Effects of the Combination of Lipopolysaccharide-Specific Monoclonal Antibodies and Sparfloxacin against 
*Pseudomonas aeruginosa* Pneumonia in Neutropenic Mice

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The effects of the combination of a murine monoclonal antibody (MAB) specific for the O side chain of *Pseudomonas aeruginosa* Fisher immunotype 1 lipopolysaccharide and sparfloxacin in a neutropenic mouse model of *P. aeruginosa* pneumonia were examined. Under the condition that neither MAB at a dose of 500 μg per mouse administered intravenously nor a suboptimal dose of oral sparfloxacin (5 mg/kg of body weight) protected mice from challenge with a fatal dose, the combination therapy with MAB and sparfloxacin caused a significant increase in the survival rate (*P* < 0.001 compared with either treatment alone). The effect of the combination was closely correlated to bacterial killing in plasma and lung tissue of infected mice. In vitro, a significant MAB-dependent, complement-mediated killing of *P. aeruginosa* was documented in the presence of sparfloxacin at one-half the MIC, while the killing was not observed in the absence of sparfloxacin. These in vivo and in vitro data suggest the usefulness of combination therapy with a lipopolysaccharide-reactive immunoglobulin G MAB and sparfloxacin in neutropenic patients with *P. aeruginosa* pneumonia.

Pneumonia caused by *Pseudomonas aeruginosa* is the highest cause of mortality in immunosuppressed or neutropenic patients (6, 17, 24). Despite recent advances in antimicrobial agents against *P. aeruginosa*, a substantial reduction in mortality from nosocomial *P. aeruginosa* pneumonia has not resulted (1, 12). Recently, considerable interest in passive immune therapy by the use of a monoclonal antibody (MAB) against *P. aeruginosa* infections has developed (20, 26, 29). Past studies have supported the possibility that MAB specific for the lipopolysaccharide (LPS) O side chain of *P. aeruginosa*, which has serotype-specific opsonic activity, is a possible candidate for the treatment of *P. aeruginosa* pneumonia (5, 16, 19, 30). Although a previous investigator (18) indicated that neutropenia adversely affects the therapeutic efficacy of antibody in pseudomonal pneumonia, hyperimmune intravenous immunoglobulin used in conjunction with tobramycin was shown to be efficacious against this disease in neutropenic animals. More recently, Collins and colleagues (2) showed a beneficial effect of combination therapy with a murine immunoglobulin G1 (IgG1) LPS-reactive MAB and oral ciprofloxacin in a leukopenic rat model of systemic pseudomonal infection. Therefore, combination therapy with LPS-specific MABs and antimicrobial agents appears to be an important strategy for treating *P. aeruginosa* pneumonia in neutropenic patients.

It has been demonstrated that several quinolone derivatives are useful in the treatment of *P. aeruginosa* pneumonia in normal and neutropenic guinea pigs (4, 9, 27). Sparfloxacin (AT-4140) is a newly developed quinolone for oral use that has broad and potent antibacterial activity (14). This compound is characterized by its excellent tissue penetration and long half-life in plasma and tissues (13). Furthermore, sparfloxacin is bactericidal for *P. aeruginosa* at a concentration near the MIC and can be used prophylactically for fatal *P. aeruginosa* pneumonia in mice (10, 14).

Our study was designed to evaluate the efficacy of an LPS-specific IgG3 MAB in conjunction with a suboptimal dose of oral sparfloxacin in a neutropenic mouse model of *P. aeruginosa* pneumonia.

**MATERIALS AND METHODS**

**Reagents.** Cyclophosphamide (Endoxan) was provided by Shionogi & Co., Ltd., Osaka, Japan. Sparfloxacin was provided in powder form by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. Prior to use, sparfloxacin was suspended in 0.5% tragacanth gum (Nacalai Tesque, Inc., Tokyo, Japan) for oral administration.

