Bispecific antibodies are a rapidly growing class of therapeutic molecules, originally developed for the treatment of cancer but recently explored for the treatment of autoimmune and infectious diseases. *Bordetella pertussis* is a reemerging pathogen, and several of the key symptoms of infection are caused by the pertussis toxin (PTx). Two humanized antibodies, hu1B7 and hu11E6, bind distinct epitopes on PTx and, when coadministered, mitigate disease severity in murine and baboon models of infection. Here we describe the generation of a bispecific human IgG1 molecule combining the hu1B7 and hu11E6 binding sites via a knobs-in-holes design. The bispecific antibody showed binding activity equivalent to that of the antibody mixture in a competition enzyme-linked immunosorbent assay (ELISA). A CHO cell neutralization assay provided preliminary evidence for synergy between the two antibodies, while a murine model of PTx-induced leukocytosis definitively showed synergistic neutralization. Notably, the bispecific antibody retained the synergy observed for the antibody mixture, supporting the conclusion that synergy is due to simultaneous blockade of both the catalytic and receptor binding activities of pertussis toxin. These data suggest that a hu1B7/hu11E6 bispecific antibody is a viable alternative to an antibody mixture for pertussis treatment.
and it shows promise as a mechanism to enhance neutralization potency against infectious targets such as antibiotic-resistant *Pseudomonas aeruginosa* (16) as well as HIV-1 (17). The applications of bispecific antibodies are rapidly expanding, and the development of better methods to make bispecific antibodies is an active area of research.

We previously observed that an equimolar mixture of the hu1B7 and hu1E6 anti-PTx antibodies was more effective in vitro than either antibody alone (9). This led us to hypothesize that a bispecific antibody containing these two binding sites would capture the therapeutic potential of the mixture in a single molecule. Importantly, this construct would also provide insight into the structural basis of synergy exhibited by these two antibodies. Here we describe the development of a bispecific anti-PTx antibody with human IgG1 architecture. The bispecific antibody was able to simultaneously bind two PTx molecules via the different epitopes and exhibited an effective affinity similar to that of the antibody mixture. A murine neutralization assay demonstrated clear synergy for the hu1B7 and hu11E6 antibody combination as well as the bispecific antibody. This evidence supports the conclusion that synergy between hu1B7 and hu11E6 is due to more complete neutralization of toxin activity by simultaneous blockade of the receptor binding and catalytic pathways, and it also suggests that a bispecific antibody may be a viable alternative to an antibody mixture for the treatment of pertussis.

**MATERIALS AND METHODS**

**Protein preparation and purification.** Large-scale preparations of hu1B7 and hu1E6 were prepared by Catalent (Somerset, NJ) by use of polyclonal CHO cell lines, followed by protein A chromatography, anion chromatography, and buffer exchange into phosphate-buffered saline (PBS), pH 7.0 (9). Fab fragments were prepared by digestion of the parent monoclonal antibodies (MAbs) by use of immobilized papain (Thermo Scientific Pierce), followed by protein A chromatography and buffer exchange into PBS, pH 7.4.

Bispecific antibody expression plasmids were generated by introducing the previously described T366Y (knob; hu1B7H*) or Y407T (hole; hu11E6H*) point mutation (18) into an antibody expression vector containing human IgG1 constant heavy domains (19). The modified heavy chain and native light chain plasmids were transfected at a 1:1 ratio into confluent T-150 flasks containing adherent CHO-K1 cells by use of Lipofectamine 2000 (Life Technologies). Supernatant was collected over 1 week, purified using protein A chromatography, and stored in PBS, pH 7.4. The bispecific antibody was prepared by incubating a 1:1 molar ratio of the hu1B7H* and hu11E6 parental antibodies at ~2 mg/ml in PBS, pH 7.4, with 10 mM EDTA and 50 mM 2-mercaptoethanolamine (2-MEA; Thermo Scientific Pierce) for 90 min at 37°C. The partially reduced sample was then buffer exchanged into PBS, pH 7.4, and stored overnight at 4°C to allow reoxidation and heterodimerization (20).

The PTx holotoxin (PTx) and its B subunit (PTxB) were obtained from List Biological Laboratories. The A subunit (PTx-220K), a version of the pertussis toxin A subunit truncated at residue 220 and appended with a terminal lysine residue and a hexahistidine tag, was expressed from the pertussis toxin A subunit truncated at residue 220 and appended with a 50-fold molar excess of EZ-Link sulfo-NHS-biotin (Thermo Scientific) at 4°C overnight, followed by buffer exchange into PBS, pH 7.4. Biotinylation was confirmed by an enzyme-linked immunosorbent assay (ELISA) in which a hu1B7 coat was used to capture PTx-220K, which was then detected with streptavidin-horseradish peroxidase (HRP) (BD Pharmingen).

**Modeling of hu1B7 and hu11E6 epitopes on PTx.** The hu11E6 antibody sequence was submitted to the public Rosetta antibody server (22, 23) for modeling. Four models with diverse structures were selected and submitted to PatchDock (24, 25) for an initial docking estimate, using the residues within 10 Å of the sugar residues on PTx structure IPTO (26) as a constraint for the binding area. The best docking run for each antibody structure was submitted to Rosetta Docking2 for refinement. The epitope for the top docking runs was defined as all residues on the PTx structure within 4 Å of the complementarity-determining regions (CDRs). The final consensus epitope was defined as residues consistently contacted in both the S2 and S3 subunits. An average plane was calculated from the coordinates of all surface-exposed residues in each epitope, and a line normal to this plane was added using a custom PyMol script.

