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Antibody-Dependent Cell-Mediated Cytotoxicity- and Complement-Dependent Cytotoxicity-Independent Bactericidal Activity of an IgG against Pseudomonas aeruginosa O6ad

Xuemei Xie,1 Michael D. McLean, and J. Christopher Hall

In addition to Ag recognition, some Abs are capable of killing target organisms in the absence of phagocytes and complement. In this study, we report that an anti-Pseudomonas aeruginosa O6ad LPS IgG1, tobacco-expressed human S20 IgG1 (te-hS20), as well as its recombinant Fab and single-chain variable fragment (scFv) fragments have cellular- and complement-independent bactericidal activity. te-hS20 and its Fab and scFv significantly reduced viability of P. aeruginosa O6ad in dose- and time-dependent manners in vitro and also showed lower levels of bactericidal activity against P. aeruginosa PAO1, but had no activity against P. aeruginosa O10, Escherichia coli TG1, and Streptococcus agalactiae. The H chain and its Fd fragment both had significant Ag-binding and bactericidal activities against P. aeruginosa O6ad. Bactericidal activity was completely inhibited with specific LPS Ag, suggesting that Ag binding is involved in the bactericidal mechanism. Live/dead cell staining and electron microscopic observations indicate that the bactericidal effect was due to disruption of the cell wall and suggest inhibition of cell division. In addition to te-hS20, the Fab and scFv were also protective in vivo, as leukopenic mice had prolonged and improved survival after administration of these Ab fragments followed by challenge with P. aeruginosa O6ad cells at 80–90% lethal dose, supporting a bactericidal mechanism independent of phagocytes and complement. Understanding of the bactericidal mechanism will allow assessment of the potential for therapeutic application of these Abs. The Journal of Immunology, 2010, 184: 000–000.

In the classical view of microbial immunity, Abs typically do not kill microorganisms directly. Ab-mediated elimination of target microorganisms normally involves specific Ag recognition and subsequent Fc region-mediated opsonic phagocytosis or cytotoxic killing via activation of immune system cells through Ab-dependent cell-mediated phagocytosis, Ab-dependent cell-mediated cytotoxicity (ADCC), or complement cascade activation through complement-dependent cytotoxicity (CDC) (1, 2). Despite this, some Abs have been found to have the capacity to kill microorganisms or inhibit their growth independent of complement and immune cells (3–5). The direct antimicrobial action of such Abs can be specific to their target microbes or nonspecific to unrelated microorganisms. The antimicrobial mechanisms of these Abs range from catalytic activities toward either target Ags or nonspecific molecules to interference with biological functions of target organisms upon Ab binding.

Because the first independent reports on catalytic Abs, also called abzymes (6, 7), a number of Abs have been discovered or designed to catalyze many distinct classes of chemical reactions (8–15). Certain chemical reactions catalyzed by abzymes result in production of toxic byproducts, therein leading to complement- and immune cell-independent killing of target organisms (12, 14, 16). For example, Abs with peroxidase activity catalyze the reaction between singlet oxygen (1O2*) and water, resulting in the production of oxidative molecules, such as H2O2, H2O2, and O3 (13–18). These bioreactive oxidants are highly cytotoxic and can destroy target cells or damage tissues (12, 14–16, 19, 20).

In addition to killing target cells via catalytic activities, some Abs manifest direct antimicrobial activity through interference with biological functions of the target microorganisms. Early reports described IgG fractions specific for the LPS of pathogenic serotypes of Escherichia coli (4, 21) and secretory IgA from human milk (22), which were both bacteriostatic; these Abs were shown to interfere with the release of an iron chelator, enterochelin, after binding to LPS, thereby inhibiting iron acquisition by the bacteria. A similar mechanism was used by a bactericidal IgM raised against the iron-regulated outer membrane proteins of Acinetobacter baumannii (23). H6831, an IgG, mAb, and CB2, an IgG2, mAb, both directed against the outer membrane protein OspB of Borrelia burgdorferi (24–26), killed the spirochete by damaging its surface protein coat (27). Identical results were also obtained with Fabs of these Abs (27, 28). CB515, an IgM mAb, and its single-chain variable fragment (scFv), both specific for Vsp protein of relapsing fever Borrelia (24, 26), killed the spirochete by damaging its surface protein coat (27). Identical results were also obtained with Fabs of these Abs (27, 28). CB515, an IgM mAb, and its single-chain variable fragment (scFv), both specific for Vsp protein of relapsing fever Borrelia, were shown to be bactericidal in vitro (29, 30) and protective against bacterial challenge in mice (29). 1D5, an mAb specific for a membrane glycoprotein of Blastocystis hominis, was cytotoxic to certain isolates of Blastocystis by an apoptosis-like mechanism associated with mitochondrial dysregulation (31–33). C7, an IgM mAb raised against cell wall mannoprotein of Candida albicans, was shown to exert fungicidal effects against
C. albicans by three different mechanisms: interference with adherence, inhibition of germination, and direct fungidal activity (5); this mAb was also protective in a murine model of systemic candidosis (34). A subset of killer toxin-like Abs (KT-Abs), produced by idiotypic vaccination with mAb KT4 that was raised to neutralize a KT produced by Pichia anomala, showed broad antifungal activities in vitro against pathogenic bacteria, protozoa, and fungi (3, 35) and provided protection in experimental models of local and systemic fungal infections (36, 37). These Abs functionally mimic the cytotoxic action of the P. anomala KT, which has wide-spectrum antifungal activity against prokaryotic and eukaryotic microorganisms presenting receptors for KT (38). Similar activities were observed with KT-mAbs and its recombinant KT-scFv (3, 35–37, 39). These studies provide strong evidence that Abs can directly destroy the target organisms and contribute to the assistance of complement and immune cells.

