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*J Immunol* 2010; 184:3725-3733; Prepublished online 26 February 2010; doi: 10.4049/jimmunol.0902732

http://www.jimmunol.org/content/184/7/3725

Supplementary Material  http://www.jimmunol.org/content/suppl/2010/03/01/jimmunol.090273 2.DC1

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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: 3725–3733.

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, thereby 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 IgG1, mAb, and CB2, an IgG2a, 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 withfabs 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 burgdorferi, 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 fungicidal activity (5); this mAb was also protective in a murine model of systemic candidiasis (34). A subset of killer toxin-like Abs (KT-Abs), produced by idotypic vaccination with mAb KT4 that was raised to neutralize a KT produced by Pichia anomala, showed broad antitemapical 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 antimicrobial activity against prokaryotic and eukaryotic microorganisms presenting receptors for KT (38). Similar and fungi (3, 35) and provided protection in experimental models of P. aeruginosa Tryptic soy, Luria-Bertani, and brain and heart infusion broths were used for subcultured in fresh broth at 37°C until OD at 600 nm reached 0.5–0.7. Furthermore, the efficacy of protecting leukopenic mice from P. aeruginosa O10 and serotype O10 (ATCC 33357) and serotype O6ad was able to mediate in vitro opsonophagocytosis of serotype 0111:B4 were from Sigma-Aldrich, whereas LPS serotypes O6ad and PAO1 (serotype O5) were isolated from P. aeruginosa O6ad LPS te-hS20 was expressed in trans-

Materials and Methods

All chemicals were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada); all culture media were from Fisher Scientific (Mississauga, Ontario, Canada) unless otherwise stated. LPS from P. aeruginosa serotype O10 and E. coli serotype 0111:B4 were from Sigma-Aldrich, whereas LPS from P. aeruginosa serotypes O6ad and PAO1 (serotype O5) were isolated using the Tri-Reagent method (41). The hybridoma for Queen’s Cancer Research Laboratory-1 IgG (QCRL-1) (murine IgG1) was provided by Dr. Susan Cole (Queen’s University, Kingston, Ontario, Canada). The expression plasmid for anti-Salmonella enterica scFv was from Dr. Roger MacKenzie (Institute for Biological Sciences, National Research Council of Ottawa, Ontario, Canada).

Bacterial strains

P. aeruginosa serotype O6ad strain was provided by Dr. John R. Schreiber (Tufts University, Boston, MA). P. aeruginosa strains PAO1 (serotype O5; ATCC BAA-47) and serotype O10 (ATCC 33357) and Streptococcus agalac-tiae (ATCC BAA-22) were from the American Type Culture Collection (Manassas, VA). E. coli TGI13 was from Stratagene (La Jolla, CA).

Bacteria from a single colony were grown overnight at 37°C and then subcultured in fresh broth at 37°C until OD at 600 nm reached 0.5–0.7. Tryptic soy, Luria-Bertani, and brain and heart infusion broths were used for P. aeruginosa strains, E. coli, and S. agalactiae, respectively. Bacteria were harvested by centrifugation (3000 × g, 5 min, room temperature [RT]), washed in sterile PBS (pH 7.4), and then diluted in PBS to obtain suitable concentrations. For ELISA, bacteria were prepared as described then heat-killed at 60°C for 1 h and stored at −20°C.

Ab cloning

The human anti-P. aeruginosa O6ad LPS te-hs20 was expressed in transgenie tobacco (40). To produce the Fab of this mAb, the κ L chain and Fd fragment of the γ H chain were amplified by PCR from pMM7 and pMM3 (40), respectively, using forward primers (5′-GATATCTTCGCAAGAA-AAGAGCTCTACTCTCGACAGTTAGTATGACACAAACTGAA-CTTCTCGAAAGAAAGAGGCTGAGATTTTGTCGAGCACTATGGTTG-3′ and 5′-TGCTCTCGAAAGAAAGAGGCTGAGATTTTGTCGAGCACTATGGTTG-3′, respectively) and reverse primers (5′-CTCTGTGTTCTAGATCTCAGTT- TCAACACCTCCTCTCCTTGAAACTCTTT-3′ and 5′-CTCTGTGTTCTAGA-

P. aeruginosa serotypes O6ad, PAO1, and O10), E. coli (TGI), and Gram-positive S. agalactiae suspensions (2 × 10^9 CFU/ml) were prepared in sterile PBS from log-phase cultures. These were incubated with equal volumes of Ab solutions in PBS in the dark at 37°C. The final concentrations tested were 0.67 μM (100 μg/ml) for IgGs and 1.34 μM for Ab fragments (67 μg/ml for Fab and 40 μg/ml for scFv) to maintain equimolar binding site concentrations. mAb QCRL-1 (IgG1) was used as a negative control. After 30 min, treatment mixtures were serially diluted 10-fold and plated on glucose medium, followed by colony enumeration after 18 h at 37°C. All treatments were run in quadruplicate, and the quantity of viable cells in treatments was expressed as a percentage of viable cells of the untreated control. Other controls included incubation with Abs (100°C for 30 min) (Supplemental Fig. 4) and treatments in the presence of 50% PBS or 2% BSA (Supplemental Fig. 10).

