Published in final edited form as:

Biochemistry. 2009 December 22; 48(50): 11982–11993. doi:10.1021/bi901532z.

Characterization of a key neutralizing epitope on pertussis toxin recognized by the monoclonal antibody 1B7

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Abstract

Despite over five decades of research and vaccination, infection by *Bordetella pertussis* remains a serious disease with no specific treatments or validated correlates of protective immunity. Of the numerous monoclonal antibodies binding pertussis toxin (PTx) that have been produced and characterized, the murine IgG2a monoclonal antibody 1B7 is uniquely neutralizing in all *in vitro* assays and *in vivo* murine models of infection. 1B7 binds an epitope on the enzymatically active S1-subunit of PTx (PTx-S1) with some linear elements but previous work with S1 scanning peptides, phage displayed peptide libraries, and S1 truncation/deletion variants were unable to more precisely define the epitope. Using computational docking algorithms, alanine scanning mutagenesis, and surface plasmon resonance, we characterize the epitope bound by 1B7 on PTx-S1 in molecular detail and define energetically important interactions between residues at the interface. Six residues on PTx-S1 and six residues on 1B7 were identified which, when altered to alanine, resulted in variants with significantly reduced affinity for the native partner. Using this information, a model of the 1B7-S1 interaction was developed, indicating a predominantly conformational epitope located on the base of S1 near S4. The location of this epitope is consistent with previous data and is shown to be conserved across several naturally occurring strain variants including PTx-S1A, B (Tohama-I), D, and E (18-323) in addition to the catalytically inactive 9K/129G variant. This highly neutralizing but poorly immunogenic epitope may represent an important target for next generation vaccine development, identification of immune correlates and passive immunization strategies in pertussis.

Keywords

pertussis toxin; 1B7; epitope; neutralizing antibody; Bordetella; antibody docking

Whooping cough, a respiratory infection caused by the bacteria *Bordetella pertussis*, remains the third major cause of infant mortality, resulting in nearly 50 million cases and 350,000 deaths per year world-wide [1]. In spite of wide-spread vaccination since the 1950s, outbreaks continue to occur in industrialized countries, with over 15,000 probable or confirmed cases in the US during 2005 [2]. To control disease, two cellular and thirteen acellular vaccines have been tested for safety and immunogenicity in large clinical trials. The pertussis toxin (PTx) is a major virulence factor and chemically or genetically detoxified PTx is a major component of all acellular vaccine formulations in combination with up to four additional virulence factors [3,4]. These vaccines are highly effective at preventing the severe manifestations of the disease, but do not, in general, prevent bacterial colonization. Vaccine stimulated immunity declines over time, allowing adults and adolescents to present a reservoir for the pathogen [5]. As a result, booster vaccines were approved for adults and adolescents in 2005 [5] and vaccine research in pertussis remains an active area of investigation.

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There is a general consensus that humoral immunity dominates protection against *Bordetella*, but controversy as to which antigens confer protection and the roles of innate and cellular immunity. Some reports claim a correlation between vaccine-induced anti-PTx IgG levels and subsequent protection against disease [6], while other groups observe a stronger correlation with anti-pertactin responses [7]. Mechanistically, only pertactin-specific antibodies in human immune sera display opsonic activity and induce bacterial phagocytosis by human leukocytes [7]. However, a monovalent vaccine consisting of only PTx is protective and passively administered anti-*Bordetella*, anti-PTx polyclonal and some anti-PTx monoclonal antibodies are able to mitigate many disease symptoms and even reverse disease in mice [4,6,8-10]. In humans, titers of anti-PTx antibodies rise upon disease onset and are predictive of typical disease [11]. Administration of human immune sera generated from immunized volunteers shortened disease duration and cough rates in a Phase 1 safety trial with hospitalized infants, but a larger Phase 3 trial was unable to confirm or refute the benefit, perhaps because the sera was not sufficiently enriched in antibodies of appropriate specificity [12].

PTx is a 105 kDa AB₅ toxin structurally similar to cholera and *E. coli* heat labile enterotoxins. The protein mediates bacterial attachment to ciliated epithelial cells and exhibits both ADP-ribosylase and NAD glycohydrolase activities. Although not expressed in the related pathogens *B. bronchiseptica* and *B. parapertussis* due to an inactive promoter, PTx is required for the long-term persistence of *B. pertussis* [13]. The B-oligomer (S2-S5) of the toxin binds to carbohydrates on many cell types resulting in endocytosis and exhibits independent adjuvant effects via ligation of the T cell receptor. Upon internalization, the toxin undergoes retrograde transport to the ER where the catalytically active A (PTx-S1) subunit is translocated to the eukaryotic cytosol. Here, PTx-S1 catalyzes ADP-ribosylation of G α subunits of G_i/G_o signaling complexes (see Figure 1). The disrupted inhibitory signaling cascade leads to transiently high intracellular cAMP levels and general immunosuppression in neutrophils and macrophages.

In an effort to understand mechanisms of protective immunity in pertussis, large numbers of PTx-specific neutralizing murine monoclonal antibodies have been created and characterized to varying degrees [14-16]. After screening a panel of ten antibodies with a series of *in vitro* and *in vivo* assays (ADP ribosylation, leukocyte promotion, islet-activation, permeability-increasing activity, CHO cell clustering, hemagglutination and both aerosol and intracerebral mouse models of infection), the monoclonal antibody 1B7 was notably protective in more assays and at lower doses than any other characterized antibody preparation, including polyclonal anti-PTx sera [8]. 1B7 was able to protect mice when administered up to seven days after infection, reducing bacterial titers in the lungs as well as PTx concentrations and PTx-related effects. Further studies determined that the monoclonal antibody 1B7 acts by binding the PTx-S1 subunit with high affinity ($K_d \sim 4.2$ nM) [17].

Analysis of monoclonal antibodies binding PTx-S1 has broadly identified a conformational neutralizing epitope near the ADP-ribosyltransferase catalytic center [18-21]. Antibody epitope specificity has been largely inferred from activity assays, for instance, *in vitro* inhibition of ADP-ribosyltransferase activity but not NAD glycosyltransferase activity was thought to be predictive of protection in the CHO cell clustering assay. This correlation suggested that protective anti-PTx-S1 antibodies act by blocking the PTx-S1 catalytic site [22]. However, the fine details of molecular recognition are critical, as a screen of anti-PTx antibodies with high *in vitro* anti-ADP ribosylation activity showed that half did not protect *in vivo* [8]. The ability of monoclonal antibody 1B7 to bind PTx-S1 on Western blot indicates that there is a linear component to this highly conformational epitope. However, experiments using 15-mer peptides covering the entire PTx-S1 sequence were unable to identify any peptide mimicking PTx-S1 binding to protective monoclonal antibodies [15]. In spite of previous

efforts with phage displayed peptides, toxin truncation and deletion variants, the precise location of this important epitope remains elusive.