Our previous studies showed that a tobacco-expressed human S20 IgG (te-hS20) specific for the LPS of Pseudomonas aeruginosa serotype O6ad was able to mediate in vitro opsonophagocytosis of target bacteria by human polymorphonuclear leukocytes (40). In this study, we discovered that te-hs20 as well as its recombinant Fab and scFv fragments, had specific bactericidal activity against P. aeruginosa O6ad. It was later observed that the te-hs20 and its fragments share the membrane integrity and cell morphology of the target bacteria was investigated by live/dead cell staining and electron microscopy. Furthermore, the efficacy of protecting leukopenic mice from infection plasmid for anti-S. aureus IgG1 (40) was used as a negative control. After 30 min, treatment with te-hS20 (200 μg/ml) was performed to determine the concentrations of LPS required for 100% binding (IC100). In vitro bactericidal assays

P. aeruginosa (serotypes O8ad, PAO1, and O10), E. coli (TG1), and Gram-positive S. agalactiae (2 × 10⁶ CFU/ml) or their LPSs (1 μg/ml) and treated with serial dilutions of Abs or fragments, which were measured with mouse anti-human IgG (H+L) conjugated with HRP (Pierce, Rockford, IL), both at 1:2000 dilutions in PBS/Tween as primary and secondary Abs, respectively. Ag binding specificities were confirmed by ELISA as previously described (40, 46). Microtiter polystyrene plates (Costar, Corning, NY) were coated with either heat-killed P. aeruginosa serotypes (1 × 10⁶ CFU/ml) or their LPSs (1 μg/ml) and treated with serial dilutions of Abs or fragments, which were purified by immobilized metal affinity chromatography (43). Because the top-6dAbs Fab fraction contained <40% of glycosylated yeast α-factor signal sequence-linked Fab, it was further treated with Con A-agarose to remove α-factor-linked Fab (44). The dissociation of te-hs20 into H and L and Fab into Fd and L chain subunits was carried out according to Sun et al. (45). The dissociated L and H chains from te-hs20 were incubated with protein G-agarose and then separated by gravity filtration and glycine elution (100 mM [pH 2.3]) and neutralization (1 M Tris-HCl [pH 8.5]), respectively, then dialyzed against PBS and concentrated. The dissociated Fd and L chains from Fab were incubated with NP-40-nitrotoluic acid-agarose, from which the L chain was separated by gravity filtration, and the Fd fragment was eluted with 250 mM imidazole; both were dialyzed in PBS and then concentrated. The purity of all Abs and fragments was analyzed by SDS-PAGE and Western immunoblotting (40), using Penta-His IgG (Qiagen, Mississauga, Ontario, Canada) and goat anti-mouse IgG conjugated with alkaline phosphatase (Pierce, Rockford, IL), both at 1:2000 dilutions in PBS/Tween as primary and secondary Abs, respectively.

Ab production, purification, and binding specificities

Tobacco-expressed te-hs20 was purified according to McLean et al. (40). The Fab and scFv were expressed as described previously (42). These fragments were purified by immobilized metal affinity chromatography (43). Because the Fab fraction contained >40% of glycosylated yeast α-factor signal sequence-linked Fab, it was further treated with Con A-agarose to remove α-factor-linked Fab (44). The dissociation of te-hs20 into H and L and Fab into Fd and L chain subunits was carried out according to Sun et al. (45). The dissociated L and H chains from te-hs20 were incubated with protein G-agarose and then separated by gravity filtration and glycine elution (100 mM [pH 2.3]) and neutralization (1 M Tris-HCl [pH 8.5]), respectively, then dialyzed against PBS and concentrated. The dissociated Fd and L chains from Fab were incubated with NP-40-nitrotoluic acid-agarose, from which the L chain was separated by gravity filtration, and the Fd fragment was eluted with 250 mM imidazole; both were dialyzed in PBS and then concentrated. The purity of all Abs and fragments was analyzed by SDS-PAGE and Western immunoblotting (40), using Penta-His IgG (Qiagen, Mississauga, Ontario, Canada) and goat anti-mouse IgG conjugated with alkaline phosphatase (Pierce, Rockford, IL), both at 1:2000 dilutions in PBS/Tween as primary and secondary Abs, respectively.

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with LPSs at IC_{100} values of 2.2 mg/ml, 279.3 μg/ml, and 222.2 μg/ml, respectively, for 1 h at RT in sterile PBS. Following preincubation, an equal volume of _P. aeruginosa_ O6ad cell suspension (2 × 10^7 CFU/ml) was added, and treatments were incubated in the dark for 30 min at 37°C. LPS_{E. coli} was used as a negative control. Following treatment, the number of viable cells was determined as above.

For the dose-response, bacterial suspension (2 × 10^7 CFU/ml) was mixed with an equal volume of Ab at final concentrations ranging from 1.3–5.36 μg/ml, and then incubated in the dark for 30 min at 37°C. Tobramycin was used at concentrations ranging from 0–107.5 μg/ml. Treatment mixtures were then enumerated as above. The EC_{50} for each Ab and tobramycin were calculated by performing regression analysis on data showing linear decreases in bacterial viability.

**Microscopic analyses**

Direct counts of viable and nonviable bacteria following Ab treatments were obtained using the live/dead BacLight Bacterial Viability Kit (Invitrogen). Log-phase cells, washed with sterile 0.85% NaCl and diluted to 2 × 10^7 CFU/ml, were mixed with an equal volume of te-hS20 (1.34 μg/ml), Fab (2.68 μg/ml), or scFv (2.68 μg/ml) in 0.85% NaCl and incubated at 37°C for over 24 h. EDTA (10 mM) and tobramycin (50 μg/ml) were used as positive controls. At 1, 3, 6, and 24 h, 100 μl samples were removed, mixed with BacLight stock solution containing 0.33 mM SYTO9 and 2 mM propidium iodide (PI), incubated in the dark at RT for 15 min, and then filtered through a 0.2-μm black polycarbonate filter (Millipore, Billerica, MA). The filters with attached bacteria were mounted in BacLight mounting oil on a clear glass slide, and the numbers of viable and dead bacteria were obtained from 10 microscopic fields at 1000× using a fluorescence microscope and three replicates per treatment.

