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Inhibitory Activity on Bacterial Motility and In Vivo Protective Activity of Human Monoclonal Antibodies against Flagella of *Pseudomonas aeruginosa*

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Three stable hybridoma cell lines, IN-2A8, IN-5D6, and ZI-3A8, that secrete human monoclonal antibodies (MAbs) specific for b-type flagella of *Pseudomonas aeruginosa* were established by fusing peripheral blood lymphocytes from healthy volunteers with murine myeloma P3X63-Ag8.653 cells. The immunoglobulin M MAbs reacted specifically with flagellin (M_r, 52,000) by Western blotting (immunoblotting) analysis and bound specifically to clinical isolates belonging to Homma serotypes A, B, H, I, and M at frequencies of 58, 50, 46, 30, and 35%, respectively, but did not bind to any serotype E or G isolates. Overall, the MAbs bound to 31% of the clinical isolates. MAb IN-2A8 strongly protected burned mice challenged with *P. aeruginosa* bearing b-type flagella from death following parenteral administration of 0.1 μg per mouse. This MAb also inhibited *P. aeruginosa* colony spreading in soft agar at a concentration of more than 1 μg/ml but only slightly enhanced opsonophagocytosis by human polymorphonuclear leukocytes. A line of evidence suggests that the potent in vivo activity of MAb IN-2A8 in the burned-mouse model is likely to be caused by its inhibition of bacterial motility after binding to flagella.

Gram-negative bacteremia and endotoxemia still cause a high risk of mortality in clinics, although new types of antibiotics have been developed and used to treat gram-negative bacterial infectious diseases. This is true especially for infections caused by *Pseudomonas aeruginosa*. Activation of the immune system of a host by active or passive immunization in combination with antibiotic therapy is needed to cure pseudomonal infections completely.

P. aeruginosa strains produce a number of extracellular products, including exotoxin A (17), elastase (20), and protease (15), which have been reported to be involved in the pathogenesis and virulence of *P. aeruginosa*. Cell surface components, such as lipopolysaccharide (5), alginate (6), pili (19), and flagella (14), are also involved in the pathogenesis and virulence of this bacterium. These virulence factors may be candidates as target molecules for vaccine and passive immunization.

Flagella help bacteria to move more rapidly from colonized sites to blood vessels and promote progress from local to systemic infection. Flagella have been reported to play an important role in pseudomonal infections of burned patients in whom the immune system is temporarily suppressed by burning (16). Furthermore, vaccination with flagellar preparations (9) and antibody therapy with antiflagellar sera (7) have been demonstrated to be protective against lethal challenges with *P. aeruginosa* strains bearing flagella in the experimental burned-mouse model.

Here we describe human monoclonal antibodies (MAbs) against flagella of *P. aeruginosa* and show that the MAbs act in a mode different from that of O antigen-specific MAbs and potently protect burned mice against *P. aeruginosa* infections.

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MATERIALS AND METHODS

***P. aeruginosa* strains.** Standard strains of *P. aeruginosa* O serotype classified by the Serotype Committee for the Japan *Pseudomonas aeruginosa* Society (10) were obtained from the Institute of Medical Science, University of Tokyo, Tokyo, Japan. Habs standard O serotype strains were obtained from the American Type Culture Collection. H antigen serotype reference strains were kindly given by R. Ansorg, Universitätsklinikum Essen, Essen. Federal Republic of Germany. Strain M-2 (18) was a kind gift from I. A. Holder, University of Cincinnati, Cincinnati, Ohio. Clinical isolates of *P. aeruginosa* were stocked in our laboratory, and the O serotype was determined with a Meiassay serotype grouping kit (Meiji Seika Co., Tokyo, Japan). All strains were grown on heart infusion agar (Nissui Pharmaceuticals, Tokyo, Japan) at 37°C.