PTx binding assays. All ELISAs followed the same general procedure, with the specific modifications detailed below. First, a high-binding 96-well ELISA plate (Costar) was incubated overnight at 4°C with the coat protein in PBS, pH 7.4. Second, the plate was blocked. Third, protein dilutions were prepared in the plate, in duplicate. After any additional protein incubation steps, the secondary antibody was added to the plate. After the final incubation and washes, the plate was developed with TMB substrate (Thermo Fisher Scientific) and quenched with 1 N HCl, and the absorbance was measured at 450 nm by using a SpectraMax M5 spectrophotometer. All incubation steps proceeded for 1 h at room temperature. Wash steps between incubation steps used PBS with 0.05% Tween 20 (PBST), while the blocking buffer and assay diluent used was PBS with 5% powdered milk (PBSTM).

For the PTx sandwich ELISA, the plate was coated with 1 µg/ml of murine 1B7 or 11E6, blocked, and then incubated with dilutions starting from 70 to 100 nM PTx-220K, PTxB, or full PTx. The sandwich was completed with 1 µg/ml human 1B7 or 11E6, labeled with a 1:2,500 dilution of goat anti-human Fc–HRP (Thermo Scientific Pierce), and developed as described above.

For the bispecific sandwich ELISA, the plate was coated with 1 µg/ml PTxB, blocked, and then incubated with antibody dilutions starting from 20 µg/ml. Following incubation with 1 µg/ml PTx-220K–biotin, binding was detected with streptavidin–HRP (BD Pharmingen) at 1:5,000 dilution.

For the PTx binding ELISA, the plate was coated with 0.2 µg/ml PTx, blocked, and then incubated with antibody dilutions starting from 5 µg/ml (hu1B7 variants) or 10 µg/ml (hu11E6 variants). Binding was detected using goat anti-human constant kappa–HRP (Thermo Scientific Pierce) at a 1:1,250 dilution. Data shown are representative of three independent experiments.

PTx binding affinity was determined by competition ELISA. A high-binding plate was coated with 0.2 µg/ml PTx and blocked as described above. While the plate was being blocked, a 3 nM (MAbs and bispecific antibody) or 6 nM (Fab fragments) solution of each protein was incubated with dilutions of PTx starting from 200 nM. After equilibration for an hour at room temperature, the PTx-antibody mixtures were transferred to the washed ELISA plate in duplicate and allowed to incubate for 15 min at room temperature to capture unbound antibody. Binding was detected with goat anti-human FC–HRP (monoclonal and bispecific antibodies) or goat anti-human constant kappa–HRP (Fab fragments), and the signal was developed as described above. The resulting curves were fit to equilibrium binding equations (27) corrected for bivalent binding (28) where appropriate. Three independent experiments were performed. Statistical significance was determined by one-way analysis of variance (ANOVA) and Tukey’s test.

**Protein biophysical characterization.** For mass spectrometry (MS) analysis, 100 µg each of the hu1B7H* and hu11E6H* antibodies and the
hu1B7/hu11E6 bispecific antibody was digested with the IdeS enzyme (Promega) for 1 h at 37°C. The digested products were run on a Superdex 200 size exclusion column on an FPLC instrument (Åkta; GE Healthcare), using PBS, pH 7.4, as the running buffer, and the peak corresponding to ~100 kDa was collected based on molecular size standards and buffer exchanged into 50 mM ammonium acetate by use of a centrifugal filter unit (Amicon). The antibodies were analyzed by liquid chromatography-mass spectrometry (LC-MS) on an Orbitrap Fusion Max mass spectrometer (Thermo Fisher). A linear gradient of 0.1% formic acid and water and 0.1% formic acid and acetonitrile over 15 min was used to elute the antibodies from a ProSwift RP-4H (1 × 50 mm) monolithic column (Thermo Fisher). The Orbitrap Fusion instrument was operated in standard pressure mode at a resolution of 15,000 from 1,800 to 4,500 m/z, with 20 microscans and a source fragmentation energy of 35 V. Following acquisition, data were deconvoluted using MagTran and Protein Deconvolution 4.0.

To assess protein size and purity, 3 μg of each purified protein was analyzed by SDS-PAGE. Proteins were incubated with reducing or nonreducing loading buffer and incubated for 5 min at 80°C or 30 s at 42°C, respectively. The protein was loaded onto a 10% acrylamide gel or 4 to 20% gradient gel (Bio-Rad) and run at 120 V prior to staining with GelCode Blue (ThermoFisher).

To assess protein stability, the antibody thermal melting temperature was measured by differential scanning fluorimetry. Purified antibodies were prepared at 400, 200, and 100 μg/ml in PBS, pH 7.4, and mixed with a protein thermal shift dye (Life Technologies) as described in the product literature. Quantification of fluorescence was measured using a ViIA-7 instrument at a ramp rate of 1°C/min, with derivative melting temperature (Tm) values calculated by use of Protein Thermal Shift software (V1.2; Applied Biosystems). Samples were run in triplicate.

**In vitro PTx neutralization assay.** Inhibition of CHO cell clustering was used to determine the in vitro neutralization ability of antibody preparations as described previously (29). Briefly, each antibody was serially diluted across a 96-well tissue plate, from 800 to ~0.4 nM, in the presence of 4 pM PTx. Antibody and PTx were incubated for 30 min at 37°C, after which 1 × 10⁶ freshly trypsinized CHO-K1 cells were added per well. After incubation at 37°C for an additional 48 h, the degree of clustering was scored as 0 (no clustering), 1 (equivocal result), 2 (positive clustering), or 3 (maximal clustering). The neutralizing dose was expressed as the lowest antibody-to-PTx molar ratio resulting in a score of 1. Samples were run in duplicate, and three independent experiments were performed. Statistical analysis was performed with the Kruskal-Wallace test (for data not coming from a Gaussian distribution) and Dunn’s post hoc test.