The 1B7 antibody potently neutralizes PTx and is able to independently ameliorate disease in the mouse model. Increased understanding of the mechanism of 1B7-mediated protection as well as the precise epitope bound by 1B7 may be crucial to understanding protective immunity in *Bordetella*. As a first step in describing the epitope, we have developed a model of the 1B7-S1 interaction, based on extensive alanine scanning mutagenesis, a homology model of 1B7 and the crystal structure of PTx [23]. To facilitate mutagenesis and expression of the binding partners, the 1B7 variable regions were reconstructed as recombinant murine (m1B7) and CDR-grafted human (hu1B7) single-chain antibodies (scAb); similarly, the S1 subunit was truncated to amino acid residues 1-220 to allow periplasmic expression in *E. coli* [24]. Molecular-level characterization of the neutralizing epitope bound by 1B7 may aid in development of passive immunotherapies or improved acellular vaccines.

Experimental procedures

PTx (holotoxin), the full length PTx-S1 (1-235) protomer, and the B oligomer were purchased from List Biological Laboratories, Inc., Campbell, CA.

Antibody and Toxin expression

The S1 subunit was amplified using oligonucleotide primers which truncated PTx-S1 from the amino terminus of the mature, processed protein to the carboxy-terminal residue 220, renamed PTx-S1-220 and sub-cloned into the expression vector pAK400 [25]. The expression vector pMoPac16, a pAK400 derivative with a α -terminal human constant κ -domain, was used to express scFvs as scAbs and the residues were numbered using the Kabat numbering system [26]. Point mutations for both scAbs and the truncated toxin S1 subunit were introduced by around the plasmid PCR [27].

Recombinant proteins were expressed in the bacterial periplasm of *E. coli* strain BL21 followed by osmotic shock and immobilized metal affinity chromatography purification was used to produce and purify all recombinant proteins as previously reported [28]. Size-exclusion chromatography with PBS as eluant was used as a polishing step for PTx-S1-220 and m1B7 scAb proteins (Superdex 75 and 200, respectively, GE Healthcare, Uppsala, Sweden). Protein L affinity chromatography was used as a second purification step for the hu1B7 scAb proteins (immobilized Protein L, Pierce, Rockford, IL) using 100 mM Na₂HPO₄, 150 mM NaCl pH 7.2 during binding and low pH IgG elution buffer (Pierce, Rockford, IL) followed by 1 M Tris pH 8.0 to neutralize eluted fractions. Micro-bicinchoninic acid assay (Pierce, Rockford, IL) was used to measure protein concentrations while SDS-PAGE with GelCode Blue stain reagent (Pierce, USA) was used to verify protein preparation homogeneity and purity.

Antibody-antigen binding analysis

High binding enzyme-linked immunosorbent assay (ELISA) plates (Costar) were coated with PTx at 1.3 μ g/mL or serial dilutions of PTx-S1-220 or its variants and incubated at 4°C overnight. The plates were then blocked with PBS + 1% milk for an hour at room temperature. After washing 3 times with PBS + 0.05% Tween 20, anti-PTx antibody (mAb 1B7, m1B7, hu1B7, or hu1B7 variants), was added either in serial dilution or at 8 μ g/mL and allowed to equilibrate at room temperature for one hour. After washing an additional three times, peroxidase conjugated anti-human C_k (Sigma) or peroxidase conjugated anti-mouse IgG (Sigma) was added for one hour at room temperature. Finally, the plate was washed 3 times and developed with tetramethylbenzidine dihydrochloride substrate (Pierce). The reaction was quenched with 1 N HCl and read using a SpectraMax M5 (Molecular Devices) at 405 nm.

EC₅₀'s were calculated as the concentration at 50% of the maximum response from the linear range of a dose-response curve ($EC_{50} = A_{405,max} - A_{405,min} / 2$). The % EC₅₀ values were calculated as the ratio of the EC₅₀ wild-type reference to the EC₅₀ variant ($EC_{50-WT} / EC_{50-variant} * 100\%$). Reported % EC₅₀'s are average values with each experiment being performed at least in triplicate and outliers, defined as values greater than three times the median, omitted.

Antibody stability analysis was performed by incubating duplicate samples of scAb variant or 1B7 IgG in PBS at 37, 50, and 4°C for 24, 2, and 24 hrs, respectively. After incubation, the fraction of active antibody remaining was determined by ELISA and calculated with reference to untreated sample maintained at 4 °C.

CHO cell clustering assays were performed by incubating 1039 pg/mL PTX with 100,000 molar excess scAb protein for ½ hr at room temperature in 96 well tissue culture plates. Freshly trypsinized confluent Chinese hamster ovary (CHO) cells were then seeded into the plates at a concentration of 10⁵ cells/well. After 24 h of incubation at 37°C, a microscope was used to examine and score the wells based on clustering morphology on a scale of 0 to 3 with 0 = no clusters and 3 = all clustered, as described by Hewlett et al [29]. Final protective molar ratios were reported as the lowest ratio resulting in no clusters. The working concentration of PTx was determined from a preliminary toxin concentration series which resulted in scoring of 1 to 3 over the concentration range of 65-1039 pg/mL PTx. In order to observe this same range with protective scAb activity, the lowest concentration resulting in complete clustering was chosen along with a 100,000x molar ratio of scAb to PTx based upon CHO cell sensitivity to any free PTx.

Surface plasmon resonance (SPR) analysis was performed using a BIAcore 3000 instrument (GE Healthcare, Uppsala, Sweden). CM5 chips (GE Healthcare, Uppsala, Sweden) which contain carboxymethylated dextran covalently attached to a gold surface were used for all SPR experiments. After activation using a 50/50 solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and N-hydroxy-succinimide, the monoclonal antibody 1B7 was immobilized on the chip surface in 100 mM sodium acetate pH 5.0 until 750 response units was obtained at which point the reaction was quenched using 1.0 M ethanolamine-HCl at pH 8.5. Samples of the B-oligomer, PTx, PTx-S1, or PTx-S1-220 and variants in HBS buffer pH 7.4 with 3 mM EDTA and 0.005% Tween at five different concentrations between 50-3500 nM were run in duplicate over the chip with a 1 minute injection, a 5 minute dissociation, and a flow rate of 50 µL/min. A 30 second injection of 2 M magnesium chloride at 30 µL/min was used to regenerate the surface in between antibody injections. Baseline correction was calculated by subtracting simultaneous runs over a second flow cell which had been activated and quenched with no protein immobilized on its surface. The off-rates were calculated using BIAevaluation software (version 3.0) from Pharmacia Biosensor. Due to the low expression levels and varying purity of the PTx-S1-220 variants, the concentration dependent association and dissociation constants, K_A and K_D , were not calculated. Instead, off-rates, which are typically very sensitive to amino acids changes and do not depend on precise concentration measurements, were compared. Reported values are the average and standard deviation of all off-rates calculated for each protein.

Fluorescence measurements were performed on a Molecular Devices SpectraMax M5 using 96 well special optics low fluorescence assay plates (Corning). Measurements were made on a 200 µL volume of 0.65 µm filtered samples diluted from stock in PBS. Excitation wavelengths of 278 and 295 nm were used for tyrosine and tryptophan, respectively. Emission spectra were recorded every nm from 330 to 460 nm. The emission spectrum for PBS was subtracted as background from all sample spectra.