For electron microscopy, log-phase bacterial suspensions (2 × 10^7 CFU/ml) in PBS (pH 7.4) were incubated in the dark for 6 h at 37°C with an equal volume of the Ab at final concentrations of 1.34 μg/ml or 6.7 μg/ml. A nonspecific scFv (anti-S. enterica scFv) served as a negative control. At 0.5, 2, and 6 h, 100 μl samples were removed and adsorbed onto carbon planchets (Canemco, Canton de Gore, Quebec) for 30 min at RT. Adsorbed samples were fixed for 30 min at RT in 2% glutaraldehyde (w/v) in 0.07 M phosphate buffer (PB) (pH 7.4), followed by another 30 min fixation at RT with 1% OsO4 (w/v) in 0.07 M PB. Fixed samples were washed with 0.07 M PB, dehydrated through a graded ethanol series (50, 70, 80, 90, and 100%), air dried, then mounted onto aluminum specimen adapters (Canemco) coated with gold-palladium (Emitech, Ashford, Kent, UK.) and viewed under a Hitachi S-570 scanning electron microscope (SEM) (Hitachi, Tokyo, Japan). Samples from 6 h treatments were also examined using a Hitachi S-4500 field emission (FE)-SEM (Hitachi).

**Evaluation of in vivo protective efficacy**

The in vivo efficacy of te-hS20, Fab, and scFv to prevent infection by _P. aeruginosa_ O6ad was investigated using a leukopenic mouse model as described previously (47, 48). Inbred wild-type CD1 female mice, 8–10 wk old and free of _P. aeruginosa_ (Charles River Laboratories, Saint-Constant, Quebec, Canada), were housed under pathogen-free barrier husbandry (Isolation Unit, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada). Leukopenia was established by i.p. administration of cyclophosphamide at 150 μg/g of mouse weight on days 1, 3, and 5. On day 5, 3 h later after i.p. administration of cyclophosphamide at time zero, 80 μg te-hS20, Fab, or scFv in 50 μl sterile PBS (pH 7.4) was injected i.v. via tail vein; 15 min later following Ab administration, _P. aeruginosa_ O6ad cells at 80–90% lethal dose (LD_{90,90}) (10^7 bacteria/mouse in 50 μl sterile PBS) (Supplemental Fig. 9) were administered i.v. via tail vein. Mice infected with bacteria and injected with the same volume of PBS or QCRL-1 at the same protein concentration were used as negative controls. Mouse survival was recorded daily for 7 d. Severely ill mice were euthanized to minimize suffering; this was considered a lethal event caused by the treatment. All mice were euthanized at the end of the experiment. Experiments were performed twice, with five and eight mice per group for each experiment, respectively. The second experiment was done blinded. Based on a heterogeneity test, results from these two experiments were pooled to yield data with 13 mice for each treatment as shown in Fig. 8. All animal work was undertaken in accordance with the Use of Life Support Animals (Canadian Council on Animal Care, Ottawa, Canada) and with protocols approved by the Animal Care Committee of the University of Guelph.

**Statistical analyses**

Statistical analyses were performed using SigmaStat for Windows software (SAS 8.2; SAS Institute, Cary, NC). All data are displayed as mean ± SD/SE, and graphs were generated using Excel software (MS Office, Microsoft, Redmond, WA). Comparisons between groups were performed using a one-way ANOVA multiple comparison test (SAS 8.2); probability values of <0.05 were considered significant.

**Results**

Ag-binding properties of te-hS20 and its fragments

The binding of te-hS20 and its recombinant Fab and scFv fragments, as well as its polypeptide components (i.e., the H, Fd, and L chains) to the homologous immunogens _P. aeruginosa_ O6ad (1 × 10^6 CFU/ml) or purified LPS_{O6ad} (1 μg/ml) was determined by ELISA (Fig. 1A–C). Te-hS20 had an EC_{50} of 0.46 nM to whole bacteria and 0.35 nM to purified LPS_{O6ad}, whereas the anti-O6ad Fab had an EC_{50} of 14.42 nM and 20.96 nM to whole bacteria and LPS_{O6ad}, respectively (Fig. 1A). The anti-O6ad scFv exhibited an EC_{50} of 45.74 nM to whole cells and had no binding to LPS_{O6ad} at lower tested concentrations (Fig. 1A); however, it did bind to LPS_{O6ad} when applied at higher concentrations (Fig. 1D). These results indicate that te-hS20 has the highest Ag binding capacity to both heat-killed O6ad cells and its corresponding LPS, followed by the Fab and then the scFv. The binding of te-hS20, Fab, and scFv was specific to _P. aeruginosa_ O6ad bacteria and LPS_{O6ad} because there was no cross-reactivity to _P. aeruginosa_ PAO1 (serotype O5) or O10 or their LPSs (Supplemental Fig. 1). Also, the Ag binding specificity of te-hS20, Fab, and scFv was further confirmed by inhibition ELISA by preincubation of Abs with increasing concentrations of LPS_{O6ad} (Supplemental Fig. 2).

In comparison with the Ag binding of te-hS20 and Fab, the binding of their polypeptide components [i.e., H (Fig. 1B), Fd (Fig. 1C), and L chains (Fig. 1B, 1C)] to either the target bacterium O6ad or its LPS_{O6ad} was much lower, with the L chain having the lowest binding ability. When compared with the binding of te-hS20 at concentrations of 15 nM, the binding of the H chain was 3-fold lower to whole bacteria and 10-fold lower to LPS_{O6ad}; whereas the L chain had 4- and 10-fold lower binding to whole bacteria and LPS_{O6ad}, respectively (Fig. 1B). Similarly, in comparison with the binding of the Fab at concentrations of 30 nM, the binding of the Fd and L chains were 2- and 3-fold lower to whole bacteria and 4- and 10-fold lower to LPS_{O6ad}, respectively (Fig. 1C). In summary, these data indicate that the H chain and Fd region play a major role in Ag recognition; however, the complex of the L chain with either the H chain or Fd region is essential to achieve maximal Ag binding.

