on FHA bound by the isolated antibodies, we assessed binding to recombinant FHA fragments produced using the pMAL-c expression system in *E. coli* (Figure 2.12). The use of the 861-1655 (pMAL 83) and 1655-2111 (pMAL 85) fragments have been previously described (Leininger, Bowen et al. 1997). The 1-435 and 1156-1295 fragments were generated in this study. The A9, B6, D12 and G10 antibodies bound the 1-435 fragment with high affinities in the range of 0.1-0.36 nM. These antibodies however bind more tightly to full length FHA with affinities in the range of 0.01-0.06 nM, suggesting a loss affinity to the 1-435 fragment. This result suggests the epitopes of A9, B6, D12 and G10 are located close to the N-terminus, but their epitopes may span amino acids not included in the 1-435 fragment. Further studies using longer recombinant FHA fragments are therefore needed to assess the full binding epitopes of these antibodies. None of the antibodies bound the 861-1655 fragment which contains the RGD sequence (Saukkonen, Cabellos et al. 1991, Ishibashi, Claus et al. 1994) nor the 1156-1295 fragment which is within the carbohydrate binding domain (Prasad, Yin et al. 1993). A9, B6, D12 and G10 however also very weakly recognized the 1655-2111 fragment located in the C-terminus of the mature FHA (Table 2.7). The strong reactivity of the A9, B6, D12 and G10 antibodies to the N-terminal 1-435 fragment, and weak reactivity with the C-terminal 1655-2111 fragment in the ELISAs was therefore consistent with the observation of binding to both N and C terminal fragments in the immunoblot. Cross reactivity of antibodies to both the N- and C-termini of FHA has also been previously observed (Leininger, Bowen et al. 1997), and suggests the antibodies may have secondary binding epitopes.

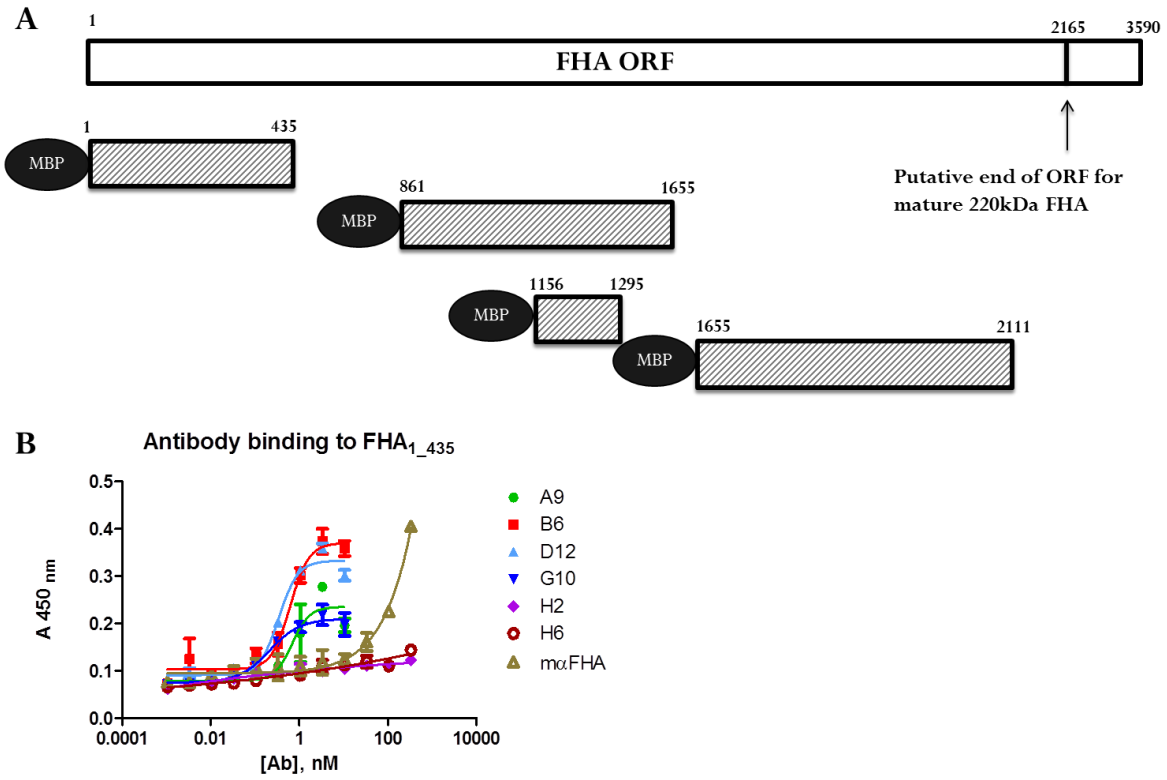


Figure 2.12: Recombinant FHA fragments used to probe the antibody binding epitopes.

A) Schematic of recombinant FHA fragments for antibody epitope mapping. The open box represents the complete ~3590 amino acid open reading frame (ORF) of the precursor FHA. The putative end of the ORF to form the mature 220 kDa FHA is indicated. Hatched smaller boxes represent the recombinant FHA fragments produced as MBP fusions in *E. coli*. Amino acid residue numbers are indicated. **B)** Representative ELISA for antibodies binding to the FHA 1-435 fragment. Serially diluted antibodies were added to a coat of the purified FHA fragment. Bound antibodies were detected with a polyclonal anti-human Fc HRP antibody.

Table 2.7: Reactivity of isolated antibodies with recombinant FHA fragments.

Antibody/FHA fragment	**1-435**	**861-1655 (pMAL 83)**	**1156-1295**	**1655-2111 (pMAL 85)**
A9	++ (0.36)	±	-	±
B6	+++ (0.36)	±	-	±
D12	+++ (0.29)	±	±	±
G10	++ (0.14)	-	-	-
H2	-	±	-	+ (0.01)
H6	-	±	-	+ (0.07)
P-IVIG	+	+	±	++
Polyclonal mαFHA	+	+	±	+++ (0.8)

Reactivity was graded as very strong (+++), strong (++), weak (+), equivocal (±) or negative (-) based on the intensity of the ELISA signal and ability to reach saturation at the highest concentration tested. The estimated affinities (EC_{50}) for antibodies which exhibited saturating binding curves are indicated in parentheses. pMAL 83 and 85 kindly provided by Dr. Camille Locht.

The secondary binding epitopes may be because of repeat sequences present in FHA. The first repeat region (R1) consists of about 38 tandem copies of a 19-residue repeat, located approximately at the N-terminal end of FHA. Next to the R1 region, is a smaller repeat region similar to R1 designated B1 (Makhov, Hannah et al. 1994, Jacob-Dubuisson, Kehoe et al. 2000, Kajava, Cheng et al. 2001). Antibodies to FHA have also been identified which recognize an amino acid sequence repeat in the R1 and B1 regions (Leininger, Bowen et al. 1997). The second repeat region (R2) consists of 13 tandem copies of a 19-residue repeat, and is located approximately at the C-terminal end of FHA (Makhov, Hannah et al. 1994, Jacob-Dubuisson, Kehoe et al. 2000, Kajava, Cheng et al.

2001). Next to R2, is the B2 region, also containing similar repeat sequences. Further studies using peptide scanning analysis can therefore be used to identify specific sequences to which the antibodies bind. The ability of the A9, B6, D12, G10 and H2 anti-FHA antibodies to recognize the SDS denatured *B. pertussis* TahomaI FHA fragments in the western blot also suggests these antibodies may recognize chemically modified forms of FHA. This is because the toxins in aP vaccines are stabilized by addition of cross-linkers such as glutaraldehdye or formalin, a process which can change the physical and immunological properties of the proteins (Nencioni, Volpini et al. 1991).

While H2 weakly recognized the FHA fragments in the western blot, H6 did not recognize any of the FHA fragments in the immunoblot, suggesting the H6 epitope may be conformational, or it only recognizes the native form of FHA. H2 and H6 also recognized the 1655-2111 fragment with affinities of approximately 0.02 and 0.07 nM respectively. The estimated binding affinities for H2 and H6 binding the 1655-2111 fragment were similar to binding the full length FHA (Table 2.7), indicating their primary epitopes lie close to the C-terminus. The polyclonal mαFHA also strongly bound the 1655-2111 fragment with an estimated affinity of 0.8 nM. The strong binding therefore suggested most of the antibodies in the polyclonal preparation predominantly bound the C-terminal end of FHA. There was a marked reduction in the absorbance at saturation for H2 and H6 compared to the polyclonal mαFHA binding to the 1655-2111 fragment (not shown). In ELISAs with the full length FHA, H2 and H6 reach high absorbance at saturation, similar to the polyclonal mαFHA. This suggested while the primary epitopes of H2 and H6 may be close to the C-terminus, they may also have other secondary epitopes on FHA. We also used a competition ELISA with biotinylated H6 to assess whether the antibodies compete with H6 for similar epitopes. The antibodies weakly competed with H6 binding for to FHA, suggesting an overlap in their epitopes, however,

H2 and the polyclonal m α FHA inhibited H6 binding the most (Figure 2.13), further suggesting H2 and H6 may share a primary epitope in close proximity. The strong competition with polyclonal m α FHA further indicates the H2 and H6 epitopes are closer to the C-terminus.

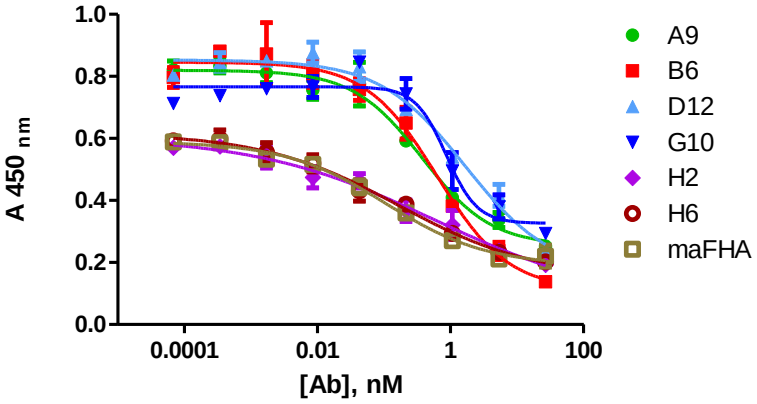


Figure 2.13: Competition of binding of the anti-FHA antibodies with biotinylated H6.

Representative ELISA for inhibition of biotinylated H6 binding to FHA. Serially diluted anti-FHA antibodies were incubated with a constant concentration of biotinylated H6 anti-FHA. Bound biotinylated H6 was detected with streptavidin-HRP.

The lack recognition of FHA fragments in the western blot by H6 could be due to the presence of conformational epitopes at/close to the C-terminus. The C-terminal domain is thought to have globular structure (Makhov, Hannah et al. 1994, Mazar and Cotter 2006), and may therefore present conformational epitopes which not detected in immunoblots due to linearization from the SDS. Alternatively, a conformational epitope may be formed by the N- and C-termini. The current model of FHA topology and maturation hypothesizes that prior to the secretion of mature FHA, the C-terminal domain of the precursor FHA remains intracellular to allow the mature C-terminus to achieve its

proper conformation. FHA therefore would initially form a hairpin which would bring the N- and C-termini close together (Mazar and Cotter 2006, Noël, Mazar et al. 2012).

2.3.8 Anti-FHA antibodies after aP vaccination bind *Bordetella* whole cells

FHA is both secreted and surface bound, and surface bound FHA is relevant for *B. pertussis* adhesion (Arico, Nuti et al. 1993), while the secreted FHA may help in the dispersal of bacteria (Coutte, Alonso et al. 2003). The presence of FHA on the surface of *B. pertussis* however has implications for antibody mediated bactericidal activity by opsonization of bacteria and/or complement mediated lysis (Aase, Herstad et al. 2011). A FACS assay was therefore used to assess whether the epitopes of the isolated anti-FHA antibodies are exposed on surface bound FHA molecules on the bacteria. Significant binding of the antibodies was detected to whole cell *B. pertussis* compared to an irrelevant antibody control (Figure 2.14). Irrelevant control antibodies and the secondary fluorescent antibodies did not show binding to *B. pertussis*. This result is consistent with the detection of monoclonal anti-FHA antibodies to surface bound FHA on *B. pertussis* (Coutte, Alonso et al. 2003). The six *B. pertussis* anti-FHA antibodies also showed binding to *B. bronchiseptica and B. parapertussis* whole cells in the FACS assays (*B. parapertussis* not shown). Binding of the anti-FHA antibodies to the *Bordetellae* however was detected only when the antibodies were incubated with the bacteria in buffer containing 5% glycerol at room temperature. In modified assays where the antibodies were incubated with *B. pertussis* on ice in PBS without glycerol, we detected minimal binding to *B. pertussis*. No significant binding of the antibodies was detected to a strain of *B. pertussis* with the FHA gene deleted, indicating the observed binding was specific to surface exposed FHA (not shown). In control assays, we however detected binding of

the anti-*B. pertusiss* lipo-oligosaccharide (LOS) antibody (D26E), to *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* (not shown). A further control testing only secondary antibodies binding to the *Bordetellae* showed no binding. Cross reactivity of *B. pertussis* anti-LOS antibodies to *B. bronchiseptica* lipopolysaccharide (LPS) has been reported. Western blot analysis however suggests the LPS of *B. pertussis* and *B. parapertussis* are serologically distinct (Amano, Fukushi et al. 1990, Archambault, Rondeau et al. 1991, Martin, Peppler et al. 1992). The LPS of *Porphyromonas gingivalis* shows glycerol conjugates and derivatives are present within the structure (Paramonov, Aduse-Opoku et al. 2009). The cross reactivity we observed for D26E binding the *B. parapertussis* in glycerol may therefore have been due to the glycerol present in the buffer modifying the LPS structures of *B. parapertussis* to make it more similar to that of *B. pertussis* and *B. bronchiseptica*. In the absence of glycerol, D26E did not bind to *B. parapertussis* (Independent assay from lab colleague, Xianzhe Wang).

Antibodies against surface exposed antigens on *B. pertussis* have been reported to promote phagocytosis (Hellwig, Rodriguez et al. 2003, Aase, Herstad et al. 2011), and anti-FHA antibodies are sometimes thought to be bactericidal (Weiss, Patton et al. 2004). We therefore assessed whether the anti-FHA antibodies induced to aP vaccination were capable of inducing bactericidal activity. Serum from a baboon recovering from *B. pertussis* infection had the highest bactericidal activity. Compared to the serum, or the absence of antibodies, the B6, H2 and H6 antibodies were however not significantly bactericidal. The lack of significant bactericidal activity may be because only a few available molecules may be available for complement mediated lysis, due to the efficient secretion of FHA (Weiss, Patton et al. 2004). In addition, *B. pertussis* expresses the BrkA (*Bordetella* resistance to killing) protein (Barnes and Weiss 2001), which inhibits complement mediated bactericidal killing.

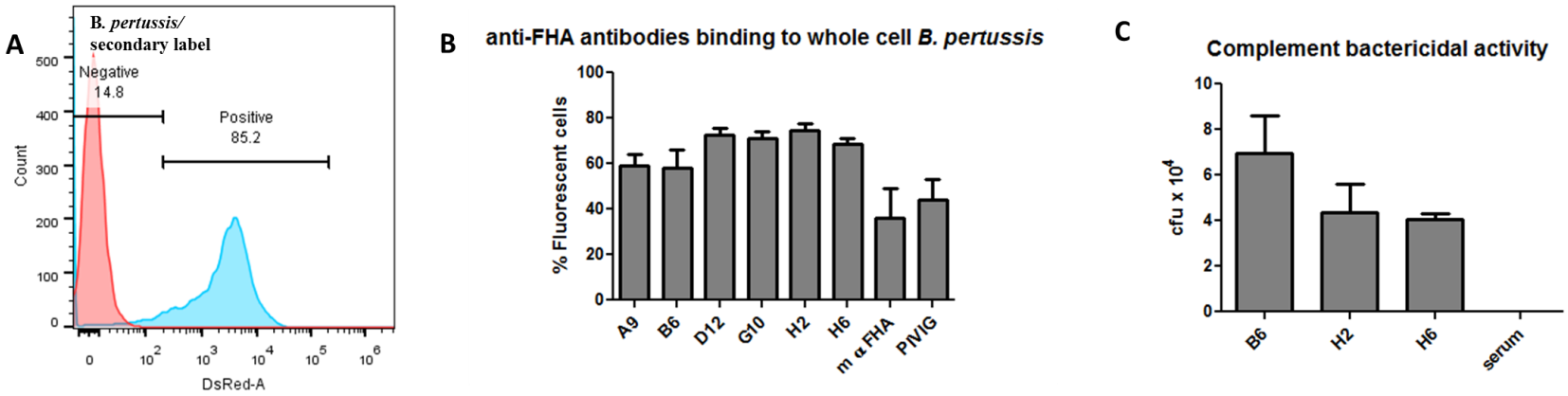


Figure 2.14: Anti-FHA antibody binding to intact *B. pertussis* TahomaI whole cells and bactericidal activity.

A) Representative FACS plot of H2 anti-FHA antibody binding to whole *B. pertussis* cells. *B. pertussis* cells grown on Bordet Gengou plates were scraped and resuspended to 1 OD_{600}/mL. The cells were washed and incubated with 1 µg of the antibodies in PBS with 5% glycerol. Bound antibodies were detected with fluorescently labeled secondary antibodies and analyzed by flow cytometry. **B)** Percentages of fluorescent *Bordetella* cells after labeling with the *B. pertussis* anti-FHA antibodies. Using the FlowJo_V10 software, % fluorescent cells were determined for a gated population of the cells using unlabeled *B. pertussis* as a negative control. Data represent the average of at least two independent experiments. Error bars represent the standard error of the mean. **C)** Complement bactericidal activity. 3 x 10^5 cfu *B. pertussis* in the presense of 10% antibody depleted serum was incubated with 25 µg/ml of the indicated antibodies or 25% serum from an infection baboon for an hour. Dilutions of bacteria were spread on Bordet Gengou blood agar plates and colonies counted after 3 days of growth at 37°C. The results represent the average and standard error of two experiments.

2.4 DISCUSSION

In this study, the spectrum of antibodies isolated from single plasmablast cells from recently vaccinated adult volunteers was analyzed. Pertussis still remains a vaccine preventable disease in industrialized countries in spite of high vaccine coverage. Understanding the immunologic mechanisms underlying enhanced protection against pertussis infection is important for development of next generation vaccines with enhanced efficacy. Greater than 50 unique anti-FHA antibodies were recovered from the single B cells, compared to only 5 anti-PTx antibodies (Chapter 3). The higher frequency of anti-FHA antibodies compared to anti-PTx antibodies is consistent with a strong anti-FHA serum response after aP vaccination (Berbers, van de Wetering et al. 2013). The higher magnitude of the anti-FHA immune response may be because of the efficient secretion of this adhesin by *B. pertussis* compared to the other virulence factors. Secreted FHA may be important for promoting adherence of phase variants which may arise *in vivo* (Mooi 1988) or for spreading the bacteria from micro-colonies after initial colonization (Coutte, Alonso et al. 2003).

There was significant similarity in the sequences forming the framework regions of the 13 antibody sequences analyzed. The biggest differences in the sequences were not surprisingly in the CDRs. Analysis of putative germline families showed the antibodies descended from different germline sequence families, suggesting the B cells responding were oligoclonal, with somatic hypermutations resulting in the identified antibodies with very similar framework regions. This result is similar to antibodies isolated B cells responding to the influenza virus, where the authors noted each individual antibody clone arose from different phylogenetic lineages, and suggested multiple clad members exist to provide antibodies that respond to drift variant virus epitopes that occur quickly in the

rapidly mutating influenza virus. (Krause, Tsibane et al. 2011). Similarly diverse, and high-affinity anti tetanus antibodies have been isolated from volunteers immunized with the tetanus toxoid (Meijer, Andersen et al. 2006). The oligoclonal nature of the B cell response to *B. pertussis* FHA may provide plasticity to respond to the large complex antigen. The small data set of antibodies characterized in this study however limits the extent of analysis of diversity. The isolated antibodies all exhibited high affinity to FHA, with diversity in the expression levels, stability and epitopes. The affinities of B6 and H6, estimated to be 0.19 and 1.27 nM respectively by SPR analysis, are consistent with affinities of 0.1-10 nM for antibodies in the circulating immune repertoire (Foote and Eisen 1995).

Two anti-FHA antibodies, B6 and H6, were identified which neutralized the *in-vitro* FHA mediated hemagglutination of red blood cells (RBC). FHA mediated hemagglutination and binding to epithelial cells *in-vitro* can be inhibited by sulfated glycoconjugates such as heparin, and the heparin binding site is located in the N-terminus of FHA (Menozzi, Gantiez et al. 1991, Menozzi, Mutombo et al. 1994). The ability of B6 and H6 to inhibit FHA mediated hemagglutination therefore suggested these antibodies may block the interaction of *B. pertussis* with glycoconjugates on the surface of host cells. Using recombinant FHA fragments, we found that B6 as well as A9, D12 and G10 preferentially bound an N-terminal fragment with high affinity, but also weakly bound other fragments. H6 on the other hand bound the C-terminal fragment more strongly, with indications that it may also have other secondary epitopes. Only B6 however prevented the attachment of *B. pertussis* to CHO-K1 epithelial cells *in-vitro*. A previous study also identified an antibody to the N-terminus of FHA which inhibited *B. pertussis* binding to epithelial cells (Leininger, Probst et al. 1993). In mice, immunization with an N-terminal recombinant FHA fragment (Fha44) protected against challenge with *B.*

pertussis, indicating the N-terminus of FHA contains protective epitopes for inhibiting bacterial adherence to epithelial cells. Fha44 by itself however was not sufficient for adherence to epithelial cells and macrophages, although it contains the complete heparin binding domain. Additionally, Fha44 could not replace FHA during colonization of the respiratory tract (Alonso, Reveneau et al. 2002).

The C-terminal ~500 amino acids on the other hand is more immunodominant after immunization or infection (Delisse-Gathoye, Locht et al. 1990, Leininger, Bowen et al. 1997, Wilson, Siebers et al. 1998), and appears very relevant for adherence and inducing protection (Knight, Huang et al. 2006, Julio, Inatsuka et al. 2009). Recent studies with *B. bronchiseptica* have also indicated the C-terminus of FHA is what is surface exposed during secretion (Mazar and Cotter 2006), in contrast to a previous model suggesting the N-terminus emerges first (Jacob-Dubuisson, Buisine et al. 1996). Information obtained from studies of *B. bronchiseptica* FHA is thought to be directly related to *B. pertussis* FHA since the two proteins are similar at the sequence level, and may be functionally interchangeable (Julio, Inatsuka et al. 2009). An extensive analysis of serum responses after natural infection showed that antibodies induced to FHA predominantly bind the C-terminus, while aP vaccination induces antibodies predominantly to the N-terminus (Leininger, Bowen et al. 1997). Our observation that 4 out of the 6 characterized monoclonal antibodies preferentially bound the N-terminus is therefore consistent with the results by Leininger et al, indicating that aP vaccination induces anti-FHA antibodies predominantly to the N-terminus. The immunodominance of the N-terminus after aP vaccination may be because FHA is tethered to the bacterium during maturation. After secretion, the N-terminus may be more accessible, as such; the use of soluble FHA in aP vaccines may therefore predispose the formation of antibodies to the more accessible N-terminus. We suggest that *B. pertussis* may also secrete FHA at

such levels to present decoy non-neutralizing epitopes. Further studies are therefore needed to further define the epitopes recognized by the neutralizing antibodies to aid in the design of relevant recombinant FHA fragments which can be included in improved aP vaccines.

Tested antibodies were also competent at inhibiting FHA binding to the macrophage CR3 receptor, with H6 being the most potent. FHA mediates *B. pertussis* binding to human monocytes, macrophages (Relman, Tuomanen et al. 1990, Ishibashi, Claus et al. 1994) and epithelial cells (Ishibashi, Relman et al. 2001). Entry and survival of *B. pertussis* within the host macrophages is thought constitute a reservoir for transmitting infection (Steed, Setareh et al. 1991, Friedman, Nordensson et al. 1992). Antibody mediated reduction of FHA binding to the CR3 receptor may therefore directly reduce *B. pertussis* invasion and persistence. FHA also exhibits immunosuppressive effects on macrophages and dendritic cells by upregulating CR3 binding activity . The CR3 complement receptor 3 ($\alpha_M\beta_2$ integrin; CD11/CD18) is also the receptor for another *B. pertussis* virulence factor, the adenylate cyclase toxin (ACT) (Guermonprez, Khelef et al. 2001, Wang and Maynard 2014). ACT binding allows translocation of its catalytic domain into the cellular cytosol to increase intracellular cAMP levels and inhibit the anti-bacterial responses (Confer and Eaton 1982). FHA mediated adherence of *B. pertussis* to macrophages may therefore facilitate delivery of ACT to the target cells (Zaretzky, Gray et al. 2002) allowing it to exert immunosuppressive effects (Henderson, Inatsuka et al. 2012). Antibody mediated reduction of FHA binding to the CR3 receptor may therefore additionally inhibit FHA and ACT mediated immunosuppression.

Although studies have suggested FHA antibodies provide protection (Storsaeter, Hallander et al. 1990, Shahin, Amsbaugh et al. 1992, He, Viljanen et al. 1994), possibly by reducing *B. pertussis* adherence (Sato, Izumiya et al. 1981), there is some debate about

whether FHA is sufficient for protection against *B. pertussis* infection *in-vivo* (Oda, Cowell et al. 1984, Cherry, Gornbein et al. 1998, Storsaeter, Hallander et al. 1998). In a vaccine efficacy trial, there appeared to be no correlation between anti-FHA antibodies induced to the aP vaccines and protection in the absence of antibodies to PTx and PRN (Cherry, Gornbein et al. 1998). Our in-vitro studies indicated that neutralizing anti-FHA antibodies may be able to reduce bacterial adherence as previously reported (Sato, Izumiya et al. 1981, Knight, Huang et al. 2006). Recent studies in baboons have however suggested aP vaccination protects against toxin mediated effects, but does not prevent transmission (Warfel, Zimmerman et al. 2014). Surface exposed antigens on *B. pertussis* could serve as targets for antibody mediated bactericidal activity by opsonization of bacteria and/or complement mediated lysis (Aase, Herstad et al. 2011). Of the antigens in currently licensed aP vaccines, FHA, PRN and FIM, which are outer membrane proteins, are more likely to serve as targets for bactericidal activity. Anti-PRN antibodies have been shown to be bactericidal *in-vitro* (Weingart, Keitel et al. 2000) and in mice (Gotto, Eckhardt et al. 1993). FHA on the other hand is both secreted and also loosely associated with the bacterial outer membrane. Although the anti-FHA antibodies were found to be competent to bind to surface bound FHA on *B. pertussis*, preliminary experiments suggest there may not be a significant bactericidal effect of the anti-FHA antibodies. This may be because of the efficient secretion of resulting in only a few available molecules for complement lysis (Weiss, Patton et al. 2004). In addition, *B. pertussis* expresses the BrkA (*Bordetella* resistance to killing) protein (Barnes and Weiss 2001), which inhibits the complement mediated bactericidal killing. Although *B. pertussis* appears to be killed specifically via the antibody dependent classical pathway (Barnes and Weiss 2003), *B. pertussis* has been shown to bind to the host inhibitor factor H, C4b binding proteins (Amdahl, Jarva et al. 2011) and C1-esterase inhibitor (Marr, Luu et al. 2007) to escape

complement mediated killing via both the alternative and classical pathways. Anti-FHA antibody mediated bactericidal mechanisms may therefore not be sufficient for clearing bacteria. Other surface exposed antigens and/or T-cell mediated bactericidal activity may be the predominant mechanism for clearing *B. pertussis*.

Low *in-vivo* protection by FHA may also be because FHA by itself may reduce the protective Th1 responses by apoptosis of activated Th1 cells (Boschwitz, Batanghari et al. 1997, McGuirk and Mills 2000, Abramson, Kedem et al. 2001, McGuirk, McCann et al. 2002, Öhman, Willén et al. 2005). However the formulation of the vaccine may increase the FHA induced apoptosis of Th1 cells. Like most vaccines, aP vaccines are formulated with alum as the adjuvant. It has however been demonstrated that alum preferentially promotes Th2 cells (Lambrecht, Kool et al. 2009) whereas the more protective Th1/17 responses from by wP vaccines or natural infection may be induced by other types of adjuvants (Ross, Sutton et al. 2013). Protective immunity to an experimental aP vaccine containing PTx and FHA was demonstrated to be enhanced by replacing alum with a different adjuvant such as CpG (Ross, Sutton et al. 2013) which promotes the induction of Th1 cells (Hemmi, Takeuchi et al. 2000). Additionally, secondary infections may reduce the effective immune responses of the aP vaccines. It has been demonstrated that co-infection with *Fasciola hepatica* suppresses the protective Th1 responses against *B. pertussis* (Brady, O'Neill et al. 1999). Prevention and treatment of such secondary infections may also improve the immune responses to aP vaccines.

The limited efficacy of anti-FHA antibodies may also be a reflection of reduced immunologic properties retained by FHA after chemical treatment in vaccines from different manufacturers. In one study, formalin treated FHA or its antibodies appeared to be less protective unless administered with pertussis toxoid or its antibodies (Sato and Sato 1984). Chemical treatment has been shown to reduce immunogenicity of PTx, a

major virulence factor in current aP vaccines (Nencioni, Volpini et al. 1991, Fowler, Byron et al. 2003). Further studies may therefore be needed to assess the immunogenicity of chemically treated FHA in current aP vaccines. Although different binding domains have been attributed to separate regions on the mature FHA, the C-terminal end of mature FHA (MCD) has been recently recognized as being critical for *B. pertussis* adherence and colonization (Julio, Inatsuka et al. 2009). A recombinant FHA fusion protein of the MCD has also been shown to be immunogenic and protective (Knight, Huang et al. 2006). Recombinant FHA fragments containing protective epitopes which would require less stabilizing agents may therefore be suitable candidates for inclusion in new aP vaccines.