Immunoblotting (western blotting) was performed using samples electrophoresed on a 12% SDS-PAGE gel followed by transfer to a nitrocellulose filter using standard methods. All toxin samples were loaded at equimolar concentrations except truncated PTx-S1 which was loaded at approximately double concentration to compensate for impurities and cleavage. After blocking with 5% PBS-Tween-milk, the membrane was incubated with 1B7 monoclonal antibody, m1B7, or hu1B7 with scAbs at double the concentration of the monoclonal antibody for 1 ½ hrs. Secondary antibody, either peroxidase conjugated anti-mouse Fc or anti-human C_k (Sigma), was then added for 1 hr with washing both before and after addition. The resulting bound protein bands were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce) coupled with exposure to X-ray film.

Circular dichroism spectra from 300 to 190 nm were recorded on a JASCO-815 chiro-optical spectrometer at room temperature. A 1 mm path length quartz cell was used to take triplicate readings on 200 µL samples in PBS. A spectrum of PBS was taken and subtracted from each sample spectrum to correct for background. The average of the three spectra for each sample was used for secondary structure analysis using the JASCO software.

Computational methods

Antibody modeling programs: *Web Antibody Modeling (WAM)*, <http://antibody.bath.ac.uk/>, predicts antibody structure from sequence data by finding template matches for the light and heavy chains separately and then fitting them together using conserved interface residues [30]. *Rosetta Antibody Beta*, <http://antibody.graylab.jhu.edu/>, searches the protein database (PDB) of crystal structures to find template matches and then utilizes Lennard-Jones potentials, a Lazaridis-Karplus solvation energy model, rotamer internal energies, and H-bonds to predict the antibody structure [31]. *Z-dock*, <http://zdock.bu.edu/>, is a Fast-Fourier Transform based docking program used to generate initial docked models of the antigen, PTx-S1, and antibody, hu1B7, from separate structural pdb files [32]. Once initial experimental data was collected, a pre-docked model was constructed manually, based on prior experimental data. *RosettaDock*, <http://graylab.jhu.edu:8088/>, was then used to refine the model by optimizing side-chain and rigid-body orientation using a Monte-Carlo coupled with an energy function to return ten different models [33]. All probable docked models were submitted for further analysis using Rosetta computational alanine point mutagenesis, <http://rosetta.bakerlab.org/>, which replaces each residue with an alanine and computes the resulting interface energies [34,35]. This results in identification of energetically important residues at the protein-protein interface for each docked model. *Pepsurf*, <http://peptide.tau.ac.il/index.html>, is a computational program that predicts both linear and conformational epitope based upon the crystal structure of the antigen and experimentally determined peptide sequences [36]. Six peptide sequences determined via phage screening of monoclonal antibody 1B7 by the Cortese lab [37] were used in conjunction with the crystal structure of PTx [23] to predict the three “best clusters” on the toxin surface most likely to contain the desired epitope.

Results

Functional characterization of truncated PTx-S1, the B-oligomer, and naturally occurring PTx-S1 variants

To facilitate mutagenesis of the toxin, PTx-S1 from strain Tohama I (variant B) was expressed recombinantly in *E. coli*. Yields of full length PTx-S1 (amino acids residues 1-235) were extremely low due to the presence of a long hydrophobic tail anchoring PTx-S1 into the B-oligomer, but a truncated version consisting of amino acid residues 1-220 was expressed and purified from the bacterial periplasm [24]. Protein purity was assessed by size exclusion chromatography and SDS-PAGE. Binding between 1B7 and the commercially available holotoxin, PTx-S1 (1-235), B-oligomer, and recombinant PTx-S1-220 were measured by

ELISA and SPR using a BIAcore3000 biosensor. PTx-S1 and PTx-S1-220 bound 1B7 with similar kinetics, k_d of 1.9 and $1.4 \times 10^{-3} \text{ sec}^{-1}$, respectively (see Table 1 and Figure 2). These data indicate that the PTx-S1 truncation does not affect the affinity of 1B7 for PTx-S1, and therefore the truncated PTx-S1 format was used to generate and analyze all subsequent site-directed toxin variants.

A systematic loss of affinity was observed for both the truncated PTx-S1-220 and the full length PTx-S1 (off-rates of 1.9 and $1.9 \times 10^{-3} \text{ sec}^{-1}$, respectively), versus the holotoxin (off-rate of $0.4 \times 10^{-3} \text{ sec}^{-1}$). To explain this loss, we first noticed weak binding between hu1B7 and the B-oligomer via ELISA (EC_{50-PTx} of 8%). More detailed analysis with SPR detected an off-rate of $2.7 \times 10^{-3} \text{ sec}^{-1}$ between purified B-oligomer and immobilized monoclonal antibody 1B7 when a high concentration of B-oligomer was used (1150-2890 nM). When the ELISA was repeated using monoclonal antibody 1B7, an EC_{50-PTx} of 30% for B-oligomer versus holotoxin was measured.

To address the potential for natural or vaccine-induced epitope drift to result in S1 variants no longer bound by 1B7, we cloned and expressed truncated PTx-S1 proteins (AA 1-220) corresponding to three remaining naturally occurring variants (PTx-S1A, PTx-S1D, and PTx-S1E) using site-directed mutagenesis of PTx-S1B. This was also performed for the catalytically inactive, genetically detoxified variant (PTx-S1 9K/129G) which is promoted for use in acellular vaccines to reduce side effects while retaining most adjuvant and protective qualities [38]. The *B. bronchiseptica* PTx-S1 subunit containing four mutations (D34E, I198T, S209P, and Y161P) was also constructed, but did not express well enough to include in the analysis [39]. Reducing and non-reducing SDS-PAGE gels confirmed the correct molecular weight and purity of the naturally occurring PTx-S1-220 variants compared with the parent PTx-S1B from the Tohama I strain. Binding analysis of these S1 variants using ELISA and SPR measured mAb 1B7 off-rates for both PTx-S1E and PTx-S1 9K/129G within error of those measured for PTx-S1-220 (1.8 and $1.6 \times 10^{-3} \text{ sec}^{-1}$, respectively). Slightly slower off-rates were measured for PTx-S1A and D (0.7 and $0.95 \times 10^{-3} \text{ sec}^{-1}$, respectively; see Table 1). Thus, the neutralizing epitope recognized by 1B7 is conserved across all known PTx-S1 variants, including laboratory-generated, catalytically inactive variants.

Functional characterization of 1B7 recombinant antibodies

Two recombinant versions of mAb 1B7, murine (m1B7) and humanized (hu1B7) single-chain Fv antibodies (scAb, a scFv with a c-terminal human kappa constant domain), were constructed by RT-PCR, overlap PCR, and CDR grafting as previously described (data not shown) [25-27]. This single-gene, single protein format facilitated rapid site-directed mutagenesis and expression of the resulting variant proteins. To characterize m1B7 and hu1B7 binding behavior, a western blot containing the B-oligomer, PTx, PTx-S1, and PTx-S1-220 in triplicate was probed with mAb 1B7, m1B7, and hu1B7 (see Figure 3). All three constructs bound PTx-S1 from the holotoxin, PTx-S1, and the truncated PTx-S1-220 with no detectable binding to any of the remaining four subunits. The intensity of the bands corresponds with the affinity of the antibody format for holotoxin or PTx-S1 (mAb 1B7 > m1B7 > hu1B7). Although hu1B7 exhibits the lowest overall binding affinity, this version expressed much better than m1B7 and was used as the parent for site-directed mutagenesis.