General methods. Methods for preparation of human mouse hybridomas, enzyme-linked immunosorbent assay (ELISA), and Western blotting (immunoblotting) analysis were exactly as previously described (21), except for the following minor modifications. Peripheral blood lymphocytes from healthy adult donors whose sera had high antibody titers against whole *P. aeruginosa* cells and were supposed to have been naturally exposed to *P. aeruginosa* at some time were stimulated in RPMI 1640 culture medium containing 0.0002% (wt/vol) Formalin-killed *P. aeruginosa* IID1002 (serotype B) cells, 0.2% (vol/vol) pokeweed mitogen, heat-inactivated fetal bovine serum, sodium pyruvate, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2-mercaptoethanol, and antibiotics, and the stimulated peripheral blood lymphocytes were fused with mouse myeloma cell line P3X63-Ag8.653 (ATCC CRL/580). Hybridoma cell line IN-2A8 was adapted to serum-free medium (Celgrosser H; Sumitomo Pharmaceuticals, Osaka, Japan) for further studies. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using a slab gel

TABLE 1. Binding activities of MAbs IN-2A8 and ZI-3A8 to standard strains of *P. aeruginosa*

Strain	H serotype	ELISA reactivity (OD_{405})[a]	
		IN-2A8	ZI-3A8
Habs			
1	a	0.02	0.01
2	b	2.35	1.76
3	b	1.81	1.39
4	b	0.82	0.61
5	b	1.99	1.43
6	a	0.08	0.03
7	b	1.35	1.07
8	a	0.06	0.02
9	a	0.05	0.03
10	b	2.35	1.78
11	b	0.93	0.62
12	b	0.99	0.84
170001	b	2.35	2.04
170018	a	0.07	0.01
None[b]		0.03	0.01

[a] Binding activity was determined by ELISA using plates coated with *P. aeruginosa* as described in Materials and Methods and is expressed as OD_{405}.
[b] Negative control in which wells were not coated with any bacterium but only blocked with 3% bovine serum albumin.

TABLE 2. Binding activity of MAb IN-2A8 to clinical isolates of *P. aeruginosa*

Homma serotype	No. of isolates bound[a]/no. tested	Frequency (%)[b]
A	11/19	58
B	10/20	50
E	0/20	0
G	0/19	0
H	6/13	46
I	6/20	30
M	7/20	35

[a] OD_{405} of more than 0.2 in an ELISA.
[b] The overall frequency was 31%.

apparatus (Bio-Rad Laboratories, Richmond, Calif.) by the method of Laemmli (11) with a slight modification. The stacking gel contained 3% acrylamide, and the separating gel contained 10% acrylamide. Proteins in the gel and on the Durapore filter were detected by staining with Coomassie brilliant blue.

Preparation of flagellin. Flagellin was isolated by the method of Montie et al. (13) with a slight modification. *P. aeruginosa* cells were cultured on heart infusion agar at 37°C for 17 h. Grown cells were gently scraped from the agar surface and suspended in phosphate-buffered saline (PBS). Cell suspensions were centrifuged at 5,000 × *g* for 15 min at 4°C. The resulting pellet was suspended in PBS and blended in a commercial blender (Automatic Mixer S-100; Taiyo-kagaku, Tokyo, Japan) for 3 min to shear off the flagella. Suspension was centrifuged at 16,000 × *g* for 15 min at 4°C, and the resulting supernatant was centrifuged again at 40,000 × *g* for 3 h at 4°C. The supernatant was carefully removed, and the pellet was suspended in a small amount of PBS and used for further experiments as a preparation of flagellin. The purity of flagellin preparations was demonstrated to be about 90% by SDS-PAGE.

The burned-mouse model. Burned mice were prepared by the method of Stieritz and Holder (18) with a slight modification. Four-week-old male ICR mice were anesthetized. A glass fiber filter was dipped into ethanol, ignited, and placed over the shaved backs of the mice for 10 s. The mice were given 0.3 ml of sterile saline intraperitoneally to prevent shock caused by burning. The mice were then given 0.2 ml of a serially diluted bacterial suspension subcutaneously at the burn site. At 1 h postchallenge, the MAb in 0.2 ml of PBS was injected intravenously. In vivo protective activity was determined from the survival rate at 1 week postchallenge. We calculated 50% lethal doses (LD_{50}s) by probit analysis.