**Murine PTx leukocytosis study.** An in vivo leukocytosis assay was performed as previously described (30, 31), with the following modifications. First, 2 μg PTx or 2 μg PTx plus 20 μg of total antibody was preincubated for 2 h at room temperature to allow for binding equilibration. These preparations were then injected into a lateral tail vein of 5- to 6-week-old female BALB/c mice. Four days later, blood was collected by terminal cardiac puncture and combined with Na₂EDTA to a final concentration of 10 mM to prevent clotting. Blood (10 μl) was lysed in 150 μl mouse red blood cell lysis buffer (Alfa Aesar) for 15 min, washed with PBS plus 2% fetal bovine serum (FBS), and stained for 1 h on ice with 2.5 μg/ml anti-mouse CD45 antibody or isotype control antibody, each labeled with Alexa Fluor 488 (BioLegend). Samples were run on a flow cytometer (BD FACSFortessa) with CountBright absolute counting beads (Life Technologies) to calculate the total number of white blood cells (WBC) per microliter of blood. Group sizes were based on power calculations from pilot experiment data, with groups of 6 (PBS group) or 10 or 11 (PTx- and antibody-treated groups) mice. In a pilot experiment, mice were administered PBS or 200 μg of hu1B7 by intraperitoneal injection (n = 3 per group), with blood taken from a lateral tail vein on day 4 and processed as described above. In addition, blood was also taken on day 0 from a lateral tail vein of these and other mice to assess the variability in baseline WBC counts. Statistical analysis was performed using one-way ANOVA and Tukey’s test.

**RESULTS**

The hu1B7 and hu11E6 antibodies can simultaneously bind the same toxin molecule. PTx is an AB₅ toxin, with the S1 subunit comprising the “active” A subunit and subunits S2 through S5 comprising the receptor “binding” B subunit. The hu11E6 antibody binds two homologous sites on the B subunit and thus prevents the initial interaction between the toxin and glycosylated receptors on host cells (33), while the hu1B7 antibody binds the A subunit, preventing ADP-glycosylation of cytoplasmic Gₐₒ receptors (21). We thus hypothesized that the synergy previously observed between hu1B7 and hu11E6 was due to a more complete neutralization of the toxin function when both the binding and catalytic activities of the toxin were blocked (9). This effect would be maximal if the hu1B7 and hu11E6 epitopes on a single PTx molecule could be bound simultaneously by their corresponding antibodies, creating a hu1B7-PTx-hu11E6 complex. To evaluate this as a possible mechanism, we wanted to confirm that binding of one hu1B7 or hu11E6 antibody did not preclude binding of a second antibody to the same toxin molecule.

We addressed this question via a series of ELISAs in which one antibody (murine 1B7 or 11E6) was used to capture PTx, which was then detected by a second antibody (hu1B7 or hu11E6) (Fig. 1A). As suggested by the known binding stoichiometry, the full-length toxin could be sandwiched between two 11E6 antibodies or any combination of 1B7 and 11E6 antibodies: both the m1B7/hu11E6 and m11E6/hu11E6 combinations gave strong signals, while the m1B7/hu11E6 combination did not. Similarly, the B subunit alone showed a positive signal only with the m1E6/hu11E6 combination, albeit with lower signals reflecting the lower affinity of 11E6. Similarly, the S1 subunit could not be sandwiched between any combination of 1B7 and 11E6. These data confirm that hu1B7 and hu11E6 can bind the same PTx molecule simultaneously and suggest that multiple bispecific antibodies can simultaneously bind a single toxin molecule.

**Cross-linking of two epitopes on a single PTx molecule is not required for synergistic neutralization.** To further understand the constraints on antibody-PTx binding, we highlighted the amino acid residues recognized by hu1B7 and hu11E6 on the crystal structure of PTx (26) (Fig. 1B). The hu1B7 epitope has been defined experimentally: it localizes primarily to the S1 subunit (21) and was finely resolved by use of a combined yeast display/high-throughput sequencing approach (34). Although experimental epitope mapping of hu11E6 has not been performed, the murine antibody has been reported to bind epitopes present in the homologous S2 and S3 subunits, thereby inhibiting PTx from binding glycosylated and sialylated cellular receptors (33). We further refined the putative hu11E6 epitopes by docking Rosetta models (22, 23) of the antibody onto the toxin, guided by the locations of oligosaccharides in a relevant crystal structure (PDB entry IPTO) (26). The resulting structure shows that the two predicted hu11E6 epitopes and the single known hu1B7 epitope are located on different faces of PTx and are oriented in opposing directions. The midpoints of any two epitopes are approximately...
50 Å apart, which is much narrower than the typical ~130-Å wingspan of a cross-linking antibody (35). A line normal to the best-fit plane calculated from the solvent-exposed residues was used to approximate the orientation of an antibody bound to the PTx surface. Even considering a large elbow angle of 125° between the variable and constant regions (36), the geometry suggests that a single antibody molecule, whether monoclonal or bispecific, would be able to interact with only a single epitope at a time. Thus, complete blockade of all three epitopes would require a stoichiometry of three antibodies for every PTx molecule.