A CHO cell neutralization assay was conducted to evaluate the ability of the antibody constructs to effectively neutralize PTx in an *in vitro* assay. A prerequisite for success in this assay is thermal stability, as antibodies must retain their binding ability for at least 24 hours in serum at 37°C . To rule out the possibility that hu1B7 scAb variants may not protect in the *in vitro* assay due to poor thermal stability, antibody samples at $37 \mu\text{g/ml}$ were heated at 37°C for 24 hrs and 50°C for 2 hrs prior to binding analysis. The fraction of functional scAb remaining was quantified by ELISA with reference to mock-treated samples. All three constructs (mAb

1B7, m1B7 and hu1B7) retained at least 70% affinity at assay conditions and less than 40% affinity after exposure to the higher temperature (see Table 2). The molar ratio of antibody to toxin required to protect CHO cells *in vitro* corresponded to the relative antibody binding affinities, with m1B7 requiring a 10-fold and hu1B7 a 30-fold increase in the molar excess of antibody versus mAb 1B7. Despite a drop in affinity relative to the mAb 1B7, both m1B7 and hu1B7 not only retained the ability to bind purified PTx with high affinity, but also to effectively neutralize the toxin *in vitro*.

Computational and experimental selection of residues for interaction analysis

To reduce the experimental workload, residues were selected for alanine point mutagenesis analysis in conjunction with two computational methods, (1) ZDock [32] coupled with Rosetta Computational Mutagenesis Alanine Scanning [33-35] and (2) Pepsurf [36] coupled with previously determined 1B7 phage peptides [37]. Due to the absence of a crystal structure of either hu1B7 or m1B7, two antibody structure prediction algorithms, Rosetta Antibody Beta [31] and Web Antibody Modeling (WAM) [30], were used to predict four possible antibody structures. All the resulting models had random mean square deviations (rmsd) of approximately 1 Å for the overall structure, the CDR loops, and a few key residues (L-W91A and H-W33A) except the Rosetta hu1B7 model. Due to the similarity of the other three models in addition to the improved expression levels of hu1B7, the hu1B7 WAM model was chosen for subsequent docking models. The crystal structure of PTx-S1 [23] and a WAM antibody predicted structure of hu1B7 were run using method 1, resulting in the prediction of an initial docked model. This was then used for *in silico* alanine point mutagenesis to guide experiments by predicting which residues would result in the highest loss of free energy, $\Delta\Delta G_{\text{complex}} \geq 1.0$ kcal/mol, for each partner. Twelve residues on hu1B7 (see Table 3) and fourteen residues on PTx-S1 (see Table 4) were chosen based upon their resulting $\Delta\Delta G_{\text{complex}}$, amino acid residues over-represented in protein-protein interactions, and spatial proximity to the binding partner.

Method 2 was performed to take advantage of previously identified peptides mimicking the PTx-S1 epitope. Pepsurf compared the peptide sequences to the holotoxin crystal structure, identifying three potential conformational epitopes on PTx. Only two of these predicted epitopes were likely candidates since the third predicted no binding of PTx-S1. The “best cluster” consisted of the following: A74, G78, R79, G80, T81, H83, and I152 on PTx-S1 and A40 on PTx-S4. Two of these predicted residues, T81 and H83, were also predicted using the first method. If the prediction of partial binding to PTx-S4 is correct, it would explain the reduction in off rates and affinity seen in PTx-S1 versus PTx binding. This weak interaction could be below the sensitivity of either western blot analysis or SPR or simply non-existent when looking at PTx-S4 alone due to its sole stabilizing role in the 1B7/PTx-S1 interaction. Unfortunately, we do not have an established expression system for production of PTx-S4 variants and are unable to directly test this interaction. The “second cluster” consisted of the following: P3, P4, A5, P175, N176, and P177 on PTx-S1. This “second cluster” is highly unlikely since previous studies have determined that the first six residues of PTx-S1 are not involved in the binding of monoclonal antibody 1B7 [19]. Amino acid residue N176 was chosen in order to confirm this hypothesis. Further analysis using the peptide sequences as linear epitopes and comparison with PTx-S1 amino acid sequence resulted in identification of one additional cluster containing R39. Overall, an additional three residues were chosen based upon this second method, resulting in a total of seventeen PTx-S1 residues for experimental alanine scanning analysis.

Experimental and computational residue analysis

After point mutagenesis to alanine of each chosen residue, each resulting variant was expressed in *E. coli* and purified on at least three separate occasions. These purified variants were first

analyzed for a change in affinity toward PTx or WT hu1B7 via ELISA. Variants were categorized as either non-binding or reduced binding based upon average EC₅₀ cutoffs of less than 1% or less than 40%, respectively. For WT hu1B7, three variants were determined to be non-binding (L-W91A, H-W33A, and H-N58A) and three were determined to be lower binding (L-F31A, L-H94A, and H-S97A). PTx-S1 had four non-binding variants (R79A, H83A, Y148A, and N150A) and six lower binding variants (E16A, R39A, T81A, T153A, T158A, and Y166A).

In order to verify these results, a second assay was performed on each of the variant types. For the hu1B7 variants, heat studies and *in vitro* CHO cell neutralization assays were performed. The heat studies resulted in all variants retaining > 70% affinity at assay conditions, except L-F31A which only retained 60% affinity. The results of the CHO cell assay showed that L-W91A, H-W33A, H-N58A, L-H94A, H-S97A, and L-F31A offer no protection *in vitro*, thereby confirming the ELISA results. SPR analysis confirmed that PTx-S1 variants R79A, H83A, Y148A, and N150A have significantly reduced affinity for monoclonal antibody 1B7 as seen by their rapid off-rates of 25, 10, 29, and $8 \times 10^{-3} \text{ sec}^{-1}$, respectively. Of the previously classified lower binding variants, R39A and T153A showed higher off rates than WT PTx-S1-220 at 3.0 and $2.0 \times 10^{-3} \text{ sec}^{-1}$, while the other four variants, E16A, T81A, T158A, and Y166A, had similar off rates of 1.6, 1.7, 1.3, and $1.4 \times 10^{-3} \text{ sec}^{-1}$ thereby changing their classification to binding. The computational methods used to guide experimental efforts correctly predicted residues with experimental $\Delta\Delta G_{(\text{complex})} > 1 \text{ kcal/mol}$ with approximately 50% accuracy. The experimental results confirm the most likely cluster predicted with Pepsurf as PTx-S1 residues R79A and H83A were non-binding in all assays.