**Bacteria and LPS.** Fisher immunotype 1 (It-1) *P. aeruginosa* was obtained from M. Fisher, Parke-Davis Division, Warner-Lambert Company, Detroit, Mich. This organism was grown overnight on brain heart infusion agar (Difco, Detroit, Mich.) at 37°C, harvested in sterile saline, resuspended in brain heart infusion broth (Difco) containing 2% skim milk, and stored at −80°C until use. The MIC of sparfloxacin for the It-1 strain was determined to be 0.39 μg/ml by an agar dilution method in Mueller-Hinton medium. *P. aeruginosa* It-1 LPS, which was purified by hot phenol-water extraction and gel filtration chromatography, was obtained from List Biological Laboratories, Campbell, Calif.

**Preparation and characterization of MABs.** MAB 1D3, a murine antibody of the IgG3 subclass, was prepared by fusing spleen cells from a mouse immunized 4 times at weekly intervals with the heat-killed *P. aeruginosa* It-1 strain with the SP/O-Ag14 mouse myeloma partner (16) and screening the resulting hybridomas by enzyme-linked immunosorbent assay with microtiter wells coated with It-1 LPS (21). The hybridoma designated 1D3 was cloned by limiting dilution and adapted as an ascitic tumor in pristane-
primed BALB/c mice (Shizuoka Agricultural Cooperation Associations for Laboratory Animals, Shizuoka, Japan). The MAb was isotyped with an isotyping kit (American Qualex, La Mirada, Calif.), and the MAb concentration in ascitic fluid (6.3 mg/ml) was determined with a radial immunodiffusion kit (Tago, Burlingame, Calif.). A control IgG3 MAb, designated R6-3F2, directed to the Re chemotype of Salmonella minnesota LPS was provided by M. Pollack, Uniformed Services University, Bethesda, Md. (21). MAb 1D3 bound purified P. aeruginosa It-1 LPS, produced multiple slower-migrating bands in a stepladder pattern on Western blots (immunoblots) produced from sodium dodecyl sulfate-polyacrylamide gels of It-1 LPS. The negative control MAb R6-3F2 did not bind It-1 LPS.

**Experimental pneumonia in neutropenic mice.** Specific-pathogen-free female Slc:ICR mice (age, 5 weeks) were obtained from Shizuoka Agricultural Cooperation Associations for Laboratory Animals. Animals were given sterile food and water. To induce a neutropenic condition in these mice, 200 mg of cyclophosphamide per kg of body weight was administered subcutaneously two times every other day. On the fourth to sixth days after the first dose of cyclophosphamide, the total leukocyte counts in the peripheral blood of the mice were less than 10% of the pretreatment counts (Fig. 1). On day 4 after the first dose of cyclophosphamide, P. aeruginosa pneumonia was produced in the neutropenic mice by intratracheal challenge with P. aeruginosa It-1 by a previously described procedure (16). Infected mice were monitored for survival for 7 days. Mice with neutropenia induced by cyclophosphamide became extremely susceptible to intratracheal challenge with P. aeruginosa It-1. The 50% lethal doses of the strain in normal and neutropenic mice were determined by probit analysis to be 1.9 × 10^3 and 62 CFU per mouse, respectively. Quantitative bacterial cultures were performed at various times on samples of venous blood and lung tissue from animals that were euthanized by ether anesthesia. The lungs of exsanguinated mice were removed aseptically and were homogenized in 9 ml of sterile saline per g of lung tissue prior to culture. We also performed bronchoalveolar lavage in this pneumonia model. The lungs that were removed from exsanguinated mice were lavaged with 1 ml of phosphate-buffered saline. The lavage fluid was resuspended in 1.0 ml of Hanks’ balanced salt solution. Morphological differentiation of the recovered cells was done on cell monolayers prepared by centrifugation, and the cell monolayers were stained with May-Giemsa solution. More than 97% of the cells in the lavage fluid from neutropenic mice obtained 12 h after challenge was found to be alveolar macrophages (AMs).