Inhibitory activities of MAbs against bacterial motility of *P. aeruginosa*. Inhibitory activities of MAbs against bacterial motility were assayed by the method of Craven and Montie (4) with a slight modification. The bacterial cell suspension (50 μl at an optical density at 600 nm [OD_{600}] of 0.2) in PBS and 50 μl of the MAb solution in PBS were mixed and incubated at 25°C for 15 min. Each 50 μl of the mixture was dispensed into wells (6 mm in diameter) of a soft agar plate containing 1% tryptone, 0.3% yeast extract, 0.5% NaCl, and 0.3% agar. The plate was incubated at 25°C. The diameters of bacterial swarms with sharp and less distorted rings were measured after incubation for 24 h.

Bacterial agglutination test. A *P. aeruginosa* cells suspension (50 μl at an OD_{600} of 1.0) was mixed with 50 μl of a serially threefold-diluted solution of the MAb. Equal volumes (100 μl) of the mixture were dispensed into the wells of 96-well round-bottom plates (Falcon). After incubation at 4°C for 24 h, agglutination was monitored.

Opsonophagocytic activity. Human polymorphonuclear leukocytes (PMNs) were used to determine the opsonophagocytic activities of the MAbs. Human PMNs were separated on Mono-poly resolving medium (Flow Laboratories, Inc., McLean, Va.) from blood obtained from healthy adult donors. Cells were washed three times with RPMI 1640 medium and suspended in RPMI 1640 with 25 mM HEPES at a concentration of 6.7×10^6 cells per ml. Three-tenths of a milliliter of human PMNs (4×10^6 cells per ml), 100 μl of a *P. aeruginosa* clinical isolate SP10052 suspension (8×10^5 or 8×10^6 cells per ml), 50 μl of the MAb (1 μg/ml) in RPMI 1640 with 25 mM HEPES, and 50 μl of normal human serum preabsorbed with whole bacterial cells, as a source of complement, were mixed. The mixtures were incubated at 37°C for 2 h in 24-well multiplates (Falcon Plastics, Oxnard, Calif.) which had previously been coated with human serum albumin (Buminate; Sumitomo Pharmaceuticals) with gentle shaking at 100 rpm on a rotary shaker. A small portion of the uniformly suspended mixture was removed, serially diluted with sterile saline, and inoculated onto heart infusion agar plates. Numbers of viable bacteria were calculated by colony counting following incubation at 37°C for 16 h and expressed in CFU. PMNs were not disrupted before plating for viable bacterial counts in the experiments shown in Table 4, but disruption of PMNs did not greatly change the number of viable bacterial cells recovered.

RESULTS

Three cell lines that produce human MAbs which bound to a wide range of standard strains consisting of several different O serotypes of *P. aeruginosa* were obtained in two independent experiments by fusing peripheral blood lymphocytes with mouse myeloma P3X63-Ag8.653. The peripheral blood lymphocytes were stimulated in vitro in the presence of pokeweed mitogen and Formalin-killed *P. aeruginosa* cells as described in Materials and Methods. The hybridoma