Although several variants of both WT hu1B7 and PTx-S1 were identified as non-binding or reduced binding, this change could be due to indirect structural effects as opposed to a reduction in binding energy. Focusing first on WT hu1B7, three of the identified residues (L-W91, H-W33, and L-H94) are considered structurally relevant in the CDR regions of antibodies [40, 41]. The *in silico* alanine scanning results were then re-analyzed focusing on the value of the calculated ΔG (partner), for which a value of greater than 1.0 indicates the residue may play a role in stabilizing protein secondary and tertiary structure. This analysis identified residues L-W91, H-W33, and L-F31 as playing structural roles. The final method to determine each residue's contribution to proper folding was experimental comparison of CD analysis of each variant with WT hu1B7. This indicated that experimentally only H-S97A and H-W33A were structurally different than the WT. Based upon these three methods, only H-W33 consistently appears to be structurally important. However, this residue has ~15% solvent accessibility and has been shown to form hydrogen and pi bonds with residues across the interface of the HEL/FabD44.1 interaction [40]. A similar method was used to determine the structural importance of the PTx-S1 residues. ΔG (partner) analysis identified all residues except R79 as structural, while CD analysis with comparison to WT PTx-S1-220 indicated only N150A and R79 as structurally different. Due to the results being method dependent, only N150 can definitely be classified as a structurally important residue. This conclusion is further cemented by structural analysis of PTx-S1 which shows N150 as 7% solvent accessible and playing a key role in a β -sheet structure with 5 hydrogen bonds with neighboring PTx-S1 residues. Despite the structural roles of both H-W33 on WT hu1B7 and N150 on PTx-S1, the docked model indicates hydrogen bonding of these residues with partner residues across the interface. The model indicates these residues serve dual roles maintaining structural conformation and mediating antigen/antibody binding.

As a final check as to whether the identified tryptophan residues (L-W91 and H-W33) are located at the complex interface, tryptophan fluorescence measurements were collected individually for the two tryptophan deficient variants, L-W91-A and H-W33-A, in addition to WT hu1B7 and PTx-S1-220 (see Figure 4A). Comparison of WT hu1B7 with L-W91-A and

H-W33-A shows relative peaks of 60% and 70%, respectively, indicative of the decrease in the total number of tryptophan residues. While L-W91-A shows no shift in peak position, a red shift of 3 nm was observed for H-W33-A, signifying tryptophan exposure. This shift is most likely due to a conformational change, which is expected due to the structural role played by this residue. Fluorescence measurements of WT hu1B7, PTx-S1-220, and the equimolar complex of the two were then collected (see Figure 4B). Comparison of the complex with WT hu1B7 and PTx-S1-220 resulted in blue shifts of 4 and 9 nm, respectively. This masking of the tryptophans [42] confirms their presence in the binding interface between WT hu1B7 and PTx-S1-220.

Development and prediction of best fit model of interaction

With these twelve “key binding residues” in mind, as well as the requirements for geometric and electrostatic complementarity to allow binding, WT hu1B7 was manually docked onto PTx-S1 using the molecular viewing programs Swiss PDB Viewer and Pymol to provide a starting complex for computational refinement. The resulting complex was submitted for computational docking using RosettaDock, which is able to manipulate the two docked partners ± 3 Å toward or away from each other, 8 Å along each other's surface, 8 Å of tilt, and 360° around the center axis between the two [33]. Of the resulting models, the one which best fit the experimental data, predicting interactions of five of the six PTx-S1 residues and also five of the six WT hu1B7 residues, was selected (see Figure 5). The model predicts hydrogen bonding between N150 and the backbone oxygen of L-W91, H-N58 and the backbone oxygen of H83, N150 and the backbone oxygen of L-S92, and T153 and the backbone oxygen of L-S92. It also predicts hydrogen bonding between several residues determined to be unimportant for binding: E16, R146, L-S92, and L-S93. Although Y148 does not form any predicted hydrogen bonds, it has a large solvation energy effect of 0.14 kcal/mol, indicating solvent interaction. Looking at the residue side chains, nearly half are aromatic residues with two tryptophans, two histidines, and one tyrosine. Since aromatic clusters are common at protein-protein binding interfaces, comprising ~30% of energetically important residues [41], the binding chemistry may involve π -bonding or stacking between the two tryptophans on the antibody and the histidine on PTx-S1. Changes in either salt or pH conditions did not significantly affect binding (data not shown), consistent with a binding interface dominated by hydrophobic interactions.

As a final check on the probability of the proposed model, the change in Gibbs free energy for the 1B7/PTx-S1 complex was calculated from experimental equilibrium affinity using the change in Gibbs free energy equation as follows (Eqn 1):

$$\Delta G = -RT \ln(K_{eq}) = \sum_{i=1}^n \Delta \Delta G_i \quad \text{Eqn 1}$$

Using the known K_d of 5 nM, the gas constant (R) of 1.987 cal/mol, and 277 K for temperature (T), the ΔG of the complex was calculated to be 10.5 kcal/mol. In the absence of non-additive co-operative effects [43], this value should also equal the sum of the $\Delta \Delta G$ for each binding residue of the complex, with any deviation between these values indicating one or more binding residues have been omitted. *In silico* computational alanine scanning of the hu1B7-Ptx-S1 complex results in a $(\Delta \Delta G)_{\text{sum}}$ of 10.5 kcal/mol, very similar to the predicted ΔG and in the predicted range for antibody-antigen complexes ($\Delta G = 10.9 - 12.3$ kcal/mol for K_d 's from 10 – 1 nM). This correspondence indicates that a majority of interacting residues have likely been identified.

Discussion

As the *B. pertussis* organism has not been detected in the blood of patients, it has been suggested that the systemic manifestations are due to toxin release and dissemination. Pittman famously hypothesized that symptoms are mediated primarily by pertussis toxin (PTx), a theory which, 30 years later, remains to be proven. It is clear that PTx is a major antigen, as transposon-insertion mutants of *B. pertussis* manifest greatly reduced virulence in mice [44] and administration of purified toxin induces numerous effects associated with infection such as histamine-sensitization, leukocytosis, and insulin secretion. In terms of protection, it has been difficult to demonstrate a definitive correlation between a humoral response to any antigen and protection against disease although qualitatively high levels of antibody to PTx are associated with a lower likelihood of developing clinical disease upon exposure to pertussis. [11,45]

After immunization with PTx, antibodies recognizing the S1 subunit are recovered at high frequency [14], indicating this subunit as a whole is strongly immunogenic. Analysis of monoclonal antibodies binding PTx have documented the presence of at least four non-overlapping epitopes on the PTx holotoxin; monoclonal antibodies binding two of these (on the S1 and S2/3 subunits) have been shown to be protective in mouse models [14-16]. Sato *et al* performed a detailed comparison of 10 anti-PTx-S1 antibodies, polyclonal anti-PTx sera, and 10 anti-B-oligomer antibodies in the mouse aerosol model [8]. Remarkably, only the 1B7 antibody conferred significant survival when administered between zero and seven days after infection while also reducing the number of bacteria and amount of PTx in the lungs [8]. This anti-PTx-S1 antibody may protect by binding an epitope which prevents substrate access to the catalytic cleft or restricts unfolding of the S1-subunit necessary for translocation to the cytoplasm, while non-protective anti-PTx-S1 antibodies bind a separate epitope and do not interfere with PTx-S1 function (see Figure 1). The infrequent recovery of antibodies displaying 1B7-like neutralizing activities suggests that (1) the antibody or the epitope recognized possess unique protective qualities and (2) that the epitope is poorly immunogenic.