**In vitro bactericidal activity**

In vitro bactericidal activities of te-hS20, Fab, and scFv against _P. aeruginosa_ O6ad were evaluated by recovered colony counts after treating bacteria with Abs in PBS. Treatments involved 30 min incubation of 10^7 CFU/ml bacteria with te-hS20 Fab, or scFv at 0.67 μM for te-hS20 and 1.34 μM for fragments, respectively. To keep the same Ag-binding molarity as that of whole IgG, the Ab fragments used in all subsequent experiments were two times higher than IgG. te-hS20 Fab, and scFv had significant bactericidal activity against _P. aeruginosa_ O6ad, as recovered colony counts were reduced to <10% of the untreated control (p < 0.0001), whereas mAb QCRL-1 had no antiinhibitory activity (Fig. 2). The Fab and scFv exhibited similar levels of bactericidal activity as te-hS20, suggesting that the V region of te-hS20 is solely responsible for the bactericidal action.

The H, Fd, and L subunits were tested for bactericidal activity at the same molarity as the Fab. The H chain of te-hS20 and the Fd chain of the Fab reduced the bacterial viability to <50% (p < 0.0001), whereas their corresponding L chains had only a slight effect on the bacterial viability (Fig. 2). These results show that the H and Fd chains play a more important role than the L chain in the bactericidal action of te-hS20 and Fab, respectively; however, their combination with the L chain is necessary for maximal antibacterial activity.

Strain susceptibility to the anti-O6ad Ab molecules was assessed using other _P. aeruginosa_ serotypes, PAO1 (serotype O5) and O10,
Gram-negative bacterium *E. coli* TG1, and the Gram-positive bacterium *S. agalactiae*, under the same conditions. Although te-hS20, Fab, and scFv did not bind to the heterologous strain *P. aeruginosa* PAO1 or its LPS (Supplemental Fig. 1), they exhibited some crossreactive bactericidal activity against PAO1, as there was a 30–60% reduction of viability (*p*, 0.01; Supplemental Fig. 3). te-hS20 and its fragments did not exhibit any effect on the viability of *P.* aeruginosa O10, *E. coli* TG1, and *S. agalactiae*.

To investigate whether the Ag-Ab binding interaction is essential for the observed bactericidal activity, te-hS20 and its recombinant derivatives were evaluated for their bactericidal ability after saturation of their Ag-binding sites with LPSO6ad. Following a 1 h preincubation with an IC100 of LPSO6ad (determined by inhibition ELISA; Supplemental Fig. 2), the bactericidal activities of te-hS20 and its fragments were fully abrogated (*p*, 0.0001), whereas preincubation with LPS *E. coli* at the same concentrations did not reduce the bactericidal activity of the Abs (Fig. 3). These data imply that the binding of the anti-O6ad Abs to the target bacteria is required for the subsequent antibacterial activity.

Dose-response of the bactericidal activity of te-hS20 and its fragments against *P. aeruginosa* O6ad was evaluated over 30 min. As expected, the bactericidal activity of te-hS20, Fab, and scFv against *P. aeruginosa* O6ad is directly proportional to the increase of their concentrations. Bacterial viability was reduced to 50% compared with untreated controls (*p*, 0.001) by te-hS20 at 25.17 nM, Fab at 170 nM, and scFv at 91.1 nM (Fig. 4). The effective concentration required to kill 50% of the cells (EC50) by tobramycin was determined to be much greater (0.65 μM; Fig. 4), suggesting that the anti-O6ad Abs are more effective bacteriocides than this antibiotic on a per-molecule basis.

Influence of anti-O6ad Abs on cell membrane integrity and morphology

Damage to the bacterial outer membrane by some antibiotics and bactericidal peptides is an essential part of the bactericidal process (49–51). To identify the mechanism of bactericidal action of te-hS20 and its fragments, the membrane integrity of *P. aeruginosa* O6ad bacteria was assessed following Ab treatment using a live/dead cell-staining assay and viewing treated cells under fluorescence microscopy. In this assay, bacteria were treated with both a green fluorescent dye (SYTO9) and a red fluorescent dye (PI). SYTO9 can penetrate all bacterial cells, regardless of their membrane integrity; in contrast, PI penetrates only bacteria with damaged membranes. Therefore, viable cells are stained green, dead cells are stained red, and injured cells are orange or yellow because they take up both stains. EDTA was used as a positive control in this study because it quickly disrupts divalent-cation crossbridges by chelation, increasing the permeability of the outer membrane of bacteria (52).

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Images from fluorescence microscopy analysis (Fig. 5) demonstrated that the viability of *P. aeruginosa* O6ad cells, in the absence of the anti-O6ad Abs, decreased from 93.5% at 1 h 74.7% at 6 h. Similar results were obtained with the bacterial cells treated with QCRL-1 (0.67 μM). In contrast, the viability of O6ad cells treated with the anti-O6ad Abs was significantly reduced. Following treatment with te-hS20 (0.67 μM), the proportion of viable cells decreased from 74.6%
at 1 h to 28.6% at 6 h. After treatment with anti-O6ad Fab (1.34 μM), the proportion of viable cells decreased from 83.6% at 1 h to 36.4% at 6 h; with the anti-O6ad scFv (1.34 μM), viable cells decreased from 77.6% at 1 h to 31.7% at 6 h. Thus, compared with untreated controls, te-hS20 and its fragments significantly reduced bacterial viability by >40% after 6 h treatment (p < 0.0001). The number of viable cells was also reduced significantly after treatment with tobramycin (50 μg/ml), whereas EDTA (10 mM) caused the death of all cells 1 h posttreatment. These data suggest that te-hS20 and its fragments may increase permeability of the outer membrane of O6ad cells, thereby resulting in cell death.

The effect of the anti-O6ad scFv on P. aeruginosa cells was examined using SEM and FE-SEM (Figs. 6A–C, 7A, 7B), although at 6 h after treatment, the cells did appear slightly shorter in length. Similarly, no morphological change was observed over time when the bacteria were incubated with a heterologous scFv (data not shown). In contrast, O6ad cells exhibited severe morphological changes after exposure to the anti-O6ad scFv at 1.34 μM and 6.7 μM. At 2 and 6 h after treatment, shape distortion, increased roughness and indentation of the surface, swelling, and elongation were observed. Furthermore, images from FE-SEM revealed that incubation with the anti-O6ad scFv (>2 h) led to the formation of membrane-bound vesicles (Fig. 7D, indicated by black arrows) and caused severe disruption of the cell wall and leakage of cellular content (Fig. 7D, indicated by black arrows). More severe morphological effects were observed with high concentrations of the anti-O6ad scFv (6.7 μM) (Figs. 6G–I, 7D) and longer incubation time (6 h) (Figs. 6F, 6L, 7D). Also, anti-O6ad scFv caused what appears to be multiple cells attached end-to-end (Fig. 6E, 6F, 6H, 6I), suggesting that cell division may have occurred but the dividing cells failed to separate; this was not observed with the nonspecific scFv (data not shown).