FHA is a common antigen produced by all the major *Bordetella* strains which infect humans. Previous studies with serum containing anti-FHA antibodies have shown that *B. pertussis* induced serum cross reacts with *B. bronchiseptica* (Goebel, Zhang et al. 2009) and *B. parapertussis* (Khelef, Danve et al. 1993) FHA. The isolated *B. pertu*ssis anti-FHA monoclonal antibodies also recognized FHA from the three major *Bordetella* species. *B. pertussis* FHA also bound *B. bronchiseptica* FHA with high nM affinity, and also bound *B. parapertussis* whole cells. While immunization with purified *B. pertussis* FHA appears to be sufficient for protection against *B. bronchiseptica* infection in mice (Goebel, Zhang et al. 2009), it is not protective against *B. parapertussis* colonization (Khelef, Danve et al. 1993). aP vaccination is even thought to enhance *B. parapertussis* infection (Long, Karanikas et al. 2010). Interestingly, immunization with whole *B. pertussis* appears to be cross protective against *B. parapertussis* infection. This suggests *B. pertussis* antigens not included in the current acellular vaccines may confer cross protection against *B. parapertussis*. It has been suggested that the cross reaction of anti *B. pertussis* FHA, but lack of cross-protection against *B. parapertussis* infection is because

the FHA from the two species are immunologically distinct. Of the six antibodies characterized, four appeared to have their major binding sites within the N-terminus of FHA. Although *B. pertussis* and *B. parapertussis* share 85.7% and 87.5% sequence identity and similarity respectively, the N-terminus of *B. parapertussis* FHA is more distinct, with the presence of additional sequences. The antibodies to the N-terminus of *B. pertussis* may therefore only bind weakly to the N-terminus of *B. parapertussis* FHA, and may not be fully protective. Further studies are therefore needed to assess the role of antibodies to the C-terminus of FHA in inducing cross-protection.

The type of immune response generated *B. pertussis* infection may additionally be required for cross protection. *B. pertussis* cross-protection with *B. parapertussis* appears to be T-cell mediated (Feunou, Bertout et al. 2010). Nasal vaccination with attenuated *B. pertussis* which induces a predominantly Th1/17 response appears adequate for cross-protection against *B. parapertussis* in mice (Watanabe and Nagai 2001, Mielcarek, Debrie et al. 2006, Feunou, Bertout et al. 2010). Natural infection and wP vaccination also induces a predominantly Th1/17 response, whereas infants primed with aP vaccines have a Th2 skewed or mixed Th2/Th1 response (Ross, Sutton et al. 2013). aP vaccine antigens which generate a Th1/17 biased immune response may therefore be useful to induce cross-protection against *B. parapertussis*.

2.5 CONCLUSIONS

The single cell approach utilized for understanding the humoral response to FHA after aP vaccination, as opposed to analysis of serum responses, shows antibodies induced to FHA after aP immunization arise from diverse germline sequences, and bind with high nM affinity. Neutralizing antibodies reduced bacterial adherence to CHO-K1

epithelial cells or the leukocyte CR3 receptor *in-vitro*. While natural infection induces antibodies predominantly to the C-terminus of FHA, aP vaccination induces antibodies predominantly to the N-terminus. The tested anti-FHA antibodies were also all competent to bind whole *B. pertussis* cells, but did not appear to induce significant bactericidal activity. To improve upon current aP vaccines, the inclusion of protective recombinant FHA fragments may reduce the induction of antibodies to non-neutralizing epitopes. Neutralizing anti-FHA monoclonal antibodies identified in this study may have prophylactic potential and are useful reagents for characterizing functional domains of FHA.

Chapter 3: Quality and diversity of antibodies isolated from single B cells induced to the pertussis toxin (PTx) after acellular pertussis booster immunization.

3.1 INTRODUCTION

PTx is a 105kDa extracellular toxin and colonizing factor produced only by *B. pertussis*. The closely related species, *B. bronchiseptica* and *B. parapertussis* which cause a milder form of whooping cough in humans, contain a transcriptionally silent PTx operon due to mutations mostly in their promoter regions (Aricò and Rappuoli 1987). PTx belongs to the AB_5 class of toxins, which are characterized by a catalytically active A subunit and a receptor binding B subunit. The initial classifications of the AB type toxins were based on sequence homology and catalytic activity. However, more toxins were added to the group as more crystal structures of these toxins were solved, revealing a high degree of structural homology, and has offered great insight into the mechanisms of these toxins (Beddoe, Paton et al. 2010). The crystal structure of PTx determined to 2.9 Å resolution (Stein, Boodhoo et al. 1994), shows significant homology with other bacterial toxins such as the cholera toxin, the *E. coli* labile toxin and the shiga toxin (Figure 3.1.1).

The single A subunit (S1) of PTx with a molecular weight (M_W) of 26 026 kDa, contains the catalytic site for the toxic ADP-ribosyltransferase activity. The B pentamer consists of four non-covalently linked subunits, S2 (M_W 21 925 Da), S3 (M_W 21 873 Da), S4 (M_W 12 059 Da) and S5 (M_W 11 013 Da) in a molar ratio of 1:1:2:1 respectively. The B oligomer contains recognition domains responsible for binding host cell surface receptors for internalization of the toxic S1 subunit. Independent of the toxic ADP-ribosyltransferase activity of the S1 subunit, the B oligomer also displays non-enzymatic

toxicity including cellular agglutination, T cell activation and mitogenicity (Tamura, Nogimori et al. 1983, Nencioni, Pizza et al. 1991, Schneider, Weiss et al. 2007), and intercellular transfer of membrane proteins and lipids (Millen, Schneider et al. 2013).

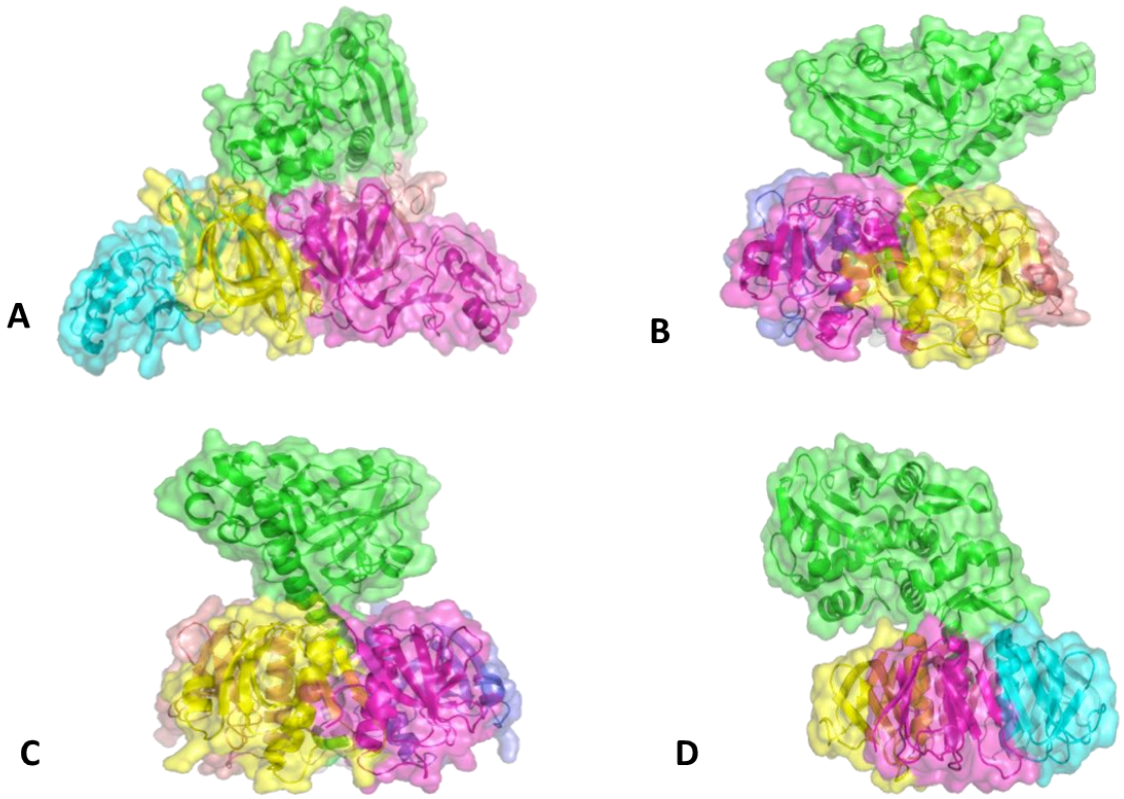


Figure 3.1: Crystal structures of bacterial AB_5 toxins.

A) *Bordetella* pertussis toxin **B)** Cholera toxin, **C)** *E. coli* heat-labile enterotoxin **D)** Shiga toxin. The enzymatically active A subunit is green for the four toxins shown with the receptor binding B pentamer in distinct colors for the different subunits.

Early in infection, PTx targets resident cells in the lung tissue such as alveolar macrophages and epithelial cells to aid in colonization of the respiratory tract (Carbonetti,

Artamonova et al. 2003). PTx binds to cell surface receptors via its B subunit, undergoes receptor-mediated endocytosis, followed by retrograde transport from the Golgi to the endoplasmic reticulum (ER). Within the ER, reduction of a disulfide bond in S1 and an ATP-induced conformational change in the B subunit, results in release of the S1 subunit from the holotoxin (Hazes, Boodhoo et al. 1996). Unfolded S1 is then transported into the cytoplasm possibly by a Sec61 dependent pathway (Hazes and Read 1997). Within the cytosol, the S1 subunit catalyzes the ADP-ribosylation of some membrane-associated $G_{i/o}$ proteins, disrupting G-protein function (Katada and Ui 1982, Katada, Tamura et al. 1983, Meade, Kind et al. 1984, Meade, Kind et al. 1984, Schaeffer and Weiss 2001, Kirimanjeswara, Agosto et al. 2005). PTx toxicity results in inhibition of neutrophil recruitment and altered circulation of mononuclear phagocytes, thus aiding in the evasion of bacterial opsonization by monocytes (Kirimanjeswara, Agosto et al. 2005, Andreasen and Carbonetti 2008). In addition to inactivating neutrophils and macrophages, PTx also confers a colonization advantage for *B. pertussis* by suppressing antibody responses to resist antibody mediated clearance (Vogel, Klein et al. 1985).

In mice, neutralizing anti-PTx antibodies elicited after immunization or administered passively are protective by reducing in-vivo PTx induced toxicity such as leukocytosis and histamine sensitization. Neutralizing anti-PTx antibodies are even able to reverse disease when administered as treatment in mice (Sato, Sato et al. 1987, Sato and Sato 1990). Studies in humans have also shown high anti-PTx antibodies in serum after household exposure (Cherry, Gornbein et al. 1998, Storsaeter, Hallander et al. 1998) or immunization (Taranger, Trollfors et al. 2000), correlate with lower incidence of severe pertussis. When present in aP vaccines however, immune responses to PTx do not appear to be dependent on its concentration, instead, the immunogenicity may be influenced by antigen derivation and formulation (Nencioni, Volpini et al. 1991,

Edwards, Meade et al. 1995, Fowler, Byron et al. 2003). The PTx responses after aP vaccination also tend to be lower than that to other components of the vaccine such as FHA or PRN with a faster decay rate (Edwards, Meade et al. 1995, Berbers, van de Wetering et al. 2013). In addition, aP vaccination induces lower neutralizing serum antibody titers to either the A or B subunit (Sutherland, Chang et al. 2011).

In this study, five anti-PTx antibodies isolated from single B cells after aP booster vaccination were characterized. The low frequency of B cells to PTx is consistent with low serum anti-PTx antibodies after aP vaccination. However, since the presence of high titers of neutralizing anti-PTx antibodies correlates with protection after exposure to *B. pertussis*, aP vaccines need to induce higher neutralizing anti-PTx antibodies through the use of the more immunogenic genetically modified PTx (PTg) (Rappuoli 1999), or formulate the vaccines with adjuvants which boost the PTx response. The isolated antibodies appeared to be derived from unique putative parental germline sequences. They also strongly bind unique epitopes on PTx with affinities in the range of 0.05 ± 0.03 to 0.3 ± 0.08 nM. The antibodies also similarly recognized genetically modified PTx (PTg) with high affinity. Antibody D8 potently neutralized PTx activity in an *in-vitro* CHO cell neutralization assay, while the rest of the antibodies were either weakly neutralizing or non-neutralizing. D8 and another antibody, A12, also inhibited PTx binding to a model receptor, fetuin, suggesting they may both block PTx-receptor interactions. The inability of A12 to inhibit PTx toxicity in the CHO assay however raises questions about the use of this assay for *in-vitro* identification of neutralizing anti-PTx antibodies. This study, the first to provide insight into the anti-PTx antibody repertoire after pertussis vaccination, also identifies useful antibodies for further elucidation of PTx function and provides promising candidates for passive immunotherapy for whooping cough infection.

3.2 MATERIALS AND METHODS

3.2.1 Antibody plasmid DNA

DNA plasmid vectors encoding antibody sequences corresponding to the single B cells responding to PTx were also kindly provided by Excelimmune Inc. The single B cells were isolated from healthy adult volunteers as described for anti-FHA antibody isolation (Chapter 2). The anti-PTx heavy and light chain antibody sequences were provided in a one vector system which contained the heavy and light chain sequences (Table 3.1). The plasmid vectors contained a neomycin/kanamycin resistance cassette and other functional elements such as a ColE1 origin of replication, and a CMV promoter for high level protein expression in mammalian cells.

Table 3.1: Plasmid vectors for expression of anti-PTx antibodies

Heavy chain antibody	Light chain antibody	Plasmid Vector	Antibody ID used in this study
42.18.E12.24F10.VH	42.18.E12.25D10.K	Single	E12
55.12.A8.33A8.VH	55.12.A8.33G4.K	Single	A8
55.15.H5.33C9.VH	55.15.H5.37C11.K	Single	H5
55.17.D8.33A10.VH	55.17.D8.33E5.K	Single	D8
42.12.A12.24G9.VH	55.17.D8.33E5.K	Single	A12

3.2.2 Bacterial strains

The *Bordetella* strain used in this study was the *B. pertussis* Tahoma I strain (ATCC # BAA-587) grown on Bordet-Gengou agar supplemented with 15% sheep blood (BD Biosciences). Plasmid vectors for transient expression of antibodies in CHO-K1 cells were purified from the *E. coli* strain DH5α. Transformation of the antibody plasmid vectors into DH5α was by electroporation. Cells successfully transformed were selected on 2XYT plates containing ampicillin.

3.2.3 Antigens and antibodies

3.2.3.1 PTx

Native PTx (in glycerol or lyophilized) or genetically modified PTx (PTg) was purchased from List Biological Laboratories, Inc. (Campbell, CA). Native PTx was also obtained through BEI Resources, NIAID, NIH: *B. pertussis* Toxin, Salt-free, NR-31826. A soluble, truncated version of the PTx S1 subunit, PTx-S1-220 expressed using the pAK400 plasmid was purified from *E. coli* as described (Sutherland and Maynard 2009).

3.2.3.2 Antibodies

The anti-PTx antibodies were transiently expressed on a laboratory scale by protein A/G affinity chromatography as described for purification of the anti-FHA antibodies (Chapter 2). Human intravenous pertussis immunoglobulin (P-IGIV) (Bruss, Malley et al. 1999) was obtained from the Massachusetts Public Health Biologic Laboratory. The previously characterized murine anti-PTx neutralizing antibodies, 1B7 and 11E6 (Sato, Sato et al. 1987, Sato and Sato 1990) were purified as described (Sutherland and Maynard 2009). Humanized versions of 1B7 and 11E6 with similar affinity and epitope specificity as the parent murine antibodies were purified from the

culture media of stably transfected CHO cells by Catalent (Somerset, NJ) (Nguyen et al., 2014. In review). HRP and fluorescent conjugated secondary antibodies were obtained from Southern Biotech and Thermoscientific respectively.

3.2.4 Phylogenetic analyses

The DNA sequences of the isolated anti-PTx antibodies were analyzed similar to the analyses done for the anti-FHA antibodies (Section 2.2.5). Briefly, NCBI/IgBLAST was used to map the VDJ or VJ sequences of each VH or Vκ respectively to the closest human germline sequence from the IMGT database by selecting the hit with the highest score. The score is determined after adding points for each match and subtracting points for each mismatch (Ye, Ma et al. 2013). The gene segment pairings (VDJ or VJ) for each genetic locus (H or K) were compared. The frequencies of pairing for the putative VDJ or VJ are presented as a heat map. The individual gene families and alleles were also analyzed by indicating the frequencies in a pie chart. To compare the full V_H or Vκ, the sequences of the isolated antibodies were aligned using Phylogeny.fr (Dereeper, Guignon et al. 2008) to generate phylogenetic trees.

3.2.5 Thermal stability analysis

Thermal stability of the purified antibodies was analyzed as described for the anti-FHA antibodies (Chapter 2). Briefly, three replicates of 100 µg/ml solutions of the antibodies were prepared in PBS using the protein thermal shift dye (Life Technologies) according to the manufacturer's protocol. The antibodies were heated from 25^{o}C to 90^{o}C with a scanning rate of 1^{o}C/min increments. The melting temperature (T_m) was determined from fitting the fluorescence data normalized to the maximum and minimum

fluorescence. The data was fit to a sigmoidal four parameter (4PL) non-linear regression model using Graph pad prism 5. For antibodies which exhibited complex melting paths, the temperatures at 10% of the maximum fluorescence, defined as the T_{onset} temperature, or the average of the minimum and maximum fluorescence, $T_{average}$ was determined.

3.2.6 Antigen specific responses

3.2.6.1 Indirect ELISA assays

Indirect capture ELISAs were performed as described for FHA (Chapter 2) with the following modifications. PTx holotoxin final concentration wase 1 µg/ml. PTx A-subunit and PTx B-subunit final concentrations were 0.25 µg/ml and 0.85 µg/ml respectively. Briefly, serially diluted antibodies were added to the plates after blocking with 5% milk in wash buffer. Subsequently, HRP-conjugated anti-human Fc antibodies (Southern Biotech) diluted 1:1000 were used to detect bound antibodies. The plates were developed with TMB substrate (ThermoScientific) and quenched with 1N HCl. Absorbances were read at 450 nm in a SpectraMax M5 instrument (Molecular Devices) using the SoftMax Pro v5 software. The relative binding affinities of antibodies to respective coated antigens were determined by estimation of the concentration of each antibody required to achieve 50% maximal binding at saturation (EC_{50}). The EC_{50} values were determined from fitting the binding curves to the 4 parameter logistic (4PL) non-linear curve model using the GraphPad Prism 5 software.

3.2.6.2 Competition ELISA assays

The ability of the antibodies to compete with competitor antibody for binding to PTx was evaluated by a competition ELISA as described for FHA (Chapter 2) with the following modifications. The competitor antibody was either a biotinylated or a murine antibody. Due to low concentrations, the anti-PTx antibodies were biotinylated with 1000 or 5000 molar excess EZ-link Sulfo-NHS-LC-Biotin following the manufacturer's instructions. Functional binding of the biotinylated antibodies to PTx was confirmed. The biotinylated antibody was detected with streptavidin-HRP, while the murine antibodies were detected with a polyclonal goat-anti-mouse antibody preparation.

3.2.6.3 Simultaneous binding of antibody to PTx bound to fetuin

Fetuin was used as model soluble receptor to detect antibodies bound to a PTx-fetuin complex using a method adapted from (Kenimer, Kim et al. 1989). High binding ELISA plates (Costar) were coated with 50 µL/well of 10 µg/mL fetuin (Sigma) at in PBS. After overnight incubation at 4˚C, the plates were blocked with 50 µL/well blocking buffer (PBS + 5% BSA) at room temperature for 1 hour. Plates were washed three times in wash buffer (PBS, + 0.05% Tween-20) after each incubation step. Serially diluted PTx was then added to the fetuin coat and incubated further overnight. Antibodies at a concentration of 1 µg/ml antibody were then added and incubated for 1 hour at room temperature. Anti-human Fc HRP (Jackson Immunoresearch) was added at a 1:1000 dilution in wash buffer and incubated at room temperature for another hour. Signal was developed using the TMB Substrate Kit (Thermo-Scientific), the reaction stopped with 1N HCl, and the plate read using a SoftMax Pro v5 (Molecular Devices) at 450 nm. All plated volumes were 50 µL/well.

3.2.6.4 Western blot

To detect antibody epitopes on PTx subunits, 0.5 µg PTx was first boiled for 2 minutes in reducing SDS buffer to dissociate PTx into its constituent subunits. PTx was then electrophoresed in a 12% gel, and then transferred to a PVDF membrane at 20V, 4^{o}C overnight. The membranes were blocked with PBS + 5 % milk, washed with PBS-tween, incubated with 25 µg of antibody, and subsequently with HRP-conjugated secondary antibodies. Binding to PTx subunits was detected using a colorimetric TMB substrate kit (Fisher scientific).

3.2.6.5 Expression of antibody epitopes on whole B. pertussis

The expression of anti-PTx epitopes on live *B. pertussis* was determined as for FHA (Chapter 2). Briefly, *B. pertussis* harvested from 3 day cultures grown on Bordet Gengou agar, were resuspended in PBS with 5% glycerol (PBSG), to an OD600/mL of 1 after washing with PBS. The cells were then incubated with 1 µg of each antibody in 100 µL at room temperature. Bound antibodies were detected after washing with a 1:500 dilution of goat anti human Fc Alexa Fluor 647 conjugate (Jackson Immunoresearch). 10 000 events per treatment were collected on a BD SLRII Fortessa flow cytometer. The cell associated fluorescence for a gated population of the cells was determined using the FlowJo_V10 software.

3.2.7 In-vitro neutralization of PTx activities

3.2.7.1 Inhibition of PTx mediated CHO cell clustering

A CHO neutralization assay was used to determine the molar neutralizing concentration of the isolated anti PTx human antibodies to PTx induced CHO cell

clustering. CHO-K1 cells (ATCC, Manassas, VA) were grown in DMEM media supplemented with 10% fetal bovine serum (complete medium) and 1X penicillin/streptomycin and maintained at 37°C with 5% CO2 in a jacketed incubator. Serial two-fold dilutions of each antibody were prepared in complete DMEM media leaving 25 µl in all the wells. Next, 25 µl of a constant concentration of 5 pM PTx was added and the complex incubated for 30 minutes at 37°C. A volume of 100 µl of trypsinized CHO cells at 1.5 x 10^5 cells/ml was then added to the PTx-1B7 solution, and the cells scored 24 hours later. Clustering due to PTx toxicity was scored from 0 to 3 as follows; 0 (no clustering), 1 (a few clusters/equivocal), 2 (positive clustering), 3 (maximum/complete clustering). Concentrations of antibodies were tested to find the molar concentration able to relieve PTx mediated clustering. Up to 500 000 molar excess antibodies were tested for neutralization of PTx mediated CHO cell clustering.

3.2.7.2 Inhibition of PTx binding to fetuin, a model receptor

PTx binds to receptors on host lung resident cells, is internalized and transported to the cytosol to exert its toxic effects. While the receptors for PTx are still unknown, PTx has specificity for sialylated glycoprotein conjugates such as fetuin, transferrin and haptoglobin (Heerze and Armstrong 1990). Using fetuin as a model receptor, the ability of the anti-PTx antibodies to inhibit PTx binding to fetuin in a competition ELISA was investigated. Fetuin was coated overnight at 4^{o}C, at a concentration of 10 µg/ml in PBS. The next day, the plates were blocked with PBS-BSA(5%). Serially diluted antibodies with a constant concentration of 0.79 nM PTx were incubated overnight at 4^{o}C. Bound PTx was detected with a home-made mixture of three murine antibodies to PTx (1B7, 11E6 and 7F2) at 0.1 µg/mL. The mouse antibodies were detected using HRP-conjugated

goat-anti mouse antibodies and the plates developed with TMB. The absorbances were read at 450nm and analyzed using Graphpad prism 5.

3.3 RESULTS

3.3.1 A low frequency of B cells respond to native PTx after aP vaccination

From the antigen specific responses of the individual B cells isolated, only 5 anti-PTx antibodies were identified, compared to the >50 anti-FHA antibodies (Chapter 2). The high number of B cells responding to FHA is consistent with serological data of a strong antibody response to FHA after infection or immunization with whole cell vaccine (Thomas, Ashworth et al. 1989, Berbers, van de Wetering et al. 2013). The low numbers of B cells responding to native PTx may be due to the immunosuppressive nature of PTx, which naturally acts to inhibit the primary antibody response by inhibiting lymphocyte proliferation (Vogel, Klein et al. 1985), thus allowing *B. pertussis* to colonize the host more easily. Additionally, reduced immunogenicity of PTx from chemical detoxification for the aP vaccine formulation may also contribute to the lower numbers of B cells responding to PTx. Since the screening used native PTx, while the vaccine uses chemically detoxified PTx, B cells responding to only native epitopes on PTx may have been preferentially isolated, thus underestimating the overall response to PTx.

3.3.2 Phylogenetic analysis of isolated anti-PTx antibodies

The sequences of the 5 anti-PTx antibodies isolated were analyzed phylogenetically. From an NCBI/IgBLAST sequence search, potential germline

sequences of the heavy and light chains were identified by selecting germline sequences in the IMGT database with the smallest e-value. Within the heavy chain sequences, two of the antibodies utilized the IgH V3, one utilized IgH V1, and another utilized the IgH V4 family. Within the light chain sequences, IgK V1 was used by two of the antibodies, and the other three used the IgK V3 family. Only the D1 and D3 gene families were used by the heavy chains, with D3 being more predominant. The heavy chain also utilized the J4-6 family while the light chain used J1-4 (Figure 3.2). Although a very small subset of anti-PTx antibodies were isolated, the pairing of the VDJ also showed paring preferences among the gene segments, with the 5' D segments showing increased frequency of pairing with the 3'- most J segments as previously suggested (Volpe and Kepler 2008). At the gene and allele level, we observed unique families were utilized by the V gene segments of the heavy and light chains, as well as the D segment of the heavy chains. The 6*02 gene and allele combination was utilized twice with the J gene segments of the heavy chain, while the rest of the three antibodies utilized other unique gene and alleles. For the light chain, the 4*01 gene and allele combination was utilized by three of the antibodies, while the other two, utilized other unique genes and allele combinations (Figure 3.3). The five isolated anti-PTx antibodies therefore appeared to each descend from unique germline sequences. Overall, the number of mutations in the amino acids of the isolated antibodies compared to the putative parental germline sequences ranged from 11 to 24 in the V gene segment. The D and J regions on the other hand, had few changes with 0 to 2 mutations compared to the putative germline sequences (Table 3.2). Combining the full V_H and V_L sequences to form a phylogenetic tree also indicated the antibodies descended from unique germline families with A12 and E12 potentially descending from (Figure 3.4).

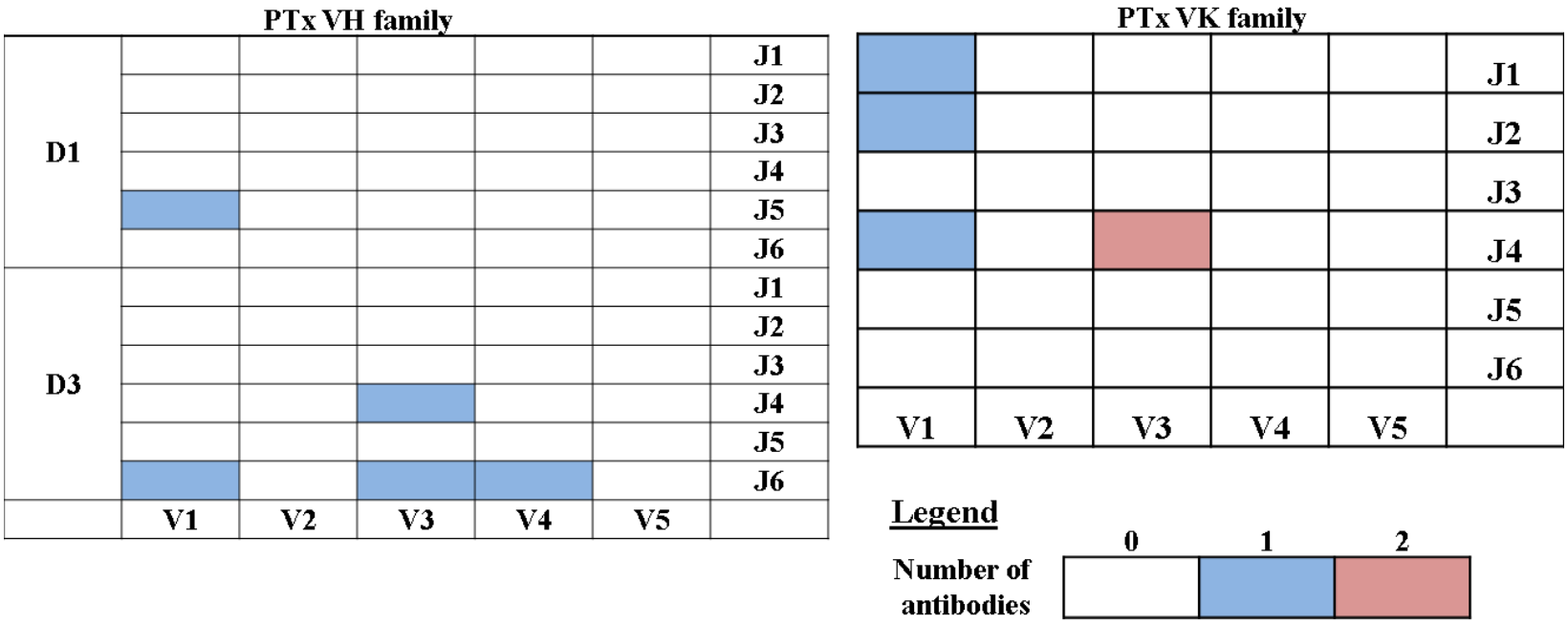


Figure 3.2: VDJ germline usage of the heavy and light chains of the five anti-PTx antibodies.