**Expression and purification of a stable hu1B7/hu11E6 bispecific antibody.** Since hu1B7 and hu11E6 previously exhibited synergy in vitro as native IgG1 antibodies, we chose to develop a bispecific antibody with natural architecture by using the knobs-in-holes platform (18), followed by in vitro assembly to form the desired heterodimer (20) (Fig. 2A). The resulting human IgG1 bispecific antibody is structurally similar to the hu1B7 and hu11E6 monoclonal antibodies, facilitating comparisons among the variants, with the added benefits of low immunogenicity and an enhanced circulating half-life due to FcRn binding (37).

To make the bispecific antibody, the T366Y “knob” mutation was introduced into the C\textsubscript{\gamma}3 domain of hu1B7 to generate hu1B7\textsuperscript{T366Y}, while the Y407T “hole” mutation was introduced into the C\textsubscript{\gamma}3 domain of hu11E6 to generate hu11E6\textsuperscript{T407Y}. These two parent antibodies were transiently expressed in separate CHO cell cultures and purified via protein A chromatography. Nonreducing SDS-PAGE of the purified proteins revealed mixtures of homodimers and half-antibodies at ~75 kDa after storage in PBS, pH 7.4 (Fig. 2B). Notably, hu11E6\textsuperscript{T407Y} ran primarily as a covalent homodimer at this pH (38), while hu1B7\textsuperscript{T366Y} showed bands for homodimers and half-antibodies, consistent with previous reports of an Fc-antiparallel knob-knob dimer (39). The hu1B7\textsuperscript{T366Y} and hu11E6\textsuperscript{T407Y} proteins were combined at an equimolar ratio and subjected to a controlled reducing step using 2-mercaptoethanol (2-MEA) to generate the heterodimeric bispecific antibody (20). Nonreducing SDS-PAGE analysis of the product after this step showed a significantly reduced presence of the half-antibody and high-molecular-weight species, suggesting successful formation of the bispecific antibody (Fig. 2B).

The hu1B7/hu11E6 bispecific antibody was digested with IdeS to generate F(ab’\textsubscript{2}) fragments, which were purified and analyzed by LC-MS to assess the purity of the bispecific antibody (Fig. 2C). The precursor antibodies hu1B7\textsuperscript{T366Y} and hu11E6\textsuperscript{T407Y} were prepared in the same way as controls. The bispecific fragment had a major peak at 97.878 kDa, which is a unique molecular mass intermediate to the hu1B7\textsuperscript{T366Y} and hu11E6\textsuperscript{T407Y} homodimers of 96.669 and 99.087 kDa, respectively. The measured masses were nearly identical to the expected values calculated from the primary amino acid sequences. The bispecific antibody also showed a small peak corresponding to hu11E6\textsuperscript{T407Y} homodimers, representing ~5% of the maximum intensity. Because the original reaction was performed at a 1:1 hu1B7\textsuperscript{T366Y}:hu11E6\textsuperscript{T407Y} ratio, we expected a similar proportion of unreacted hu1B7\textsuperscript{T366Y}. Based on SDS-PAGE of the hu1B7\textsuperscript{T366Y} precursor, the unreacted hu1B7\textsuperscript{T366Y} fraction likely contained a significant amount of half-antibodies, which would not show up in our IdeS analysis. Thus, assuming that there was a similar amount of unreacted hu1B7\textsuperscript{T366Y} also present, the final purity of the assembled bispecific antibody was ~90%, consistent with other bispecific antibodies generated using the T366Y/Y407T mutations (18).

**Biophysical characterization of monoclonal, Fab, and bispecific antibodies.** To provide a point of reference in our functional assays and to assess valency effects, we also produced monoclonal hu1B7 and hu11E6 and their Fab fragments. The monoclonal antibodies were expressed in transient CHO cell cultures and purified by protein A and anion exchange chromatography as previously described (9). The Fab fragments were generated by subsequent digestion of purified antibody with immobilized pa-
pain, followed by protein A chromatography to remove undigested antibody and free Fc domains. Reducing and nonreducing SDS-PAGE analyses determined that the proteins were highly pure (≥90% or higher) and of the expected sizes (Fig. 3A). The reducing lanes in Fig. 3A show characteristic bands at ~50 kDa (intact immunoglobulin heavy chain) and ~25 kDa (light chain and Fab heavy chain), while the nonreducing lanes show single bands at ~150 kDa (intact immunoglobulin and bispecific antibody) and ~50 kDa (Fabs).

Antibody thermal stability was assessed using a differential scanning fluorimetry assay to monitor protein unfolding in PBS, pH 7.4 (Fig. 3B). The full-length hu1B7 profile shows two melting regimes, with the first peak (~71°C) likely representing CH2 unfolding and the second peak (~79°C) representing CH3 and Fab unfolding (40). In this buffer, the hu11E6 domains unfolded in a more continuous manner, with the most rapid unfolding occurring around 74°C. The Fab formats showed similar or slightly higher melting temperatures than those of their full-length counterparts, with derivative melting points of 78°C for hu1B7 Fab and ~76°C for hu11E6 Fab. The bispecific antibody exhibited a more complex profile, making it difficult to identify specific melting regimes, but major transitions were observed at ~67°C and ~77°C. The introduction of the knob and hole mutations is expected to have a slight destabilizing effect on the CH3 domain of the bispecific antibody. Derivative traces are also shown to aid in identification of melting transitions (Fig. 3B).