Four groups, each containing 14 neutropenic mice, were challenged with the It-1 strain at doses of 10^3, 10^4, 10^5, and 10^6 CFU per mouse and were intravenously given 0.2 ml of appropriately diluted mouse ascitic fluid containing MAb 1D3 or MAb R6-3F2 (as control) in sterile saline, so that a final dose of 500 μg of MAb was delivered 2 h after bacterial challenge. The efficacy of combination therapy with MAb and sparfloxacin was evaluated in neutropenic mice challenged with P. aeruginosa It-1 at a dose of 10^5 CFU per mouse. This experiment involved four groups of 22 mice each. The mice received no treatment, MAb 1D3 alone, sparfloxacin alone, or both MAb 1D3 and sparfloxacin. A total of 0.2 ml of appropriately diluted mouse ascitic fluid containing 500 μg of MAb 1D3 was administered intravenously 2 h following bacterial challenge. Sparfloxacin was also initiated orally 2 h after challenge and was continued once every 12 h for 3 days (total of six doses). In a preliminary experiment, oral sparfloxacin at an oral dose of 250 μg per mouse (10 mg/kg) afforded complete protection against pneumonia-associated mortality. Thus, a dose of 125 μg per mouse (5 mg/kg) was chosen as a suboptimal dose of sparfloxacin. Mouse survival data from three experiments were pooled. To show the specificity of the additive effect between MAb 1D3 and a suboptimal dose of sparfloxacin, we also compared the survival rate of 10 mice that received MAb R6-3F2 at a single dose of 500 μg and sparfloxacin with that of 10 mice that received sparfloxacin alone.

**Determination of sparfloxacin levels.** Levels of sparfloxacin in plasma and lung tissue after oral administration in neutropenic mice challenged with P. aeruginosa It-1 at doses of 10^5 CFU were measured by bioassay by the procedures of Nakamura et al. (13). The plasma and lung tissues were harvested from exsanguinated mice at 0.5, 1, 2, 4, 6, and 12 h postadministration. The lungs were homogenized in 5 ml of 1/15 M phosphate buffer (pH 7) per g of lung tissue, and the homogenate was incubated at 80°C for 15 min. The plasma and lung homogenate were kept at −80°C until use. The maximum concentrations observed and the elimination half-lives of sparfloxacin in plasma and lung tissue were calculated by linear least-squares regression (13).

**Opsonophagocytic assays.** Bacteria that were presensitized with heat-inactivated mouse ascitic fluid containing MAb 1D3 (final concentration, 5 μg/ml) were resuspended in Hanks’ balanced salt solution containing 0.1% gelatin, 0.15 mM CaCl_2, and 1.0 mM MgCl_2 (GHBSS*) to a cell density of 5 × 10^5 CFU/ml. Human polymorphonuclear leukocytes (PMNs) were isolated from blood by dextran sedimentation and Ficoll-Hypaque density gradient centrifugation. Human AMs were collected from healthy smokers by bronchoalveolar lavage and were prepared as described previously (23). The AMs contained PMNs in less than 5% of total cells. AMs and PMNs were suspended in GHBSS at a concentration of 2.5 × 10^7/ml. Fresh absorbed normal human serum (AbsNHS) was prepared by a previously published method by using live homologous bacteria (15). AbsNHS (final concentration, 4%) was used as a complement source be-
cause of the low levels of complement in bronchial secretions (25). Reaction mixtures run in triplicate and added to plastic tubes (12 by 75 mm) contained 0.1 ml of the suspension of presensitized bacteria, 0.2 ml of AM (or PMN) suspension, and 0.2 ml of AbsNHS. Control tubes contained presensitized or nonpresensitized bacteria plus AbsNHS, presensitized bacteria, and AMs (or PMNs) plus heat-inactivated AbsNHS. The culture tubes were incubated by continuous rotation at 37°C for 2 h. Aliquots (30 µl) were removed at time zero and 2 h, added to sterile distilled water, allowed to stand for 10 min in PMNs and 20 min in AMs, and diluted in sterile saline. The number of viable bacteria was determined by quantitative culture. Opsonophagocytic killing was expressed as the mean log₁₀ CFU per milliliter in samples at 0 h minus the mean log₁₀ CFU per milliliter in samples at 2 h.