Location of the neutralizing epitope on PTx recognized by monoclonal antibody 1B7

Prior efforts to characterize the conformational epitope on PTx-S1 bound by 1B7 using truncations, deletions, and peptide fragments identified two linear sub-epitopes involved in the binding (AA 8-14 and 124-186) [18,21]. These appeared to be independent linear sub-epitopes as mutations in either region was associated with loss of binding on a Western blot. Since 1B7 inhibits toxin-mediated ADP ribosylation of G proteins *in vitro*, early reports suggested the antibody may directly and predominately interact with the catalytic residues located in the linear region between AA 8-14 which shows homology with both cholera toxin and *E. coli* heat labile toxin. The PTx holotoxin structure, published subsequently [23], revealed that residues 9-13 are mostly buried ($\leq 13\%$ solvent accessible), making it unlikely that antibodies could interact directly with these in a properly folded protein. However, Western blot analysis showed that 1B7 was unable to bind Y8A and had a reduced ability to bind R13A (see Table 5). The relevance of this linear interaction is unclear since 15-mer PTx-S1 peptides capable of recapitulating this linear binding site could not be identified [15]. Furthermore, antibody conformational epitope prediction programs including ElliPro, Discotope, and Pepsurf did not predict binding residues in this region.

Using a combination of experimental and computational techniques, the PTx holotoxin structure and prior data, we have developed a model of the interaction between PTx and the neutralizing antibody 1B7. Using primarily CDR loops L3 and H3, 1B7 binds the base of PTx-S1, possibly engaging in weak interactions with the S4 subunit (see Figure 5). This region of PTx-S1 appears readily accessible to antibodies and is fairly flat, consisting of three anti-parallel β -sheets and two different turns: one between the aforementioned β -sheets and another prior to an α -helix. The energetically important PTx-S1 residues, assessed by alanine scanning

mutagenesis, are R79, H83, N150, and Y148. Chemically, the surface is mostly hydrophobic with one arginine, nine hydrogen bonds, and a solvent-accessible area of $\sim 1000 \text{ \AA}^2$, typical for antibody-antigen interactions. Interestingly, both the model and data support a weak stabilizing interaction with the S4 subunit of the B-oligomer involving a separate turn between β -sheets. Although only S4 residue A40 was suggested by Pepsurf, due to their close proximity, residues S42 and S43 may also be involved in this interaction. Overall, this is a conformational epitope which includes a short linear sequence (Y148 to N150 along a β -sheet strand), consistent with 1B7's ability to bind reduced and denatured PTx-S1 on a Western blot. This is consistent with a linear sub-epitope between AA124-186. Binding of monoclonal antibody 1B7 to the PTx-S1-220 variants, N150A and Y148A, was not detected in ELISA with up to $1.6 \text{ }\mu\text{M}$ antibody. Similarly, 1B7 monoclonal antibody had greatly reduced ability to bind N150A and no ability to bind Y148A on a Western blot using very sensitive chemiluminescence detection (see Table 5).

Involvement of the B-oligomer in this protective epitope is indicated by the marked reduction in mAb 1B7 binding to PTx holotoxin (k_d of $0.4 \pm 0.6 \times 10^3 \text{ sec}^{-1}$) versus PTx-S1 (k_d of $1.9 \pm 0.3 \times 10^3 \text{ sec}^{-1}$). This reduction in binding affinity is observed with both the commercially prepared S1-235 and the recombinant, truncated S1-220, indicating it is not an artifact of the recombinant version (see Table 1). Interestingly, although both ELISA and SPR analysis measured a weak interaction between the B-oligomer and mAb 1B7 (see Figure 6), no recognition of the B-oligomer was detected by chemiluminescent Western (see Figure 3). There are three possibilities to explain this result: (1) 1B7 recognizes a separate conformational epitope on the B-oligomer at significantly lower affinity than PTx-S1; (2) 1B7 recognizes a single epitope dominated by PTx-S1 but supplemented by a weak B-oligomer interaction; (3) PTx-S1 is stabilized by its interaction with the B-oligomer resulting in reduced entropic costs of 1B7 binding. The model is consistent with the latter two possibilities but we were unable to directly test these hypotheses due to difficulties in recombinant expression of S4 and reconstitution of holotoxin with PTx-S1 variants. If 1B7 does interact with both the S1 and S4 subunits, this may explain both the low frequency at which 1B7-like antibodies are recovered, since the antibody would need to span the A-B subunit interface. Moreover, it suggests a mechanism of protection: 1B7 may act as a molecular staple linking PTx-S1 to the B-oligomer, slowing PTx-S1 unfolding and dissociation from the B-oligomer in the ER, reducing the ability of PTx-S1 to escape to the cytosol and perform catalysis. Validation of this model by co-crystallization of 1B7 with PTx is on-going to provide direct experimental evidence of the 1B7 binding site.

Contribution of mAb 1B7 to PTx recognition

Although previous studies have focused on the 1B7 epitope on PTx-S1, the antibody itself may possess unique qualities which result in effective neutralization. In addition to blocking toxin catalysis, 1B7 binding may, for instance, slow toxin unfolding necessary for escape from the ER into the cytoplasm (see Figure 1). To understand the antibody's role in binding and to aid in creating a docked model of the interaction, twelve hu1B7 residues predicted to contribute significantly to the energetics of binding were individually altered to alanine. These experiments identified six key residues required for high-affinity binding to S1, L: F31, H94, W91 and H: S97, W33 and N58. These six residues are equally distributed over the heavy and light chains, with a slight bias towards CDR L3. Of the CDRs, only L2 contains no key residues and does not appear to contribute significantly to the interaction. This is consistent with most antibody-antigen contacts wherein CDR-L2 only has the least involvement of all the CDR loops with contacting residues on 42% of the time (www.bioinf.org.uk). Of the six key residues, H-W33, L-W91, H-S97 and L-H94 are common interacting residues with contacts in over 70% of complexes. The other two residues, L-F31 and H-N58, are less common at 15 and 38% respectively. However, L-F31 mutation to alanine only resulted in reduced binding and is thus

not thought to be a large contributor to the interaction. Overall, the residues chosen on 1B7 for analysis using RosettaDock and ZDock were correctly predicted in 50% of the cases. Based on these results, further optimization of 1B7 binding could be achieved by targeted mutagenesis of CDRs L3 and H3, which often dominate binding in antibody complexes.

Epitope conservation across naturally occurring and engineered strains

A consequence of broad vaccination programs is the potential to select for escape variants of the major virulence factors, which may be accelerated by the use of acellular vaccines. Detailed genotyping of strains, primarily in the Netherlands, has aimed to track naturally occurring and vaccine-induced variation in pertussis antigens [46]. The naturally occurring mutations in these strains (PTx-S1A, PTx-S1D, and PTx-S1E) are mainly found in the S1 subunit with each variant containing up to three amino acid substitutions with respect to PTx-S1B (Tohama-I) [47]. The most divergent PTx-S1 gene is found in *B. bronchiseptica* with four amino acid mutations but is not expressed due to promoter mutations [39,46].