In vivo protection

The in vivo protective efficacy of the anti-O6ad Abs against P. aeruginosa O6ad was investigated using a leukopenic mouse model of bacterial infection. As compared with the controls that were treated with PBS or an irrelevant IgG1, the pretreatment of the infected mice with anti-O6ad Abs prolonged animal survival, with more animals remaining alive at the end of the experiment (Fig. 8). Seventy-two hours after i.v. infection with LD_{50–90} live O6ad bacteria (10^3 CFU/mouse; Supplemental Fig. 9), most of the control mice (i.e., 10 of 13 for PBS and 11 of 13 for QCRL-1) were dead, whereas significantly less mice treated with a specific Ab had died (i.e., 2 of 14 for IgG, 2 of 13 for Fab, and 6 of 13 for scFv). After seven days, 12 of 14, 9 of 13, and 7 of 13 mice that received single i.v. doses (80 μg/mouse) of te-hS20, Fab, and scFv, respectively, were still alive. Therefore, when compared with QCRL-1– and PBS-treated controls, survival of infected mice was significantly improved (p < 0.05) following treatments with te-hS20, Fab, and scFv, suggesting that the anti-O6ad Abs provided in vivo protection against P. aeruginosa O6ad infection.
content (indicated by black arrows) was observed. Vesicles (indicated by white arrows) were visible, and leakage of cellular contents, cell swelling, and elongation (Figs. 6, 7) were observed. Furthermore, a number of Abs have been found to have complement-independent antimicrobial activities against bacteria, fungi, protozoa, or viruses (3, 5, 24–26, 29–31, 33, 35–37, 39). These Abs kill their target organisms via different mechanisms: 1) production of oxidant species by catalyzing water oxidation reactions (14, 16); 2) interference with biological activities of target cells (4, 5, 21–23); and 3) induction of apoptosis-like cell death (31). In this study, it is shown that a tobacco-expressed anti-P. aeruginosa O6ad LPS IgG1 Ab, te-hS20, as well as its recombinant Fab and scFv fragments, also have the capacity of performing Ag-specific bacterial killing independent of immune cells and complement.

Treatment of P. aeruginosa O6ad with te-hS20 or with its Fab or scFv resulted in nearly >90% reduction of bacterial CFU counts (Fig. 2). The fact that the Fab and scFv both had comparable bactericidal activities when compared with their parent IgG1 suggests that the reduction in bacterial colonies following Ab treatments is a result of coupled specific target binding and killing, independent of functions attributed to the Fc region. Agglutination tests were negative for both anti-O6ad Fab and scFv, ruling this out as the cause of reduced CFU counts, although te-hS20 did induce some bacterial agglutination under conditions used for the killing assays (Supplemental Tables I and II). Furthermore, the utilization of different, nonmammalian Ab expression systems (transgenic tobacco for te-hS20; yeast for Fab and scFv) and purification methods (immobilized protein G for te-hS20; immobilized nickel for Fab and scFv) exclude the possibilities that contaminating serum-derived molecules interact with IgGs in vivo or that nonspecific toxic contaminants from a single expression system accounted for killing.

Live/dead staining demonstrated directly that specific Ab treatments caused increased cell death over a 6-h time course (Fig. 5). This experiment strongly suggests that increasing membrane permeability occurs after target binding by te-hS20, Fab, or scFv. This is further supported by the cell-wall damage observed using electron microscopy following treatment with the anti-O6ad scFv (Figs. 6, 7). The scFv treatment caused surface roughness and indentation, release of flagella, formation of membrane blebs or vesicles, leakage of cellular contents, cell swelling, and elongation. Thus, the results from Ab treatments enumerated by CFU counts, live/dead cell staining, and electron microscopy suggest that the anti-O6ad Abs exert bactericidal activity through disruption of the cell wall. In addition, it appeared that the anti-O6ad scFv also inhibited cell division, as indicated by observations of cells attached end-to-end (Fig. 6E, 6F, 6H, and 6I). This may have...
resulted from inhibition of division septum formation between dividing cells by the scFv after binding to LPS on the bacteria.

The detailed mechanism of bactericidal action of te-hS20 and its Ab fragments is presently unknown and warrants further investigation. Some cytotoxic Abs to microorganisms or mammalian cells trigger target cell death by DNA or protein hydrolysis (53–59) or by production of toxic oxidative molecules (i.e., H₂O₂, O₂⁻, and hydroxide radicals) via catalyzing water oxidation reactions (13–15). Our data exclude the possibility that the anti-O6ad Abs exert their bactericidal action through DNase (Supplemental Fig. 5), proteinase (Supplemental Fig. 6), or peroxidase activities (Supplemental Fig. 7). It is possible that the anti-O6ad Abs may exert bactericidal effects through degradative hydrolysis of LPS, because IgG, IgM, and/or secretory IgA from the milk of healthy women and from the sera of cancer and autoimmune patients as well as pregnant women have been shown to have polysaccharide-hydrolyzing activities (11, 60–62) or lipase activities. Future work is needed to address whether the anti-O6ad Abs kill target bacteria by enzymatic LPS catalysis.