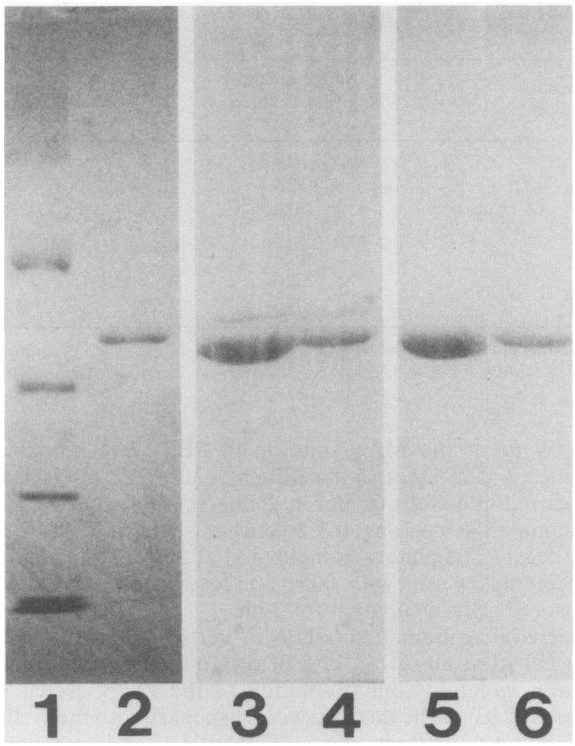

FIG. 1. SDS-PAGE of flagellin preparations and Western blotting analysis with MAb IN-2A8. Flagellin preparations from both strains M2 (15 μg of protein per lane; lanes 3 and 5) and SP6818 (5 μg of protein per lane; lanes 2, 4, and 6) and molecular weight standards (lane 1) were subjected to SDS-PAGE and then electrophoretically transferred to a Durapore filter (lanes 3 to 6). Proteins were detected by staining with Coomassie brilliant blue (lanes 1 to 4) or immunostaining with 1 μg of immunoglobulin M MAb IN-2A8 per ml (lanes 5 and 6).

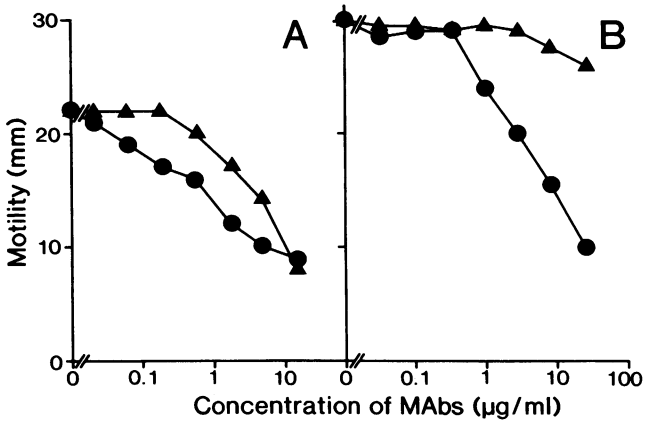

FIG. 2. Inhibitory activities of MAbs against motility of *P. aeruginosa*. Each concentration of MAbs IN-2A8 (●) and KO-2F2 (▲) was mixed with *P. aeruginosa* IID1002 (A) and M2 (B) as described in Materials and Methods. The mixtures were dispensed into the wells of a soft agar plate, and the bacterial swarms were measured. The experiments were done in duplicate. The average diameter of the rings formed by bacterial swarming minus the diameter of each well (6 mm) is expressed as motility. The deviations were less than 1 mm.

cell lines and their specific MAbs were named IN-2A8, IN-5D6, and ZI-3A8. Their isotypes were immunoglobulin M(λ) for MAb IN-2A8, immunoglobulin M(λ) for MAb IN-5D6, and immunoglobulin M(κ) for MAb ZI-3A8. Hybridoma cell line IN-2A8 was subjected to extensive cloning and adaptation to serum-free medium. The resulting hybridoma cell line IN-2A8 stably produced a specific MAb at the rate of 25 μg/10^6 cells per day for at least 6 months.