Each panel represents the number of unique VDJ patterns existing in the heavy and light chains of the five isolated anti-PTx antibody sequences. The color legend shows the number of antibodies with the corresponding VDJ genes. The white space represents absent VDJ recombination types in the repertoire.

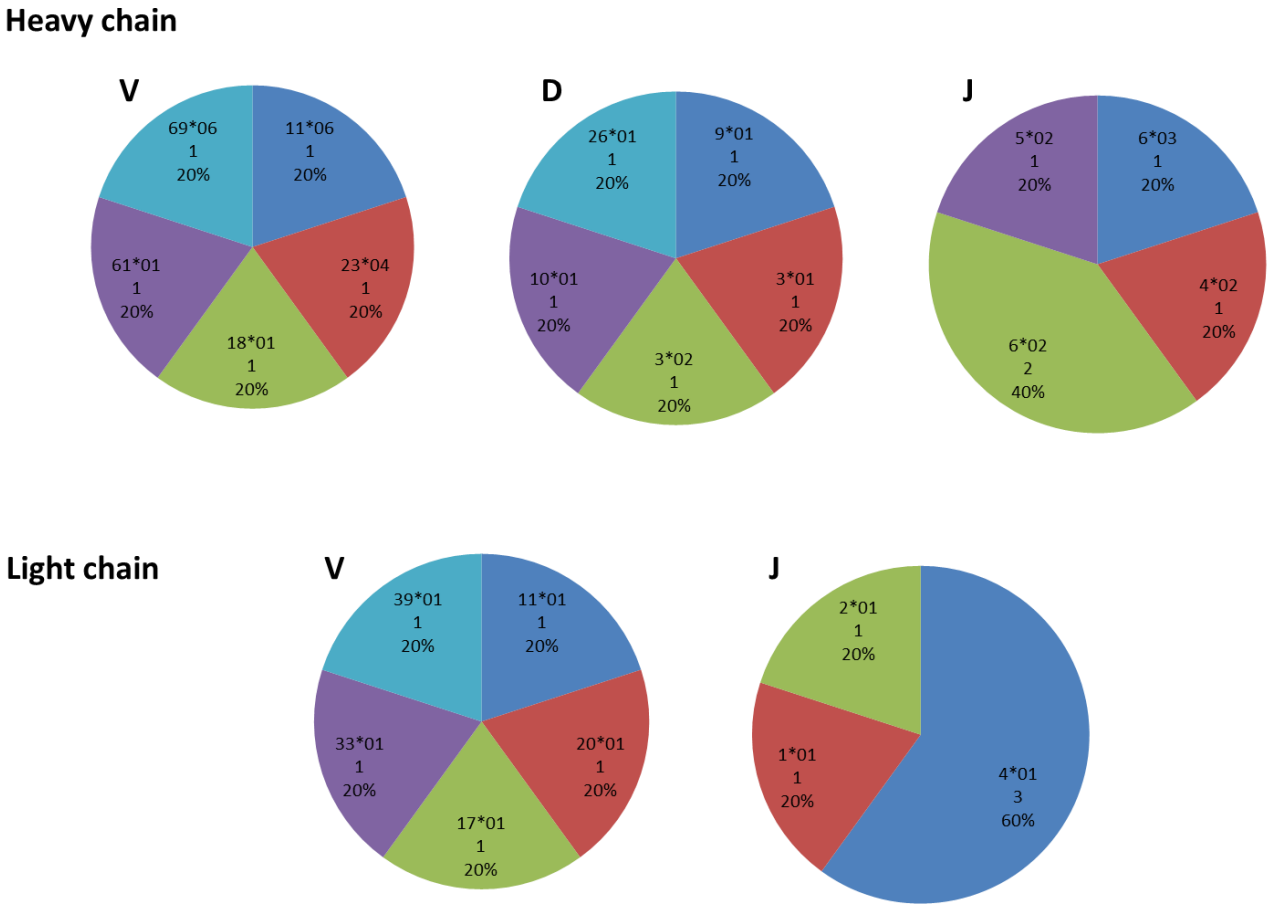


Figure 3.3: Gene and allele family usage of the isolated anti-PTx antibodies.

Genes and alleles are shown in the pie chart in the a*b format. The number and percentage of antibodies represented is indicated below each gene and allele used.

Table 3.2: Anti-PTx antibody gene diversity

Antibody	Heavy/Light	V	D	J	Mismatches
A12.K	Light	IGKV3-11*01	-	IGKJ4*01	11,1
E12.K	Light	IGKV3-20*01	-	IGKJ4*01	22,2
A8.K	Light	IGKV1-17*01	-	IGKJ1*01	12,2
H5. K	Light	IGKV1D-33*01,IGKV1-33*01	-	IGKJ4*01	11,0
D8.K	Light	IGKV1-39*01,IGKV1D-39*01	-	IGKJ2*01	11,2
A12.VH	Heavy	IGHV3-11*06	IGHD3-9*01	IGHJ6*03	11,0,2
E12.VH	Heavy	IGHV3-23*04	IGHD3-3*01	IGHJ4*02	12,0,1
A8.VH	Heavy	IGHV1-18*01	IGHD3-3*02	IGHJ6*02	19,0,1
H5.VH	Heavy	IGHV4-61*01	IGHD3-10*01	IGHJ6*02	24,0,2
D8.VH	Heavy	IGHV1-69*06, IGHV1-69*14	IGHD1-26*01	IGHJ5*02	13,0,0

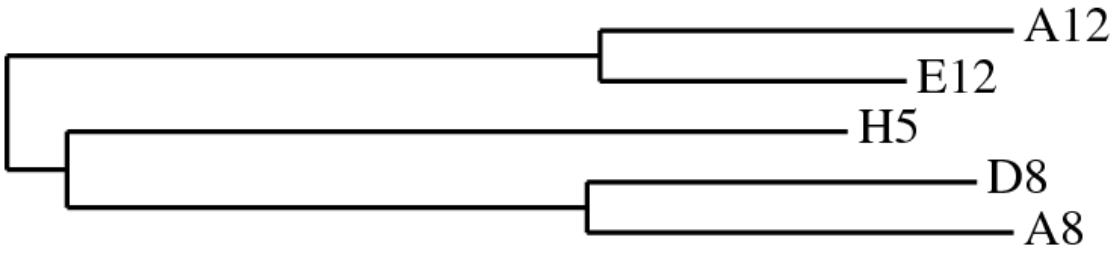


Figure 3.4: Phylogenetic tree of isolated anti-FHA antibodies.

The phylogenetic tree was constructed from the VH and Vk gene sequences of the isolated anti-PTx antibodies using phylogeny.fr. The tree shows the diversity of germline families of the antibodies. The length of a branch is proportional to the number of mutations in the antibody compared to a putative germline.

3.3.3 Antibody expression and stability

The isolated human anti-PTx antibodies were expressed in adherent CHO-K1 cells using Lipofectamine 2000 (Life Technologies) following the manufacturer's instructions. Expression levels of the anti-PTx antibodies in 6 well culture flasks ranged 0.01 to 1.32 µg/mL (Table 2). A12 and D8 had the lowest expression levels of 0.26 ± 0.04 and 0.01 ± 0.003 µg/ml respectively. The two antibodies were however the most interesting in terms of neutralization of PTx binding to a model receptor, fetuin (Section 3.3.6). The purified antibodies (Figure 3.5) were stable with melting temperatures ranging from 54.41 ± 0.03 to 74.29 ^{o}C (Table 3.3). For A12, A8 and H5 which exhibited complex melting paths, the $T_{average}$ and T_{onset} temperatures as defined were determined. The complex unfolding of A12, A8 and H5 may reflect differences in stability of the variable regions. The D8 antibody which was potently neutralizing in the in-vitro CHO clustering assay, exhibited the least stable melting temperature. From a therapeutic

standpoint, the A12 and D8 antibodies may therefore need further engineering for increased expression levels and stability (D8).

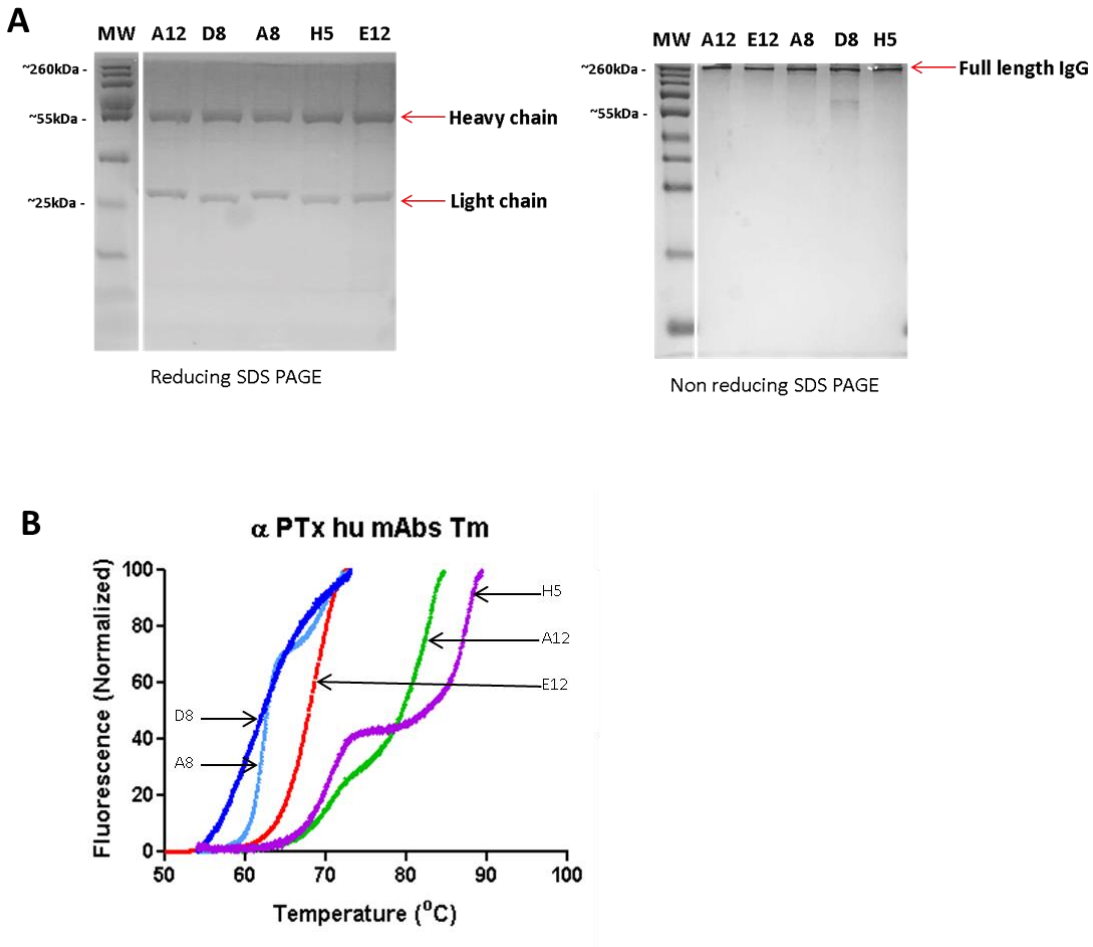


Figure 3.5: Purity and stability of purified anti-PTx antibodies.

A) SDS-PAGE of purified anti-PTx antibodies. Each well contained 1 µg of protein boiled for two minutes under reducing or non-reducing conditions. **B)** Melting temperature profile of purified anti-PTx antibodies. Each antibody at a concentration of 100 µg/ml, was heated up to 90°C in PBS with the protein thermal shift dye. The T_m was determined from fitting the data to a sigmoidal four parameter logistic (4PL) non-linear regression model after normalizing the data to the maximum and minimum fluorescence. Data after the maximum fluorescence are not shown.

Table 3.3: Expression levels and stability of anti-PTx antibodies

Antibody ID	Expression level (µg/ml)	Melting temperature, T_m (^oC)
A12	0.26 ± 0.04	69.76^, 8.47#
E12	1.26 ± 0.05	64.07 ± 0.01
A8	1.32 ± 0.13	63.68^, 7.25#
D8	0.01 ± 0.003	54.41 ± 0.03
H5	0.9 ± 0.14	74.29^, 8.95#

^ - $T_{average}$: Average of the maximum and minimum temperature of unfolding.
# - T_{onset}: 10% of mean maximum temperature for the unfolding curve.

3.3.4 Anti-PTx antibodies after aP vaccination recognize unique epitopes with high affinity

The isolated anti-PTx antibodies exhibited strongly bound PTx as well as genetically modified PTx (PTg) with high affinities <0.5 nM (Figure 3.6, Table 3.4). PTg has two amino acid substitutions (R9K and E129G) in the enzymatically active S1 subunit and has been proposed as a replacement for chemically detoxified PTx in current acellular vaccines due to its reduced toxicity but high immunogenicity (Nencioni, Pizza et al. 1990). The binding affinities of D8 and H5 to PTx or PTg appeared to remain intact without significant changes. Since PTg has the amino acid changes in the S1 subunit, the lack of change in the affinities of these two antibodies to PTg is consistent with their epitopes being primarily on the B-oligomer (epitope specificities discussed in next paragraph). On the other hand, the affinity of E12 to PTg appeared to be slightly improved, while the affinities of A12 and A8 were slightly reduced about 2-fold

compared to native PTx. The epitope of A8 appears to lie primarily on the S1 subunit, however the slightly reduced affinity of A8 binding to PTg, suggests the amino acid changes in PTg may slightly perturb the A8 binding site. The A12 epitope, although Previous reports of antibody binding to PTx or PTg also observed affinities which either improved or remained intact (Ibsen 1996). For the antibodies with improved affinities, the author suggested the amino acid changes may make the epitope more accessible leading to the enhanced binding.

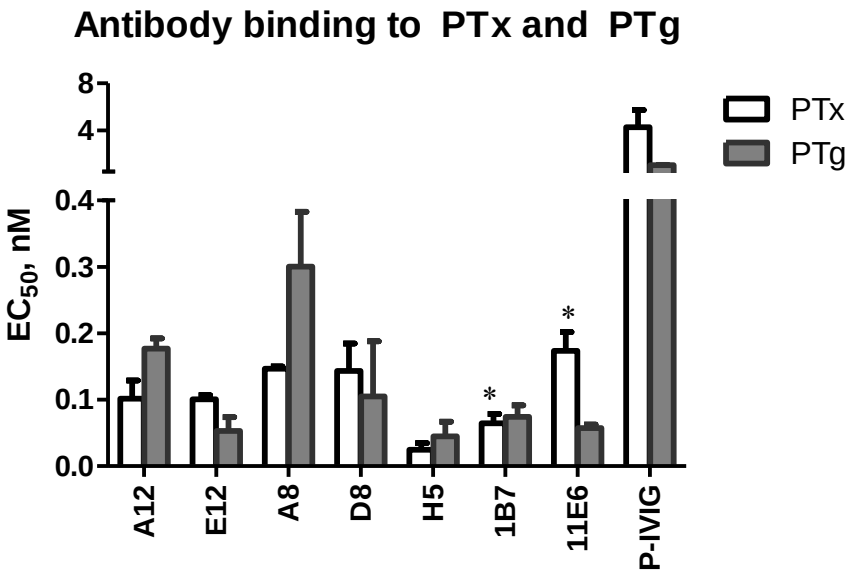


Figure 3.6: Binding affinity of the isolated anti-PTx antibodies to native PTx and genetically modified PTx (PTg).

Serially diluted antibodies were added to purified PTx coated at 1 µg/mL. Antibody binding was detected with HRP conjugated anti-human Fc antibodies and TMB substrate development. The estimated binding affinity (EC50) was determined after fitting the binding curves to the four parameter logistic (4PL) model using GraphPad prism 5. The ELISAs were repeated at least twice in duplicate. Error bars represent the standard error from replicate experiments. The murine 1B7 and 11E6 were used as controls.

* Humanized versions of 1B7 or 11E6

The epitope specificities of the isolated antibodies were then assessed by subunit ELISAs. Two of the anti-PTx mAbs, A8 and E12, bound a recombinant version of the PTx S1-subunit, S1-220, with affinities of 0.05 ±0.03 and 0.02 ± 0.006 nM respectively (Figure 3.7 A, Table 3.4). The full length PTx S1 subunit has 235 amino acid residues, but is more difficult to express due to the presence of a hydrophobic C-terminal tail (Sutherland and Maynard 2009). A PTx S1-219 truncated version maintains the ADP-ribosyltransferase catalytic activity of the full length PTx S1 (Krueger and Barbieri 1994), while a PTx S1-220 version has better expression levels (Sutherland and Maynard 2009). The PTx S1-220 recombinant version has also been shown previously to maintain the binding affinity of a previously characterized anti S1-antibody, 1B7 (Sutherland and Maynard 2009). We observed high binding of A8 and E12 to the A subunit similar to binding the holotoxin, with affinities of 0.05 ± 0.03 and 0.02 ± 0.006 nM. The results indicate the PTx S1-220 truncation does not affect the E12 and A8 binding, and their epitopes lie primarily on the S1 subunit. Two of the anti-PTx antibodies, D8 and H5, also bound the B-subunit of PTx with affinities of 0.78 ± 0.22 and 0.11 ± 0.05 nM (Figure 3.7 B, Table 3.4). D8 and H5 therefore maintained high sub nM affinity to the B-oligomer suggesting their epitopes lie primarily on the B-pentamer. However, the approximately 7 and 5 fold decrease in affinities of D8 and H5 respectively for binding to the B oligomer indicated their epitopes may be stabilized by the presence of the S1 subunit, or they may have weak interactions with the S1 subunit. The binding affinities of the antibodies with specificity for the S1 subunit or the B-oligomer were comparable to the affinities of the previously characterized S1 or B-oligomer antibodies, 1B7 and 11E6 respectively. For the A12 antibody however, we observed loss of binding to either the A or B subunit in

the subunit ELISAs, suggesting the A12 epitope may span an interface on the A and B subunits, or it may be stabilized by the presence of the other subunit.

The ability of the antibodies to detect linear epitopes on the PTx subunits using western blots was also used to further analyze the epitope specificity of the antibodies. PTx dissociates into its component five subunits under the denaturing conditions of SDS-PAGE. Western blots confirmed A8 and E12 recognize an ~26 kDa fragment, most likely the A subunit of PTx (Figure 3.7 C). E12 and A8 also recognized a smaller band less than 25kDa. A previously characterized antibody, 11E6, which binds the S2 and S3 subunits of 21.93 kDa and 21.83 kDa respectively, did not recognize the smaller fragment, suggesting it is more likely a processed form of the S1 subunit. In addition, another previously characterized antibody, 1B7, which binds the S1 subunit of ~26 kDa, recognized the same two bands in the western blot, suggesting the smaller band may be a processed form of the S1 subunit. Binding of A12, D8 and H5 antibodies to the PTx subunits was not detectable on the western blot. The lack of binding of D8 and H5 is likely due to insensitivity of the technique to detect subunits of the B oligomer. The difficulty of western blotting for detecting antibodies to the PTx-B oligomer has been previously demonstrated (Lynn, Burnette et al. 1994). Higher concentrations of PTx in western blots which increases the sensitivity of the detection of the B oligomer (Lynn, Burnette et al. 1994) were however not tested due to cost limitations. A12 showed a loss of binding to either the A or B subunit in the subunit ELISAs, suggesting the A12 epitope may span an interface on the A and B subunits, or it may be stabilized by the presence of the other subunit. The lack of A12 binding to the S1 subunit in the western blot also supports the indication that its epitope may be conformational.

Table 3.4: Binding affinities of the isolated anti-PTx antibodies to the native PTx or its subunits, and to genetically modified PTx (PTg).

Antibody	**PTx EC_{50}, nM ± SE**	**PTg EC_{50}, nM ± SE**	**PTx S1-220 EC_{50}, nM ± SE**	**PTx B-oligomer EC_{50}, nM ± SE**
A12	0.10 ± 0.03	0.18 ± 0.02	ND	ND
E12	0.13 ± 0.006	0.05 ± 0.02	0.05 ± 0.03	ND
A8	0.15 ± 0.003	0.3 ± 0.08	0.02 ± 0.006	ND
D8	0.14 ± 0.04	0.11 ± 0.08	ND	0.78 ± 0.22
H5	0.02 ± 0.01	0.06± 0.03	ND	0.11 ± 0.05
Mouse 1B7	0.06 ± 0.01*	0.07 ± 0.03	0.008 ± 0.002*	ND
Mouse 11E6	0.17 ± 0.03*	0.06 ± 0.007	ND	1.18 ± 0.19
P-IVIG	4.26 ± 1.27	1.05 ± 0.04	9.39 ± 1.67	ND

PTg- genetically modified PTx with the 9K/129G amino acid substitutions in the S1 subunit (Burnette, Cieplak et al. 1988, Pizza, Covacci et al. 1989).
PTx S1-220 – A truncated variant of the PTx S1 subunit (Sutherland and Maynard 2009).
* Humanized antibody (Nguyen et al. In review).

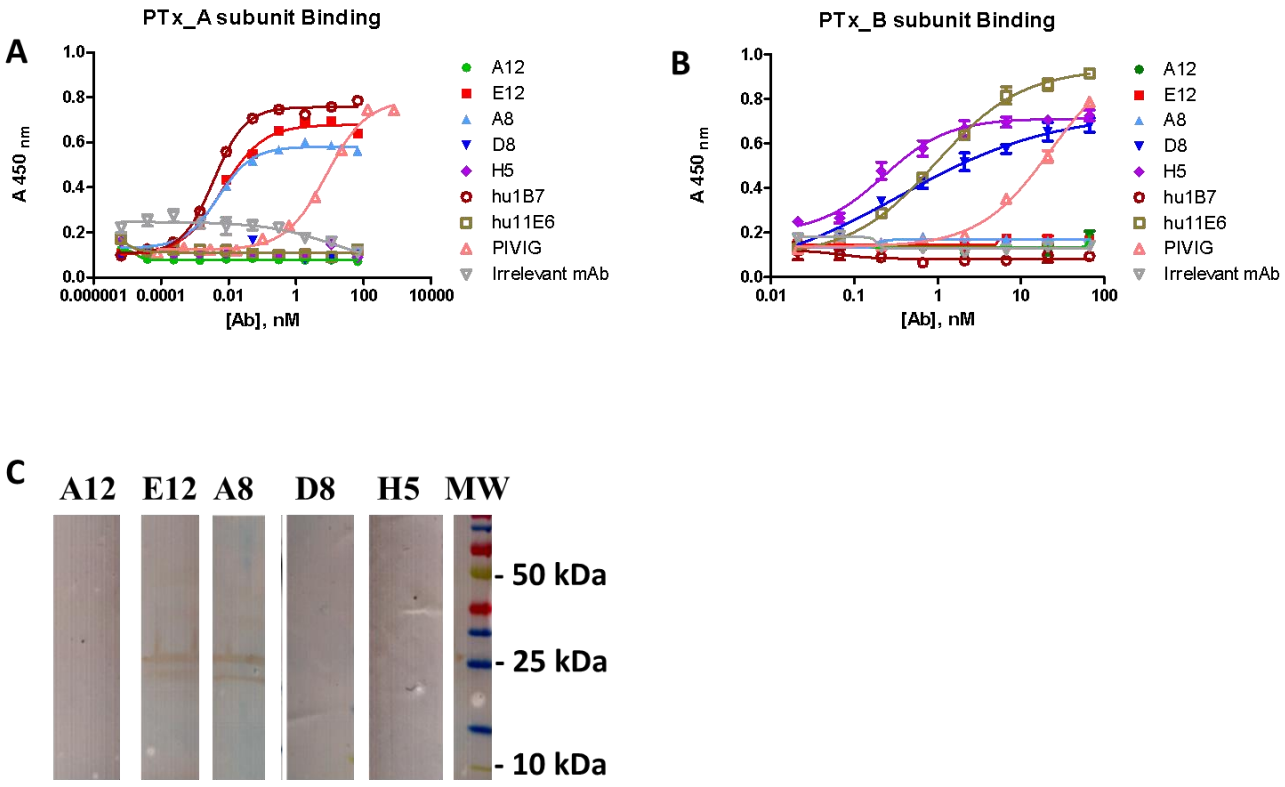


Figure 3.7: Epitope specificity of the isolated anti-PTx mAbs.

Representative ELISA plots for antibody binding to the **A)** PTx-A and **B)** PTx B subunits. A truncated form of the PTx-A subunit, PTx S1-220, or the B-oligomer was coated in ELISA plates. Serially diluted antibodies were added to the plates after blocking and detected with goat-anti-human Fc HRP antibody. **C)** Immunoblots of the antibodies binding to PTx after electrophoresis under reducing and denaturing conditions.

The epitopes of the isolated anti-PTx antibodies were further analyzed by comparison with two previously characterized neutralizing antibodies 1B7 and 11E6. The epitope of 1B7 was previously shown to be primarily on the A subunit of PTx (Sato, Sato et al. 1987, Sato and Sato 1990), and recent analysis suggest it may also span the B subunit (Sutherland and Maynard 2009). The 11E6 epitope on the other hand, is on the S2 and S3 subunits in the B pentamer of PTx (Sato, Sato et al. 1987, Rambow-Larsen and Weiss 2004). The ability of the anti-PTx antibodies to inhibit 1B7 or 11E6 binding to PTx was then assessed in a competition ELISA. In the 1B7 competition, A12 weakly inhibited 1B7 binding to PTx with an IC_{50} of 0.39 ± 0.15 μM (Figure 3.8 A). The other four antibodies however did not appear to inhibit 1B7 binding to PTx. The ability of A12 to weakly compete with 1B7 for binding to PTx suggests an overlap in their epitopes. Alternatively binding of one may affect weakly affect the conformation needed for binding of the other. In the 11E6 competition, none of the five isolated antibodies inhibited binding to PTx, suggesting they bind unique epitopes not overlapping with that of 11E6 (Figure 3.8 B).

Competition between the five antibodies for binding to PTx was also assessed using biotinylated forms of the antibodies incubated with unlabeled antibody. Biotinylated A8 inhibited unlabeled A8 and E12 binding to PTx (Table 3.5), indicating A8 and E12 may share a similar epitope. The rest of the antibodies however did not inhibit each other's binding to PTx, suggesting they bind unique epitopes. Competition between biotinylated D8 and the rest of the antibodies was not tested due to low express yields and ineffective biotinylation of D8. Further studies are ongoing to define the epitopes of the S1-subunit antibodies using yeast display libraries with mutations in the S1 subunit.

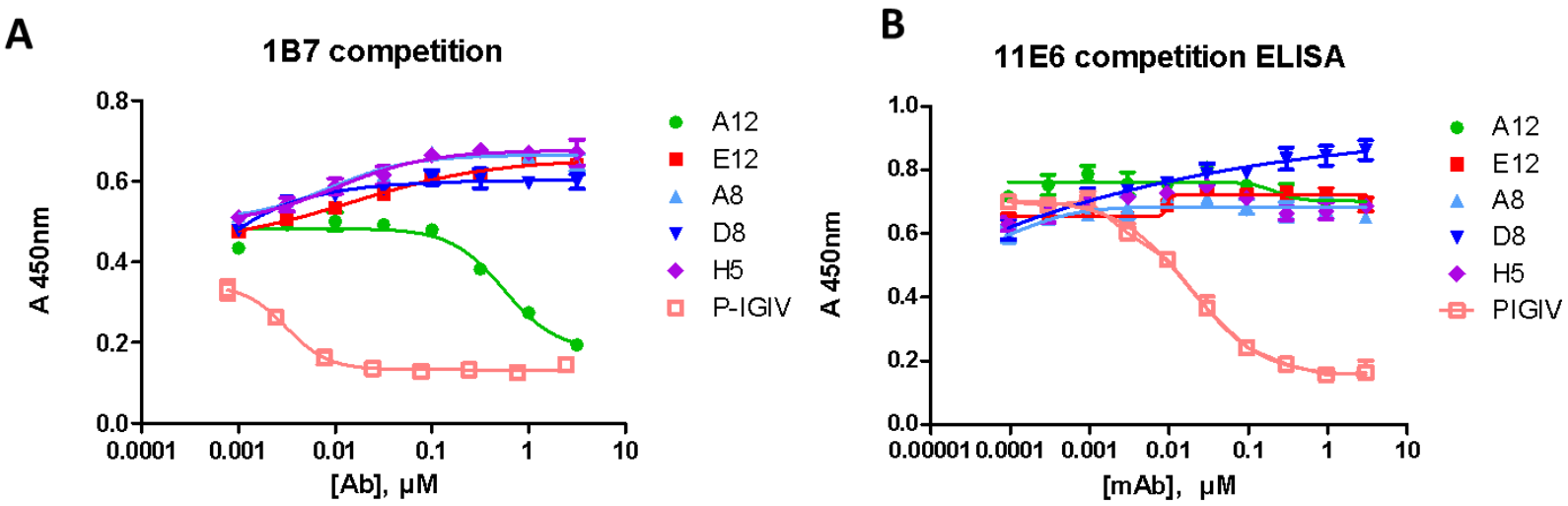


Figure 3.8: Representative competition ELISAs for epitope specificity of the isolated antibodies with the previously characterized antibodies, 1B7 and 11E6.