Given this modest level of natural variation, a key question is whether variation impacts in the 1B7 epitope, and if so, whether 1B7 bind these variants with the same affinity. After expression and purification of truncated versions (1-220) of three of the four naturally occurring strain variants, it was found that PTx-S1E is bound by 1B7 with identical (within error) off-rates versus WT PTx-S1-220 (see Table 1). However, 1B7 had approximately a two-fold decrease in off-rates with respect to PTx-S1A and PTx-S1D, indicating an increase in affinity. A second question is whether the catalytically inactive PTx-S1 variant used in some acellular vaccines (containing amino acid substitutions R9K and E129G) will also be recognized by 1B7. 1B7 binds this PTx-S1 variant with identical affinity as WT (within error), suggesting that acellular vaccines containing the R9K, E129G variant will be able to elicit 1B7-like protective antibodies. The value of the 1B7 epitope as a target for passive immunization and a potential correlate of protective immunity will depend both on its ability to elicit neutralizing 1B7-like antibodies and conservation within the pool of circulating clinical strains. The ability of hu1B7 to bind existing PTx-S1 variants indicates that the epitope recognized by 1B7 is conserved in the presence of natural antigenic drift.

Mechanism of PTx neutralization by mAb 1B7

When originally characterized, the 1B7 monoclonal antibody was thought to protect against toxin effects by blocking catalysis, either by directly associating with catalytic residues or blocking substrate access to these residues. However, several other monoclonal antibodies have been characterized which block ADP ribosylation *in vitro* but are not neutralizing *in vivo* [14]. Furthermore, the mechanism of toxin cellular binding and internalization has been characterized in detail and it is not clear whether an antibody would remain associated with PTx-S1 during retrograde transport - in the low pH of the endosome, the reducing environment of the ER, or during translocation into the cytoplasm (see Figure 1). The fact that 1B7 can bind reduced and unfolded PTx-S1 on a Western blot and under the cytoplasmic conditions replicated during *in vitro* ADP ribosylation assays suggests that it could block catalysis in the cytoplasm if present in that compartment, but 1B7 ligation may protect by altering PTx trafficking within the cell.

Burns and colleagues [39] demonstrated that PTx is transiently expressed on the bacterial cell surface during secretion and *B. pertussis* knock-out mutants have shown that along with FHA, PTx is necessary and sufficient for bacterial adherence. Thus, 1B7 may bind whole bacteria, blocking bacterial adhesion to respiratory epithelial cells or potentially mediating effector functions. 1B7 was isolated as a murine IgG2a antibody, an isotype able to opsonize bacteria and fix complement, while murine IgG1 isotypes are more commonly associated with toxin neutralization or blocking bacterial adherence. It is not clear if the isotype has mechanistic

relevance or is a result of the Th1/ Th2 bias induced by the toxin itself. F(ab')₂ fragments of anti-PTx antibodies [48] and Fab fragments of 1B7 displayed identical *in vitro* CHO clustering effects as the parental monoclonal antibody. While the requirement for an Fc has not been directly tested *in vivo*, anti-PTx antibodies in human immune sera do not display opsonic activity or induce phagocytosis by human leukocytes [7,11]. Similar to other toxin-mediated diseases, such as botulinum and anthrax [49], toxin neutralization may not require Fc-mediated effector functions, but remains an intriguing area of future investigation.

Conclusions

We have identified a uniquely neutralizing epitope on PTx using PTx-S1 variants and humanized scAb versions of the 1B7 monoclonal antibody. This epitope is adjacent to but does not include the catalytically active residues on PTx-S1 and spans the junction between the S1 and S4 subunits. This model suggests a mechanism of 1B7 antibody protection: antibody ligation may anchor the catalytically active S1 subunit to the B-oligomer, thereby preventing PTx-S1 dissociation and subsequent transport into the cytosol, where PTx-S1 disrupts G-protein signaling. Confirmation of this model awaits additional co-crystallization and cellular experiments of antibody-toxin complex trafficking within cell. Notably, numerous anti-PTx-S1 antibodies have been produced with the ability to neutralize *in vitro* ADP-ribosylation activity, but most perform poorly in *in vivo* mouse models of disease. Thus we propose that the molecular details of epitope recognition are critical in discriminating between antibodies capable of *in vitro* versus *in vivo* protection and that this epitope is a unique target to exploit for passive immunotherapy and acellular vaccine design. Moreover, it suggests that the search for immune correlates in pertussis may require careful examination of serum responses to specific epitopes, not just individual virulence factors [20,22].

Acknowledgments

We gratefully acknowledge transfer of the 1B7 and 11E6 hybridomas from Jerry Keith, NIH.

Financial support from NIH grant #AI066239 (to JAM) and the University of Texas at Austin.

Abbreviations

CD	circular dichroism
CDR	complementary determining region
CHO	Chinese hamster ovary
EC ₅₀	50% effective concentration
ELISA	enzyme-linked immunosorbent assay
hu1B7	humanized scFv version of 1B7 monoclonal antibody
K _d	equilibrium dissociation constant
m1B7	murine scFv version of 1B7 monoclonal antibody
mAb	monoclonal antibody
PTx	pertussis toxin
PTx-S1	pertussis toxin subunit 1
scFv	single-chain antibody
SPR	surface plasmon resonance

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Figure 1.

Model of pertussis toxin function. The 1B7 antibody neutralizes toxin catalytic function while the 11E6 antibody competes with the cellular receptor for B-oligomer binding. After the toxin binds glycoproteins or glycolipids on the host cell, it undergoes receptor-mediated endocytosis and retrograde transport through the endosome to the Golgi apparatus and finally the ER. In the ER, ATP binds to the central pore of the B-oligomer, resulting in the release of the S1 subunit which is subsequently reduced and exposed to the cytosol (it may remain associated with the membrane associated via the hydrophobic tail) [50]. In the cytosol, PTx-S1 ADP-ribosylates G proteins, disrupting normal signaling and increasing cAMP levels.

Figure 2. Representative BIAcore 3000 (GE Healthcare, Uppsala, Sweden) SPR runs on a CM5 chip with mAb 1B7 coupled at ~1000 RU. A) PTx holotoxin at 300, 200, 150, 100, and 50 nM; B) PTx-S1 at 200, 150, 100, and 50 nM; C) PTx-S1-220 at 300, 200, 150, 100, and 50 nM; and D) B-oligomer at 2890, 2300, 2000, 1730, and 1150 nM.

Figure 3.

Immunoblot analysis of 1B7 antibody constructs. After electrophoresis of 23 pmol of the B-oligomer, PTx, and PTx-S1 and 46 pmol of PTx-S1-220 in three identical sections on a 12% SDS-PAGE gel, proteins were electrophoretically transferred to nitrocellulose paper. The antibody constructs mAb 1B7 at 2.9 mg, m1B7 at 5.9 mg, and hu1B7 and 5.9 mg were used to probe a section each with subsequent detection using equivalent amounts of anti-mouse-Fc HRP, anti-human-C_k HRP, and anti-human-C_k HRP, respectively.