P. aeruginosa, a Gram-negative opportunistic pathogen, produces a number of virulence factors (63). One such factor is LPS, which is the major constituent (>90%) of the outer leaflet of the outer membrane of Gram-negative bacteria (64) and serves as the first protective layer for bacterial resistance to a variety of host defense molecules (50, 63, 65). The Abs described in this paper bind to the O-Ag, which is the major antigenic determinant of LPS (66). Many bactericidal peptides have been reported to kill bacteria by induction of membrane depolarization (50) or LPS release from the bacterial surface (67). Our study showed that the anti-O6ad Ab treatments did not induce LPS release (Supplemental Fig. 8). Because the specific LPS completely abolished the bactericidal activities of the anti-O6ad Abs (Fig. 3), their biological activities are assumed to be related to specific binding to LPS. Unexpectedly, the anti-O6ad Ab molecules also displayed minor nonspecific bactericidal activity to P. aeruginosa PAO1 (Supplemental Fig. 3), but not to P. aeruginosa O10, E. coli TG1, or S. agalactiae. There is no apparent structural similarity between LPSO6ad and LPSPAO1 (66); thus, the reason for the minor nonspecific bactericidal activity of te-hS20, Fab, and scFv toward P. aeruginosa PAO1 is not understood.

It is likely that the binding of the anti-O6ad Abs to cell wall LPSs resulted in the alteration in its conformational and/or dynamic properties, which could in turn cause disordered packing of LPS and membrane disintegration. These changes may lead to the formation of transient fissures in the outer membrane of the bacteria that allow the leakage of a variety of essential molecules, eventually leading to cell death. The anti-O6ad Abs might use a mechanism similar to pore-forming mechanisms called the barrel-stave or carpet-like models, which have been proposed for the action of many antimicrobial peptides (49–51, 68–71). Alternatively, the carpetlike model, suggested for the antimicrobial activity of peptide K₁₂ against Gram-negative bacteria, provides another plausible mechanism; in this model, the K₁₂ peptide binds and penetrates the LPS layer, eventually inducing LPS mis-cellularization and cell death (50). Because the variable H chain region of te-hS20 plays a major role in Ag binding and is highly positively charged with a theoretical PI of 8.96, having a predominance of the basic amino acids arginine and lysine, it is possible that the anti-O6ad Abs may functionally mimic the bactericidal activity of cationic antimicrobial peptides. Additional studies to clarify the bactericidal mechanism of the anti-O6ad Abs are needed.

The bactericidal function of these Abs may offer valuable insights for developing a new class of therapeutic applications for bacterial infections. Our in vitro study demonstrated that these Abs are more effective than the antibiotic tobramycin at killing target bacteria on a per-molecule basis, as indicated by their EC₅₀ (i.e., 25.17 nM versus 0.65 μM for te-hS20 and tobramycin, respectively) (Fig. 4); however, for therapeutic applications, tobramycin is a more practical bactericid for because it is inexpensive, has high solubility and stability, and is widely available commercially for in vivo administration in large amounts. Our in vivo study showed that P. aeruginosa O6ad cells were highly virulent, being able to kill 80–90% of leukopenic mice at a dose of 10⁶ CFU/mouse and resulting in 100% death at 10⁵ CFU/mouse (Supplemental Fig. 9). Although 50% FBS and 2% BSA slightly inhibited the bactericidal activities of these Abs in vitro (Supplemental Fig. 10), when challenging leukopenic mice with LD₅₀–₉₀ (10⁵ CFU/mouse) dosages, te-hS20 and, more importantly, its Fab and scFv fragments effectively provided protection (Fig. 8). The difference in the protective efficacies among te-hS20, Fab, and scFv may result from differences in pharmacokinetic properties due to their different m.w., differences in their functional Ag-binding affinities, and Fe region-mediated effector functions in the specific case of te-hS20. In vitro studies showed that te-hS20 had the highest functional Ag-binding affinity, followed by Fab and then scFv (Fig. 1A), and that there was a strong correlation between the Ag-binding affinity and bactericidal efficiency. Thus, te-hS20 may have the best protective efficacy because it has the highest binding avidity and because it might further mediate bacterial elimination through Fe region-mediated effector functions such as phagocytosis (40). Furthermore, the longer in vivo t₁/₂ (22–120 h) of intact IgGs in mice (72, 73), when compared with those of Fab (4–8 h) (73) and scFv molecules (2–3.5 h) (74, 75), also likely contributes to its better protective efficacy. Nonetheless, despite lacking the Fe region, the protection provided by the anti-O6ad Fab and scFv was clearly profound, adding strong support in demonstrating the bactericidal activity of these Abs independent of ADCC and CDC.

On the basis of our results, te-hS20 has two independent functions in target bacterial elimination: phagocytosis (40) and direct bactericidal activity. Although Abs against Lyme disease or relapsing fever Borreliae have previously been described that bind a 19 kDa outer membrane protein and have also been shown to be bactericidal per se (24–30), our work is unique because te-hS20 binds LPS instead of a protein Ag, causes disruption of cell membrane integrity, and possibly inhibits cell division. te-hS20 and its fragments are bactericidal to a serotype of P. aeruginosa, a bacterial species that is an important source of nosocomial infections and is of great concern for immunocompromised patients. Because there is currently no vaccine available for Pseudomonas infections, uncovering the specific and detailed mechanism involved in this bactericidal activity would be of extreme importance. However, future study is needed to further evaluate its therapeutic potential to provide new concepts for understanding antimicrobial immunity.

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Disclosures
The authors have no financial conflicts of interest.

References
BACTERIAL ACTIVITY OF AN ANTI-P. AERUGINOSA IgG


Intrinsic Bactericidal Activity of an IgG against *Pseudomonas aeruginosa* O6ad

**Data Supplement**

Files in this Data Supplement:

- **Supplementary Tables 1-2**

Table 1. Agglutination of *P. aeruginosa* O6ad bacteria following 30 min incubation with te-hS20 (IgG1), its fragments, or QCRL-1.

Table 2. Agglutination of *P. aeruginosa* O6ad bacteria (10^7 CFU/ml) following 30 min incubation with te-hS20 (IgG1), its fragments, or QCRL-1 over a concentration range of 0.003 to 5.36 μM.

- **Supplementary Figures 1-10**

Figure 1. Antigen binding specificity of te-hS20 (IgG1), Fab, and scFv to heat-killed O6ad, PAO1, and O10 and their corresponding LPSs.

Figure 2. Inhibition of antigen binding of te-hS20 (IgG1), Fab, and scFv by LPS_{O6ad}.