A constant concentration of the murine **A)** 1B7 or **B)** 11E6 was incubated with serially diluted human antibodies. The antibody mixtures were then added to PTx coated at 1 µg/ml. Bound murine 1B7 or 11E6 was detected using a polyclonal goat-anti-mouse HRP antibody. The inhibition curves were fit to the 4PL non-linear model using GraphPad prism 5 to estimate the concentration needed to achieve 50% inhibition (IC_{50}).

Table 3.5: Competition of the isolated anti-PTx antibodies for binding to similar epitopes on PTx.

Antibody	Human A12	Human E12	Human A8	Human D8	Human H5	Humanized 1B7	Humanized 11E6
Biotin A12	+	-	-	-	-	+	-
Biotin E12	-	+	+	-	-	-	-
Biotin A8	-	+	+	-	-	+	-
Biotin D8	-	-	-	+	-	-	-
Biotin H5	-	-	-	-	+	-	-
Mouse 1B7	+	-	-	-	-	+	-
Mouse 11E6	-	-	-	-	-	-	+

3.3.5 D8 anti-PTx antibody potently neutralizes PTx mediated CHO cell clustering

In the presence of PTx, CHO cells grow in a clustered morphology (Hewlett, Sauer et al. 1983), due to the toxicity of the A-subunit catalyzed ADP-ribosyltransferase activity (Burns, Kenimer et al. 1987). In the presence of neutralizing antibodies, the clustered CHO cell morphology is rescued to the normal elongated non-clustered phenotype [25]. Neutralization of CHO cell clustering is therefore a simple *in-vitro* assay commonly used to assess the *in-vivo* toxicity of the A-subunit. Of the five antibodies, only D8 potently neutralized PTx mediated CHO cell toxicity, approximately 10-fold better than the previously characterized neutralizing anti-PTx antibodies, 1B7 and 11E6 (Sato, Sato et al. 1987) or the polyclonal anti-PTx preparation (P-IVIG) (Bruss, Malley et al. 1999) (Figure 3.9, Table 3.6).

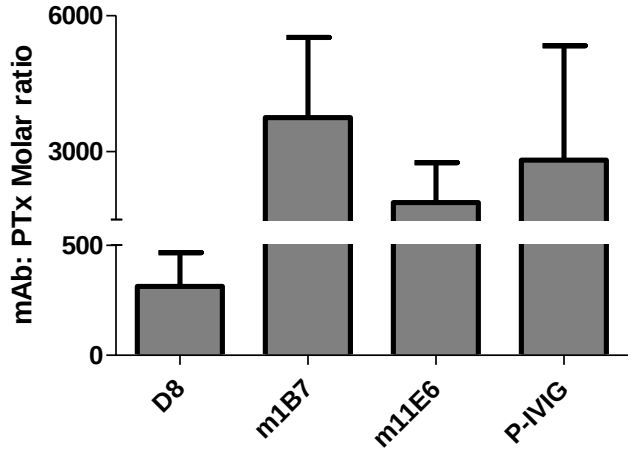


Figure 3.9: Molar neutralization of PTx mediated CHO cell clustering.

Serial dilutions of the antibodies were pre-incubated with a constant concentration of PTx. The PTx-antibody complexes were then added to CHO cells in 96 wells and clustering scored 24 hours later. The molar concentration of antibody needed to neutralize PTx mediate CHO cell clustering was determined from the concentration amount of antibody required to change the clustering score from 1 to 0.

Table 3.6: Molar neutralization of PTx mediated CHO cell clustering.

Antibody	Neutralizing ratio (molar [Ab: PTx])
A12	> 500 000
E12	~100 000
A8	ND
D8	313 ± 77
H5	~100 000
m1B7	3750 ± 1250
m11E6	1875 ± 625
P-IVIG	2813 ±1263

ND – not determined.

3.3.6 A12 and D8 anti-PTx antibodies inhibit PTx binding to a PTx model receptor, fetuin

While the *in vivo* target receptors for PTx are unknown, *in vitro* it promiscuously binds proteins with terminal sialylated oligosaccharide sequences. These are found on glycoproteins such as transferrin, haptoglobin and fetuin, making these useful model receptors for *in vitro* studies, including co-crystallization (Heerze and Armstrong 1990). The ability of the anti-PTx antibodies to inhibit PTx-receptor interactions was therefore tested in an inhibition assay using fetuin as a model receptor. The IC_{50} values were determined from fitting the inhibition curves to the four parameter logistic (4PL) model in GraphPad prism 5. The potently neutralizing antibody in the CHO clustering assay, D8, was able to inhibit PTx binding to fetuin with an IC_{50} of 1.31 ± 0.54 nM. The neutralizing antibody A12 which showed almost no neutralization in the CHO assay however also showed inhibition of PTx binding to fetuin with an IC_{50} of 1.86 ± 0.31 nM.

A12 and D8 were however approximately 10-fold less potent at inhibiting PTx binding to fetuin compared to the previously characterized B subunit antibody 11E6. A12 and D8 were also approximately 5-fold less potent at inhibiting PTx binding to fetuin compared to the previously characterized A subunit antibody 1B7 (Figure 3.10, Table 3.7). A12 and D8 were however approximately 7-fold more potent than the polyclonal pertussis immunoglobulin (P-IVIG) (Table 3.7). The other three antibodies, A8, E12 and H5 also appeared to weakly inhibit PTx binding to fetuin, however the inhibition was not significant compared to an irrelevant antibody. IC_{50} values were therefore not determined for these antibodies.

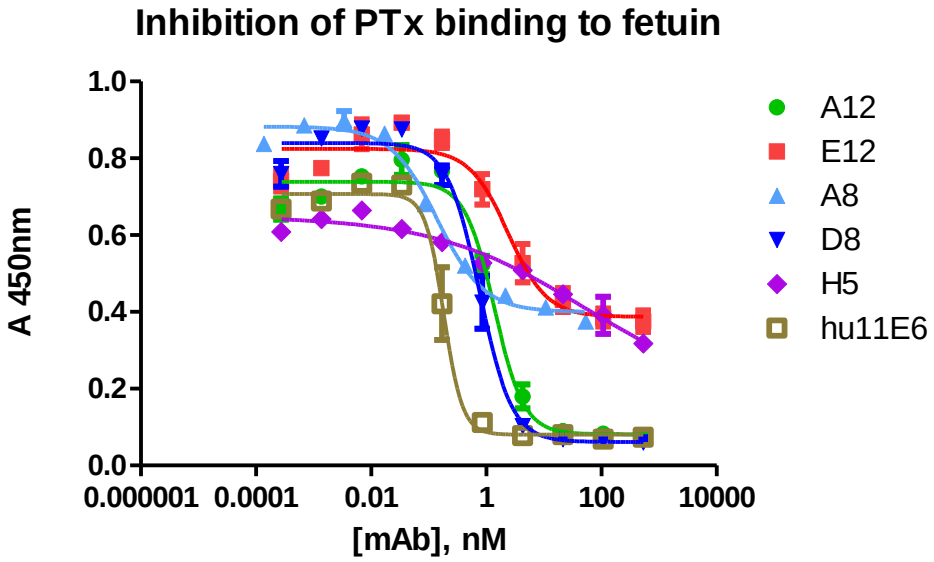


Figure 3.10: Competition ELISA for inhibition of PTx binding to soluble fetuin.

Serially antibodies with a constant concentration of 0.79 nM PTx were added to ELISA plates with fetuin coated at 10 µg/ml. Bound PTx was detected using a mixture of three murine anti-PTx antibodies at 0.1 µg/ml. Inhibition of PTx binding by E12 A8 and H5 were not significant compared to an irrelevant antibody. IC_{50}s were determined from fitting the curves to the 4PL model using GraphPad prism 5.

Table 3.7: IC_{50} values for inhibition of PTx binding to fetuin.

Antibody	**IC_{50}, nM ± SE**
A12	1.86 ± 0.31
E12	ND
A8	ND
D8	1.31 ± 0.54
H5	ND
1B7*	0.26 ± 0.05
11E6*	0.11 ± 0.07
P-IVIG	13.87 ± 3.1

ND – not determined.
* – humanized version

Fetuin and other glycoconjugates with terminally sialylated oligosaccharides are thought to closely resemble PTx receptors on host cells. The results from the PTx mediated CHO clustering assay and fetuin inhibition suggests that A12 may block PTx interactions with receptors containing glycoconjugates resembling fetuin. Fetuin has also been indicated to be unable to block PTx intoxication of CHO cells (Armstrong and Peppler 1987, Tuomanen, Towbin et al. 1988), further suggesting that the PTx receptors on CHO cells may not be representative of PTx receptors in humans. Other studies have also indicated that the CHO cell clustering assay does not correlate with other *in-vivo* assays for detecting residual PTx activity in detoxified aP vaccines (Kataoka, Toyoizumi et al. 2002). In addition, the murine monoclonal antibody, 1B7, with a high CHO cell neutralization titer has been found to be potently neutralizing *in-vivo* (Sato and Sato 1990). The CHO cell neutralization assay alone may therefore be limited in identifying

and fully assessing *in-vitro* efficacy of the isolated anti-PTx antibodies. Addition of other *in-vitro* assays such as the ELISA for inhibition of PTx binding to model receptors may be more practical.

To assess whether the inhibition PTx binding to fetuin by A12 and D8 was due to antibody binding at the fetuin binding site, we assessed whether the antibodies could simultaneously bind to PTx already bound to fetuin. We found that A12 and D8 were competent to bind to PTx already bound to fetuin (Figure 3.11). E12 and A8 almost completely lost binding to PTx already bound to fetuin, while H5 exhibited markedly reduced binding. The polyclonal P-IVIG was also competent to bind PTx already bound to fetuin. This result suggests the primary epitopes recognized by A12 and D8 remain accessible when PTx is bound to fetuin. Alternatively, A12 and D8 may have secondary binding sites which are not affected after PTx is bound to fetuin. E12, A8 and H5 on the other hand which exhibited loss of binding to PTx bound to fetuin, may either bind close to the fetuin binding site, or the conformation of PTx after binding fetuin may be altered such that it inhibits their binding.

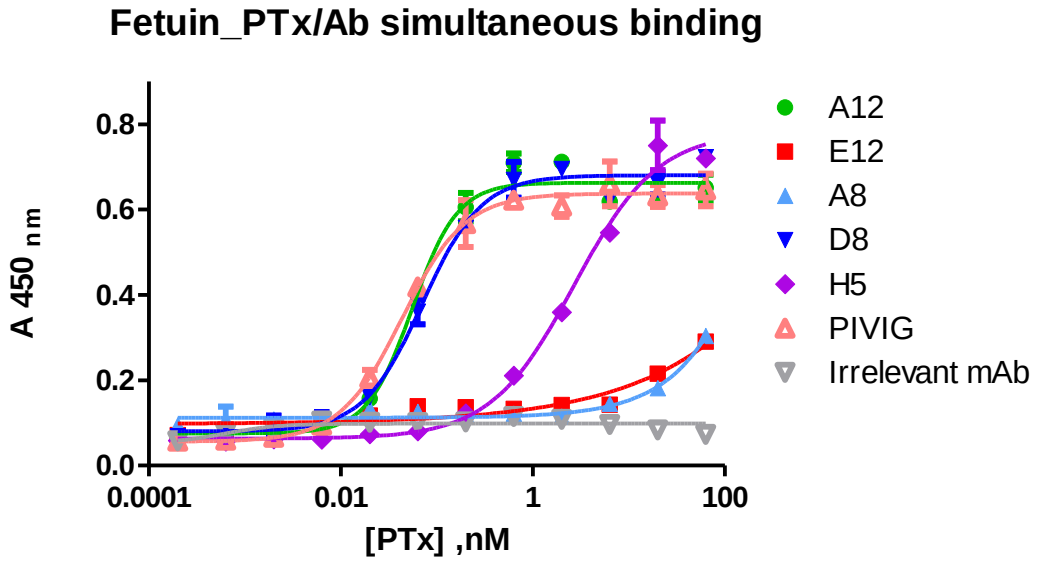


Figure 3.11: Simultaneous binding of antibodies to PTx bound to fetuin receptor.

Serially diluted PTx was added to a coat of fetuin, after which a constant concentration of antibodies was added. Bound antibodies were detected using secondary goat-anti-mouse HRP antibody. Error bars indicated are the standard error of the mean.

3.3.7 Anti-PTx antibodies bind whole *B. pertussis*

PTx is a secreted *B. pertussis* toxin, but may be transiently associated with the bacterial surface during secretion (Farizo, Huang et al. 2000, Farizo, Fiddner et al. 2002). Binding to the whole bacteria was assessed by incubating the antibodies with live *B. pertussis,* followed by washing, signal development with a fluorescent secondary antibody conjugate and analysis by flow cytometry. Binding of the anti-PTx antibodies to live *B. pertussis* was detected (Figure 3.12). There was lower binding of the anti-PTx antibodies to *B. pertussis* compared to the polyclonal P-IVIG and a control anti-FHA antibody, H2. Expression of anti-PTx antibody epitopes on the whole bacteria suggests some molecules of PTx could possibly serve as targets for antibody mediated bactericidal

activity since antibodies against surface exposed antigens on *B. pertussis* are reported to promote phagocytosis (Hellwig, Rodriguez et al. 2003, Aase, Herstad et al. 2011). Moreover, PTx has also been implicated in bacterial adherence *in-vivo* (Tuomanen and Weiss 1985, van't Wout, Burnette et al. 1992) , as such, anti-PTx antibodies recognizing surface bound PTx may be able to block relevant interactions needed for bacterial adherence. Further studies would be needed to evaluate the role of anti-PTx antibodies in bacterial adherence.

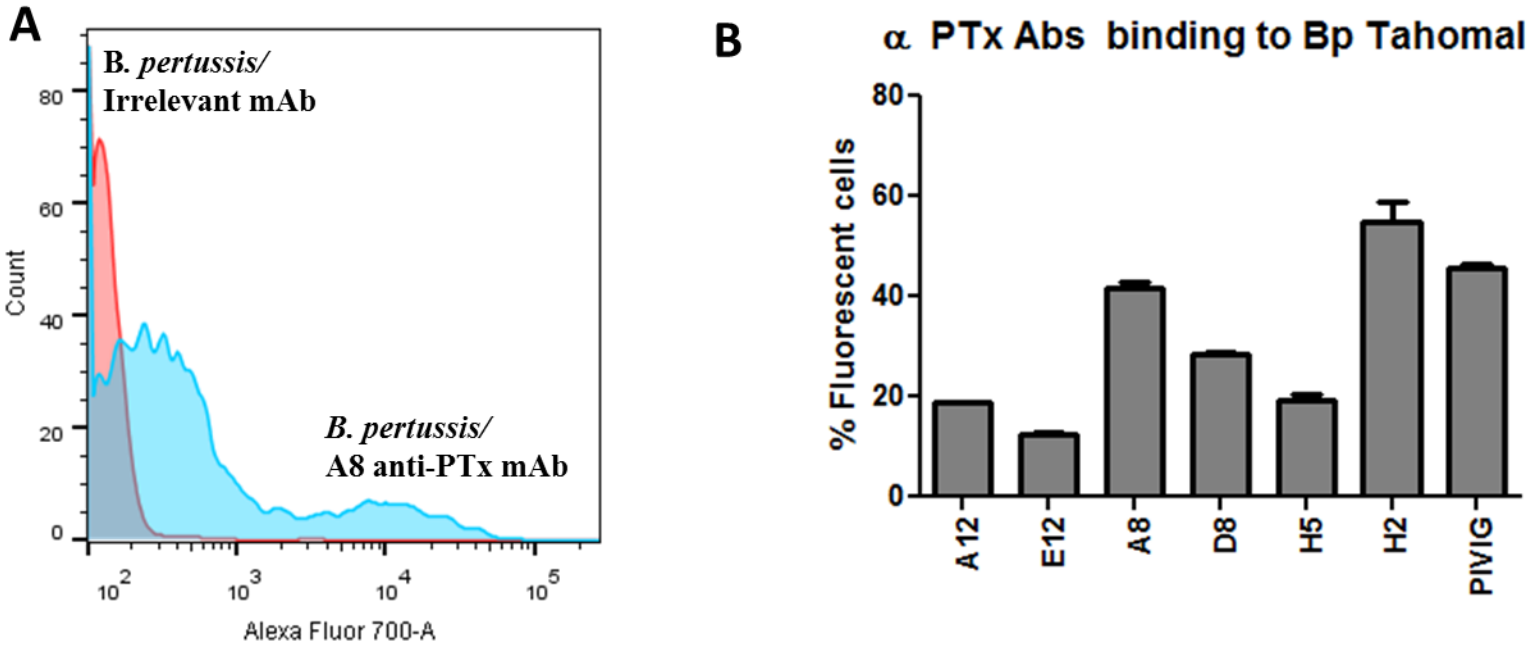


Figure 3.12: Detection of anti-PTx antibody epitopes on *B. pertussis* TahomaI cell surface.

A) Representative FACS plot of antibody binding to whole *B. pertussis* cells. *B. pertussis* cells grown on Bordet Gengou plates were scraped and resuspended to 1 OD_{600}/mL. The cells were washed and incubated with 1 µg of the antibodies in PBS with 5% glycerol. Bound antibodies were detected with fluorescently labeled anti-human Alexa Fuor 647 secondary antibody and analyzed by flow cytometry. **B)** Percentages of fluorescent *Bordetella* cells after labeling with the anti-PTx antibodies. Using the FlowJo_V10 software, % fluorescent cells were determined for a gated population of the cells using unlabeled *B. pertussis* as a negative control. Data represent the average of two independent experiments. The error bars represent the standard error of the mean.

3.4 DISCUSSION

In this study, 5 antibodies isolated from single plasmablast cells responding to PTx after adult booster aP vaccination were characterized. Of the numerous virulence factors produced by *B. pertussis*, PTx is a major antigen which may mediate most of the symptoms associated with severe pertussis infection (Pittman 1984, Weiss, Hewlett et al. 1985) and induces a humoral response sufficient for protection (Granstrom, Granstrom et al. 1985). High levels of neutralizing anti-PTx antibodies correlate with protection against severe disease in humans (Cherry, Gornbein et al. 1998, Storsaeter, Hallander et al. 1998, Storsaeter, Hallander et al. 2003), and neutralizing antibodies administered passively are able to alleviate and even reverse disease symptoms in mice (Sato, Sato et al. 1987).

The lower frequency of anti-PTx antibodies isolated compared to anti-FHA antibodies (Chapter 2) is consistent with a lower anti-PTx serum response after aP vaccination (Berbers, van de Wetering et al. 2013). A ten-fold difference in the frequency of anti-PTx antibodies may be the result of the immunosuppression due to residual active in the vaccine, reduced immunogenicity of PTx in the vaccine, or the presence of memory B cells biasing new responses. PTx in aP vaccines are detoxified by chemical modification, a process which can change the physical and immunological properties of the toxin (Nencioni, Volpini et al. 1991). While natural infection induces high titers of antibodies recognizing native or genetically modified PTx, acellular vaccines produce higher titers of antibodies recognizing detoxified PTx (Sutherland, Chang et al. 2011). The lower anti-PTx responses observed may possibly be because of reduced immunogenicity of PTx from chemical detoxification processes and that only antibodies recognizing only the native conformation of PTx were isolated. Genetically modified PTx (PTg) with the 9K/129G amino acid substitutions in the catalytic S1 subunit of PTx has

reduced toxicity (Nencioni, Pizza et al. 1990), therefore requires less chemical treatment for stabilization, and is therefore a viable alternative to chemically detoxified PTx in acellular pertussis vaccines.

In addition to inactivating neutrophils and macrophages, PTx confers an additional colonization advantage for *B. pertussis* by suppressing antibody responses to resist antibody mediated clearance (Vogel, Klein et al. 1985). Proteomic analysis of *B. pertussis* clinical isolates associated with the 2010 California state pertussis outbreak, showed isolates with reduced pertactin (PRN) activity, but increased PTx S1 production (Williamson, Moura et al. 2015). A previous study in the Netherlands also identified *B. pertussis* strains with increased PTx S1 production due to a novel allele in the PTx promoter (Mooi, van Loo et al. 2009). The higher secreted amounts of PTx S1 by current circulating *B. pertussis* strains have been suggested that vaccination may have driven selection of such strains to lead to increased virulence (Mooi, van Loo et al. 2009, Williamson, Moura et al. 2015). As such, the lower frequency of anti-PTx antibodies recovered may be a direct result of increased anti-S1 toxicity resulting in lower anti-PTx antibody responses. That the isolated anti-PTx antibodies also bound PTg with high nM affinity, further suggests PTg is therefore clearly a compelling replacement for PTx in new acellular vaccines, since it has reduced toxicity, induces neutralizing anti-PTx antibodies, (Nencioni, Pizza et al. 1990) and are similarly recognized by anti-PTx antibodies.

On the other hand, the subjects from whom the B cells were isolated for this study were adults who may have been immunized with the whole cell vaccine as infants, or been exposed to infection. While the number of memory B cells responding to pertussis antigens is lower than for other antigens such as tetanus (Hendrikx, Ozturk et al. 2011), the presence of memory B cells, could bias future immune responses. As such, the low

anti-PTx response isolated from subjects may alternatively and/or additionally be a bias of only a few B cells responding to the PTx antigen due to previous vaccinations or infections.

Many studies have identified anti-PTx antibodies with different epitope specificities and neutralization capacities (Anwar, Ashworth et al. 1987, Sato, Sato et al. 1987, Schou, Au-Jensen et al. 1987, Kenimer, Kim et al. 1989, Kim, Burnette et al. 1989, Lang, Ganss et al. 1989, Sato, Sato et al. 1990, Halperin, Issekutz et al. 1991, Halperin, Issekutz et al. 1991, Sato, Sato et al. 1991). Such studies have shown that not only the catalytic A-subunit is immunogenic (Raupach and Schmidt 1994) but that the B-subunit also contains protective epitopes (Arciniega, Shahin et al. 1991, Nencioni, Pizza et al. 1991). In analysis of serum responses to PTx, the B-subunit antibodies are however more readily identified with ELISAs using the purified B oligomer, than from recognition of the individual B subunits in western blots (Lynn, Burnette et al. 1994). Specific protective epitopes to antigens in aP vaccines may be used as serological correlates of immunity to pertussis infection. Two potently neutralizing epitopes recognized by the antibodies, 1B7 and 11E6 may be used as anti-PTx correlates of protection. The protective 1B7 and 11E6-like epitopes, more often induced after natural infection, than after vaccination with aP vaccines, are also more susceptible to chemical modification (Sutherland, Chang et al. 2011). The combination of results from the subunit ELISAs, western blots and competition ELISAs demonstrated the isolated antibodies bind unique epitopes. A8 and E12 possibly share a similar epitope, or epitopes in close proximity on the S1 subunit, while D8 and H5 primarily bound the B-oligomer. The A12 epitope was however elusive, since it bound neither the truncated S1 subunit alone, nor the B-oligomer. The epitope of A12 may however overlap the potently neutralizing 1B7

epitope. Conversely, none of the antibodies appeared to compete with the neutralizing 11E6 epitope.

Although the A12 antibody was not neutralizing in the CHO assay, it is of interest first because its epitope appears to overlap with the previously characterized anti-PTx S1-subunit antibody, 1B7 and secondly, it was able to inhibit PTx binding to fetuin. 1B7 is also less potent than 11E6 in the CHO clustering assay, but is more potently neutralizing *in-vivo* (Sato, Sato et al. 1987, Sato and Sato 1990), and also inhibits PTx binding to fetuin. Fetuin and other glycoconjugates have been shown to contain the carbohydrate groups on receptors recognized by PTx (Heerze and Armstrong 1990). However, as a model receptor, fetuin does not block the intoxication of CHO cells by PTx (Tuomanen, Towbin et al. 1988). The weak neutralization of PTx mediated CHO cell clustering by A12, but an ability to inhibit PTx binding to fetuin suggests the types of glycoconjugates on PTx receptors on CHO cells may be different from the glycoconjugates on fetuin to which PTx binds. The potent neutralization of PTx toxicity by 1B7 in mice (Sato, Sato et al. 1987, Sato and Sato 1990) and baboons (Nguyen et al. 2014, in prep), further suggests CHO cell receptors may not fully represent relevant receptors on host cells. Recent epitope mapping has suggested the epitope of 1B7 may span the A/B interface (Sutherland and Maynard 2009). Such A/B anti-PTx antibodies are interesting because purified recombinant S1 subunit by itself appeared incapable of inducing protective antibodies in mice (Nicosia, Bartoloni et al. 1987), and the B oligomer alone induced immunity that was less potent than protection induced to the holotoxin (Nencioni, Pizza et al. 1991), suggesting that more potent protective epitopes involve both the A-subunit and B-oligomer. Further characterization of antibodies spanning the A/B interface can additionally guide efforts in the development of recombinant PTx in next generation aP vaccines.

The B-oligomer antibody, D8, was also interesting because it potently neutralized PTx toxicity in the *in-vitro* CHO neutralization assay. D8 was approximately 10-fold more potent in the CHO assay than the previously characterized B-oligomer antibody, 11E6 (Sato, Sato et al. 1987, Sato and Sato 1990). While the S1 subunit is thought to have one main neutralizing epitope (Cieplak, Burnette et al. 1988), the B-oligomer may have multiple neutralizing epitopes due to having four different subunits. D8 may bind a unique neutralizing epitope, different from the 11E6 epitope, since it did not compete with 11E6 for binding to PTx. From the receptor inhibition assay, it appears the mechanism of D8 neutralization is by inhibiting PTx interaction with the CHO cell receptors. It must be noted however that D8 inhibition of PTx binding to fetuin, was less potent than 11E6.

Previous analysis of monoclonal antibodies binding PTx has suggested at least four non-overlapping epitopes on PTx (Bartoloni, Pizza et al. 1988, Cieplak, Burnette et al. 1988, Kenimer, Kim et al. 1989, Kim, Burnette et al. 1989). The anti-PTx antibodies in this study provide more reagents for elucidating both neutralizing and non-neutralizing PTx epitopes which can be used to further define serological correlates of PTx immunity. Neutralizing antibodies identified in this study after confirmation of *in-vivo* efficacy are also potentially useful for passive immunization therapies.

3.5 CONCLUSIONS

The single cell characterization of the humoral response to PTx, indicates only a few B cells may be induced to PTx after aP vaccination. We suggest the frequency of anti-PTx antibodies may be the result of immunosuppression due to residual active PTx after dextoxification, reduced immunogenicity of PTx in the booster vaccine, or the

presence of memory B cells biasing new responses. Antibodies induced to native PTx are diverse and recognize epitopes on either the A or B subunits with high affinity. The anti-PTx antibodies also recognize genetically modified PTx (PTg) with amino acid changes in the catalytic A-subunit. PTg would require less chemical treatment for detoxification, and with reduced toxicity is therefore a viable alternative to chemically detoxified PTx in acellular pertussis vaccines. We identified a neutralizing anti B-subunit antibody which may inhibit PTx binding to cell surface receptors to prevent internalization and subsequent toxicity. We also identified an antibody, A12 with an elusive epitope which inhibited PTx binding to a model receptor. A12 also competed with a previously characterized neutralizing antibody, 1B7, which has a unique protective epitope. Such rare neutralizing antibodies may be useful as serological correlates of protection during development of next-generation vaccines and for passive immunization strategies for pertussis.

Chapter 4: *In-vitro* neutralization mechansims of two potently neutralizing anti-PTx antibodies, 1B7 and 11E6.

4.1 INTRODUCTION

Of the numerous virulence factors produced by *B. pertussis*, PTx is thought to be a major antigen which elicits a humoral response sufficient for protection (Granstrom, Granstrom et al. 1985). Mice lacking PTx genes have reduced virulence (Weiss, Hewlett et al. 1985) and neutralizing anti-PTx antibodies administered passively are able to alleviate and even reverse disease symptoms in mice (Sato, Sato et al. 1987). Due to its immunogenicity and clear role as a major protective antigen, several anti-PTx antibodies have been generated and characterized for subunit specificity and protection in *in vitro* and *in vivo* assays (Sato, Ito et al. 1984, Sato, Sato et al. 1987, Schou, Au-Jensen et al. 1987, Sato and Sato 1990, Halperin, Issekutz et al. 1991). Through extensive mouse studies, rare highly neutralizing anti-PTx antibodies have been isolated and characterized. Antibodies recognizing neutralizing epitopes are thought to either block the binding of the B-oligomer to the cell surface receptors or block catalysis of the A subunit (Sato, Sato et al. 1987, Kaslow, Schlotterbeck et al. 1990). Two antibodies in particular, named 1B7 and 11E6, have proven to be neutralizing in in-vitro assays and exhibit synergistic effects when administered together (Sato and Sato 1990). In addition to demonstrating protection in a series of *in vitro* assays, 1B7 and 11E6 were able to protect mice prophylactically and even therapeutically, reversing disease symptoms when administered days after infection (Sato, Ito et al. 1984).

The epitope recognized by 1B7 was initially mapped near the S1 catalytic site, based on a series of experiments with truncated toxin variants (Bartoloni, Pizza et al.