MAbs IN-2A8, ZI-3A8, and IN-5D6 reacted with Habs standard strains 2, 3, 4, 5, 7, 10, 11, and 12 and H serotype reference strain 170001 (Table 1). All of the strains bore b-type flagella as shown by the slide coagglutination method with polyclonal antisera (3). They showed no reactivity with Habs standard strains 1, 6, 8, and 9 and H serotype reference strain 170018, which is known to bear a-type flagella (data not shown for MAb IN-5D6). These results indicate that the three MAbs specifically bind to b-type flagella of *P. aeruginosa*. The three MAbs reacted with Homma standard strains IID1002 (serotype B), IID5004 (B), IID1009 (H), IID5141 (L), and IID1015 (M) and also with Fisher standard strains 2, 6, and 7.

The reactivity of MAb IN-2A8 with 131 clinical isolates of *P. aeruginosa* Homma serotypes A, B, E, G, H, I, and M, which are isolated in clinics at high frequencies, was examined by ELISA (Table 2). MAb IN-2A8 reacted with clinical isolates of serotypes A, B, H, I, and M at frequencies of 35 to 58% but reacted with no serotype E or G clinical isolates. Overall, MAb IN-2A8 bound to 31% of the clinical isolates tested. MAbs IN-5D6 and ZI-3A8 had the same specificities and frequencies of binding to clinical isolates as MAb IN-2A8.

Next, the antigen molecule recognized by MAb IN-2A8 was determined by Western blotting analysis using preparations of flagellin, a flagellar protein (Fig. 1). As shown by Coomassie brilliant blue staining of the Durapore filter following SDS-PAGE (lanes 3 and 4), a major band with an M_r of 52,000 and a faint band of impurity with an M_r of 60,000 were detected in preparations of flagellin isolated from both strain M2 and clinical isolate SP6818 (Homma serotype A), both of which were bound by MAb IN-2A8 in an ELISA. The major band was likely to be identical to the flagellin preparation of strain M2 with an M_r of 53,000 reported by Montie et al. (13). MAb IN-2A8 specifically immunoreacted with the major band in the flagellin preparation but not with the minor band (Fig. 1, lanes 5 and 6), indicating that MAb IN-2A8 specifically binds to flagellin.

The inhibitory activity of MAb IN-2A8 against the motility of *P. aeruginosa* cells was examined by measuring the rate of inhibition of colony spreading in soft agar compared with that of a specific human immunoglobulin M MAb to the O antigen of Homma serotype B (MAb KO-2F2), which was established in our laboratory by the human-mouse hybridoma method. MAb IN-2A8 partly inhibited the motility of *P. aeruginosa* Homma serotype B standard strain IID1002, which had low virulence in the burned-mouse model, at a concentration lower than 0.2 μg/ml (Fig. 2). Both MAbs inhibited bacterial motility in a concentration-dependent manner at a concentration higher than 0.5 μg/ml, but MAb IN-2A8 showed relatively stronger inhibition than MAb KO-2F2. Against highly motile strain M2 (Homma serotype B), which was highly virulent in the burned-mouse model, on the other hand, more than 1 μg of MAb IN-2A8 per ml was required for inhibition of bacterial motility. In contrast, however, MAb KO-2F2 showed only a slight effect at a concentration of 25 μg/ml. The inhibitory activities of MAbs against bacterial motility in soft agar should be tightly

TABLE 3. Agglutinating activities of MAbs on viable and killed *P. aeruginosa*

Strain and condition	MAb	Agglutinating activity[a] (μg/ml)
IID1002		
Viable	IN-2A8	>15[b]
	KO-2F2	0.55
Killed[c]	IN-2A8	0.062
	KO-2F2	0.55
M2		
Viable	IN-2A8	0.55
	KO-2F2	5
Killed	IN-2A8	0.062
	KO-2F2	0.55

[a] The minimum concentrations of the MAbs required for bacterial agglutination are shown. Experiments were done in duplicate, and the results were highly reproducible.
[b] No bacterial agglutination was observed, even at 15 μg of the MAb per ml.
[c] Bacteria were killed by incubation with 0.5% Formalin at 25°C for 15 h.