Figure 4.

Tryptophan fluorescence spectra of hu1B7 ($\square$), L-W91A ($\blacklozenge$), H-W33A ($\times$), PTx-S1-220 (Δ), and the hu1B7/PTx-S1-220 complex ($\blacklozenge$) in PBS. A) hu1B7, L-W91A, and H-W33A comparison at 4.5 μ M at room temperature. Lines indicate spectral peaks at 352 nm, 352 nm, and 355 nm, respectively. B) hu1B7, PTx-S1-220, and the hu1B7/PTx-S1-220 complex at a 1:1 molar ratio comparison at 4.5 μ M at room temperature. Lines indicate spectral peaks at 357 nm, 362 nm, and 353 nm, respectively.

Figure 5.

The 1B7-PTx best fit interaction model generated by RosettaDock using the crystal structure of PTx and the WAM predicted structure of hu1B7. S1 is green; B-oligomer is teal; the heavy chain is red; and the light chain is purple. Experimentally determined important interacting residues on both PTx-S1 and hu1B7 are shown. Black are interacting residues on hu1B7 and blue are interacting residues on PTx-S1.

Figure 6. ELISA of PTx in red and B-oligomer in blue with primary antibody of 1B7 ($\blacktriangle$), 11E6 (x), or the negative control, c-myc, representing non-specific antibody binding (no symbol).

Table 1

Binding between PTx, its subunits, and naturally occurring variants to 1B7

PTx	Mutations wt Tohama I (PTx-SIB)	ELISA * %EC ₅₀ PTx	m1B7 mAb ~ 1000 RU k _d × 10 ⁻³ (sec ⁻¹)
PTx	-	100	0.4 +/- 0.6
B-oligomer	-	8	2.7 +/- 0.6
PTx-S1 235	-	20 **	1.9 +/- 0.3
PTx-S1 220	-	90 +/- 10 ***	1.4 +/- 0.1
PTx-S1A	M194I	50 +/- 40	0.71 +/- 0.04
PTx-S1D	D34E, I198V	90 +/- 50	0.95 +/- 0.05
PTx-S1E	D34E, S162P, I198M	80 +/- 30	1.8 +/- 0.5
PTx-S1 9K/129G	R9K, E129G	60 +/- 30	1.6 +/- 0.7

* All ELISAs were run using hu1B7 as the primary antibody.

** S1 235 maintained in soluble form in 0.03% CHAPS, 0.1 mM Na₂EDTA, and 10 mM Tris

*** Due to low expression, only the purest samples were used for these numbers

Table 2

Binding and neutralizing activity of 1B7 variants

Antibody	Heat Studies %EC ₅₀ 4°C		CHO cell Neutralization
	37°C/ 24 hr	50°C/ 2hr	Assay (µg)
mAb 1B7	100	20	0.09
M1B7	100	40	3
Hu1B7	70	10	9

Table 3

In silico and experimental characterization of hu1B7 and variants

Antibody	CDR	$\Delta\Delta G_{(\text{complex})}$ (kcal/mol)	Alanine Scan Highest $\Delta G_{(\text{partner})}$ (kcal/mol)	*CD spec % α	% β	ELISA %EC ₅₀ hu1B7	Heat Studies EC ₅₀ 4°C 37°C 24 hr	50°C 2 hr	CHO Cell Neutralization Assay μg
Binding									
hu1B7	-	-	-	34	38	100	70	10	9
S30A	L1	2.9	0.6	33	49	100 +/- 0	100	2	9
N53A	L2	1.2	0.4	23	37	390 +/- 30	70	30	9
S92A	L3	1.2	0.3	31	68	50 +/- 10	100	5	9
S93A	L3	2.8	1.5	34	28	80 +/- 60	100	20	9
S31A	H1	0.2	-0.2	25	19	70 +/- 20	100	5	9
F52A	H2	2	2.9	31	44	120 +/- 50	70	9	9
Reduced Binding									
F31A	L1	1.8	2.4	28	28	2 +/- 3	60	<1%	NP
H94A	L3	3.1	0.8	27	45	3 +/- 4	90	<1%	NP
S97A	H3	2.5	-0.2	29	19	5 +/- 4	100	30	NP
Non-Binding									
W91A	L3	2	3.6	27	44	<1%	-	-	NP
W33A	H1	2	3.5	54	32	<1%	80	<1%	NP
N58A	H2	4.2	0.5	32	23	<1%	90	<1%	NP

• These secondary structure values are typical for scFv-huCk in our hands.

• NP, no protection

Table 4

In silico and experimental characterization of PTx-S1-220 and variants

PTx	Alanine Scan Average		CD spec		ELISA %EC ₅₀ PTX	m1B7 mAb ~1000 RU k _d × 10 ⁻³ sec ⁻¹
	ΔΔG _(complex) (kcal/mol)	ΔG _(partner) (kcal/mol)	%α	%β		
Binding						
PTx-S1-220	-	-	11	40	90 +/- 10	1.4 +/- 0.1
R146A	10.9	3.8	12	42	60 +/- 40	1.5 +/- 0.1
E155A	0.4	0.6	8	44.1	50 +/- 40	1.1 +/- 0.1
T156A	0.5	2.5	12	41.6	70 +/- 30	1.1 +/- 0.1
T159A	3.7	2.1	6	49.8	90 +/- 30	1.4 +/- 0.2
Y161A	6.5	2.3	11	41.1	50 +/- 20	1.3 +/- 0.6
N176	0.6	-0.4	13	54	50 +/- 20	1.1 +/- 0.1
E210A	2.2	0.4	17	33.7	70 +/- 40	1.6 +/- 0.2*
E16A	2.9	-0.3	9	44	20 +/- 10	1.6 +/- 0.1
T81A	2.8	1.1	9	58	30 +/- 10	1.7 +/- 0.9
T158A	1.5	1.2	10	42.8	30 +/- 20	1.3 +/- 0.2
Y166A	4.9	6.8	29	27.2	30 +/- 10	1.4 +/- 0.2
Reduced Binding						
R39A	1.1	-0.3	15	43	30 +/- 10	3.0 +/- 0.4
T153A	1.2	1.0	14	39.2	11 +/- 5	2.0 +/- 0.4
Non-Binding						
R79A	0.8	0.1	9	61	1 +/- 1	25 +/- 4
H83A	3.3	1.4	12	41	<1%	10 +/- 2
Y148A	6.6	4.7	9	42	<1%	29 +/- 6
N150A	2.1	2.2	6	46	<1%	8 +/- 1

Table 5

Binding analysis of PTx-S1-220 variants

PTx	ELISA %EC ₅₀ PTx	Western Blot mAb 1B7
PTx-S1-220	90 +/- 10	+++
9K/129G	60 +/- 30	+++
Y8A	<1%	–
R9A	9	+++
Y10A	5	+
D11A	7	+
S12A	20	++
R13A	8	+/-
E16A	20 +/- 10	++
R79A	1 +/- 1	+/-
T81A	30 +/- 10	+++
H83A	<1%	+
Y148A	<1%	–
N150A	<1%	+/-
T153A	11 +/- 5	++