1988, Cieplak, Burnette et al. 1988). Although the mechanism of neutralization is unknown, the ability of 1B7 to inhibit the ADP-ribosyltransferase (ADPR) activity of PTx *in-vitro*, suggests 1B7 blocks S1 subunit-mediated catalytic activity (Sato, Ito et al. 1984, Kaslow, Schlotterbeck et al. 1990, Kaslow, Schlotterbeck et al. 1991). However, no correlation has been shown between high *in-vitro* anti ADPR activity and *in-vivo* neutralizing activity of anti-S1 antibodies (Sato, Sato et al. 1991). In addition, purified S1 by itself is unable to induce protective antibodies in mice (Nicosia, Bartoloni et al. 1987, Bartoloni, Pizza et al. 1988); suggesting neutralizing anti-S1 antibodies may inhibit PTx activity independently of the S1 catalytic site. Furthermore, the S1 subunit-mediated catalytic activity occurs in the cytosol after PTx undergoes retrograde trafficking as an ER escape route to bypass degradation (Hazes and Read 1997, Worthington and Carbonetti 2007).

To traffic the retrograde pathway, PTx first binds a cellular receptor with carbohydrate moieties via its B-oligomer (Heerze and Armstrong 1990, Heerze, Chong et al. 1992), undergoes receptor-mediated endocytosis to enter the early/recycling endosomes, followed by retrograde transport through the Golgi and the endoplasmic reticulum (ER) (Xu and Barbieri 1996, el Baya, Linnermann et al. 1997, Plaut and Carbonetti 2008). Within the ER, release of the PTx S1 subunit is dependent upon reduction of a disulfide bond in S1 by the presence of a reducing potential and protein disulfide isomerase (PDI), and an ATP-induced conformational change in the B subunit (Burns and Manclark 1986, Hazes, Boodhoo et al. 1996). Although the molecular details of the membrane translocation step for subsequent transport of the S1-subunit out of the ER into the cytosol remains unclear, it is thought to involve the Sec61 channel (Hazes and Read 1997). Retrotranslocation from the ER, across the Sec61 channel for entry into the cytosol, has been implicated for ricin, shiga and cholera toxins (Simpson, Roberts et

al. 1999, Schmitz, Herrgen et al. 2000, Yu and Haslam 2005) and demonstrated to be exploited by certain viruses for host immune evasion (Wiertz, Tortorella et al. 1996). Within the cytosol, the S1 subunit catalyzes the transfer of the ADP-ribosyl moiety from NAD to membrane-associated $G_{i/o}$ proteins, disrupting G-protein function (Figure 4.1) (Katada and Ui 1982, Katada, Tamura et al. 1983, Burns, Kenimer et al. 1987).

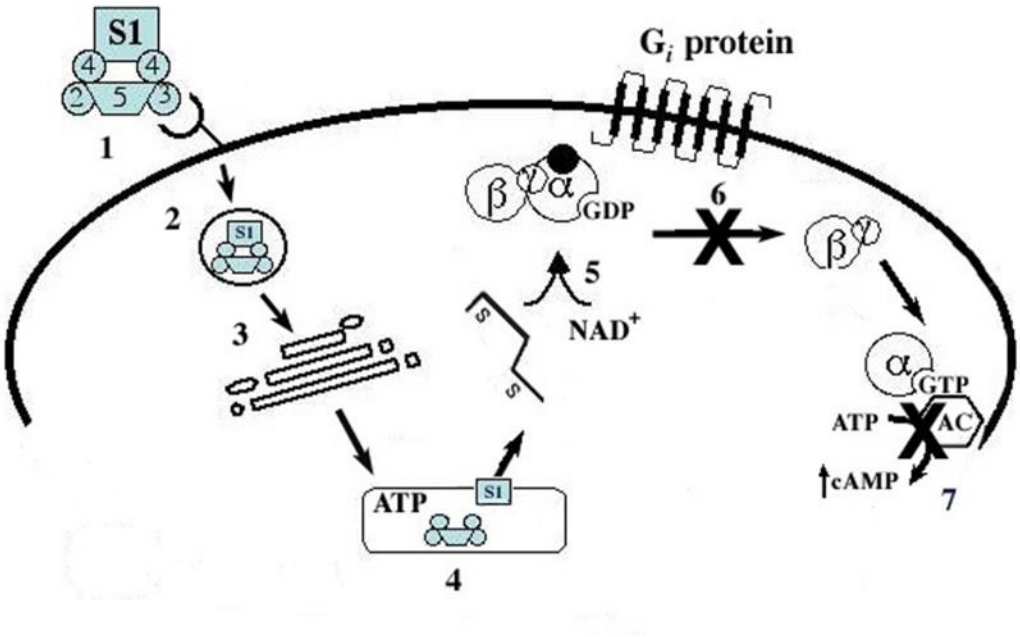


Figure 4.1: PTx retrograde trafficking and cell toxicity.

After binding a cellular receptor **1)**, PTx traffics retrogradely from the early endosomes **2)** through the Golgi **3)**, to the endoplasmic reticulum **4)**. Within the ER, ATP and a reducing environment facilitate the unfolding of the S1 subunit, from where it is translocated to the cytoplasm. In the cytoplasm, the S1 subunit catalyzes the transfer of the ADP moiety from NAD to membrane associated G-proteins **5)**. The inactivated G-proteins **6)** are unable to inhibit activated **7)** adenylyl cylase which forms cAMP from ATP, leading to increased cellular cAMP concencentrations.
Figure adapted from (Sutherland and Maynard 2009).

Considering the complexity of the retrograde transport mechanism of PTx and a requirement for unfolding of the S1 subunit for translocation potentially through the sec61 pore, it is not clear that 1B7 can directly have access to the PTx catalytic site after trafficking of the large immune complex through the retrograde organelles to reach the highly reductive and proteolytic cytosol to impact catalysis. Detailed epitope mapping using computational docking and site-directed mutagenesis, has specified the 1B7 unique epitope to lie primarily on the S1 subunit, and possibly interacting with S4 subunit (Sutherland and Maynard 2009), suggesting 1B7 may neutralize PTx toxicity without trafficking retrogradely. The epitope of 11E6 on the other hand, found on the S2 and S3 subunits (Sato, Sato et al. 1987, Sato, Sato et al. 1990, Rambow-Larsen and Weiss 2004) is thought to directly inhibit PTx binding to receptors (Sato, Sato et al. 1987).

In this study, the mechanisms of 1B7 and 11E6-mediated PTx neutralization were further characterized. Using FACS analysis, binding of 1B7 and 11E6 to live *B. pertussis* was detected, demonstrating their epitopes are exposed on *B. pertussis*, possibly during PTx secretion. 1B7 and 11E6 however showed no significant opsonophagocytic activity, indicating that these antibodies directly interfere with the toxin's activities after secretion. 1B7 with its epitope on the S1 subunit was competent to bind receptor bound PTx. 11E6 with two binding sites, one on either the S2 or S3 subunits was also able to bind receptor bound PTx Both antibodies however inhibited PTx binding to a model receptor, although it was expected only 11E6 would be able to do so. 1B7 effects on PTx trafficking in CHO-K1 cells were also analyzed to assess the possibility of 1B7 intracellular neutralization. Remarkably, although PTx can simultaneously bind both 1B7 and a model receptor, 1B7 appears to inhibit PTx internalization, and instead most of PTx remains at the cellular membrane. This suggests the epitope of 1B7 on the A/B interface impacts the

interaction of PTx with receptors to affect internalization or intracellular trafficking steps of the holotoxin.

PTx in current acellular pertussis vaccines is chemically detoxified, a process which damages the protective epitope on the S1-subunit recognized by 1B7 and 11E6 (Nencioni, Volpini et al. 1991, Sutherland, Chang et al. 2011). Destruction of such neutralizing epitopes results in lower protective antibody titers upon vaccination with aP vaccines than upon actual infection (Sutherland, Chang et al. 2011). The induction of such rare and unique epitopes, induced mostly after *B. pertussis* infection, is a goal for future pertussis vaccines and their corresponding antibodies have potential for use in passive immunotherapy.

4.2 MATERIALS AND METHODS

4.2.1 Antigens and antibodies

PTx (holotoxin) in glycerol was purchased from List Biological Laboratories, Inc. (Campbell, CA) or obtained through BEI Resources, NIAID, NIH. A soluble, truncated version of the PTx S1 subunit, PTx-S1-220 was expressed and purified from the pAK400 plasmid in *E. coli,* as described previously (Sutherland and Maynard 2009). The expression and purification details for full length murine 1B7 has been described previously (Sutherland and Maynard 2009). Humanized 1B7 and 11E6 versions of the murine monoclonal antibodies, with similar affinity and epitope specificity as the parent murine antibodies, were purified from CHO cells using protein A and anion chromatography by Catalent (Somerset, NJ) (Nguyen et al., 2014. In review). Purification of the human anti-PTx antibodies A8 and D8, specific for the A and B subunits

respectively is described in Chapter 3. Isotype control antibodies (IgG1 and IgG2a) were purchased from Santa Cruz Biotech or R&D systems. Commercial fluorescent secondary antibodies used are indicated.

4.2.2 Antibody binding to whole *B. pertussis*

The expression of PTx-epitopes on live *B. pertussis* was analyzed by indirect immunofluorescence using flow cytometry as previously described in Chapters 2 and 3 (Aase, Herstad et al. 2007). The bacteria were grown for 72 hours on Bordet-Gengou, harvested and washed, and adjusted to an OD_{600}/mL of 1 in PBS with 5% glycerol (PBSG). The bacteria were incubated with 1 µg of each antibody in 100 µL at room temperature and bound antibodies detected after washing with a 1:500 dilution of goat anti human Fc Alexa Fluor 647 conjugate (Jackson Immunoresearch). 10 000 events per treatment were collected on a BD SLRII Fortessa flow cytometer. The cell associated fluorescence for a gated population of the cells was determined using the FlowJo_V10 software.

4.2.3 Opsonophagocytic activity of PTx-specific antibodies

Opsonophagocytic activity (OPA) performed by the Aase group, was measured as a respiratory burst, as previously described (Aase, Herstad et al. 2007). Briefly, hybridoma cell supernatants of anti-PTx antibodies 1B7 or 11E6, pooled anti-pertussis antibodies from vaccinated volunteers (P-IVIG), or purified monoclonal antibodies against PorA protein of *Neisseiria meningitidis* (Michaelsen, Kolberg et al. 2004) were diluted twofold in HBSS/BSA (2 mg/ml). Live *B. pertussis* (105/06), were used as targets, and dihydrorhodamine 123 (Invitrogen) primed PMNs from a donor

heterozygous for the FcgammaRIIa allotype were used as effector cells. Dihydrorhodamine 123 was used as a probe for respiratory burst. Human serum (10 %) was used as complement source, after being passed through a protein G column (HiTrap protein G HP; GE Healthcare, Oslo, Norway) to remove antibodies.

4.2.4 CHO neutralization assay

A CHO neutralization assay was used to determine the concentration of 1B7 which rescues CHO cells from PTx induced clustering. CHO-K1 cells (ATCC, Manassas, VA) were grown in complete DMEM media (DMEM supplemented with 10% fetal bovine serum and 1X penicillin/streptomycin). The cells were grown and maintained at 37 °C with 5% CO_2 in a jacketed incubator. The working cell concentration of 1.5 x 10^4 cells/96 well was first determined from a preliminary cell concentration series. The working concentration of PTx was determined from a preliminary toxin concentration series to find the lowest concentration resulting in complete CHO cell clustering. Clustering due to PTx toxicity was scored from 0 to 3 as follows; 0(no clustering), 1(a few clusters/equivocal), 2(positive clustering), 3(maximum/complete clustering). A PTx concentration of 0.25ng/ml (150µl culture volume) was determined to be the minimum concentration causing maximum clustering with a score of 3. A test dose of 1.05ng/ml PTx (150µl culture volume) (approximately four times higher than the minimum PTx dose showing a score of 3) was used to allow maximum clustering to be observed and reduce PTx lot-to-lot variability. To determine the neutralizing concentration of 1B7 and 11E6, serial two-fold dilutions of antibody were prepared in complete DMEM media leaving 25 µl in all the wells. Next, 25 µl of PTx was added to all wells and the complex incubated for 30 minutes at 37˚C. CHO cells were trypsinized and diluted to 1.5 x 10^5

cells/ml in complete media. 100 µl of cells were added to the PTx-antibody solution to achieve the final cell concentration of 1.5 x 10^4 total cells/96 well.

4.2.5 ELISAs

4.2.5.1 Indirect PTx ELISA

Indirect capture ELISAs to assess the binding affinity and epitope specificity to the PTx holotoxin or subunits were performed as described in Chapter 3 with no modifications. PTx holotoxin final concentration was 1 µg/ml. PTx A-subunit and PTx B-subunit final concentrations were 0.25 µg/ml and 0.85 µg/ml respectively.

4.2.5.2 Simultaneous binding of 1B7 and 11E6 to PTx

Simultaneous binding of 1B7 and 11E6 to PTx was evaluated in a capture ELISA. High binding ELISA plates were coated with 1 µg/ml of the humanized versions of 1B7 or 11E6. After blocking with PBST-milk, serial dilutions of PTx were added and 1 µg/ml of the murine parental versions of 1B7 or 11E6 added. Binding of the murine antibody was detected with a goat-anti-mouse HRP. The plates were developed using TMB and quenched with 1N HCl.

4.2.5.3 Simultaneous binding of antibody PTx bound to fetuin

As described in section 3.2.6.3, fetuin was used as model soluble receptor to detect antibodies bound to a PTx-fetuin complex using a method adapted from (Kenimer, Kim et al. 1989). High binding ELISA plates (Costar) were coated with 50 µL/well of 10 µg/mL fetuin (Sigma) at in PBS. After overnight incubation at 4˚C, the plates were

blocked with 50 µL/well blocking buffer (PBS + 5% BSA) at room temperature for 1 hour. Plates were washed three times in wash buffer (PBS, + 0.05% Tween-20) after each incubation step. Serially diluted PTx was then added to the fetuin coat and incubated further overnight. Antibodies at a concentration of 1 µg/ml antibody were then added and incubated for 1 hour at room temperature. Anti-human Fc HRP (Jackson Immunoresearch) was added at a 1:1000 dilution in wash buffer and incubated at room temperature for another hour. Signal was developed using the TMB Substrate Kit (Thermo-Scientific), the reaction stopped with 1N HCl, and the plate read using a SoftMax Pro v5 (Molecular Devices) at 450 nm. All plated volumes were 50 µL/well.

4.2.6 Competition ELISA assays

4.2.6.1 Antibody competition for binding to PTx

The ability of 1B7 and 11E6 to compete for binding to PTx was evaluated by a competition ELISA as described for the human anti-PTx antibodies (Chapter 3). Briefly, the humanized 1B7 and 11E6 were biotinylated, and serial dilutions of the non-biotinylated 1B7 or 11E6 competitor antibody incubated with a constant concentration the biotinylated antibody. The biotinylated antibody was detected after incubation of the antibody mixtures on a coat of PTx, using streptavidin-HRP, and the reaction developed using TMB substrate as described above.

4.2.6.2 Antibody Inhibition of PTx binding to fetuin

Using fetuin as a model receptor, the ability of 1B7 and 11E6 to inhibit PTx binding to fetuin in a competition ELISA was investigated as described for the human anti-PTx antibodies in Chapter 3. Briefly, fetuin coated overnight at 4^{o}C, at a

concentration of 10 µg/ml in PBS was blocked the next day with PBS-BSA (5%). Serially diluted antibodies with a constant concentration of 0.79 nM PTx were incubated overnight at 4^{o}C. Bound PTx was detected with a home-made mixture of three murine antibodies to PTx (1B7, 11E6 and 7F2) at 0.1 µg/mL. The mouse antibodies were detected using HRP-conjugated goat-anti mouse antibodies and the plates developed with TMB. The absorbances were read at 450nm and analyzed using Graphpad prism 5. The relative ability of the antibodies to inhibit PTx binding to fetuin was determined by estimation of the concentration of each antibody required to achieve 50% maximal binding at saturation (IC_{50}).

4.2.7 PTx intracellular trafficking via immunofluorescence microscopy

Coverslips were seeded with at 5 x 10^5 CHO cells/ ml in a 6 well plate and allowed to grow for 48 hours. To visualize the early endosomes and Golgi, 3 µg of Rab5-GFP plasmid DNA or 50 µL of CellLight^{TM} Golgi-GFP Bacman 2.0 (Invitrogen) respectively were transfected into CHO cells grown on covers slips in a 6 well a day prior to the PTx trafficking assay. All samples were washed twice with PBS prior to further processing. The cells were then incubated in blocking buffer (serum and antibiotic-free DMEM media containing 2 mg/mL BSA, and 1µg/ml of an irrelevant murine IgG1 antibody) at 37˚C with 5% CO_2 for an additional 30 min. To visualize PTx trafficking, cells were incubated with 100µl of 10 nM PTx at 37^{o}C 5% CO_2 for varying times (15 minutes to 4 hours). At select time points, the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. After fixation, the cells were washed with PBS three times, permeabilized with PBS containing 0.1% Triton X-100 for 30 minutes at room temperature and subsequently blocked with blocking buffer for 1 hr at 37˚C. PTx was detected using 100µl of 0.5 µg/mL of a human monoclonal anti-PTx

antibody to the A subunit (A8) or to the B subunit (D8) in blocking buffer at 37˚C for 1 hour or at 4^{o}C overnight in humid conditions. The endoplasmic reticulum was detected after PTx intoxication and fixing by primary antibody labeling to the ER-resident protein PDI using 100µl of anti-rabbit PDI (Sigma-Aldrich) at a 1:1000 dilution and detected with 100µl of 1:1000 anti-rabbit-IgG-Alexa488 (Molecular Probes). Next, the cells were washed three times with PBS and secondary antibody labeling done with 100µl of 1:1000 dilutions of anti-human-IgG-AlexaFluor 594 (Molecular Probes) in blocking buffer in the dark at 37˚C for 1 hr. After three final washes with PBS, the coverslips were mounted on slides using a drop of DAPI-fluoromount-G (SouthernBiotech, Birmingham, AL). To examine the effects of 1B7 on PTx trafficking, PTx was pre-incubated with 1000 or 10,000-fold molar excess of 1B7 at 37˚C for 30 min prior to incubation with cells on at 37˚C with 5% CO_2. Imaging was performed with a Zeiss Axiovert fluorescent microscope (Carl Zeiss, Inc) and AxioVision software or a Leica SP2 AOBS confocal microscope (Leica, Bannockburn, IL). Images were enhanced by adjusting brightness and contrast using the ImageJ software (http://rsbweb.nih.gov/ij).

4.3 RESULTS

4.3.1 1B7 and 11E6 bind to live *B. pertussis* but may not induce opsonophagocytic activity

Prior to secretion of PTx, the S1 subunit localizes to the outer membrane of the bacterium to serve as a nucleation site for assembly with the B oligomer to form the holotoxin (Pizza, Bugnoli et al. 1990, Farizo, Huang et al. 2000, Farizo, Fiddner et al. 2002). Antibodies against surface exposed antigens on *B. pertussis* are reported to

promote phagocytosis (Hellwig, Rodriguez et al. 2003, Aase, Herstad et al. 2011). If either the 1B7 or 11E6 epitopes on PTx is transiently expressed on the bacterial cell surface during secretion, then antibodies could mediate opsonization of bacteria and/or complement-dependent lysis.

To determine whether 1B7 and 11E6 are able to bind whole bacteria, the antibodies were incubated with live *B. pertussis,* followed by washing and signal development with a fluorescent secondary antibody conjugate and analysis by flow cytometry. Binding of 1B7 and 11E6 to live bacteria was detected, whereas a human polyclonal immunoglobulin preparation, which includes antibodies recognizing multiple pertussis antigens, showed strong binding (Figure 4.2).

This result suggests the 1B7 and 11E6 epitopes are exposed during PTx secretion and therefore suggested that PTx may serve as a target for killing of *B. pertussis* through opsonophagocytic (OPA) or complement activity. In collaboration with Audun Aase, 1B7 and 11E6 were tested for OPA activity. A polyclonal immunoglobulin preparation (P-IVIG) and convalescent serum (KjHa) both revealed high OPA activity, however, no OPA activity was observed when 1B7 or 11E6 was incubated with live *B. pertussis* strain 105/06 (Figure 4.3).

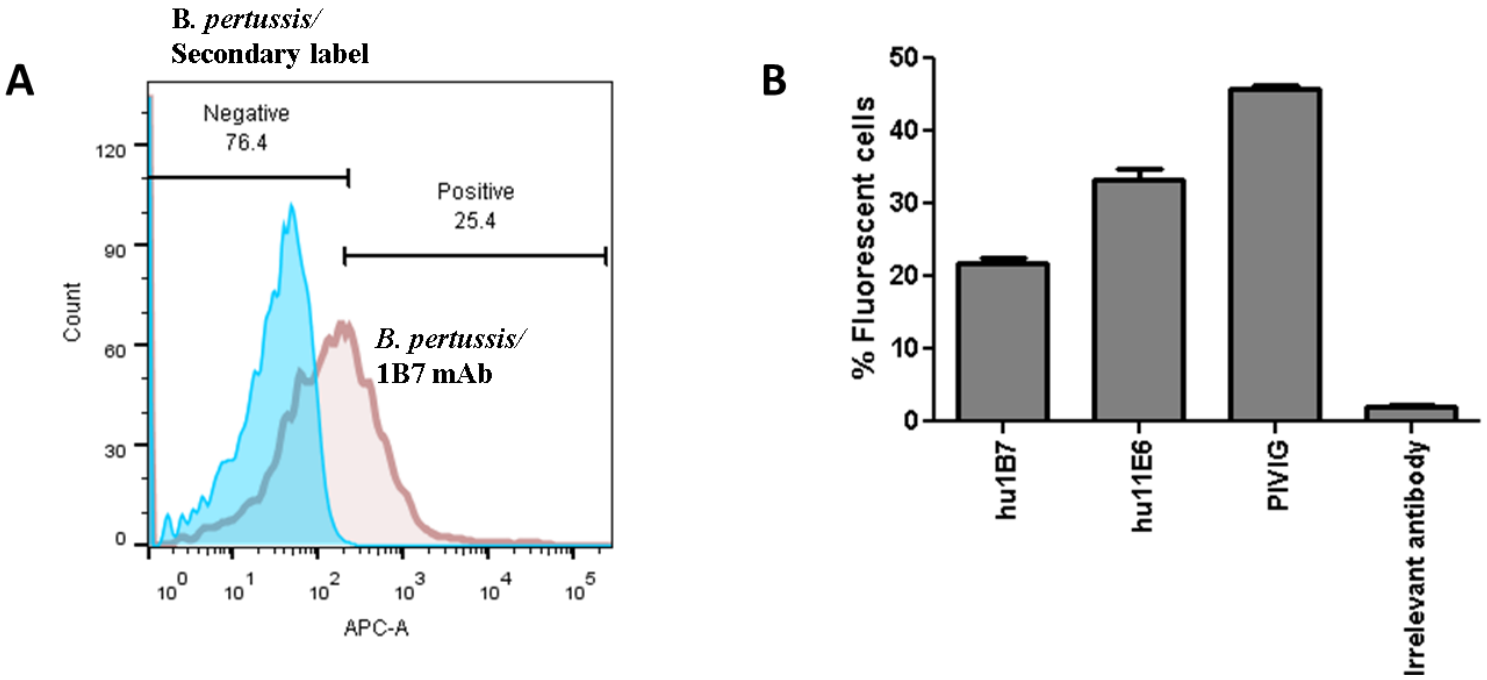


Figure 4.2: 1B7 and 11E6 binding to whole *B. pertussis*.

A) Representative FACS plot of 1B7 antibody binding to whole *B. pertussis* cells. *B. pertussis* cells grown on Bordet Gengou plates were scraped and resuspended to 1 OD_{600}/mL. The cells were washed and incubated with 1 µg of the antibodies in PBS with 5% glycerol. Bound antibodies were detected with fluorescently labeled anti-human Alexa Fuor 647 secondary antibody and analyzed by flow cytometry. **B)** Percentages of fluorescent *Bordetella* cells after incubation with the indicated antibodies. Using the FlowJo_V10 software, % fluorescent cells were determined for a gated population of the cells using unlabeled *B. pertussis* as a negative control. Data represent the average of two independent experiments. The error bars represent the standard error of the mean.

These results are in agreement with previous data showing that while polyclonal serum containing antibodies to PTx, FHA and pertactin (PRN) from vaccinated individuals exhibit binding to whole *B. pertussis* (Weingart, Keitel et al. 2000, Hellwig, Rodriguez et al. 2003, Aase, Herstad et al. 2011), opsonophagocytic activity is mostly due to anti-PRN antibodies (Gotto, Eckhardt et al. 1993, Hellwig, Rodriguez et al. 2003) and possibly anti-FHA antibodies (Aase, Herstad et al. 2011). Antibody-mediated complement killing of bacteria also requires a complex of the antigen on the surface of the bacteria with complement-fixing antibody (Weiss, Patton et al. 2004). Since PTx is mostly secreted, there may only be a few PTx molecules on the surface of the bacteria to mediate complement bactericidal activity.

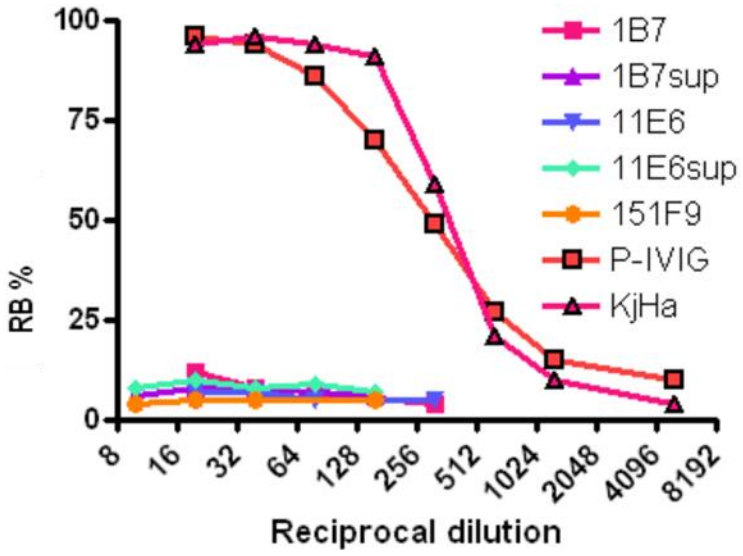


Figure 4.3: Opsonophagocytic activity of anti-pertussis antibodies.

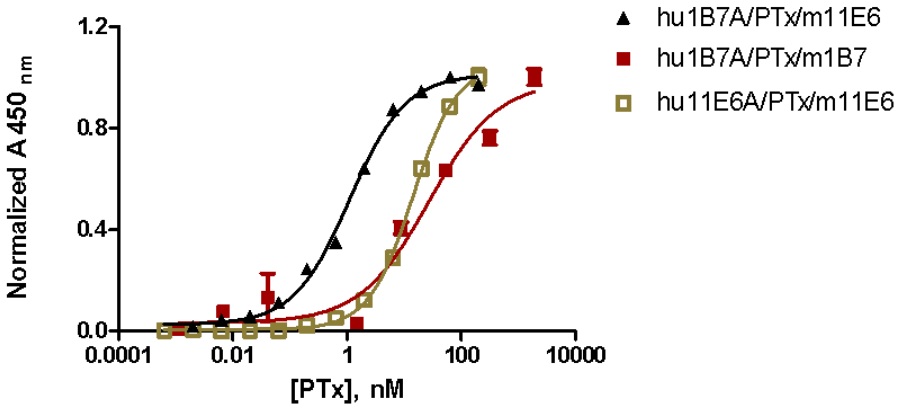
Serially diluted antibodies were incubated with live *B. pertussis* and added to (DHR) primed peripheral mononuclear cells. The mixture was incubated in the presence of 10% IgG depleted human serum. Respiratory burst was measured as the increase in green fluorescence due to oxidation of DHR to rhodamine. The experiment was repeated twice with similar results by the Aase group as indicated in the materials and methods section.

4.3.2 1B7 and 11E6 bind non-overlapping epitopes on the A and B subunits of PTx with a 2:1 stoichiometry

Using indirect ELISAs to probe antibody binding to the holotoxin, we confirmed 1B7 and 11E6 binding to the PTx holotoxin with affinities of 0.27 ± 0.14 and 1.24 ± 0.14 nM respectively (Table 3.4). 1B7 and 11E6 binding to the PTx A subunit or the B oligomer respectively were also similarly confirmed. 1B7 binding was confirmed using a truncated recombinant version of the A-subunit, PTx S1-220, or the B-oligomer. Humanized 1B7 binds the truncated S1 variant, PTx S1-220 with an affinity of 0.008 ± 0.002 nM. The murine 11E6 binds the PTx B-oligomer with a lower affinity of 1.18 ± 0.19 nM respectively (Table 3.4). The high affinity binding of 1B7 and 11E6 to the S1 subunit and B-oligomer respectively confirms their epitopes primarily lie on these subunits.

The epitope of 1B7 however was recently specified to overlap the S1 and S4 subunits (Sutherland and Maynard 2009), suggesting a weaker affinity site on the second S4 subunit in the B oligomer. The 11E6 epitope has also previously been shown to lie to on the S2 and S3 subunits. This suggests both 1B7 and 11E6 bind PTx with a 2:1 stoichiometry. We attempted to determine the stoichiometry of 1B7 binding to PTx using various techniques such as analytical ultracentrifugation and column chromatography. However, due to the susceptibility of PTx to aggregation (Tamura, Nogimori et al. 1982), and non-specific binding through its lectin like properties to various chromatography columns (Fowler, Byron et al. 2003), we were unable to determine the stoichiometry of 1B7 binding to PTx using biophysical methods such as analytical ultracentrifugation and gel filtration. To assess the 2:1 antibody:PTx binding stoichiometry, we used an antibody capture ELISA with the humanized version of 1B7 or 11E6 coated, to which serially diluted PTx was added. Detection of the parental murine antibodies added to the PTx coat

suggested 1B7 and 11E6 do bind two unique epitopes on the PTx holotoxin. The binding affinity of 11E6 to PTx already bound to 1B7 was estimated to be 1.11 ± 0.05 nM, similar to 11E6 binding to free PTx. The binding affinities of 1B7 and 11E6 binding a second epitope were estimated to be 26.75 ± 0.19 and 14.18 ± 0.02 nM respectively (Figure 4.4), suggesting a lower affinity binding of 1B7 and 11E6 to their succeeding epitopes.