The hu1B7/hu11E6 bispecific antibody has in vitro PTx binding activity similar to that of the binary mixture. To assess the PTx binding activity of the bispecific antibody and to compare this with the activity of the binary antibody mixture, we performed a series of ELISAs. To verify that the protein was functionally bispecific, we compared the hu1B7/hu11E6 bispecific and hu1B7H and hu11E6H parent antibodies by using a sandwich ELISA (Fig. 4A). The plate was coated with the PTx-B subunit and used to capture any antibodies with the hu11E6 paratope. This was followed by incubation with S1-220K– biotin and streptavidin-HRP to detect molecules also containing the hu1B7 binding site. Because hu1B7H binds only S1-220K–biotin, it did not bind to the PTx-B coating, while hu11E6H– bound PTx-B but could not be detected with S1-220K–biotin. In contrast, the bispecific antibody was able to simultaneously bind both PTx-B and S1-220K–biotin, exhibiting a characteristic logarithmic binding curve with a 50% effective concentration (EC50) of ~4 nM.

We next assessed the binding characteristics of all individual antibodies and fragments by using a direct PTx binding ELISA (Fig. 4B). All variants displayed strong binding to PTx, with the bispecific antibody showing a binding profile nearly identical to those of full-length hu1B7 and hu11E6. While the hu1B7 Fab fragment had a curve similar to that for full-length hu1B7, the hu11E6 Fab fragment showed an ~2-fold increase in EC50 compared to the full-length version, suggesting that there are significant avidity effects for hu11E6.
For a more thorough investigation into the effective binding affinities of different antibody formats, we performed a quantitative competition ELISA wherein antibody dilutions were incubated with a constant amount of PTx, after which unbound antibody was detected by a direct PTx binding ELISA (Fig. 4C). We fit curves to these data to calculate the equilibrium dissociation constants ($K_D$), with bivalency corrections used for the full-length proteins (28). Due to a simple 1:1 interaction with toxin and a binary “bound” or “not bound” structure, Fabs provide more accurate affinity measurements. The hu1B7 Fab $K_D$ was calculated to be $3.1 \pm 0.9$ nM, while the hu11E6 Fab fragment had a significantly weaker affinity, with a $K_D$ of $20 \pm 8$ nM. The full-length formats showed an approximately 3-fold improvement in effective affinity compared to the Fabs, likely due to avidity effects. The $K_D$ values calculated for the full-length hu1B7 and hu11E6 antibodies were $0.8 \pm 0.1$ nM and $6 \pm 2$ nM, respectively, and are comparable to our previous competition ELISA and surface plasmon resonance (SPR) measurements (9).

Both Fab and full-length mixtures as well as the bispecific antibody were tested in this assay, although in these cases $K_D$ represents an effective affinity. The mixture of full-length antibodies and the bispecific antibody exhibited effective affinities intermediate to those of the two parents, at $2.1 \pm 0.8$ nM and $1.1 \pm 0.4$ nM, respectively, while the Fab mixture had an effective affinity of $8 \pm 4$ nM. These affinities are skewed more toward the higher affinity of hu1B7, which in this solution-based assay may capture a larger fraction of available PTx molecules than the hu11E6 binding site. Notably, the bispecific antibody also showed a significant improvement in effective affinity compared to the Fab mixture, supporting the importance of avidity effects. The data from these three ELISAs in aggregate demonstrate that the hu1B7/hu11E6 bispecific antibody is functionally bispecific and binds PTx similarly to the antibody mixture in a solution-based assay.

The bispecific antibody neutralizes PTx activity in vitro. We next wanted to assess the neutralization capacity of the hu1B7/hu11E6 bispecific antibody in a simple in vitro model. The CHO cell assay is a commonly used method to quantify PTx activity and neutralization by observing morphological changes in CHO-K1 cells after incubation with toxin or toxin plus antibody (29). This method was previously used to observe synergy between hu1B7 and hu11E6, which was maximal in an equimolar mixture (9).

A constant amount of PTx (4 pM) was equilibrated with a series of antibody concentrations (up to 800 nM) in a 96-well plate. CHO-K1 cells were seeded into the wells and incubated for 48 h prior to scoring for the degree of cell clustering. Here we report the neutralizing ratio as the lowest antibody-to-toxin ratio which was able to completely prevent the clustered morphology, corresponding to a score of 1 (equivocal) on the established scale of 0 to 3 (33), which is a more rigorous analysis than previously applied (9) (Fig. 5). Under these conditions, full-length hu11E6 and hu1B7 completely neutralized the toxin at ratios of $\sim 1,100:1$ and $\sim 34,000:1$, respectively. This large discrepancy is consistent with previous studies, despite the superior performance of hu1B7 in a mouse aerosol challenge (9). In addition, a similar study using a panel of murine anti-PTx antibodies, including 1B7 and 11E6, also observed enhanced neutralization in the CHO cell clustering assay by anti-B-subunit antibodies, which did not necessarily correlate with protection in vivo (30). The hu1B7-hu11E6 mixture and hu1B7/hu11E6 bispecific antibody were also able to fully neutralize the toxin, with antibody-to-PTx ratios of $\sim 700:1$ and

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**FIG 3 Biophysical characterization of bispecific and parent antibodies.** (A) Reducing and nonreducing SDS-PAGE analyses were used to assess the size and purity of the hu1B7 and hu11E6 full-length and Fab antibody fragments as well as the hu1B7/hu11E6 bispecific antibody. A total of 3 µg of protein was loaded per lane. (B) Differential scanning fluorimetry was performed to determine the melting profiles of full-length hu1B7 and hu11E6 (blue and green solid lines, respectively), the hu1B7 and hu11E6 Fabs (blue and green dashed lines, respectively), and the hu1B7/hu11E6 bispecific antibody (gray line). Samples were prepared in PBS, pH 7.4. Derivative data [d(fluorescence)/dt] are aligned below the raw melting curves to facilitate identification of transition temperatures. Curves show averages for three replicates at 400 µg/ml.