**Complement-mediated killing.** Complement-mediated bacterial killing of *P. aeruginosa* It-1 that was presensitized or nonpresensitized with MAb 1D3 (final concentration, 5 µg/ml) was determined in the presence or absence of sparfloxacin at one-half the MIC by using 4% AbsNHS. Presensitized or nonsensitized bacteria were prepared as described above. The reaction mixtures were run in triplicate and were added to plastic tubes (12 by 75 mm). The reaction mixtures contained 0.1 ml of the presensitized or nonpresensitized bacterial suspension, 0.2 ml of sparfloxacin solution (final concentration, 0.2 µg/ml) or medium alone, and 0.2 ml of AbsNHS (or heat-inactivated AbsNHS). The culture tubes were incubated by continuous rotation at 37°C for 2 h. Aliquots (30 µl) were removed from the reaction mixtures at 2 h for quantitative culture, and the complement-mediated killing was expressed as the mean log₁₀ CFU per milliliter in samples containing heat-inactivated AbsNHS minus the mean log₁₀ CFU per milliliter in samples containing fresh AbsNHS.

**Statistical methods.** The significance of differences in mouse survival in the treatments with MAb alone were analyzed by the Fisher exact test. A Kruskal-Wallis test (11) and a generalized Wilcoxon test were used to determine the significance of differences in mouse survival after mice received the combination therapy. The comparison of bacterial densities in blood and lung tissue, lung weights, and complement-mediated killing were analyzed by a Student t test. Data were considered statistically significant if P values were less than 0.05.

**RESULTS**

**Treatment with MAb alone.** In cases of intratracheal challenge of the It-1 strain at a dose of 10³ or 10⁴ CFU, MAb 1D3 significantly enhanced mouse survival compared with that in the control treated with R6-3F2 MAb (P < 0.01 for challenge with 10³ and 10⁴ CFU; Fig. 2). On the other hand, no significant difference between the two groups was observed for a challenge dose of 10⁵ or 10⁶ CFU.

**Combination treatment with MAb and sparfloxacin.** The survival curves for mice challenged with 10⁵ CFU of the It-1 strain receiving MAb 1D3 and sparfloxacin, sparfloxacin alone, MAb 1D3 alone, or no treatment are presented in Fig. 3. All (100%) untreated neutropenic mice were killed within 2 days. All (100%) neutropenic mice treated with MAb 1D3 alone and sparfloxacin alone were killed within 3 and 5 days, respectively. In contrast, combination therapy with MAb 1D3 and sparfloxacin provided a significant enhancement of survival from *P. aeruginosa* pneumonia in neutropenic mice compared with survival after either treatment alone or no treatment (P < 0.001). This combination effect was confirmed to be MAb 1D3 specific, because the addition of a control MAb (MAb R6-3F2) did not alter the effect of sparfloxacin alone (data not shown). We next evaluated bacterial titers in lung tissue and blood as well as the weights of the mice.  

FIG. 2. Passive protection by MAb 1D3 against intratracheal challenge with *P. aeruginosa* It-1 in neutropenic mice. Groups of 14 mice each received 500 µg of MAb 1D3 (○) or the control MAb R6-3F2 (□) intravenously 2 h after challenge with doses of 10³, 10⁴, 10⁵, or 10⁶ CFU.