Figure 3. Bactericidal activity of te-hS20 (IgG1), Fab and scFv against *P. aeruginosa* serotypes O6ad, PAO1 and O10, as well as *E. coli* TG1 and *S. agalactiae*.

Figure 4. Effect of denaturation by heating on the bactericidal activity of te-hS20 (IgG1), Fab and scFv against *P. aeruginosa* serotypes O6ad (A) and PAO1 (B).

Figure 5. Assessment of DNase activity of te-hS20 (IgG1) and Fab.

Figure 6. Assessment of proteolytic activity of te-hS20 (IgG1), Fab, and scFv.

Figure 7. Generation of H₂O₂ by te-hS20 (IgG1), Fab and scFv, and impact of near-UV or visible lights on their bactericidal activities.

Figure 8. Measurement of LPS in supernatants and cell pellets after antibody treatments.
Figure 9. Infectious dose-response of *P. aeruginosa* O6ad in a leukopenic mouse model.

Figure 10. Bactericidal activity of te-hS20 (IgG1), Fab, and scFv against *P. aeruginosa* O6ad in the presence of 50% FBS (A) or 2% BSA (B).
**Supplementary Table 1.** Agglutination of *P. aeruginosa* O6ad bacteria following 30 min incubation with te-hS20 (IgG1), its Fab and scFv fragments, or QCRL-1. Results were scored according to the standardized degrees of agglutination established by Brokopp et al., 1977 (1), where 0 means no bacterial agglutination, and 1, 2 and 3 mean less than 25%, 25-50% and 50-75% bacterial agglutination, respectively. The experiment was performed twice with three replicates each time.

<table>
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<th>Fab (1.34 μM)</th>
<th>scFv (1.34 μM)</th>
<th>QCRL-1 (0.67 μM)</th>
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**Supplementary Table 2.** Agglutination of *P. aeruginosa* O6ad bacteria (10⁷ CFU/ml) following 30 min incubation with te-hS20 (IgG1), its Fab and scFv fragments, or QCRL-1 over a concentration range of 0.003 to 5.36 μM. Antibodies were diluted in 2-fold steps from left to right. Antibody fragment concentrations are twice the mAb concentrations for each column; the starting antibody fragment and mAb concentrations were 5.36 μM and 2.68 μM, respectively, and the finishing concentrations were 0.005 μM and 0.003 μM, respectively. Results were scored according to the standardized degrees of agglutination established by Brokopp et al., 1977 (1), where 0 means no bacterial agglutination, and 1, 2 and 3 mean less than 25%, 25-50% and 50-75% bacterial agglutination, respectively. The experiment was performed twice with three replicates each time.

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Supplemental Figure Legends

Supplementary Figure 1. Antigen binding specificity of te-hS20 (IgG\textsubscript{1}) (A), Fab (B), and scFv (C) to heat-killed O6ad, PAO1, and O10 (1 x 10\textsuperscript{8} CFU/ml), and their corresponding LPSs (1 μg/ml), as determined by ELISA. Data represent background-subtracted means of triplicates ± SD.

Supplementary Figure 2. Inhibition of antigen binding of te-hS20 (IgG\textsubscript{1}), Fab, and scFv by LPS\textsubscript{O6ad}. Te-hS20, Fab and scFv were pre-incubated for 1 h at RT with soluble LPS at concentrations ranging from 0 to 100 μg/ml prior to addition to LPS\textsubscript{O6ad}-coated microtitre plates. The antibody concentrations used (te-hS20 at 1.13 μg/ml, Fab at 12 μg/ml, and scFv at 18 μg/ml) were determined by cross-serial dilution analysis (a.k.a. “checkerboard titration”) with varying concentrations of LPS coating microtiter plate wells (not shown). Binding of te-hS20 (IgG\textsubscript{1}), Fab and scFv to solid-phase immobilized \textit{P. aeruginosa} O6ad (1 x 10\textsuperscript{8} CFU/ml) was completely inhibited by preincubation with LPS\textsubscript{O6ad} at concentrations of 25, 50, and 75 μg/ml, respectively. Data represent background-subtracted means of triplicates ± SD.

Supplementary Figure 3. Bactericidal activity of te-hS20 (IgG\textsubscript{1}) (A), Fab (B) and scFv (C) against \textit{P. aeruginosa} serotypes O6ad, PAO1 and O10, as well as \textit{E. coli} TG1 and \textit{S. agalactiae}. The bactericidal activity was determined by CFU counts following 30 min incubation of the bacteria (1 x 10\textsuperscript{7} CFU/ml) with the antibodies (0.67 μM for te-hS20 and 1.34 μM for Fab and scFv) in PBS at 37\textdegree C. Untreated bacteria were used as controls.
This experiment was performed twice, and data represent means of four replicates ± SE. Statistical differences within a strain or species are indicated by * (P<0.0001), ** (P<0.001) and *** (P<0.01).

**Supplementary Figure 4.** Effect of denaturation by heating on the bactericidal activity of te-hS20 (IgG1), Fab and scFv against *P. aeruginosa* serotypes O6ad (A) and PAO1 (B). The bactericidal activities were determined by CFU counts following a 30-min incubation of the bacteria (1 x 10^7 CFU/ml) with the active or heat-inactivated antibodies (0.67 μM for te-hS20 and 1.34 μM for Fab and scFv) in PBS at 37°C. Data represent means of four replicates ± SE. Statistical differences within a treatment are indicated by * (P<0.0001) and *** (P<0.01).

**Supplementary Figure 5.** Assessment of DNase activity of te-hS20 (IgG1) and Fab. DNase activity was assessed by incubation of te-hS20 (IgG1) (2.5 μM) or Fab (5 μM) with unmethylated-λ phage DNA (32 μg/ml) for 24 h at 37°C, followed by electrophoresis in 0.8% agarose gel. DNase I (25 kU/ml) was used as a positive control, and QCRL-1 (2.5 μM) was used as a negative control. The experiment was performed twice with three replicates each time.