Antibody	EC_{50}, nM ± SE
hu1B7/m11E6	1.11 ± 0.05
hu1B7/m1B7	26.75 ± 0.09
hu11E6/m11E6	14.18 ± 0.02

Figure 4.4: Simultaneous binding of 1B7 and 11E6 to PTx.

Serially diluted PTx was added to the humanized versions of 1B7 or 11E6 coated at 1µg/ml. A concentration of 1µg/ml of the murine 1B7 or 11E6 was then added to the antibody-PTx sandwich. Bound murine antibodies were detected using secondary goat-anti-mouse HRP antibody. The absorbances at 450_{nm} were normalized for each data set using GraphPad prism 5 by setting the highest absorbance to 1. Estimates of the affinity (EC_{50}) of the murine antibody binding to PTx were determined from the fit of the binding curves to the 4PL non-linear equation using GraphPad prism 5. SE - standard error of the mean.

4.3.3 PTx bound to a model receptor can simultaneously bind 1B7 or 11E6

PTx binds to receptors on host lung resident cells, is internalized and transported to the cytosol to exert its toxic effects. While the receptors for PTx are still unknown, transferrin and haptoglobin (Heerze and Armstrong 1990, Heerze, Chong et al. 1992) and has been co-crystallized with a soluble oligosaccharide from transferrin (Stein, Boodhoo et al. 1994). The receptor-binding sites have also been localized to subunits S2 and S3 (Schmidt and Schmidt 1989, Schmidt, Raupach et al. 1991, Schmidt, Seitz et al. 1991, Heerze, Chong et al. 1992, Stein, Boodhoo et al. 1994). The 1B7 epitope, located primarily on the S1 subunit (Bartoloni, Pizza et al. 1988, Cieplak, Burnette et al. 1988) and possibly overlapping the S4 subunit (Sutherland and Maynard 2009) is distal to the characterized receptor-binding sites. The 11E6 epitope on the other hand is found on subunits S2 and S3 (Sato, Sato et al. 1987, Sato, Sato et al. 1990, Rambow-Larsen and Weiss 2004). Consequently, it would be expected that 1B7 would be able to bind PTx when already bound to a receptor, while 11E6 would be unable to bind.

To test the ability of 1B7 and 11E6 to bind PTx and a model receptor, an indirect ELISA was used to assess simultaneous binding of the PTx-antibody complex to fetuin. Fetuin was immobilized on the plate, incubated with PTx, followed by addition of antibody to the PTx bound to fetuin and detection of bound antibody. The PTx-1B7 complex was able to bind fetuin in a dose-response manner, similarly, the PTx-11E6 complex also showed specific, but slightly weaker, dose-response binding, while none was detected for an irrelevant isotype control antibody (Figure 4.5). 1B7 is expected to bind PTx already bound to fetuin is as expected, since its primary epitope is located on the S1 subunit, distal from the receptor binding sites in S2 and S3. However, the results indicate that while the 11E6 has been hypothesized to directly inhibit PTx receptor

interactions, it may only partially block the PTx-receptor interaction, since there are two receptor sites are present on each PTx molecule. With one site binding immobilized fetuin, the other site is available for 11E6 binding. Two additional PTx-specific, non-neutralizing antibodies, 3F10 and G9A (Sato, Sato et al. 1987), also exhibited specific binding to PTx bound to another model receptor, transferrin (not shown). This data shows that an antibody-PTx complex is able to bind a model receptor and may be likely to bind a cellular receptor. Similar results have been previously observed for anti-Shiga toxin antibodies using flow cytometry analysis (Krautz-Peterson, Chapman-Bonofiglio et al. 2008). Krautz-Peterson group observed their anti-A chain antibody was competent to bind Shiga toxin already bound to receptors on cells. In their studies however, their anti-B oligomer antibody was not able to bind to Shiga toxin already bound to cells.

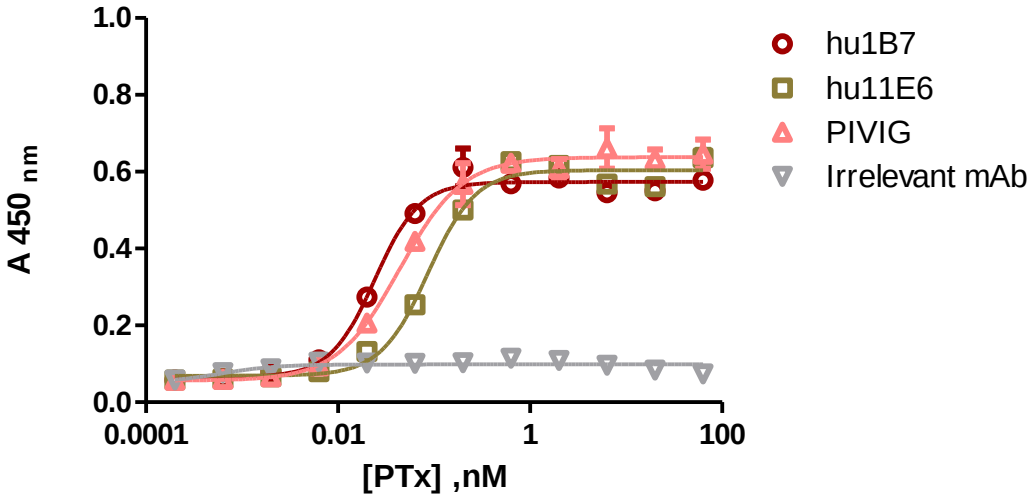


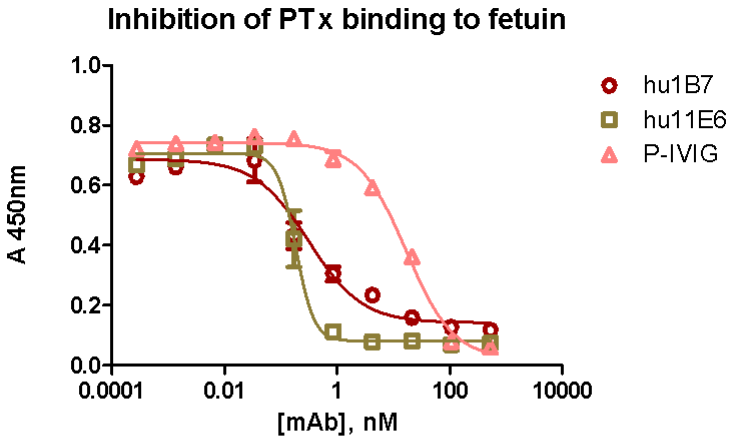
Figure 4.5: Simultaneous binding of antibodies to PTx bound to fetuin.

Serially diluted antibodies were added to PTx previously incubated with a coat of fetuin at 10 µg/ml. Bound antibodies were detected using secondary goat-anti-mouse HRP antibody. Error bars indicated are the standard error of the mean.

4.3.4 The PTx-1B7 or PTx-11E6 complex inhibits binding to fetuin

Many anti toxin antibodies neutralize toxic activity extracellularly by directly binding the toxin and sterically blocking key interactions, in particular, initial toxin-receptor interactions or toxin oligomerization steps occurring prior to receptor mediated endocytosis (Pai, Sutherland et al. 2009). For instance, the 14B7 antibody binds an epitope on anthrax toxin that overlaps with that of the cellular receptor to block the toxin-receptor interactions (Leysath, Monzingo et al. 2009). The 11E6 epitope found on the receptor-binding subunits on S2 and S3 (Sato, Sato et al. 1987, Sato, Sato et al. 1990, Rambow-Larsen and Weiss 2004), was expected to efficiently inhibit PTx binding to fetuin. The 1B7 epitope on the hand, found primarily on the S1 subunit and possibly overlapping the S4 subunit (Sutherland and Maynard 2009), was not expected to inhibit PTx binding to fetuin.

To our surprise, we found both 1B7 and 11E6 were able to block PTx binding to fetuin with IC_{50}'s of 0.26 ± 0.05 and 0.11 ± 0.07 nM respectively (Figure 4.6). The surprising ability of an anti-PTx S1 subunit antibody blocking PTx binding to fetuin although it also readily binds PTx already bound to fetuin has been reported previously (Kenimer, Kim et al. 1989). It has been suggested that the orientation of PTx after binding a cellular receptor may not completely obscure the S1 epitope (Kaslow and Burns 1992), however such anti-S1 antibodies which inhibit PTx binding to receptors may interfere with conformational changes induced when PTx binds to a receptor (Kenimer, Kim et al. 1989). Further studies would need to be done to elucidate the nature of any conformational changes induced in PTx after receptor binding, and how 1B7 may interfere with such conformational changes.



Antibody	IC_{50}, nM ± SE
hu1B7	0.26 ± 0.05
hu11E6	0.11 ± 0.07
P-IVIG	13.87 ± 3.1

Figure 4.6: Competition ELISA for 1B7 and 11E6 inhibition of PTx binding to fetuin.

Serially diluted antibodies with a constant concentration of PTx were added to ELISA wells coated with 10 µg/ml fetuin. Bound PTx was detected with a mixture of murine anti-PTx antibodies. The IC_{50} were determined from fitting the inhibition curves to the four parameter logistic (4PL) curve using GraphPad Prism 5. The IC_{50} data represent the average of two independent experiments. SE - standard error of the mean.

4.3.5 1B7 hinders PTx internalization in CHO cells

CHO-K1 cells are usually utilized for studies of pertussis toxin toxicity and neutralization due to easily observed morphological changes induced in the cells upon exposure to the toxin (Hewlett, Sauer et al. 1983). The concentration of 1B7 and 11E6 able to fully neutralize PTx induced CHO clustering, determined from the change in the score from 1 to 0 was approximately 3750 ± 1250 and 1875 ± 625 molar excess of the

PTx test dose respectively (Figure 4.7). The higher molar neutralization ratio of 1B7 compared to 11E6 for the complete neutralization of PTx induced clustering in CHO cells is consistent with previous results (Sato, Sato et al. 1987). It is important to note however that in *in-vivo* mice studies, 1B7 was as potent as 11E6 at neutralizing PTx toxicity (Sato, Sato et al. 1987, Sato and Sato 1990). In the aerosol challenge 1B7 protected 100% of the mice, while 11E6 protected 85%. In the intracerebral challenge, 1B7 protected 83% of the mice, while 11E6 protected only 7%. These results may suggest that 1B7 and 11E6 neutralize PTx differently depending on the cell type. PTx binds to oligosaccharide receptors on a variety of mammalian cells, and there are at least two glycoprotein binding sites on PTx with variable affinities, one on the S2 subunit (Francotte, Locht et al. 1989, Heerze, Chong et al. 1992), and another on the S3 subunit (Schmidt, Raupach et al. 1991). In spite of the greater than 80% sequence homology between the S2 and S3 subunits, they each appear to be carbohydrate and cell type specific (Saukkonen, Burnette et al. 1992). For instance, residues within the S2 subunit have been shown to have a higher affinity for sialic acid containing glycoconjugates such as fetuin (Heerze, Chong et al. 1992).

We observed no neutralization of CHO cell clustering when PTx was first added to CHO cells on ice, prior to the addition of 1B7 or 11E6 (not shown), further suggesting both 1B7 inhibit PTx toxicity in CHO cells by inhibiting the interaction of PTx interaction receptors as the fetuin inhibition assay suggested. The lower neutralization capacity of 1B7 compared to 11E6 in the CHO assay may be due to the differences in the antibody binding sites and which receptor subunits on PTx interact with receptors on CHO cells.

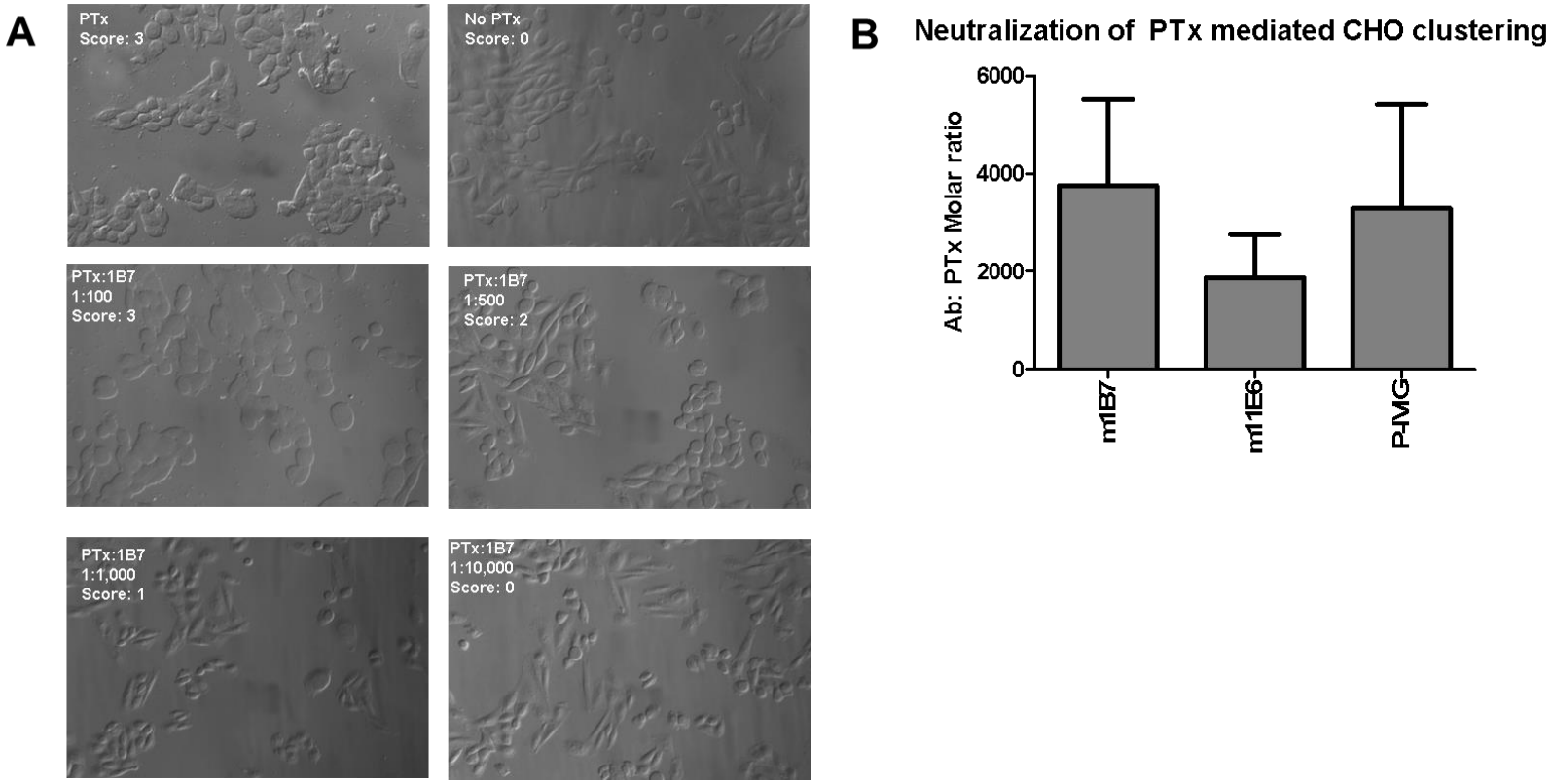


Figure 4.7: Antibody neutralization of PTx mediated CHO cell clustering.

A) A constant concentration of PTx was pre-incubated with serial dilutions of the indicated antibodies and added to a constant concentration of CHO cells. The cells were scored 24 hours later. **B)** Molar neutralization of PTx mediated CHO cell clustering by 1B7 11E6 and P-IVIG. The minimum molar concentration of antibody:PTx needed to change the CHO clustering score from 1 to 0.

Retrograde transport of PTx has previously been demonstrated in CHO-K1 epithelial cells and subsequently in AMJ2-C8 and A549 (alveolar macrophages and lung epithelial cell lines respectively) (Plaut and Carbonetti 2008). After binding to a cellular receptor, PTx undergoes receptor-mediated endocytosis and follows a path of retrograde transport, traveling from the early/recycling endosomes to the Golgi and then to the ER (Xu and Barbieri 1996, el Baya, Linnermann et al. 1997, Plaut and Carbonetti 2008). *In-vitro* ADPR activity of PTx has been shown to occur by 1 hour suggesting PTx is internalized and retrograde transport occurs within this time (Xu and Barbieri 1995). Previous co-localization experiments with the Golgi have indicated that PTx begins accumulating in this organelle after ~20 minutes (Kugler, Bocker et al. 2007). In other studies, tagged PTx-S1 was found to be modified by tyrosine sulfation which occurs in the Golgi network within 2 hours suggesting Golgi transport is the slow step in the retrograde trafficking (Plaut and Carbonetti 2008). Tagged PTx-S1 has also been found to be N-glycosylated, a modification which occurs in the ER, within 3 hours (Plaut and Carbonetti 2008).

We first visualized normal PTx transport in cells via immunofluorescence microscopy using CHO-K1 cells as model epithelial cells. Monoclonal antibodies A8 and D8, recognizing epitopes on the S1 and B subunits of PTx respectively, were used to detect PTx at various times after incubation. Simultaneously, individual organelles were visualized with endosome GFP constructs, CellLight® Golgi reagents or anti PDI antibodies to the endoplasmic reticulum. Similar PTx localization was observed when either the A or B subunit of PTx was used as a detection handle. In the absence of antibodies, PTx was observed to co-localize with the early endosomes within 15 to 60 minutes, started accumulating in the Golgi by 1 hour and started dispersing from the

Golgi to co-localize with the ER by ~3 hours (Figure 4.8). Our results are therefore consistent with the kinetics of PTx retrograde trafficking (Plaut and Carbonetti 2008).

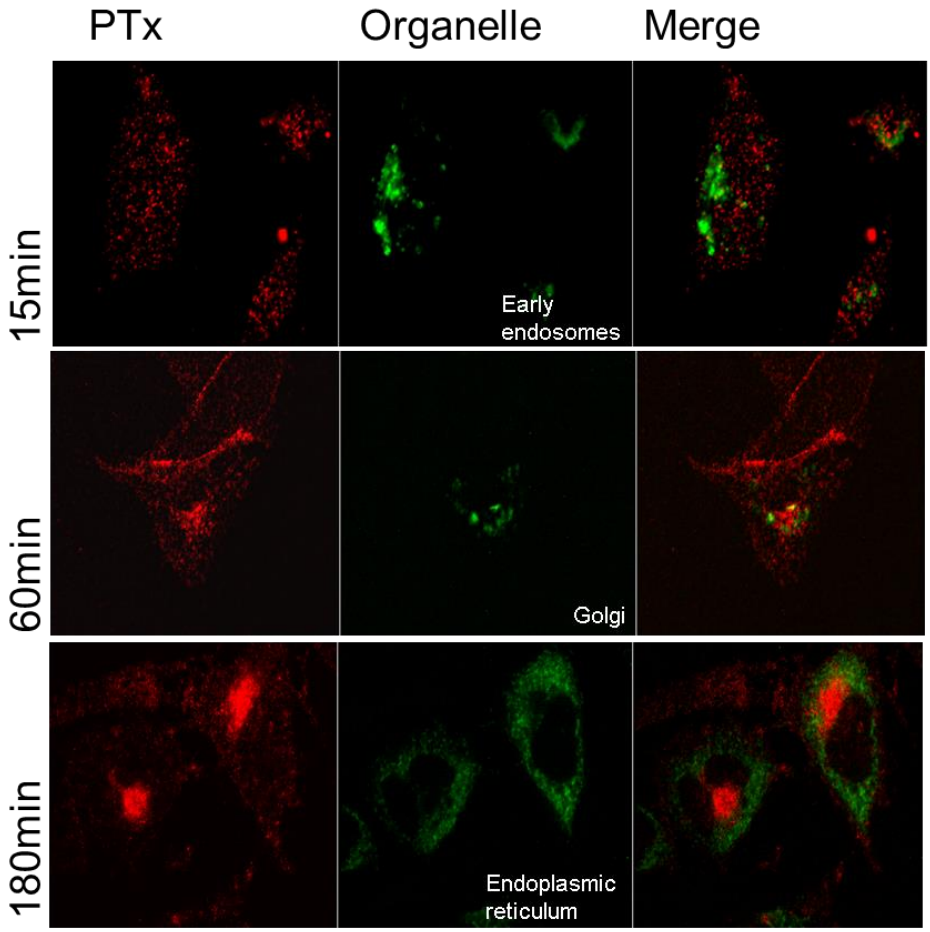


Figure 4.8: Retrograde trafficking of PTx in CHO cells.

PTx was incubated with CHO cells at 37^{o}C for the indicated time points. Intracellular PTx was detected by microscopy after fixing and permeabilization with the monoclonal antibody, D8, to the B-oligomer. D8 bound to PTx was labeled with a fluorescent anti-human-IgG-AlexaFluor 594 secondary antibody before imaging. The early endosomes and golgi were detected by transfecting the CHO cells a day prior to the trafficking experiments with a Rab5-GFP plasmid or CellLight^{TM} Golgi-GFP baculovirus respectively. The endoplasmic reticulum was detected after PTx intoxication and fixing by primary antibody labeling to the ER-resident protein PDI and detected anti-rabbit-IgG-Alexa488. The experiments were repeated at least twice with similar results.

We note that the early endosomes and Golgi are not completely labeled possibly due to fixation with the paraformaldehyde. Further studies are ongoing to test fixation methods to improve the visualization of the early endosomes and Golgi.

In order to directly visualize how 1B7 affects PTx intracellular trafficking, the CHO cells were incubated with PTx and 10 000 molar excess 1B7, a concentration determined to be approximately 3-fold higher than the neutralizing concentration determined from the CHO assay. At this concentration of 1B7, receptor mediated endocytosis of the 1B7-PTx complex appeared to be hindered, as PTx remained associated with the cell surface/plasma membrane of most cells for the time period tested (Figure 4.9). Cells incubated with up to a 10,000 molar excess mIgG2a isotype control mouse antibody showed full internalization of PTx with trafficking similar that of toxin in the absence of antibodies (not shown). A previous study in our lab has also shown that neutralizing concentrations of 1B7 incubated with PTx are also detected at the plasma membrane in the presence of PTx, and does not co-localize with any of the retrograde organelles (Jamie Sutherland dissertation, 2010). The presence of PTx at the plasma membrane suggests two models of 1B7 neutralization of PTx toxicity. In the first model, 1B7 inhibit PTx internalization by sterically hindering the interaction of PTx with the receptors on the surface of the cell. 1B7 primarily binds the S1 subunit, and has been hypothesized to bind the S4 subunit as well. We also show in this study that 1B7 does have a second lower affinity binding site on PTx. Although the S1 and S4 subunits do not have the glycan receptor binding sites (Millen, Schneider et al. 2010), the S4 subunit has been implicated in interacting with the cell membrane during the penetration step of PTx (Montecucco, Tomasi et al. 1986). The S4 subunit has also been implicated in stabilizing the S2 subunit upon formation of the S2S4 dimer (Francotte, Locht et al. 1989).

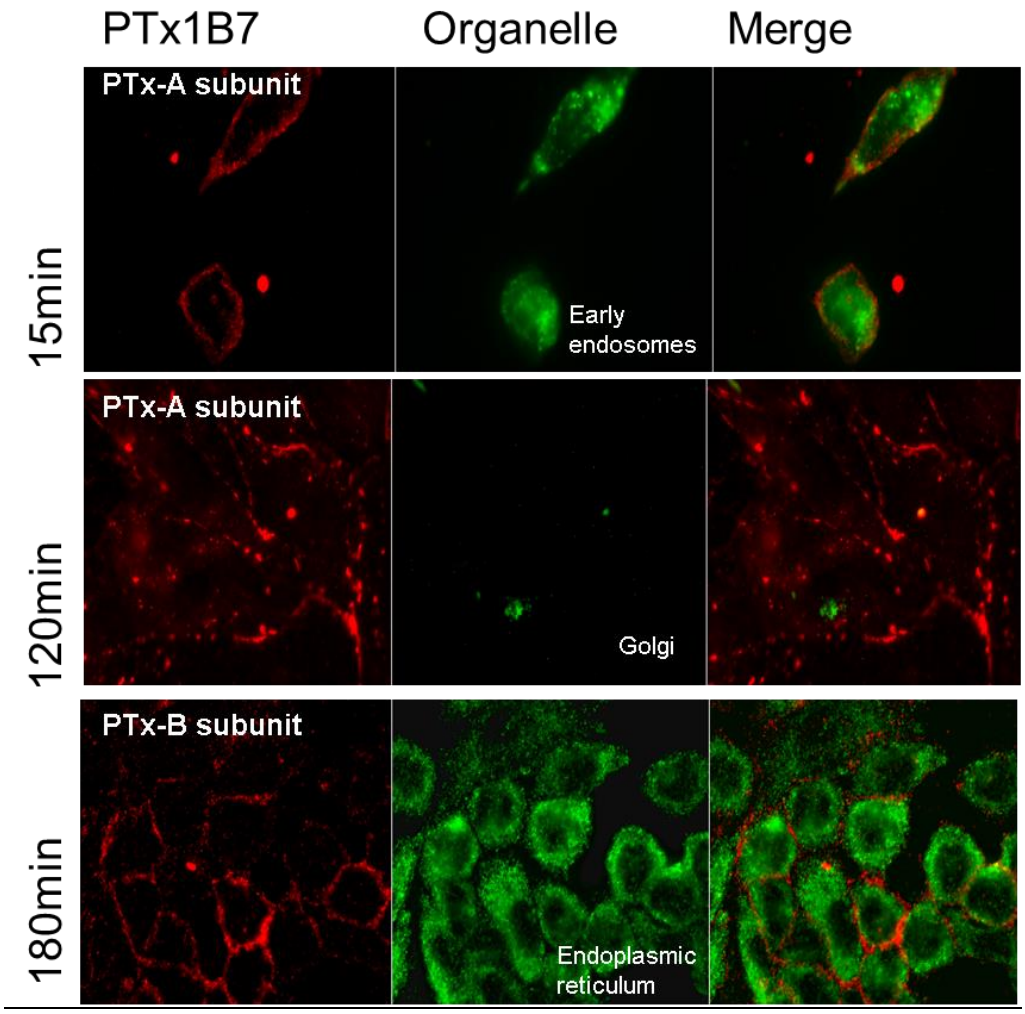


Figure 4.9: 1B7 inhibition of PTx retrograde trafficking in CHO cells.

PTx incubated with 1B7 was added to CHO cells at 37^{o}C for the indicated time points. PTx was detected with the monoclonal antibodies, A8, to the A-subunit, and D8, to the B subunit after fixing and permeabilization. The antibodies bound to PTx were detected with a fluorescent anti-human-IgG-AlexaFluor 594 secondary antibody before imaging. The early endosomes and golgi were detected by transfecting the CHO cells with a Rab5-GFP construct or CellLight^{TM} Golgi-GFP respectively, a day before the trafficking experiments. The endoplasmic reticulum was detected after PTx intoxication and fixing by primary antibody labeling to the ER-resident protein PDI and detected anti-rabbit-IgG-Alexa488. The experiments were repeated at least twice with similar results.

1B7 binding the high affinity S1-S4 epitope and possibly the second lower affinity S4 site could therefore sterically affect toxin-receptor interactions to hinder receptor mediated endocytosis. In the second model of 1B7 neutralization, PTx bound to 1B7 may co-internalize, but be inhibited from the normal retrograde trafficking pathway. Recycling of the PTx-1B7 complex back to the cell surface would then lead to accumulation of PTx at the surface. Neutralizing antibodies to the Shiga toxin and other small molecules to ricin have been shown to neutralize toxicity by inhibiting retrograde trafficking after co-internalization. Previous studies in our lab suggest the PTx-1B7 complex can remain bound in conditions mimicking the retrograde organelles. However, the ability of 1B7 to inhibit PTx binding to fetuin also suggests 1B7 may sterically hinder PTx binding to receptors. Further studies using methods such as sub-cellular fractionation may therefore be needed to fully elucidate the mechanism of 1B7 neutralization in CHO cells.

4.4 DISCUSSION

Recent studies clarifying the potently neutralizing 1B7 epitope in our laboratory show the anti S1 antibody overlaps the S4 subunit (Sutherland and Maynard 2009), thus possibly "stapling" the A and B subunits. In-vivo, 1B7 neutralizes most of the toxic activities attributed to the catalytic activity of the PTx S1-subunit such as leukocytosis-promoting and islet-activating (Burns et al., 1987). Additionally, 1B7 neutralizes the ADP ribosyltransferase (ADPR) activity of S1 *in-vitro*, suggesting that *in-vivo*; 1B7 could competitively inhibit substrate access at the S1 catalytic site. High *in-vitro* anti ADPR activity of S1 antibodies however does not correlate with *in vivo* neutralizing activity (Sato, Sato et al. 1991). Also, it appears unlikely for antibodies to directly

interfere with the ADPR activity in the cytosol in living cells, as this would require escape from the ER of the antibody toxin complex and re-formation of an antibody-S1 complex in the cytoplasm. In light of the additional information regarding the interfacial epitope of 1B7 spanning the S1 and S4 subunits, we sought to further characterize the mechanism of 1B7 neutralization of PTx. We also further characterized the mechanism of neutralization of 11E6 whose epitope is found on the S2 and S3 subunits (Sato, Sato et al. 1987, Sato, Sato et al. 1990, Rambow-Larsen and Weiss 2004) and is thought to directly inhibit PTx binding to receptors (Sato, Sato et al. 1987). Better understanding of the mechanisms of 1B7 and 11E6 neutralization of PTx may aid in antigen engineering of the epitope to develop PTx variants inducing stronger protective responses.