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**B**

1. Differential scanning fluorimetry was performed to determine the melting profiles of the full-length proteins and their Fabs.
2. The bispecific antibody was detected by a direct PTx binding ELISA.
3. The $K_D$ values calculated for the full-length proteins were $0.8 \pm 0.1$ nM and $6 \pm 2$ nM.

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**A**

1. Reducing and nonreducing SDS-PAGE analyses were used to assess the size and purity of the antibodies.
2. The bispecific antibody was detected by a direct PTx binding ELISA.
3. The $K_D$ values calculated for the bispecific antibody were $2.1 \pm 0.8$ nM and $1.1 \pm 0.4$ nM.
These values are more similar to those observed for hu11E6 alone than for hu1B7 alone, despite the presence of fewer hu11E6 binding arms in the mixture or bispecific formulation. This is preliminary evidence of synergy and also demonstrates that the bispecific format does not compromise B subunit neutralization.

We included the hu1B7 and hu11E6 Fabs as well as a mixture of the two to assess the role of valency in PTx neutralization. In particular, we did not know a priori whether the bispecific antibody would better mimic a mixture of monoclonal antibodies or a mixture of Fabs. In this assay, the antibody was present at a significant molar excess over PTx, favoring binding of multiple antibodies to a single PTx. For this reason, we did not correct for valency in our ratio calculations. In general, the Fabs required approximately 3-fold more protein to achieve the same neutralization level as the full-length antibodies (3,600:1 for the hu11E6 Fab and 2,900:1 for the Fab mix) (Fig. 5), and we were unable to

FIG 5 In vitro PTx neutralization measured by inhibition of CHO cell clustering. Adherent CHO-K1 cells were grown in the presence of 4 pM PTx preequilibrated with antibody dilutions. The lowest molar antibody-to-toxin ratio able to fully prevent clustering was recorded as the neutralizing ratio. Data are shown for neutralization with the full-length and bispecific antibodies (solid bars) and their Fab fragments (striped bars). The hu1B7 Fab was not able to fully neutralize the toxin at any concentration tested. Data are presented as geometric means for six replicates over three experiments, with error bars indicating 95% confidence intervals. Statistical analysis was performed using Kruskal-Wallis and Dunn’s post hoc tests. Statistical significance is indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001.

~900:1, respectively. These values are more similar to those observed for hu11E6 alone than for hu1B7 alone, despite the presence of fewer hu11E6 binding arms in the mixture or bispecific formulation. This is preliminary evidence of synergy and also demonstrates that the bispecific format does not compromise B subunit neutralization.

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antibody variants and controls. A PTx-coated plate was incubated with dilutions of full-length hu1B7 or hu11E6 (solid circles with blue line and hollow circles with green line, respectively), the hu1B7 or hu11E6 Fab (solid triangles with dashed blue line and hollow triangles with dashed green line, respectively) or the hu1B7/hu11E6 bispecific antibody (squares with solid gray line), followed by anti-human kappa–HRP to detect bound chains. (C) A competition ELISA was used to determine the solution-based equilibrium dissociation constants (K_D) for all variants. Data are shown for the full-length (solid bars) and Fab (striped bars) antibody formats. For all panels, the errors shown are standard deviations. The competition ELISA data were averaged for six replicates over three experiments. Statistical significance was determined using one-way ANOVA and Tukey’s test. Statistical significance is indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001. In addition to the comparisons shown, the hu11E6 Fab fragment was significantly different from all other groups (****), and the hu1B7 Fab-hu11E6 Fab mixture was significantly different from the hu1B7 antibody (*).
detect neutralization by the hu1B7 Fab under these stringent assay conditions. These results correlate well with the increase in effective affinity seen with the full-length immunoglobulins in the competition ELISA.

In this plate-based cellular assay, there is no mechanism for clearance of antibody-toxin complexes; thus, in order to prevent cellular intoxication, antibody molecules must sequester every PTx molecule for the 48-h duration of the experiment. Since transiently unbound PTx molecules are able to bind cellular receptors and enter the cell via receptor-mediated endocytosis, even high-affinity antibodies, such as hu1B7 and hu11E6, require very high ratios to fully neutralize PTx. Thus, the neutralizing ratios reported here are much higher than would be expected for an *in vivo* experiment.

**The antibody mixture and the bispecific antibody synergistically inhibit PTx-induced leukocytosis in vivo.** Systemic leukocytosis is a primary outcome of *B. pertussis* infection and is predictive of severe disease in infants (7). As a proof-of-concept study to assess the therapeutic potential of the hu1B7/hu11E6 bispecific antibody, we turned to an *in vivo* leukocytosis assay which measures the ability of an antibody to neutralize the increase in white blood cell (WBC) count induced by the injection of purified pertussis toxin (30, 31). This assay has previously been shown to be more predictive of protection during bacterial infection than the CHO cell assay (30).

In a pilot experiment, juvenile BALB/c mice were injected with 200 μg hu1B7 antibody or PBS. Whole blood was collected from a lateral tail vein and assayed for white blood cells by staining for CD45 and measuring fluorescent cells via flow cytometry. In addition, WBC counts were taken from these and other mice on day 0 to assess the baseline variability. Treatment with a high dose of hu1B7 did not elevate the WBC count compared to the baseline level or the level with PBS treatment (Fig. 6A).