TABLE 4. Opsonophagocytic killing activity of MAbs IN-2A8 and KO-2F2

MAb	Concn of viable bacteria (CFU/ml)		% of bacteria left after incubation[b]
	Before incubation (mean ± SEM, 10^4)[a]	After incubation with or without MAb (mean ± SEM, 10^4)[a]	
None	80 ± 0	200 ± 10	100
IN-2A8	80 ± 0	110 ± 10	55
KO-2F2	80 ± 0	1.4 ± 0.1	0.70
None	800 ± 0	1,200 ± 100	100
IN-2A8	800 ± 0	590 ± 40	49
KO-2F2	800 ± 0	44 ± 6	3.7

[a] Experiments were done in duplicate.
[b] The number of viable bacteria postincubation with a MAb for 2 h at 37°C was divided by that postincubation without a MAb for 2 h at 37°C and is expressed as a percentage.

associated with their bacterial agglutinating activity, because agglutinated bacteria cannot migrate rapidly in soft agar. Therefore, we examined the agglutinating activities of MAbs IN-2A8 and KO-2F2. The minimum concentrations of the MAbs required for agglutination of viable and killed bacteria are shown in Table 3. MAb IN-2A8 at a low concentration strongly agglutinated Formalin-killed *P. aeruginosa* IID1002 and M2 cells, but it showed no or weak agglutinating activity against viable bacteria. MAb KO-2F2 agglutinated viable bacteria at more than 5 μg/ml for M2 and 0.5 μg/ml for IID1002.

The PMN-mediated opsonophagocytic killing activity of MAb IN-2A8 was very low (Table 4). MAb IN-2A8 mediated bacterial killing at a rate of only 45% after incubation of bacteria at 8×10^5 CFU/ml with 1 μg of MAb per ml at 37°C for 2 h, while human MAb KO-2F2 to O antigen mediated 99.3% killing of bacteria under the same conditions.

MAb IN-2A8 at 0.1 μg per mouse (5 μg/kg of body weight) showed potent protective activity in burned mice challenged with 380 and 330 LD_{50}s of *P. aeruginosa* SP10052 and M2 (Homma serotype B), respectively. Administration of the MAb at 1 μg per mouse saved mice challenged with 10^4 LD_{50}s of *P. aeruginosa* SP10052 from death. Although protective activity was influenced by the number of bacteria used for the challenge, the protective dose giving a 50% survival rate which was calculated 7 days after administration of a serially 10-fold-increasing dosage of MAb IN-2A8 was 0.05 μg per mouse when mice were challenged with about 200 LD_{50} of SP10052 in the other series of experiments. MAb IN-2A8 (1 μg per mouse) also protected mice challenged with 15 LD_{50}s of SP6830 (Homma serotype A) and 17 LD_{50}s of SP6853 (Homma serotype I). The apparently low protective activity of MAb IN-2A8 against SP6830 and SP6853 was likely caused by the high challenge doses of low-virulence bacteria (10^5 to 10^6 CFU per mouse) which were required to kill control mice. All of the strains killed by MAb IN-2A8 in this experiment belong to H serotype b. In contrast, MAb IN-2A8 showed no protective activity against strains belonging to H serotype a (data not shown). Finally, MAb IN-2A8 showed protective activity against *P. aeruginosa* bearing b-type flagella, independently of the O serotype of the bacteria, and its protective activity was greatly dependent upon the number of bacteria used for the challenge.

DISCUSSION

We isolated human MAbs against flagella of *P. aeruginosa* and demonstrated that 0.1 μg of MAb IN-2A8 per mouse protected mice from death in the burned-mouse model.