We determined that both the 1B7 and 11E6 epitopes were weakly expressed on the surface of live bacteria, but did not observe significant opsonophagocytic activity due to the antibodies. Consequently, the potent prophylactic/therapeutic properties of 1B7 must rely on recognition of secreted PTx. Although 1B7 and 11E6 do not appear to induce bactericidal activity, the neutralization of either secreted or cell surface bound PTx toxicity, may have implications for PTx mediated adherence. PTx and FHA are important for bacterial adherence to eukaryotic macrophages or cilia (Tuomanen and Weiss 1985, van't Wout, Burnette et al. 1992). In the absence of either PTx or FHA, no adherence to the eukaryotic cells was observed (Tuomanen and Weiss 1985). PTx is a however a secreted toxin, and the apparent redundancy of PTx and FHA in bacterial adherence has been shown to be due to the PTx mediated release of TNF-α, which may damage the pulmonary epithelium to allow more efficient colonization (Alonso, Pethe et al. 2001). Through neutralization of PTx toxicity, 1B7 and 11E6 may therefore be able to block relevant TNF-α release needed for bacterial adherence. Interestingly, it has been

observed that treatment with 1B7 and 11E6 reduces bacterial load in infected mice (Sato, Sato et al. 1990) or baboons (Nguyen, et al. 2014, in review).

To inhibit PTx function, a block in any of the retrograde transport steps can lead to toxin neutralization. The first step of the retrograde transport is the interaction of PTx with the host cell receptors. Antibodies that block this interaction would most likely interact with PTx at its receptor binding sites. We determined that PTx can be simultaneously bound to a model receptor, transferrin, and 1B7 or 11E6. This result was as expected for 1B7, since its primary epitope is located on the S1 subunit, and not on the receptor binding subunits in S2 and S3. This therefore suggested 1B7 may neutralize PTx activity after it interacts with a receptor. The weaker but specific simultaneous binding of 11E6 with the model receptor also indicated 11E6 can bind its second site while bound to a receptor. We subsequently confirmed that 11E6 was able to inhibit PTx binding to the model receptor, fetuin. Surprisingly we also found that 1B7 was also competent to inhibit PTx binding to fetuin, although its binding sites are not on the receptor binding subunits.

Upon directly visualizing 1B7 effects on PTx trafficking in CHO cells, we observed that 1B7 appeared to hinder endocytosis of PTx. Over the time period of 4 hours of PTx trafficking in the presence of neutralizing 1B7 concentrations, PTx was observed be associated with the plasma membrane in the cells imaged. Cells incubated with up to a 10,000 molar excess mIgG2a isotype control mouse antibody however showed full internalization of PTx with trafficking similar that of toxin in the absence of antibodies, further suggesting accumulation of PTx at the plasma membrane to be due to 1B7-mediated inhibition/hindrance of receptor mediated endocytosis. This result was therefore complementary to our observation that 1B7 could inhibit PTx binding to the model receptor, fetuin. 1B7 is thought to have a second low affinity site the S4 subunit (Sutherland and Maynard 2009). Although the characterized receptor-binding sites of

PTx are contained in subunits S2 and S3, possibly interact the S4 subunit is thought to interact with the cell membrane during the penetration step (Montecucco, Tomasi et al. 1986). At least two anti PTx antibodies (1B7 and 7F2) recognizing epitopes spanning the A/B interface are highly neutralizing *in vivo* and *in vitro* (Sato, Sato et al. 1991). While the 1B7 and 7F2 antibodies both bridge the S1/S4 interface, 1B7 predominantly recognizes S1 and 7F2 predominantly recognizes S4 (Sato, Sato et al. 1991, Sutherland and Maynard 2009). With two copies of S4 on PTx, 1B7 may bind the high affinity S1-S4 epitope as well as the second low affinity S4 site to possibly affect PTx interactions with receptor molecules to hinder endocytosis of PTx. Further studies are needed to assess the nature of any conformational changes induced in PTx after receptor binding, and how 1B7 may interfere with such conformational changes.

PTx has a relatively fast internalization time, entering cells within 30 minutes (Xu and Barbieri 1995), thus escaping into the cell before uptake by phagocytes. Inhibition of endocytosis of PTx by 1B7 could therefore allow cells with PTx trapped on the surface to be more easily captured by other professional antigen presenting cells (including dendritic cells, B lymphocytes and macrophages), for presentation to conventional $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells (Cohen et al., 1973; Bilsborough and Viney 2004). Also, PTx induces receptor cross linking to initiate signal transduction in cells (Schneider, Weiss et al. 2007, Schneider, Millen et al. 2012) which contributes to its toxicity. Antibodies spanning the A/B interface may therefore additionally/alternatively inhibit PTx toxicity by preventing such receptor cross linking.

The primary epitope of 1B7 being on the S1 subunit, and its ability to inhibit the ADP-ribosyl transferase activity of PTx *in-vitro*, suggests 1B7 directly blocks S1 subunit-mediated catalytic activity in the cytosol (Sato, Ito et al. 1984, Kaslow, Schlotterbeck et al. 1990, Kaslow, Schlotterbeck et al. 1991). For 1B7 to neutralize PTx intracellularly, it

would need to undergo receptor-mediated endocytosis with PTx and retain high affinity binding in the unique biochemical conditions corresponding to each sub-cellular compartment. Rare, yet protective antibodies, and even novel small molecules have been identified which intracellularly protect against Shiga toxins (Krautz-Peterson, Chapman-Bonofiglio et al. 2008), ricin toxin (Song, Mize et al. 2013), and anthrax toxin (Gillespie, Ho et al. 2013). These molecules block toxin retrograde transport, although at the early endosome-Golgi step, resulting in toxin transfer to the plasma membrane possibly through the recycling endosomes. In the study by Krautz-Peterson et al. (Krautz-Peterson, Chapman-Bonofiglio et al. 2008), the neutralizing A-subunit specific antibody, 5C12, bound and co-internalized with cell bound Shiga toxin 2 (Stx2). 5C12 also did not prevent Stx2 binding to the Gb3 receptor, and protected Stx2 bound HeLa cells. In contrast to 5C12, 1B7 inhibits PTx binding to a model receptor, fetuin, and is also unable to protect CHO cells pre-incubated with PTx, further suggesting 1B7 protects prior to internalization of PTx.

For 1B7 to directly neutralize PTx activity in the cytosol, it would require translocation of the PTx-1B7 complex through the retrograde organelles to reach the cytosol. Previous studies in our lab evaluating the affinity of the 1B7-toxin complex under varying conditions mimicking the retrograde organelles indicated there was no significant change in PTx-1B7 binding. These data suggested that the PTx1B7 complex could remain bound as the complex co-traffics through the acidic endosomes. The analysis also indicated the EC_{50} of the complex was unaffected by incubation at 37˚C with 8μM PDI and up to 0.5 mM ATP suggesting that 1B7 would be able to remain in complex with PTx upon binding of PDI and ATP in the ER. (Jamie Sutherland thesis, 2010). Stability of the PTx1B7 complex in a reducing environment with ATP is in agreement with previous *in-vitro* studies which showed that 1B7 was able to neutralize

the toxin's ADP-ribosylase activity in the presence of ATP and DTT (Sato, Sato et al. 1987). 1B7 also recognizes reduced PTx on a Western blot. In addition, 1B7 recognizes truncated PTx S1 variants with deletions of the C terminus containing the second cysteine needed for forming the disulfide bond holding the S1 subunit together (Bartoloni, Pizza et al. 1988, Sutherland and Maynard 2009). These results support some linearity to the 1B7 epitope or suggest folding due to the disulfide bond does not appear to be important in forming the 1B7 epitope. It has also been suggested that Western blot and ELISA analyses may represent a heterogeneous mixture of undefined forms of PTx (Kaslow, Schlotterbeck et al. 1990). As such, the indication of linearity to the 1B7 epitope may therefore not be a clear indication that 1B7 neutralizes PTx toxicity intracellularly.

Furthermore, for 1B7 to directly neutralize PTx activity in the cytosol, it would require translocation of both PTx and 1B7 through the ER to reach the cytosol. The molecular details of the membrane translocation step for subsequent transport of the S1 subunit out of the ER into the cytosol remains unclear, however, it is thought retrotranslocation across the ER lipid bilayer may involve the Sec61 channel (Hazes and Read 1997). It appears improbable for the immune complex of the large IgG molecule, consisting of four polypeptides and 17 di-sulfide bonds, and the S1 subunit to dissociate and unfold for transport out of the ER, then refold and re-bind S1 in the cytoplasm. If any PTx1B7 complex reaches the ER, it is more likely the complex would be trapped in the ER. Antibody-toxin immune complexes however have been shown to be able to be delivered to the cytosol (Calafat, Molthoff et al. 1988, Raso, Brown et al. 1997), and such antibody-toxin conjugates are being explored for use as therapeutics (Teicher and Chari 2011). It is therefore possible that sec independent pathways may be utilized for transport of a PTx1B7 complex to the cytosol. It has also been suggested that PTx can cross the plasma membrane directly without the need for endocytosis, since it does not require an

acidic environment for entry into eukaryotic cells (Kaslow and Burns 1992), unlike the cholera toxin (Janicot, Fouque et al. 1991). The S1 subunit has also been suggested to become more structured following binding to NAD in the cytosol (Pande et al, 2006). As such, if 1B7 is able to reach the cytosol in complex with PTx, the presence of NAD may further facilitate stability of any PTx1B7 complex that reaches the cytosol to enhance 1B7 neutralization. Further studies using sub-cellular fractionation are therefore needed to assess 1B7 co-trafficking with PTx into the cytosol for intracellular neutralization. Live cell imaging with fluorescent PTx may also reveal differences in PTx trafficking in the presence or absence of 1B7.

Intracellular antibody mediated neutralization mechanism could also utilize lysosomes for degradation (Mellman and Plutner 1984, Joller, Weber et al. 2010) or degradation of the immune complex through other Fc receptor mediated mechanisms such as TRIM21 (McEwan, Mallery et al. 2011, McEwan, Tam et al. 2013). Various constructs of 1B7, including Fabs, scFvs, and scAbs which all lack Fc regions are however able to protect in *in vitro* and *in vivo* assays, (Sato, Sato et al. 1987, Sato, Sato et al. 1990, Sato, Sato et al. 1991, Sutherland and Maynard 2009) suggesting that any Fc receptor functions of 1B7 mediated neutralization may be involved secondarily in other target cells such as macrophages, APCs, or neutrophils which do contain Fc receptors. 1B7 also neutralizes PTx activity in CHO cells, which do not have Fc receptors, further consistent with a lack of a role for Fc receptor mediated neutralization.

In other infectious diseases, notably HIV, potently neutralizing epitopes have been identified for which corresponding antibodies protect even at modest serum concentrations (Walker, Phogat et al. 2009). Currently licensed acellular vaccines for pertussis include chemically detoxified PTx, a process which damages the protective epitope recognized by 1B7 (Nencioni, Volpini et al. 1991) resulting in lower protective

antibody titers upon vaccination than upon actual infection (Sutherland, Chang et al. 2011). Antibodies recognizing the potently neutralizing 1B7 11E6 epitope are more common in naturally infected than vaccinated individuals (Sutherland, Chang et al. 2011), suggesting that this epitope may important for protection. New directions in pertussis vaccine design which include S1 subunit DNA and other protein vaccines, also have lower protective activity most likely because they too lack this protective epitope (Kamachi, Konda et al. 2003, Kamachi and Arakawa 2004, Kamachi and Arakawa 2007). Improvements in vaccine design can be made to elicit 1B7-like antibodies for this unique epitope similar to current HIV research (Walker, Phogat et al. 2009). One possibility would be to use catalytically inactive genetically detoxified PTx (9K/129G) (Burnette, Cieplak et al. 1988, Pizza, Covacci et al. 1989) with some additional genetic modifications to the B-subunit in order to block cellular binding and other side-effects such as T cell proliferation and cell agglutination (Nencioni, Pizza et al. 1991, Millen, Schneider et al. 2013) while retaining protective epitopes. Further research either into engineering or isolating unique mAbs which span A/B subunit interfaces may be the future in developing passive immunization strategies for pertussis.

4.5 CONCLUSIONS

The antibodies 1B7 and 11E6 recognize unique and potently neutralizing epitopes on PTx. Although their epitopes are exposed on whole bacteria, they appear to lack opsonophagocytic activity, indicating their potency lies in neutralizing secreted toxin. While PTx is competent to simultaneously bind a model receptor and the 1B7 or 11E6 antibodies, both the PTx1B7 and PTx11E6 antibody complexes are able to inhibit PTx binding to a receptor. This conundrum is likely because both antibodies have multiple

binding sites which allow the antibody to be simultaneously bound to PTx and a receptor. The observation that 11E6 inhibits PTx binding to a model receptor, is in agreement with its epitopes being on the receptor binding subunits, S2 and S3. 1B7 on the other hand, which binds on the S1 and possibly S4 subunits, also inhibits PTx binding to a model receptor, and hinders typical toxin receptor-mediated endocytosis, trapping the immune complex at the plasma membrane. We hypothesize 1B7 inhibits PTx internalization by altering the conformation of PTx required to bind the receptors, thus hindering the internalization of PTx and subsequent toxicity. The unique non-overlapping epitopes of 1B7 and 11E6 are useful as serological correlates of protection during development of next-generation vaccines, and are candidates for passive immunization strategies.

APPENDIX

A.1: LABORATORY PROTOCOLS

A.1.1 Eukaryotic cell growth and maintenance

A.1.1.1 Starting up a culture from frozen cells

Reagents needed
Complete growth media - DMEM + 10% FBS + 1X P/S
T-25 flask

- Equilibrate the media to 37^{o}C
- Remove the frozen cells from the liquid nitrogen storage wearing the appropriate protective equipment
- Place the frozen cells in a 37^{o}C water bath to thaw
- Aseptically transfer the thawed cells into the cell culture hood by spraying down with 70% ethanol
- Transfer the thawed cells to a 15 mL conical tube and dilute the cells stored in DMSO 10-fold with complete media.
- Spin down gently at 300g for 5 minutes and aspirate all media from the culture
- Resuspend the cells with 5 mL of pre-warmed complete media and seed into the T-25 flask
- Incubator at 37^{o}C, 5% CO_2 and change the media after overnight incubation
- Passage the cells when they reach 80-90% confluence

A.1.1.2 CHO-K1 adherent cells passaging protocol

Cells need to be passaged every 2-4 days or when they reach up to 90% confluence. This protocol is for expanding cells from a T-25 into a T-75 flask. The reagent volumes can be scaled up for larger flasks.

Reagents needed
Complete growth media - DMEM + 10% FBS + 1X P/S
PBS
Trypsin-EDTA

- Equilibrate all media to 37^{o}C

- Aspirate all media from the culture
- Wash cells 2X with pre-warmed PBS
- Add 1 mL of pre-warmed Trypsin-EDTA into the T-25 flask
- Return cells to incubator for 2-10 minutes to allow the adherent cells to detach
- Tap the bottom of the flask to allow cells to completely detach
- Add 4mls of pre-warmed complete media to the cells. The FBS in the complete media quenches the trypsin
- Re-seed the cells using the desired dilution into a T-75 flask (~12mL per T-75 flask).
    - Eg: To re-seed at a 1:12 dilution, add 1mL of the cells into 12 mL of media in the T-75 flask
    - To re-seed at a desired cell concentration, count the cells using the hemacytometer and dilute to the desired concentration

A.1.1.3 Hemacytometer cell counting and determination of cell viability

Reagents needed
0.2% trypan blue
Hemacytometer and cover slip
Microscope
Hand counter

- Add 10µl of 0.2% trypan blue to 10µl of the cell suspension.
- Load one side of the hemacytometer with 10µl of the 2X diluted cell suspension and place on the microscope.
- Count the number of viable cells in 5 out of 9 squares of the hemacytometer. Viable cells are impermeable to the trypan blue dye and remain bright, while non-viable cells take up the dye and are blue.
- Divide the total number of cells counted by 5 to get the average number of cells per square.
- Multiply the average count per square by 10^4 to get the number of cells per ml of the diluted cell suspension.
- Determine the concentration of cells before dilution by multiplying by 2 since the cells were diluted into trypan blue.
    - To determine cell viability Eg for suspension cells, count the total number of cells, and total number of viable cells.

$$\% \text{ viable cells} = \frac{\text{number of viable cells (unstained cells)}}{\text{Total number of cells}} \times 100\%$$

- Clean the cover slip and hemacytometer with 70% ethanol

A.1.1.4 Preparation of 0.2% trypan blue for determining cell viability

Reagents needed
Trypan blue powder
Distilled water

- Dissolve 0.1 g of trypan blue in 50 mL of distilled water
- Spin down at 3000g for 5 minutes to remove large precipitates.
- Clarify by filtering with a 0.45 µm or 0.22 µm filter to remove particulates

A.1.1.5 Cryopreservation of CHO-K1 cells

Reagents needed
Complete growth media - DMEM + 10% FBS + 1X P/S
Freezing media - DMEM + 10% FBS + 1X P/S + 10% DMSO
PBS
Trypsin-EDTA
0.2% trypan blue
Hemacytometer and cover slip
Microscope
Hand counter
Cryovials
Ethanol freezing container

- Equilibrate all media to 37^{o}C
- Aspirate all media from the culture and trypsinize as for passaging
- After cells are detached, dilute 10-fold with complete media
- Count the cells using trypan blue staining. Cell viability to be greater than 90%
- Spin the cells down at 300g for 5 minutes and aspirate off the growth medium
- Resuspend the cells in freezing medium to a concentration of 2 x 10^{6} cells/mL
- Aliquot the cells into cryovials and transfer the cells immediately into the -80^{o}C freezer in an ethanol slow freezing container
- Freeze the cells overnight at -80^{o}C and then transfer to the liquid nitrogen storage

Adapted from
- Abcam cell culture protocols online.

A.1.1.6 Transfection of CHO-K1 cells for transient antibody expression

Materials needed

Low passage umber (<10 passages) CHO-K1 Cells
150 µg Midi-prepped single vector plasmid DNA containing the heavy and light chains
or
75 µg midi-prepped light chain plasmid DNA and 75 µg midi-prepped heavy chain plasmid DNA
Opti-MEM (Life Technologies)
Lipofectamine 2000 (Life Technologies)
Complete growth media: DMEM + 10% FBS + 1X Penicillin/Streptomyin
Antibody growth media: DMEM + 10% Low IgG FBS + 1X Penicillin/Streptomyin
1 M Tris pH 8.0, sterile

Day 1 - Cell preparation:

- Trypsinize CHO-K1 cells
- Count using the hemocytometer
- Plate 5 x 10^6 cells per T-150 flask (3 flasks total) in 24 mL of complete growth media
- Grow overnight in jacketed incubator at 37C and 5% CO_2
- Transfect when the cells reach ~95% confluency

Day 2 - Transfection:

- Mix 12 mL Opti-MEM with 75 µg of the heavy chain and 75 µg of the light chain plasmid
    - For single plasmid vectors, dilute 150 µg of the vector in 12.5 mL of Opti-MEM
- Mix 12 mL Opti-MEM with 375 µL Lipofectamine 2000
- Allow reagents to sit at RT 5 min
- Mix the diluted DNA and Lipofectamine (24 mL total)
- Allow to sit at RT for 20 minutes
- While reagents are sitting, remove media from each flask of cells and replace with 16 mL antibody growth media
- Add 8 mL of the transfection mix (DNA + Lipofectamine) to each flask
- Mix gently and incubate overnight at 37^{o}C, 5% CO_2

Day 3 - Media change:

- Remove the old media from the cells
- Spin down the old media at 5000g for 5 minutes to pellet the cells
- Save the clarified culture supernatant for further antibody purification
- Add fresh antibody growth media to the cells and return to the incubator
- To increase yields, the cells from the old flasks can be reseeded into two new flasks with fresh antibody growth media.

- Replace the media on the cells with fresh antibody growth media every other day for a total of three media changes. Saved the media for further purification.

A.1.1.7 Guidelines for scaling up or down transfection of DNA into different tissue culture vessels

Table A.1: Required volumes for transfection reagents

Scale up/down the concentration of cells plated depending on the culture vessel so that the cells will be approximately 95% confluent at the time of transfection.

Culture vessel	Low IgG media	Opti-MEM (2 tubes)	DNA in µg, (L+H), Dilution volume (µL)	Lipofectamine-2000, dilution volume (µL)	Complex added
24-Well	0.4 mL	50 µL	0.6 µg in 50 µL	1.5 µL in 50 µL	100 µL
12-Well	0.8 mL	100 µL	1.2 µg in 100 µL	3 µL in 100 µL	200 µL
6-Well	1.5 mL	250 µL	3 µg in 250 µL	7.5 µL in 250 µL	500 µL
T-150	16 mL	4 mL	50 µg in 4 mL	125 in 4 mL	8 mL

Adapted from
- Lipofectamine® reagent protocols.
- Optimized transfection protocols from Ellen Wagner

A.1.2 Bacteria growth and maintenance

A.1.2.1 Bordetella growth conditions

Reagents needed
Bordet Gengou blood agar plates
Compplete Stainer Scholte modified medium

- Streak bacteria from frozen stock onto Bordet Gengou blood agar plates and incubate at 37˚C. Plates show good hemolysis after 2-3 days of growth.
- To grow a liquid culture, inoculate a 2 mL starter culture in complete Stainer Scholte media.

- Incubate shaking at 100 rpm at 37˚C for 2-5 days.
- Inoculate larger cultures >100 mL of SSM with added supplements and incubate at 37˚C for an additional 2-5 days.

A.1.2.2 Stainer-Sholte Modified growth medium (SSM)

Complete Stainer Scholte media (SSM) contain the following supplements with basal medium (BM)

- 20 µL 100x supplements / 2 mL BM
- 20 µL 100x proline / 2 mL BM
- 30 µL casamino acids / 2 mL BM
- 8 µL heptakis* / 2 mL BM

* Heptakis is added for production of FHA and PTx (Imaizumi, Suzuki et al. 1983)

Basal Medium (BM)

- Glutamic acid, monosodium salt 5.35 g
- Tris base - 0.76 g
- NaCl - 1.25 g
- KH_2PO_4 - 0.25 g
- KCl - 0.1 g
- $MgCl_2.6H_2O$ - 0.05 g
- CaCl2–0 H2O - 0.01 g
- dH_2O to 500 mL

Adjust with HCl to pH 7.6 (0.25-0.35 mL).
Autoclave and store at 4°C

Supplements (filter sterilize, aliquot <15 mL, and store at -20°C)

100x SSM Supplement

- HCl - 2 mL
- dH_2O - 6 mL
- L-cysteine - 0.8 g
- ddH2O to 200 mL
- Add more HCl if cysteine precipitates.
- $FeSO_4$-$7H_2O$ 0.2 g
- Ascorbic acid - 0.4 g
- Nicotinic Acid (niacin) - 0.08 g
- Glutathione (reduced) 2 g

100x proline supplement - 4.8 g L-proline / 200 mL dH_2O
Casamino acids supplement - 20 g casamino acids / 200 mL dH_2O
Heptakis supplement *- 5 g heptakis / 20 mL ddH_2O

*Blood agar plates**
SSM basal media with 100 mL less ddH_2O/L.

- Add 15 g/L bacto agar.
- Autoclave and cool to 50˚C.
- Add 10 mL of 100x SSM supplement and proline supplement.
- Heat sheep blood to 37˚C.
- Pour blood down side of flask, swirl gently and pour plates (~20mL/plate).

*Bordet Gengou blood agar plates can also be ordered from BD Biosciences (Fisher Catalog No.: B97876X)

Adapted from

- Sutherland J. S. 2010. The Potently Neutralizing Monoclonal Antibody 1B7: its Unique Epitope, Effects on Intracellular Trafficking, and Elicitation upon Infection with Pertussis. PhD Dissertation to UT, Austin.
- Menozzi, F. D., R. Mutombo, G. Renauld, C. Gantiez, J. H. Hannah, E. Leininger, M. J. Brennan and C. Locht (1994). "Heparin-inhibitable lectin activity of the filamentous hemagglutinin adhesin of Bordetella pertussis." Infect Immun **62**(3): 769-778.
- Imaizumi, A., Y. Suzuki, S. Ono, H. Sato and Y. Sato (1983). "Heptakis(2,6-O-dimethyl)beta-cyclodextrin: a novel growth stimulant for Bordetella pertussis phase I." J Clin Microbiol **17**(5): 781-786.

A.1.2.3 Bordetella glycerol stocks

Reagents needed
50% sterile glycerol
Cryovials

- Add 500 µL of the bacterial culture to 500 µL of the sterile 50% glycerol
- Freeze the glycerol stock tubes at -80^{o}C

A.1.3 Protein purification

A.1.3.1 Antibody purification

Antibodies purified from the cell culture supernatant or ascitic fluid are described.

Materials needed
Ammonium sulfate
1 M Tris pH 8.0, sterile
Binding buffer: 100 mM sodium phosphate buffer, 150 mM NaCl, pH 7
Elution buffer: 0.1 M Glycine, pH 2.7
Neutralization buffer: 1M Tris, pH 8

Cell culture supernatant
Culture supernatant volumes less than 200 mL can be directly applied to the column after centrifugation and filtration through a 0.22 µm filter. For culture supernatant volumes greater than 200 mL, initially concentrate using ammonium sulfate precipitation or tangential flow filtration.

- Clarify supernatant by centrifuging at 10 000 rpm for 30 minutes.
- Add 25 mLs of Tris–HCl, pH 8 per 500 mLs of culture supernatant.
- Precipitate antibody using 50% (w/v) ammonium sulfate (313 g/L of culture), gradually, in four parts (1 part per hour) while the supernatant is being slowly stirred at 4˚C.
- Mix the supernatant slowly overnight.
- After overnight concentration with the 50% ammonium sulfate, spin down the supernatant at 10 000 rpm for 30 min.
- Pour off the supernatant and resuspend the pellet in binding buffer (0.1*volume of starting culture volume).
- Transfer into dialysis membrane and dialyze against 2 L of binding buffer overnight at 4^{o}C.
- Spin down the resuspended pellet at 18 000 rpm for 30 min to remove all particles.
- Filter sterilize using a 0.22 µm filter and load onto a protein A/G column (See Column purification procedure).
- Estimate protein concentrations of of mAb containing fractions
- Determine purity of mAb fractions by SDS-PAGE
- Pool desired fractions
- Determine activity using an ELISA
- Filter sterilize pooled fractions under sterile conditions.
- Store aliquots at -20^{o}C

Ascites fluid

- Dilute the ascites fluid with the binding buffer. Purify antibodies from 2-3mL of the ascitic fluid.
- Clarify ascites fluid by centrifuging at 12 000 rpm for 15 minutes.
    - Filter through glass wool to remove lipids.
- Filter using a 0.22 µm filter before loading onto the protein A/G column (See Column purification procedure)
- Estimate protein concentrations of mAb containing fractions
- Determine purity of mAb fractions by SDS-PAGE
- Pool desired fractions
- Determine activity using an ELISA
- Filter sterilize pooled fractions under sterile conditions
- Store aliquots at -20^{o}C

Adapted from

- Harlow E., and Lane D. 1988. Antibodies: A laboratory manual. Cold spring harbor laboratory press. Cold spring harbor, New York.
- Sutherland J. S. 2010. The Potently Neutralizing Monoclonal Antibody 1B7: its Unique Epitope, Effects on Intracellular Trafficking, and Elicitation upon Infection with Pertussis. PhD Dissertation to UT, Austin.
- Antibody purification handbook. Amersham Biosciences.

A.1.3.2 Full length FHA purification

FHA is purified from the culture supernatant of *B. pertussis* and *B. bronchiseptica* using heparin affinity chromatography as described. FHA from *B. parapertussis* however does not bind heparin. For FHA production, the Bordetella is grown in Stainer Sholte media containing 1g/L heptakis. Culture supernatant volumes less than 200 mL can be directly applied to the column after centrifugation and filtration through a 0.22 µm filter. For culture supernatant volumes greater than 200 mL, initially concentrate using tangential flow filtration.

Materials needed

Binding buffer: 50 mM Tris, pH 8, 50mM NaCl

Elution buffer: 50 mM Tris, pH 8, 500mM NaCl

- Clarify supernatant by centrifuging at 10 000 rpm for 30 minutes.
- Filter sterilize using a 0.22 µm filter and
- Load supernatant onto the heparin column and elute purified FHA (See Column purification procedure)

Adapted from

- Menozzi, F. D., R. Mutombo, G. Renauld, C. Gantiez, J. H. Hannah, E. Leininger, M. J. Brennan and C. Locht (1994). "Heparin-inhibitable lectin activity of the filamentous hemagglutinin adhesin of Bordetella pertussis." Infect Immun **62**(3): 769-778.
- Stainer, D. W. and M. J. Scholte (1970). "A simple chemically defined medium for the production of phase I Bordetella pertussis." J Gen Microbiol **63**(2): 211-220.
- Imaizumi, A., Y. Suzuki, S. Ono, H. Sato and Y. Sato (1983). "Heptakis(2,6-O-dimethyl)beta-cyclodextrin: a novel growth stimulant for Bordetella pertussis phase I." J Clin Microbiol **17**(5): 781-786.