In the neutralization experiment, mice were injected with PBS, preequilibrated mixtures of 2 μg PTx plus 20 μg antibody, or 2 μg of the toxin alone. Four days later, whole blood was collected via cardiac puncture and assayed for white blood cells as described for the pilot experiment. The toxin alone induced an ~14-fold increase in WBC count compared to that for PBS-treated mice, while the full-length hu1B7 and hu11E6 antibodies were each able to significantly suppress this WBC rise (*P < 0.0001* and *P < 0.01*, respectively) (Fig. 6B). hu1B7 was significantly more effective than hu11E6 (*P < 0.001*), consistent with previous data showing enhanced *in vivo* protection against bacterial challenge by hu1B7 over hu11E6 (9).

A relatively low antibody dose (7:1 molar ratio of antibody to PTx) was selected to provide only partial suppression of leukocytosis by hu1B7 or hu11E6 to allow for the detection of synergy based on pilot experiments (data not shown). Under these conditions, an equimolar antibody mixture (10 μg of each antibody) significantly reduced the WBC count compared to that with treatment with the same dose of either hu1B7 or hu11E6 alone (20 μg of antibody) (*P < 0.0001*). The resulting WBC count was not significantly different from that for PBS-treated naive mice. Since the antibody mixture contained equimolar amounts of both hu1B7 and hu11E6 specificities, simple additivity predicts that the mixture would provide a result intermediate to that of either antibody used alone. Instead, the efficacy of the mixture was significantly enhanced over that of either monoclonal preparation, indicating synergy between the antibodies.

Synergy of the bispecific antibody can be assessed in a similar way, although avidity effects complicate the analysis. As there is only a single hu1B7 epitope on a PTx molecule, the bispecific antibody can be expected to retain all of the hu1B7 functionality. In contrast, while hu11E6 can also bind only a single epitope at a time, bivalency was beneficial in ELISA and CHO assays, and thus
the bispecific construct may not be able to capture the full neutralization potential of hu11E6 with only a single paratope. However, treatment with 20 μg of the bispecific antibody still significantly reduced the WBC count compared to that with either of the monoclonal preparations ($P < 0.01$ versus hu1B7 and $P < 0.0001$ versus hu11E6). Notably, there was no difference in WBC count for mice treated with the antibody mixture compared to those treated with the bispecific antibody at this power level. These data show that the bispecific antibody was able to capture the synergy observed in the mixture.

**DISCUSSION**

Three of the 52 currently approved antibodies are indicated for the treatment or prevention of infectious diseases (41), with dozens more in clinical and preclinical development (10, 42, 43). Palivizumab was approved in 1998 to prevent respiratory syncytial virus (RSV) infection in high-risk patients (44), while raxibacumab and obiltoxaximab were approved in 2011 and 2016, respectively, for the treatment of anthrax (45, 46). Antibodies may be appropriate therapeutics for a variety of other infectious diseases that cause serious disease in high-risk populations, including pertussis. An antibody therapeutic could be used to treat seriously ill infants in the developing world and prevent disease in high-risk areas, a strategy that is being pursued for pediatric HIV-1 (47). In this work, we further explored the mechanism of synergy between the anti-PTx antibodies hu1B7 and hu11E6 and showed that a bispecific antibody captures this synergy in a single molecule.

We previously demonstrated that an equimolar mixture of hu1B7 and hu11E6 exhibited synergistic PTx neutralization in vitro, requiring a lower antibody-to-toxin ratio to achieve the same effect as that with either hu1B7 or hu11E6 alone (9). In the present study, we showed that while multiple antibodies can simultaneously bind the same toxin molecule (Fig. 1A), a single antibody is unlikely to cross-link two of those epitopes (Fig. 1B). Thus, if synergy depends upon simultaneous blockade of the hu1B7 and hu11E6 epitopes on PTx, a bispecific antibody with native architecture would be expected to exhibit PTx-neutralizing abilities similar to those of the mixture. This was confirmed in several in vitro assays to assess PTx binding and in vitro neutralization (Fig. 3 and 4). Finally, in vivo PTx neutralization data for a 7:1 molar excess of antibody not only demonstrated significant synergy between hu1B7 and hu11E6 but also confirmed that the bispecific format fully recapitulated this effect (Fig. 6). These results confirm the hypothesis that synergy is due to more complete neutralization of the toxin when both the binding and catalytic activities are simultaneously blocked, which can be achieved by simultaneous binding of hu1B7 and hu11E6 or several bispecific antibodies.

A similar approach was recently reported for ricin toxin, another A–B toxin with an intoxication pathway analogous to that of PTxs (48). In that work, camelid variable heavy domains blocking the active and binding activities were only partially neutralizing on their own, and a mixture of the top two performers did not exhibit synergy (49). In contrast, a flexible bispecific construct was developed that was able to cross-link the two epitopes and in doing so significantly change the dynamics of toxin binding and trafficking (50). Thus, even in the context of bacterial toxin neutralization, bispecific antibodies can be applied in several ways.

Avidity effects were difficult to deconvolute in this system, with the paratope-epitope stoichiometry, spatial presentation of the antigen, and relative amounts of toxin and antibody all having different impacts on the results of each assay. While structural modeling suggests that simultaneous engagement of two epitopes on the same toxin molecule by a single antibody is unlikely, it is possible that the presence of a second epitope nearby makes it easier for a bivalent construct to rebind the same toxin, essentially “wobbling” between the two epitopes. Avidity effects of multivalent and bispecific formats have been modeled (51) and have been proposed as a way to drive higher-affinity and higher-specificity binding of targets with multiple epitopes. Alternatively, bridging of two toxin molecules by one monoclonal antibody can allow bivalent binding of a second antibody to a different epitope, which has been proposed as a mechanism for synergy in the neutralization of anthrax toxin (52). Regardless of the exact mechanism, bivalency enhanced the effective binding affinity (Fig. 4C) and in vitro neutralization (Fig. 5). In a therapeutic setting, Fabs would not typically be used due to their significantly shorter circulating half-life.