The bactericidal activity inhibition assay was performed by pre-incubating te-hs20 (200 μg/ml), Fab (134 μg/ml), and scFv (80 μg/ml)
with LPs at IC\textsubscript{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 \textit{P. aeruginosa} O6ad cell suspension (2 × 10\textsuperscript{7} CFU/ml) was added, and treatments were incubated in the dark for 30 min at 37°C. LPS\textsubscript{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\textsuperscript{7} CFU/ml) was mixed with an equal volume of Ab at final concentrations ranging from 1.3–5.3 μM and then incubated in the dark for 30 min at 37°C. Tobramycin was used at concentrations ranging from 0–107.52 μM. Treatment mixtures were then enumerated as above. The EC\textsubscript{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\textsuperscript{7} CFU/ml, were mixed with an equal volume of te-hS20 (1.34 μM), Fab (2.68 μM), or scFv (2.68 μM) 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 0.3 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\textsuperscript{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 μM or 6.7 μM. 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 planchetts (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% OsO\textsubscript{4} (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, U.K.) 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 \textit{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 \textit{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, \textit{P. aeruginosa} O6ad cells at 80–90% lethal dose (LD\textsubscript{50,90}) (10\textsuperscript{10} bacteria/mouse in 50 μl sterile PBS) (Supplementary 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; all other mice were euthanized by 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 for the Care and Use of Laboratory 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 \textit{P. aeruginosa} O6ad (1 × 10\textsuperscript{8} CFU/ml) or purified LPS\textsubscript{O6ad} (1 μg/ml) was determined by ELISA (Fig. 1A–C). Te-hS20 had an EC\textsubscript{50} of 0.46 nM to whole bacteria and 0.35 nM to purified LPS\textsubscript{O6ad} whereas the anti-O6ad Fab had EC\textsubscript{50} of 14.42 nM and 20.96 nM to whole bacteria and LPS\textsubscript{O6ad} respectively (Fig. 1A). The anti-O6ad scFv exhibited an EC\textsubscript{50} of 45.74 nM to whole cells and had no binding to LPS\textsubscript{O6ad} at lower tested concentrations (Fig. 1A); however, it did bind to LPS\textsubscript{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 \textit{P. aeruginosa} O6ad bacteria and LPS\textsubscript{O6ad} because there was no cross-reactivity to \textit{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\textsubscript{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\textsubscript{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\textsubscript{O6ad} whereas the L chain had 4- and 10-fold lower binding to whole bacteria and LPS\textsubscript{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\textsubscript{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 \textit{P. aeruginosa} O6ad were evaluated by recovered colony counts after treating bacteria with Abs in PBS. Treatments involved 30 min incubation of 10\textsuperscript{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 \textit{P. aeruginosa} O6ad, as recovered colony counts were reduced to <10% of the untreated control (p < 0.0001), whereas mAb QCRL-1 had no antimicrobial 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 \textit{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 LPS. Following a 1 h preincubation with an IC100 of LPS (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 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).

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 for longer than 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 white 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, 6H, 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 LD₉₀ live O6ad bacteria (10⁷ 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.

**FIGURE 3.** Inhibition of the bactericidal activity of te-hS20 (IgG1), Fab, and scFv against P. aeruginosa O6ad by free LPS. Bactericidal activity was determined by CFU counts following a 30 min incubation of the bacteria (1 × 10⁷ CFU/ml) with Abs (0.67 μM for te-hS20 and 1.34 μM for Fab and scFv) that were preincubated with O6ad-derived LPS or E. coli-derived LPS. Data represent means of four replicates ± SE.

**FIGURE 4.** Bactericidal dose-response of te-hS20 (IgG1), Fab, scFv, and tobramycin against P. aeruginosa O6ad (1 × 10⁷ CFU/ml). A, Ab dose-response over a period of 30 min; Ab concentrations are given on the x-axis. B, Tobramycin dose-response over a period of 30 min; tobramycin concentrations are given on the x-axis. Data represent means of four replicates ± SE.

**FIGURE 5.** Fluorescence microscopic analysis of P. aeruginosa O6ad populations of viable and dead cells following treatment with anti-O6ad Abs. The proportion of viable and dead cells were determined at 1, 3, and 6 h after treatments of live P. aeruginosa O6ad cells (1 × 10⁷ CFU/ml) with te-hS20 (IgG1) (0.67 μM), Fab (1.34 μM), and scFv (1.34 μM) in PBS at 37°C. Untreated bacteria and those treated with QCRL-1 (0.67 μM) were used as negative controls, whereas the bacteria treated with tobramycin (50 μg/ml) or EDTA (10 mM) were used as positive controls. Data represent means of three replicates ± SE.
m 1.2 scFv at 37˚C for 0.5, 2, and 6 h, respectively. Magnification bars represent respectively. Cells were used at 1
content (indicated by black arrows) was observed. vesicles (indicated by white arrows) were visible, and leakage of cellular
for 6 h, severe disruption of the outer membrane occurred, membrane-bound
were observed (Fig. 6). This experiment strongly suggests that increasing membrane permeability occurs after target binding by 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.

Discussion
Ab-mediated clearance of microorganisms classically involves phagocytic or cytotoxic killing by activation of immune cells or the complement cascade through the Fc region of Abs (1, 2). However, 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 LPS₀₆₆₇ and LPS₀₈₀₁ (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 K₁₋₇ against Gram-negative bacteria, provides another plausible mechanism; in this model, the K₁₋₇ peptide binds and penetrates the LPS layer, eventually inducing LPS mis- 

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