**Supplementary Figure 6.** Assessment of proteolytic activity of te-hS20 (IgG1), Fab, and scFv. Proteolytic activity was examined by incubation of te-hS20 (IgG1; 0.67 μM), Fab (1.34 μM), or scFv (1.34 μM) with the protease substrate fluorescein diacetate (FDA) (15 μg/ml) for 24 h at 30°C. The amounts of FDA hydrolyzed were measured as absorbance
at 490 nm. Aminopeptidase M (APM) (0.13 U/ml) was used as a positive control, and QCRL-1 (0.67 μM) as a negative control. Data represent means of three replicates ± SD. The letters a and b indicate statistical differences among treatments (P<0.0001).

Supplementary Figure 7. Generation of H$_2$O$_2$ by te-hS20 (IgG1), Fab and scFv, and impact of near-UV or visible lights on their bactericidal activities. A, H$_2$O$_2$ generation by anti-O6ad antibodies. The amounts of H$_2$O$_2$ generated by te-hS20 (0.67 μM), Fab (1.34 μM) and scFv (1.34 μM) were measured at 2, 4, and 24 h after exposure to near-UV irradiation (800 uW/cm$^2$). QCRL-1 (0.67 μM) and BSA (1.34 μM) were used as protein controls. Statistical differences (P<0.001) for all time points within a treatments are indicated by *. B, Bactericidal activity of H$_2$O$_2$ against P. aeruginosa O6ad. The bactericidal activity was determined by CFU counts following a 30-min incubation of the bacteria (1 x 10$^7$ CFU/ml) with H$_2$O$_2$ over a range of 0 to 50 mM at 37°C. C, Impact of near-UV irradiation on the bactericidal activities of anti-O6ad antibodies against P. aeruginosa O6ad. Following exposure to near-UV irradiation (800 uW/cm$^2$) of te-hS20 (0.34 μM), Fab (0.67 μM), or scFv (0.67 μM), their bactericidal activities were determined by CFU counts following a 30-min incubation with P. aeruginosa O6ad (1 x 10$^7$ CFU/ml) in PBS at 37°C. Letters a-c above bars indicate statistical differences among treatments (P<0.01); note that “ab” indicates neither different from “a” nor “b”. D, Impact of visible light exposure on the bactericidal activity of anti-O6ad antibodies against P. aeruginosa O6ad. Following 2 h exposure to visible light (1000 candlepower) of te-hS20 (0.34 μM), Fab (0.67 μM), or scFv (0.67 μM), their bactericidal activities
were determined by CFU counts following a 30-min incubation with *P. aeruginosa* O6ad (1 x 10^7 CFU/ml) in PBS at 37°C. Data represent means of three replicates ± SE.

**Supplementary Figure 8.** Measurement of LPS in supernatants and cell pellets after antibody treatments. LPS was measured by ELISA after a 4-h incubation of *P. aeruginosa* O6ad bacteria (1 x 10^7 CFU/ml) with te-hS20 (IgG1; 0.67 μM), Fab (1.34 μM), or scFv (1.34 μM) in PBS at 37°C. Bacteria treated without antibody and with QCRL-1 (0.67 μM) were used as controls. Data represent means of triplicates ± SD. There were no statistically significant differences within each treatment.

**Supplementary Figure 9.** Infectious dose-response of *P. aeruginosa* O6ad in a leukopenic mouse model. Mice (n = 4/group) were challenged *i.v.* with bacteria at concentrations ranging from 10 to 10^6 CFU/mouse. Animals receiving the same volume of PBS were used as negative controls. Mortality was recorded daily for 7 days.

**Supplementary Figure 10.** Bactericidal activity of te-hS20 (IgG1), Fab, and scFv against *P. aeruginosa* O6ad in the presence of 50% FBS (A) or 2% BSA (B). Bactericidal activity was determined by CFU counts following a 30-min incubation of the bacteria (1 x 10^7 CFU/ml) with the antibodies (0.67 μM for te-hS20 and 1.34 μM for Fab and scFv) in the presence of 50% FBS or 2% BSA. Bacteria treated with QCRL-1 (0.67 μM) were used as a negative control. Data represent means of four replicates ± SE. Statistical differences within an antibody treatment are indicated by * (P<0.001) and ** (P<0.01).
Xie et al. Supplementary Figure 1

A

Absorbance (450 nm)

IgG1 (nM)

B

Absorbance (450 nm)

Fab (nM)

C

Absorbance (450 nm)

scFv (nM)
Xie et al. Supplementary Figure 2
Xie et al. Supplementary Figure 3

A

B

C

% live bacteria

0 20 40 60 80 100 120

no IgG

IgG

O6ad PAO1 10 TG1 S. agalactiae

% live bacteria

0 20 40 60 80 100 120

no Fab

Fab

O6ad PAO1 10 TG1 S. agalactiae

% live bacteria

0 20 40 60 80 100 120

no scFv

scFv

O6ad PAO1 10 TG1 S. agalactiae
Xie et al. Supplementary Figure 4

A

% live bacteria

0 20 40 60 80 100 120

Fab scFv IgG

Active Inactive

* * *

B

% live bacteria

0 20 40 60 80 100 120

Fab scFv IgG

Active Inactive

*** *** ***
Xie et al. Supplementary Figure 5
Xie et al. Supplementary Figure 6

Absorbance (490 nm)
Xie et al. Supplementary Figure 7

A

Graph showing the effect of antibody concentration (nM) on H2O2 levels.

B

Graph showing the percentage of live O6ad cells as a function of H2O2 concentration (mM).

C

Bar graph showing the percentage of live O6ad cells at different antibody concentrations (nM) in the presence of H2O2.

D

Bar graph showing the percentage of live O6ad cells at different antibody concentrations (nM) in the presence of H2O2 under light and dark conditions.
Xie et al. Supplementary Figure 8

![Supplementary Figure 8](image-url)

- Absorbance (450 nm)
  - O6ad
  - IgG
  - Fab
  - scFv
  - QCRL-1

- Supernatant
- Pellet
Xie et al. Supplementary Figure 10

A

![Bar graph comparing % of live O6ad with IgG Fab scFv QCRL-1, PBS, and FBS]

B

![Bar graph comparing % of live O6ad with IgG Fab scFv QCRL-1, PBS, and BSA]
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3 Pseudomonas aeruginosa: use of commercial antisera and live antigens. *J Clin