A.1.3.3 Purification of recombinant FHA fragments

(Using the pMAL c5X vector from BL21 cells)

Materials needed

TB media (500ml) per prep, autoclaved; adjust with appropriate antibiotic in 2L Flask
1M IPTG, filter sterile (stored at -20C)
Binding buffer: 50mM Hepes, 250mM NaCl, 2mM $CaCl_2$, 40mM imidazole, pH 8 (filter sterilize)
Elution buffer: 50mM Hepes, 250mM NaCl, 2mM $CaCl_2$, 40mM imidazole, pH 8, 10mM maltose (filter sterilize)

Day 1: Growth

- Start a 2mL starter culture with antibiotic from a single colony from an agar plate at the end of the day at 37^{o}C shaking at 225RPM

Day 2: Expression

- Use 500 µL of the starter culture to inoculate 500 mL of TB with antibiotic
- Grow the cells to an OD_{600} of approximately 0.5
- Induce by adding IPTG to a concentration of 1 mM and grow for an additional 2 hours at 37^{o}C
- Harvest the cells by centrifuging for 30 minutes at 10K rpm
- Resuspend the cell pellet in 25 mL binding buffer per L of culture
- Lyse the cells using the French Press
- Clarify the lysed cells by centrifugation at 18K rpm for 30 minutes
- Filter using a 0.22 µm filter before loading onto the MBP column (See Column purification procedure)

- Determine purity by SDS-PAGE

Adapted from
- NEB pMAL Protein Fusion & Purification System

A.1.3.4 HiTrap affinity column purification

- Wash the column with 5-10 column volumes (CVs) of water (max back pressure 0.3 MPa).
- Pre-equilibrate the column with 5-10 column volumes (CVs) of binding buffer until A_{280} returns to baseline.
- Apply sample.
- Wash the column with binding buffer until A_{280} returns to baseline (~5-10 CVs).
- Elute the protein with elution buffer until A_{280} returns to baseline.
- After the purification, wash the column with water and then 20% ethanol until baseline (~ 5 CV).
- Store the column at 4˚C.

Adapted from
- HiTrap column manual

A.1.4 Protein characterization assays

A.1.4.1 ELISA

A.1.4.1.1 Indirect ELISA for estimation of binding affinity

Coating antigen to microplate

- Dilute the antigen (PTx) to 1 μg/ml in PBS
- Coat the wells of an ELISA high binding microtiter plate with the antigen by pipeting 50 μl of the antigen dilution into all the wells of the plate.
- Cover the plate with an adhesive plastic and incubate overnight at 4^{o}C.
- Wash the plate 3X with PBST.

Blocking

- Block the remaining protein-binding sites in the coated wells by adding 50 μl blocking buffer (5% non-fat dry milk in PBST), per well.
- Cover the plate with an adhesive plastic and incubate for at least 1h at room temperature.
- Wash the plate 3X with PBST.

Incubation with the primary antibody

- Add 50 µl of the primary antibody (Eg. m1B7), diluted to 10 µg/ml in blocking buffer. Dilute down the plate as required (Eg. 1:5).
- Cover the plate with an adhesive plastic and incubate for 1 h at room temperature.
- Wash the plate 3X with PBST.

Incubation with the secondary antibody

- Add 50 µl per well of the secondary antibody (G α M IgG - HRP), diluted to1:1000 in blocking buffer.
- Cover the plate with an adhesive plastic and incubate for 1 h at room temperature.
- Wash the plate 3X with PBST.

Detection

- Dispense 50 µl of the substrate solution (TMB:Peroxide 1:1) per well.
- After sufficient color development (~1-10mins), add 50 µl of stop solution (1N HCl) to the wells.
- Read the absorbance (optical density) of each well with a plate reader. Softmax software.

Analysis of data

- Prepare a curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs absorbance on the Y axis (linear).
- Estimate the binding affinity fitting the curve to a non-linear equation (Eg 4 parameter logistic equation).

A.1.4.1.2 Competition ELISA

Eg 1: Antibodies competing for binding to epitopes on PTx

Coating antigen to microplate

- Dilute the antigen (PTx) to a final concentration of 1 µg/ml in PBS.
- Coat the wells of an ELISA high binding microtiter plate with the antigen and wash as described above for an indirect ELISA.

Blocking

- Block and wash the plates as described above for an indirect ELISA above.
    - When using biotinylated antibodies, use 5% BSA in PBST to block since milk contains free biotin.

Incubation with the primary antibody

- Add the competing antibody (Eg. hu1B7), diluted to the optimal starting concentration (Eg 50 µg/ml) in blocking buffer. Dilute down the plate.
- Add 25 µl of the primary antibody (Eg. biotinylated D8) to a final concentration of 0.6 µg/ml to all the wells,
    - Adjust molar ratio of the competing and primary antibodies as desired.
- Cover the plate with an adhesive plastic and incubate for 1 h at room temperature.
- Wash the plate 3X with PBST.

Incubation with the secondary antibody

- Add 50 µl per well of the secondary antibody (Streptavidin-HRP), diluted to 1:1000 in blocking buffer.
- Cover the plate with an adhesive plastic and incubate for 1 h at room temperature.
- Wash the plate 3X with PBST.

Detection and data analysis

- Dispense 50 µl of the substrate solution (TMB:Peroxide 1:1) per well.
- After sufficient color development (~1-10mins), add 50 µl of stop solution (1N HCl) to the wells.
- Read the absorbance (optical density) of each well with a plate reader. Softmax software.
- Prepare a curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs absorbance on the Y axis (linear).
- If desired, estimate the IC_{50} by fitting the curve to a non-linear equation (Eg. 4 parameter logistic equation).

Eg 2: Inhibition of PTx binding to fetuin model receptor

Coating receptor to microplate

- Dilute the fetuin receptor to a final concentration of 10 µg/ml in PBS.
- Coat the wells of an ELISA high binding microtiter plate with the receptor by pipeting 50 µl into all the wells of the plate.
- Cover the plate with an adhesive plastic and incubate at $4^{o}C$ overnight.
- Wash the plate 3X with PBST.

Blocking

- Block the remaining protein-binding sites in the coated wells by adding 50 µl blocking buffer (5% BSA in PBST), per well.
- Cover the plate with an adhesive plastic and incubate for at least 1h at room temperature
- Wash the plate 3X with PBST.

Incubation of PTx and competing antibodies

- Add the competing antibody (Eg. hu1B7), diluted to 100 µg/ml) in blocking buffer to the first well only. Dilute down the plate 1:5 leaving (This leaves 40 µl solution per well).
- Add 10 µl of 0.5 µg/mPTx to all the wells (PTx final concentration - 0.1 µg/ml.
    - Adjust molar ratio of competing antibody: PTx as desired.
- Cover the plate with an adhesive plastic and incubate overnight at 4^{o}C.
- After overnight incubation, wash the plate 3X with PBST.

Incubation with the primary antibody

- Add 50 µl of primary antibody to detect residual PTx, (m1B7 + m11E6 + m7F2 each at a concentration of 0.1 µg/ml).
- Cover the plate with an adhesive plastic and incubate for 1 h at room temperature.
- Wash the plate 3X with PBST.

Incubation with the secondary antibody

- Add 50 µl of the secondary antibody (G α M IgG - HRP), diluted to 1:1000 in blocking buffer.
- Cover the plate with an adhesive plastic and incubate for 1 hour at room temperature.
- Wash the plate 3X with PBST.

Detection and data analysis

- Dispense 50 µl of the substrate solution (TMB:Peroxide 1:1) per well.
- After sufficient color development (~1-10mins), add 50 µl of stop solution (1N HCl) to the wells.
- Read the absorbance (optical density) of each well with a plate reader. Softmax software.
- Prepare a curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs absorbance on the Y axis (linear).
- Estimate the IC_{50} by fitting the curve to a non-linear equation (Eg. 4 parameter logistic equation).

A.1.4.2.3 ELISA to detect simultaneous binding of antibody to PTx bound to receptor

Coating receptor to microplate

- Dilute the fetuin receptor to a final concentration of 10 µg/ml in PBS.
- Coat the wells of an ELISA high binding microtiter plate with the receptor by pipeting 50 µl into all the wells of the plate.
- Cover the plate with an adhesive plastic and incubate at 4^{o}C overnight.
- After overnight incubation, wash the plate 3X with PBST.

Blocking

- Block the pates with 5% BSA, and wash 3X with PBST.

Incubation with the antigen

- Add the PTx diluted to 21 µg/ml (200 nM) in blocking buffer to the first well. Dilute down the plate as required (Eg. 1:5).
- Cover the plate with an adhesive plastic and incubate for 1 h at room temperature.
- Wash the plate 3X with PBST.

Incubation with the primary antibody

- Add 50 µl of the primary antibody (Eg. hu1B7), diluted to 1 µg/ml in blocking buffer (5% BSA in PBS) to all the wells.
- Cover the plate with an adhesive plastic and incubate for 1 h at 37^{o}C.
- Wash the plate 3X with PBST.

Incubation with the secondary antibody

- Add 50 µl of the secondary antibody (Eg. G α Hu Fc - HRP), diluted to the optimal concentration (1:1000) in blocking buffer.
- Cover the plate with an adhesive plastic and incubate for 1 h at 37^{o}C.
- Wash the plate 3X with PBST.

Detection and data analysis

- Dispense 50 µl of the substrate solution (TMB:Peroxide 1:1) per well.
- After sufficient color development (~1-10mins), add 50 µl of stop solution (1N HCl) to the wells.
- Read the absorbance (optical density) of each well with a plate reader. Softmax software.
- Prepare a curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs absorbance on the Y axis (linear).
- Estimate the EC_{50} by fitting the curve to a non-linear equation (Eg. 4 parameter logistic equation).

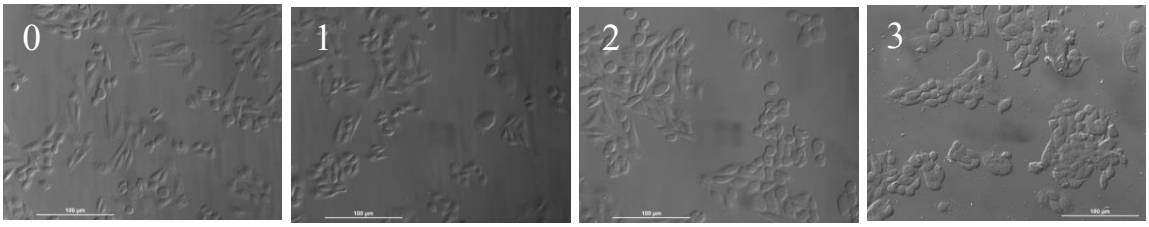
Adapted from

- Schmidt, M. A. and W. Schmidt (1989). "Inhibition of pertussis toxin binding to model receptors by antipeptide antibodies directed at an antigenic domain of the S2 subunit." Infect Immun **57**(12): 3828-3833.
- Sutherland J. S. 2010. The Potently Neutralizing Monoclonal Antibody 1B7: its Unique Epitope, Effects on Intracellular Trafficking, and Elicitation upon Infection with Pertussis. PhD Dissertation to UT, Austin.

A.1.4.2 CHO cell neutralization assay

This assay is used to determine the *in vitro* neutralization ability of anti-PTx antibodies. CHO cells normally grow in an elongated morphology. When exposed to PTx, the CHO cell morphology changes and the cells grow in clusters. The CHO neutralization assay is used to determine the concentration of antibodies which rescues CHO cells from PTx induced clustering.

Morphology is rated on a scale of 0 to 3:
0 – no clustering 1 – few clusters/equivocal
2 – Positive clustering 3 – complete/maximal clustering



Materials needed
PTx
Sterile 96 well tissue culture plate
37^{o}C incubator with 5% CO_2
Complete growth medium (DMEM + 10% FBS + 1X P/S)
PBS
Antibody
Trypsin-EDTA
Microscope

- Add 50 µl of antibody in duplicate starting at 60 000 molar excess of PTx into the top row of the plate. Dilute down serially, 1:2 into 25 µl of complete growth media (DMEM with 10% FBS and 1X P/S. This leaves 25 µl in each well.
- Prepare a stock of 10 µg/ml dilute PTx in complete growth media.
    - Prepare a working dilution of 60 pM PTx from the 10 µg/ml stock, add 25µl/well to the 96 well plate containing the antibody dilutions.
- Incubate the plate for ½ hr at 37˚C with 5% CO_2.
- While the PTx-antibody complexes are incubating, wash confluent CHO cells twice with PBS.
- Detach cells with Trypsin-EDTA at 37˚C for 1-10 min.
    - Resuspend and dilute the cells to a concentration of 1.5 x 10^5 cells/ml.
- Seed 100 µl/well of the diluted CHO cells into the plates containing the incubated PTx and antibodies.
    - This results in

        - a final PTx concentration of 10 pM PTx/well,
        - concentration of antibodies starting at 10 000 molar excess, and
        - a final cell concentration of 1.5 x 10^4 cells/well.
- Incubate at 37˚C, 5% CO_2 overnight (~17 hours).
- Score the wells with a microscope based on clustering morphology (Sample pictures above).

Notes:

- The working concentration of PTx was determined from a preliminary toxin concentration series over the range of 12 - 0.094 ng/mL PTx which resulted in scoring of 3 to 0. A test dose of 10 pM PTx in the 150µl culture volume (approximately four times higher than the minimum PTx dose showing a score of 3) was used to reduce lot- to - lot variability.
- A lower concentration of PTx can be used to conserve PTx and antibodies.
- A lower cell concentration (Eg: 5 x 10^3 cells/well) is more stringent for assessing neutralization. At lower cell concentrations, score cells after 24-48 hours.
- Neutralization to PTx toxicity is recorded as the lowest molar concentration of antibody to PTx that completely inhibits clustering (concentration at which the score changes from a 0 to a 1).
- If no neutralization is observed at 10 000M excess, test higher concentrations of antibodies if possible.
- To score at a later time, cells can be fixed with 4% paraformaldehyde (PFA) at RT for 20mins, washed with PBS, and stored at 4^{o}C in 1% (PFA).
- For imaging, cells can be stained with a giemsa stain if using a brightfield microscope. The cells however don't need to be stained when using a phase contrast or DIC microscope as the contrast allows them to be more visible.
    - The assay can also be performed on coverslips in 6 well plates, placed unto glass-slides with mounting media and imaged. DAPI Fluoromount G (Southern biotech) can be used to mount the slides to stain the nucleus and makes it easy to locate the cells if using a fluorescent microscope.

<u>Adapted from</u>

- Sutherland J. S. 2010. The Potently Neutralizing Monoclonal Antibody 1B7: its Unique Epitope, Effects on Intracellular Trafficking, and Elicitation upon Infection with Pertussis. PhD Dissertation to UT, Austin.
- Sato, H., Sato, Y., Ito, A., Ohishi, I. Infect Immun. 1987. 55(4): 909-15.
- Hewlett, E. L.,Sauer, K. T., Myers, G. A., Cowell, J. L., Guerrant, R. L. Induction of a Novel Morphological Response in Chinese Hamster Ovary Cells by Pertussis Toxin. Infect Immun. 1983 40 (3): 1198-203.

A.1.4.3 Hemagglutination Assay and Inhibition of Hemagglutination

The lowest amount of FHA that caused hemagglutination was qualitatively determined from hemagglutination of 0.5% sheep red blood cells in a round bottom 96 well micro-titer plate. Hemagglutination of the RBCs prevent them from settling out of suspension while in control wells, the RBCs settle in a pellet at the bottom of the round bottom well.

Materials needed
Round bottom 96 well micro-titer plate
PBS

FHA hemagglutination assay
The least amount of FHA that caused complete hemagglutination of 0.5% sheep red blood cells was first determined by

- Serially diluting FHA 1:2 in the 96 well round bottom plate.
- Add sheep red blood cells (RBC) to 0.5%, and top the volume up to 100 µl per well.
- Incubate the mixture kept at room temperature for approximately 3 hours.
- The last well showing complete hemagglutination by visual inspection is determined to be the limit of detection.

Inhibition of FHA mediated hemagglutination

- Serially dilute the antibodies first in the micro-titre plate.
- Add FHA to the antibodies and incubate the mixture at 25°C for 30 minutes.
- Add sheep RBC to a final concentration of 0.5% and top up the volume to 100 µl.
- Incubate the mixture at 25°C for approximately 3 hours.
- The lowest amount of antibody that inhibits hemagglutination is taken as the neutralizing dose.

Adapted from

- Colombi, D., D. S. Horton, M. L. Oliveira, M. A. Sakauchi and P. L. Ho (2004). "Antibodies produced against a fragment of filamentous haemagglutinin (FHA) of Bordetella pertussis are able to inhibit hemagglutination induced by the whole adhesin." FEMS Microbiol Lett **240**(1): 41-47.

A.1.4.4 PTx trafficking using immunofluorescence microscopy

Day 1

- Seed CHO cells on autoclaved cover slips in a 6 well tissue culture plate.

- Incubate at 37˚C with 5% CO_2 until cells are ~70% confluents (1-2 days).

Day 2

- Wash 2X with HBSS.
- Aspirate and block irrelevant antigens with blocking buffer for 30mins at 37˚C, 5% CO_2.

Blocking buffer
DMEM base (-FBS, -antibiotic)
2mg/ml BSA
1-5µg/ml irrelevant antibody (Eg. mIgG1)

- Incubate 10 nM PTx with 10,000 nM 1B7 or other co-trafficking antibody in 50µL 37˚C with 5% CO_2 for ½ hour.
- Place cover slip cell side down on the 50µL drop on parafilm and incubate at 37˚C for desired trafficking time.
- Rinse with PBS for 5 min in the 6 well plate. Remove slide 5 min before the end of the desired time point to rinse.
- Fix for 20 min at room temperature.

Fixing buffer
4% Paraformaldehyde in PBS

- Wash 3X with PBS for 10 min each.
- Permeate cells with permeabilization buffer for 20 mins at room temperature.

Permeabilization buffer
1X PBS
0.1% Triton X-100

- Block intracellular irrelevant antigens with blocking buffer for 1 hr at 37˚.
- Label by placing the cover slip cell side down on a 50µL drop of primary antibody in blocking buffer on parafilm.
- Incubate in a humid container at 4˚C overnight.

Day 3

- Wash 3X with PBS for 10 min each.
- Label by placing the cover slip cell side down on a 50µL drop of secondary antibody (fluorescent label) in blocking buffer on parafilm.
- Incubate in the dark at 37˚C for 1 hr.
- Wash 3X with PBS for 15 min each.
- Mount on slides with 1 drop of fluoromount-G or DAPI fluoromount-G (SouthernBiotech).

- Press gently with Kimwipe to remove excess and to seal the cover slip.
- Allow the preps to air dry for 5 minutes before examination or storage at 4˚C.

Widefield fluorescence microscope (UT Core facilities)

Startup

- Turn on the arc lamp followed by the microscope.
    - Wait 2 min.
- Boot up the Zeiss software.
-
- Add a small drop of oil onto your slide and focus manually on the microscope.
- Enter the Zeiss software, and change the light path from the eye to the camera.
- Set gain to minimize background fluorescence.
    - Do not change this setting once it is set for the experiment.
- Set up multi-dimensional analysis for different fluorophores to be detected. Set exposure time to minimize oversaturation and show maximum pixels.
- Save files as .tif. Export file to save in color as a .jpeg.

Shutdown

- Close the Zeiss software.
- Wait 1 min.
- Turn off the microscope followed by the arc lamp.
- Clean the oil objective with optical tissue and MeOH only wiping once

Confocal scanning fluorescence microscope (UT Core facilities)

Startup

- Turn on confocal microscope using instructions posted.
- Login into computer.
- Startup Zen software
- Choose objective. If using an oil objective, add a drop of oil to the top of the objective.
- Place sample on the stage, cover slip down.
- Focus on sample using locate tab.
- Switch to acquisition for imaging.
- For multiple dyes, use smart set up to configure acquisition settings depending on dyes used.
    - For a brightfield/DIC image, click T-PMT.
- Proceed to acquiring images
- Save image at 16 bit .tif for high resolution

Shutdown

- Close the Zeiss software.
- See instructions posted.
- Clean the oil objective with optical tissue if used.

Adapted from

- Sutherland J. S. 2010. The Potently Neutralizing Monoclonal Antibody 1B7: its Unique Epitope, Effects on Intracellular Trafficking, and Elicitation upon Infection with Pertussis. PhD Dissertation to UT, Austin.
- Zeiss 710 user guide.

A.1.4.5 Cell adhesion assay

*FACS method

Materials needed

FITC
Bacteria
12-well tissue culture plate
Cell scrapers
PBS
DMEM + 10% FBS
CHO cells

FITC labeling of *B. pertussis*

- Dissolve FITC (Thermoscientific) in DMSO to a concentration of 10 mg/mL.
- Label 1 x 10^8 cfu/mL *B. pertussis* with a concentration of 1 mg/ml FITC in 100 µL of 100 mM carbonate buffer, pH 9.2.
- Incubate the bacteria with the FITC at room temperature for 30 minutes
- Wash the cells three times with PBS to remove excess FITC.

Adherence of Bp-FITC to CHO-K1 cells

- Add 1 x 10^6 CHO-K1 cells per well to 12-well plates and allow to adhere for 1 hour at 37^{o}C, 5% CO_2.
- Maintain in media without antibiotics (DMEM buffer with 10% FBS).
- Pre-incubate the Bp-FITC in the presence of 25 µg/ml antibodies to assess antibody inhibition of cell adhesion.
    - Adjust concentrations of antibodies as needed to assess effects of concentration on adhesion.
- Add the Bp-FITC + antibody to the CHO-K1 cells and spin down gently at 300g for 2 minutes to promote contact between the bacteria and CHO cells.

- Incubate the plates for 1 hour at 37°C.
- Wash the wells with PBS to remove unbound bacteria.
- Scrape off adherent cells and resuspend into FACS buffer (PBS + 2% FBS).
- Analyze bacteria bound to the CHO cells by flow cytometry.
    - The analysis does not differentiate between bound and internalized *B. pertussis*.

* To analyze visually by light microscopy, cells are seeded on coverslips in 12-well tissue culture plates. After the assay, the cells on the coverslips are fixed, stained and visualized without scraping the cells off.

Adapted from

- Hazenbos, W. L., B. M. van den Berg, J. W. van't Wout, F. R. Mooi and R. van Furth (1994). "Virulence factors determine attachment and ingestion of nonopsonized and opsonized Bordetella pertussis by human monocytes." Infect Immun **62**(11): 4818-4824.
- Cotter, P. A., M. H. Yuk, S. Mattoo, B. J. Akerley, J. Boschwitz, D. A. Relman and J. F. Miller (1998). "Filamentous hemagglutinin of Bordetella bronchiseptica is required for efficient establishment of tracheal colonization." Infect Immun **66**(12): 5921-5929.

A.1.4.6 Bactericidal assay

Materials needed
Bordet Gengou blood agar plates
Inoculation loop
96-well round bottom plate
Stainer Scholte media
Bacterial plating beads

- Streak out *B. pertussis* unto fresh Bordet Gengou plates. Grow for 2-3 days at 37°C.
- Scrape off *B. pertussis* showing good hemolysis using inoculation loop into Stainer Scholte media.
- Strain to remove cell clumps.
- Determine the OD_{600} of the resuspended bacteria.
- Convert the OD_{600} of the resuspended bacteria to cfu/ml.
    - OD_{600} of $0.9 \equiv 5 \times 10^9$ cfu/ml (Warfel, Zimmerman et al. 2013)
- Dilute the *B. pertussis* to 3×10^5 cfu in 20 µl.
- Add the antibodies to be assayed to a concentration of 25 µg/m in 20 µl to the bacteria in the round bottom 96 well plate.
- Incubate the bacteria + antibody mixture for 30 minutes at 37°C.

- Add IgG depleted complement to a concentration of 10%, and top off the total volume to 20 µl.
- Incubate the bacteria + antibody + complement for 1 hour at 37^{o}C.
- Add 80 µl of 10% EDTA in PBS to stop the complement reaction.
- Dilute down the bacteria mixture 1:2 in the 96 well plate.
- Plate selected dilutions on fresh Bordet Gengou blood agar plates.
    - Eg: 1:16, 1:256, 1:1024
- Incubate the plates at 37^{o}C and check for growth after 2-5 days.
- Use the dilution that shows countable the colonies to estimate the bactericidal activity. 1:1024 dilution usually has countable colonies.
- Control plates
    - Bacteria only, without complement
    - Bacteria + complement, no antibody
    - Bacteria + complement + isotype control/irrelevant antibody

Adapted from

- Barnes, M. G. and A. A. Weiss (2003). "Activation of the complement cascade by Bordetella pertussis." FEMS Microbiol Lett **220**(2): 271-275.

A.2 DNA protocols

A.2.1 Colony pcr for amplification of DNA sequences from Bordetella pertussis

The pcr reaction mixture includes 5% DMSO for amplification from the GC rich genomic DNA of the *Bordetella* strains.

PCR reaction mix (50 µl total reaction):

- 2.5 µl 5' Primer (10 µM)
- 2.5 µl 3' Primer (10 µM)
- 5 µl Thermopol Buffer
- 1 µl dNTPs (10 µM)
- 5% DMSO
- 0.25 µl Vent DNA Polymerase
- dH_2O to 50 µl

PCR cycling conditions

Use the NEB Tm calculator to estimate the annealing temperature for the reaction.

- 94^{o}C for 2 minutes (disrupt cells, separate DNA)
- Cycle 35 times:

    - 95°C for 30 s (melting)
    - 55°C for 45 s (annealing)
    - 72°C 1 minute per kb (elongation)
- 72 C for 10 minutes (final elongation)
- 15 C forever

Vary the time for elongation depending on the size of the DNA fragment. Generally 1 minute per kb of DNA. Check product by running 5 µl PCR sample + 2 µl 6x DNA dye on 1% agarose gel

Adapted from
NEB online protocols

A.2.2 Restriction enzyme cloning

- Digest the insert DNA and vector DNA separately using the appropriate enzymes and reaction buffers.

Restriction enzyme digest mix

DNA (Insert or vector)
1 µL of each Restriction Enzyme
5 µL 10x Buffer
0.5 µL 100x BSA (if recommended)
dH_2O to 50 µL

- Incubate tubes at the recommended temperature for the enzymes used. Typically 37°C for 1 hour.
- Isolate the bands of interest from a 0.8% agarose gel.
- Ligate the purified insert and vector. Adjust vector to insert ratios to optimize ligation.
- Transform the ligated DNA into competent cells.

Ligation reaction

1 µL Vector DNA
3 µL Insert DNA
1 µL 10x Ligase Buffer
1 µL T4 DNA Ligase
dH_2O to 10 µL

Adapted from
NEB online protocols

A.2 SEQUENCES

A.2.1 Bordetella FHA sequences

Sequence information for *B. pertussis* TahomaI FHA
http://www.uniprot.org/uniprot/Q8VV99

Sequence information for *B. bronchiseptica* FHA
http://www.uniprot.org/uniparc/UPI00001B8D7C

Sequence information for *B. parapertussis* strain Bpp5
http://www.genome.jp/dbget-bin/www_bget?tr:K0MH93_BORPB

ABBREVIATIONS

4PL: 4 parameter logistic

ACT: Adenylate cyclase toxin

ADPR: Adenosine diphosphate ribosyl transferase

aP: acellular pertussis

BrkA: Bordetella resistance to killing protein

BSA: Bovine serum albumin

CBD: Carbohydrate binding domain

CDC: Centers for disease control and prevention

CDR: Complementarity determining region

CHO: Chinese hamster ovary

CM: Carboxymethylated

CMV: Cytomegalovirus

CO_2: Carbon dioxide

ColE1: *Escherichia coli* colicin E1 gene

CR3: Complement receptor 3

DMEM: Dulbeco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

DNT: Dermonecrotic toxin

EC_{50}: Half maximal Effective Concentration

ELISA: Enzyme linked immunosorbent assay

ER: Endoplasmic reticulum

FACS: Fluorescence activated cell sorting

FHA: Filamentous hemagglutinin

FIM: Fimbrae

FITC: Fluorescein isothiocyanate

HBD: Heparin binding domain

HCl: Hydrogen chloride

HEK: Human embryonic kidney

HIV: Human immunodeficiency virus

HRP: Horse radish peroxidase

IAP: Integrin associated protein

IC_{50}: Half maximal Inhibitory Concentration

IMGT: International ImMunoGeneTics information system

LOS: Lipooligosaccharide

LRI: Leukocyte response integrin

Mac1: Macrophage receptor 1

MCD: Mature C-terminal domain

M_w: Molecular weight

NCBI: National Center for Biotechnology Information

NIAID: National Institute of Allergy and Infectious diseases

NIBSC: National Institute for Biological Standards and Control

NIH: National Institute of Health

OPA: Opsonophagocytic activity

PBS: Phosphate buffered saline

pcr: Polymerase chain reaction

P-IVIG: Pertussis intravenous immunoglobulin

PTg: Genetically modified Pertussis toxin (9K/129G) in the S1 subunit

PTx: Pertussis toxin

PVDF: Polyvinylidene fluoride

RAD: Arginine-Alanine-Aspartate tripeptide

RBC: Red blood cells

RGD: Arginine-Glycine-Aspartate tripeptide

SPR: Surface plasmon resonance

TCT: tracheal cytotoxin

Th cell: T helper cell

T_m: Melting temperature

TMB: Tetramethylbenzidine

wP: Whole cell pertussis

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