FIG. 3. Combination therapy with MAb 1D3 and oral sparfloxacin against intratracheal challenge with *P. aeruginosa* It-1 at a dose of 10⁵ CFU in neutropenic mice. Groups of 22 mice each received MAb 1D3 and oral sparfloxacin (●), oral sparfloxacin alone (▲), MAb 1D3 alone (○), or no treatment (□). MAb 1D3, at a dose of 500 µg, was administered intravenously 2 h after bacterial challenge. Oral sparfloxacin, at a dose of 125 µg (5 mg/kg), was initiated 2 h after challenge and was continued every 12 h for 3 days.
of the lungs of infected mice in each treatment group at various times up to 30 h after challenge at a dose of 10³ CFU (Table 1). The bacterial density reached approximately 2 × 10⁷ CFU/g of lung tissue 6 h after challenge and 1 × 10¹⁰ CFU/g of lung tissue by 18 h postchallenge in untreated mice. Untreated mice became bacteremic 18 h after challenge. MAb 1D3 (500 μg per mouse) alone caused a slight but significant decrease in bacterial numbers in lung and blood (P < 0.05) and of lung weight (P < 0.01) compared with those in the untreated group. The reduction in bacterial counts in blood was greater than that in the lungs. Sparfloxacin (125 μg per mouse) alone rendered a greater killing of bacteria in lung and blood than did MAb 1D3 alone. The reduction in bacterial number in lung and blood and of lung weight in mice treated with sparfloxacin alone was also significant compared with the results for the untreated group (P < 0.01). Moreover, the combination therapy with MAb 1D3 and sparfloxacin exerted the highest level of killing of bacteria in lungs and blood. The decrease of bacterial numbers in lungs and the lung weights of mice resulting from the MAb 1D3 and sparfloxacin combination therapy was significant compared with the results for mice that received MAb 1D3 (P < 0.01) or sparfloxacin (P < 0.05) alone. The number of bacteria in the blood of mice that received the combination therapy was similarly reduced.

**Sparfloxacin levels.** After oral administration of sparfloxacin at a dose of 125 μg (5 mg/kg), maximum concentrations of 0.9 ± 0.1 μg/ml in plasma and 3.3 ± 0.2 μg/g in lung tissue were achieved. The half-lives of sparfloxacin were 0.91 h in plasma and 0.43 h in lung. At 4 h postadministration, sparfloxacin levels in plasma and lungs were less than 0.1 μg/ml and 1 μg/g, respectively. There was no accumulation of sparfloxacin in plasma or lungs after serial oral administrations every 12 h.

**Opsonophagocytic assays.** Incubation of the MAb 1D3-presensitized or nonpresensitized It-1 strain with 4% AbsNHS produced no complement-mediated bacteriolysis. A marked killing of presensitized bacteria by PMNs with log₁₀ killing (mean ± standard deviation) of 0.95 ± 0.13 was observed in the presence of complement. In contrast, we noted a slight killing of presensitized bacteria by AMs, with log₁₀ killing (mean ± standard deviation) of 0.04 ± 0.01 in the presence of complement. Heat inactivation of the complement resulted in the disappearance of these opsonophagocytic killings of presensitized bacteria by PMNs and AMs, with mean log₁₀ killings of −0.18 and −0.60, respectively.

**Complement-mediated killing.** One-half the MIC of sparfloxacin alone did not kill the presensitized or nonpresensitized *P. aeruginosa* It-1 cells. However, mild but significant killing of MAb-presensitized bacteria by 4% AbsNHS was shown in the presence of one-half the MIC of sparfloxacin, with log₁₀ killing (mean ± standard deviation) of 0.61 ± 0.13 (P < 0.05) compared with log₁₀ killing (mean ± standard deviation) of nonpresensitized bacteria of 0.12 ± 0.08.

**DISCUSSION**

There is conflicting evidence regarding the protective activity of anti-LPS MAbs in *P. aeruginosa* pneumonia in neutropenic animals. Pennington and Small (18) previously reported the inefficacy of a murine IgG2a MAb to *P. aeruginosa* It-1 LPS in a neutropenic guinea pig model of *P. aeruginosa* pneumonia. In contrast, Zweerink et al. (30) demonstrated the therapeutic effect of a human IgM MAb specific for *P. aeruginosa* immunotype 11 LPS in neutropenic mice. The effects of the LPS-reactive MAb on the survival rate of infected animals was dependent on the dose of bacterial challenge. In the present study, we demonstrated a slight killing of *P. aeruginosa* It-1 by human AMs in the presence of MAb 1D3 and complement. These in vitro data support the protective effects of MAb 1D3 against intratracheal challenge with lower doses of It-1, because almost all of the phagocytic cells in the bronchoalveolar fluid from infected mice were AMs in the pneumonia model described here. Moreover, of particular interest was the fact that MAb 1D3 provided a reduction of bacterial counts that was greater in the blood than in the lungs of mice. This may have resulted from the low levels of complement present in bronchial secretions (25), since the opsonophagocytic activity of LPS-specific MAbs is largely complement dependent (16). However, the dose of MAb required for protection (500 μg per mouse) was quite high compared with the protective doses of LPS-specific MAbs in other animal models of extrapulmonary sites of infection (15, 20, 26, 29). Although substantial lung penetration of an IgG3 MAb with similar specificity has
been demonstrated (16), effective treatment of *P. aeruginosa* pneumonia with LPS-specific MAb alone is difficult in neutropenic hosts.