Flagella are known to be an important apparatus for bacterial movement and are composed of flagellar proteinous molecules, so-called flagellin. Flagellin has been classified into two major types, a and b, by serological methods (3). It has been reported that b-type flagellin is homogeneous, while a-type flagellin is heterogeneous and further classified into at least five subtypes. MAb IN-2A8, as well as the other two MAbs, recognized b-type flagellin. The binding specificities of the MAbs for clinical isolates showed that 31 to 58% of the clinical isolates belonging to Homma O serotype A, B, H, I, and M possessed b-type flagella but no or very few clinical isolates of Homma O serotypes E and G possessed it, suggesting that some correlation between the O serotype and the flagellar serotype (H serotype) exists, as suggested by Ansorg et al. (3) and Lányi (12), who used antisera to determine H serotypes.

MAb IN-2A8 strongly protected burned mice challenged with *P. aeruginosa* bearing b-type flagella (Table 5), independently of the O serotype, and the MAb effectively inhibited the motility of *P. aeruginosa* in soft agar (Fig. 2). However, the MAb very slightly enhanced the ability of PMNs to kill bacteria in the presence of complement (Table 4). A line of evidence suggests that the in vivo protective activity of the MAb in burned mice is likely to be due mainly to binding of the MAb to flagella and resulting inhibition of bacterial motility and to be less associated with opsonophagocytic activity. In contrast, Drake and Montie and Anderson and Montie reported that antiflagellar serum was protective against lethal challenges with *P. aeruginosa* in the burned-mouse model (7) and had opsonophagocytic activity in vitro (1, 2), concluding that the in vivo activity of flagellar antiserum was dependent on both its inhibitory activity against bacterial motility and its opsonophagocytic activity. The gap between the conclusions of this study and those of the earlier studies by Montie et al. may be due to both the different antibodies used (monoclonal antibody and polyclonal antiserum) and the different methods used to measure opsonophagocytic activity. Our human MAbs blocked bacterial movement from the local infection site to blood in burned mice (data not shown) and inhibited bacterial motility in vitro. Our result is compatible with that of the previous

TABLE 5. In vivo protective activity of MAb IN-2A8 in the burned-mouse model

Strain used for challenge[a]	Homma sersotype	Drug	Dosage (μg/mouse)	LD_{50}[b]
SP10052	B	None	0	2.6×10^2 (1)
		IN-2A8	0.01	1.2×10^3 (5)
			0.1	1.0×10^5 (380)
			1	2.8×10^6 (11,000)
M2	B	None	0	$<1.3 \times 10^3$ (1)
		BSA[c]	0.1	5.7×10^3 (4)
		IN-2A8	0.1	4.3×10^5 (330)
SP6830	A	None	0	1.5×10^6 (1)
		IN-2A8	1	2.6×10^7 (17)
SP6853	I	None	0	1.0×10^5 (1)
		IN-2A8	1	1.5×10^6 (15)

[a] All of the strains used for this experiment bound to IN-2A8.
[b] Nine or ten mice were used for each group. The bacterial cell number (CFU) that killed 50% of the mice to which bacteria were administered with or without a MAb is given. The in vivo protective activity of the MAb is expressed as a relative LD_{50} in parentheses.
[c] BSA, Bovine serum albumin.

report (14) showing that bacterial motility was one of the most important virulence factors in the burned-mouse system, by comparing the virulence of strain M2 with that of its nonflagellar mutant.

Many investigators have reported on MAbs to the O antigen of *P. aeruginosa*. The O-antigen-specific MAbs showed potent protective activities in vivo and enhanced opsonophagocytosis in vitro. However, these MAbs to the O antigen possess a narrow range of binding and protective activities. In other words, the MAbs have no activities against either strains belonging to serotypes other than a specific O serotype or rough strains, such as Homma serotype M (10), which seem to be important pathogens for respiratory tract infections in patients with cystic fibrosis (8). In contrast, MAbs against flagella of *P. aeruginosa* bound to a wide range of strains independently of O serotypes, including rough strains, and inhibited bacterial motility, resulting in prevention of bacterial invasion from colonized sites to blood vessels. Thus, they might be useful for curing complicated local infections with *P. aeruginosa*, such as burn and respiratory tract infections.

More detailed studies on the in vivo protective activities of these MAbs are in progress.

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