While the synergy between hu1B7 and hu11E6 is beneficial for potency and for reducing the cost of goods, the inclusion of multiple specificities is also important for long-term safety and efficacy of a therapeutic. Suppression of escape variants is a particular concern for pertussis, as circulating *B. pertussis* strains appear to have changed in response to vaccine-induced immunity. Widespread use of acellular vaccines containing the adhesin pertactin appears to have provided a considerable fitness advantage for naturally occurring pertactin-deficient strains, with the result that 100% of clinical isolates in the United States now lack this protein (53). Thus, there is basis for concern that widespread use of anti-PTx antibodies could lead to evolution or selection of escape variants. Fortunately, PTx appears to be more essential for pathogenesis than pertactin, as *B. pertussis* PTx knockout strains are minimally infective and severely reduced in virulence (54, 55). Moreover, PTx is a key component of all acellular vaccines, including a PTx-only vaccine in Denmark, yet only two PTx-deficient strains have been isolated in the past 8 years worldwide. Both of these strains were recovered from unvaccinated individuals coinfected with other *Bordetella* strains (56), suggesting that PTx-deficient strains are unlikely to be successful pathogens on their own. The majority of sequence variation occurs in the A subunit, which is immunodominant and has five distinct circulating alleles (5). It is possible that antigenic drift could lead to PTx variants with reduced affinity for neutralizing antibodies, although changes in the A subunit are unlikely to affect hu11E6 binding and have previously been shown to have a minimal effect on hu1B7 binding (21). However, an antibody therapeutic with multiple specificities further mitigates this concern.

The main drawbacks to antibody mixtures are the increased complexity and costs of manufacturing and regulatory approval processes. The binary mixture described here would likely be produced by separately manufacturing hu1B7 and hu11E6 and adding a final coformulation step. Although this method is simple to implement, it is susceptible to batch failure for either individual antibody and requires the development of two separate manufacturing chains (57). In addition, antibody mixtures are subject to the same strict FDA approval processes as monoclonal preparations, and documentation of the safety of each individual component as well as the mixture is needed (14). While bispecific antibodies have their own manufacturing challenges, detailed below, the regulatory pathway is expected to be simpler. We explored the
use of a bispecific antibody as an alternative way to package the therapeutic potential of the antibody mixture.

Over 60 distinct bispecific architectures have been reported, ranging from constructs with a near-native immunoglobulin structure to various single-chain variable-fragment (scFv) fusions (37). As our eventual goal is to use this antibody for passive immunization in high-risk infants, stability and a long-circulating half-life are essential characteristics, which suggested the use of a format including an Fc region for FcRn-mediated recycling. We chose to use the knobs-in-holes mutations (18) on a stabilized 4D5 framework, which is a validated approach with a simple protocol. The resulting human IgG1 (hulgG1) antibody has a structure nearly identical to those of the parental hu1B7 and hu11E6 antibodies, facilitating direct comparisons with the antibody mixture. In several other applications of bispecific antibodies for infectious disease, it was found that the chosen bispecific format had a significant impact on potency, with full neutralization occurring only when the molecule was engineered to be flexible enough to cross-link two epitopes (16, 17). As hu1B7 and hu11E6 are able to neutralize PTx and exhibit synergy without cross-linking epitopes on a single PTx molecule, we did not pursue alternative formats.

Bispecific antibodies with native architectures are appealing for their high stability, long half-life, and low immunogenicity but are among the most difficult to manufacture. These designs require correct assembly of two unique heavy chains and two unique light chains into a single heterodimeric protein. In this case, the modular antibody domains that allow for diverse immune responses due to unbiased pairing of many heavy and light chain gene products are a detriment: there is minimal thermodynamic driving force to favor correct pairing, for instance, between a hu1B7 heavy chain and a hu1B7 light chain versus a hu11E6 light chain. The simple knobs-in-holes design utilized here results in over 90% correct assembly of the modified heavy chains (18) but requires separate expression and purification of the half-antibodies to maintain proper pairing between the heavy and light chains. Several processing strategies using knobs-in-holes designs have been developed (20, 58), while engineering of orthogonal C4b/C2 Fab interfaces to drive proper heavy and light chain pairing (59) has paved the way for production of a bispecific antibody from a single cell line. Bispecific antibody manufacturing is currently a very active area of research, and it is likely that future improvements will be included in a second generation of the bispecific antibody described here.

The treatment of infectious diseases presents unique challenges, but antibody mixtures and bispecific formats have the potential to address currently unmet clinical needs in this area. We have quantitatively demonstrated synergy in vivo between the two anti-pertussis toxin antibodies hu1B7 and hu11E6 and have shown that this synergy is retained when these specificities are expressed as a knobs-in-holes bispecific antibody. Since the antibody mixture was previously shown to be effective in both murine prophylactic and weaning baboon therapeutic models (9), we expect a bispecific antibody to recapitulate the efficacy of the mixture in an infection setting. This bispecific antibody is particularly appealing because the format simplifies the regulatory path while retaining the synergy and broader neutralization potential of the binary combination. We expect that bispecific and other multivalent antibody formats will see increased development for infectious disease applications.

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