Oral administration of a suboptimal dose of sparflxacin (5 mg/kg) achieved 3.6 times higher levels in lung tissue than in plasma (0.9 μg/ml) with the present model. The elimination half-life of this drug in lungs was shorter than that in plasma. In addition, the half-life of sparflxacin in the plasma of infected mice was shorter than that in the plasma of uninfected mice (13). This shortened half-life of sparflxacin in plasma in the animal model described here may be related to the infected site. Although sparflxacin levels in plasma and lung in the pneumonia model were less than 0.1 μg/ml at 4 h postadministration, sparflxacin in combination with MAb 1D3 was bactericidal against *P. aeruginosa* It-1 in vivo. The fact that sparflxacin at concentrations greater than and near the MIC is bactericidal for *P. aeruginosa* (10) may have something to do with this result.

In the present study, we documented a significant increase in the survival rates of mice given combination therapy with MAb 1D3 and a suboptimal dose of oral sparflxacin against intratracheal challenge with the It-1 strain at a dose of 10⁶ CFU, while 100% of infected mice that received MAB 1D3 alone or sparflxacin alone were killed. This combination effect of MAB 1D3 and sparflxacin on the survival rate was supported by bacterial killing in the plasma and lung tissues of infected mice. The combination therapy provided intrapulmonary killing of bacteria at a magnitude of greater than 5.0 log units, while MAB 1D3 alone and sparflxacin alone exerted 0.5 and 3.0 log units of killing, respectively. Additionally, the reduction in the weight of the lungs of treated mice was closely correlated to the intrapulmonary killing of bacteria. These in vivo effects of the combination of MAB 1D3 and a suboptimal dose of sparflxacin may be partly caused by MAB-dependent, complement-mediated killing of bacteria in the presence of sub-MICs of sparflxacin. Joiner and coworkers (7, 8, 28) reported on the mechanism of serum resistance for *Salmonella minnesota*, *Escherichia coli*, and *P. aeruginosa*. These bacteria activated complement efficiently, and C5b-9 membrane attack complexes were formed on the bacterial surfaces. The C5b-9 complexes themselves were not bactericidal, because they did not insert into the hydrophobic outer membrane domains. However, the MABS specific for the O side chain of *E. coli* O111:B4 LPS efficiently fixed complement and resulted in MAB-dependent, complement-mediated lysis on serum-resistant homologous bacteria (15). Furthermore, a recent report described the bacteriolyis of *P. aeruginosa* It-1 that was presensitized with a MAB specific for the O side chain by using higher concentrations of AbsNHS (22). On the other hand, previous investigators showed that *E. coli* treated with a DNA gyrase inhibitor, nalidixic acid, became susceptible to detergent-mediated lysis (3). After the addition of nalidixic acid, there were only slight changes in the synthesis of a few outer membrane proteins. Therefore, there was apparently a significant functional interaction between an LPS-reactive MAB and sub-MICs of sparflxacin that resulted in enhanced complement-mediated bacterial killing of *P. aeruginosa* It-1.

The data presented in this report suggest an important therapeutic interaction between LPS-reactive MABS and antibiotics in a neutropenic mouse model of *P. aeruginosa* pneumonia and appear to provide a basis for the therapeutic strategy of LPS-reactive MABS combined with antibiotics against life-threatening *P. aeruginosa* pneumonia in neutropenic patients.

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