Immunoprotective Human Monoclonal Antibodies against Five Major Serotypes of *Pseudomonas aeruginosa*

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Human monoclonal antibodies (Mabs) against the O antigens of *Pseudomonas aeruginosa* lipopolysaccharides (LPS) were produced by cell fusion between human tonsillar lymphocytes and P3-X63-Ag8-U1 (P3U1) mouse myeloma cells. To obtain human Mabs efficiently, 6 d culture supernatants of pokeweed-mitogen-stimulated lymphocytes (21 cultures from peripheral blood and 76 from tonsils) were assayed by ELISA. Five tonsillar lymphocytes which produced IgG antibody specific for *P. aeruginosa* LPS were preselected for fusion. The human Mabs, named P1-1 (IgG2, κ), P5-1 (IgG2, λ), P7-1 (IgG2, λ), P8-1 (IgG2, λ) and P10-1 (IgG2, κ), bound with high specificity to Homma standard serotype strains A, E, B, G and I, respectively, and recognized O antigens. Each Mab showed opsonophagocytic killing activity of the corresponding serotype strain. Four of the Mabs caused agglutination at a very low concentration; a rather higher concentration of P7-1 was required for this effect. Although all the Mabs conferred type-specific protection against peritoneal infection, the strongly agglutinating Mabs provided better protection than the moderately agglutinating P7-1. The protective activity of P8-1 was estimated in compromised mice. A low dose (PD₅₀ 0.5–0.6 μ g per mouse) of P8-1 prevented subcutaneous infection in burned mice and peritoneal infection in leucopenic mice. All the hybridomas described here could be cultured in serum-free medium, and they have continued to secrete human Mabs for more than 14 months at rates of 10–20 μ g per 10⁶ cells in 24 h. These results suggested that these five human Mabs specific for O antigens might be useful in the prophylaxis and treatment of *P. aeruginosa* infections.

INTRODUCTION

Pseudomonas aeruginosa infections frequently occur in compromised hosts (Hoiby & Olling, 1977; Pruitt, 1974; Bodey & Rodriguez, 1973), and a high incidence of morbidity and mortality are associated with these infections. Antibiotic therapy of these patients is not always successful because the causative organisms are often resistant to drugs (Lowbury & Jones, 1975; King *et al.*, 1983), and alternative methods for treatment and prevention of *Pseudomonas* infections are increasingly being sought. Passive immunotherapy with antibodies is thought to have potential (Fisher, 1977; Cryz *et al.*, 1983).

We have been interested in the protective activity of monoclonal antibodies (Mabs) against *P. aeruginosa* infection, and have demonstrated the protective activity of several mouse Mabs that recognize the lipopolysaccharide (LPS) of *P. aeruginosa* (Sawada *et al.*, 1984). Recently, other investigators have also reported high protective activity of mouse Mabs specific for *P. aeruginosa* LPS (Stoll *et al.*, 1986; Barclay *et al.*, 1986). For potential treatment or prophylaxis,

Abbreviations: A-PS, acid-treated LPS; FCS, foetal calf serum; HBSS-Gel, Hanks' balanced salt solution containing 0.1% gelatin; Mab, monoclonal antibody; NHlgG, normal human serum IgG; PBL, peripheral blood lymphocytes; PD₅₀, mean protective dose; PMN, polymorphonuclear leucocytes; PWM, pokeweed mitogen; i.p., intraperitoneal(ly); s.c., subcutaneous(ly).

however, a human Mab should be developed, because a mouse Mab is a foreign protein in man. We first developed a human Mab against the LPS of the Homma standard serotype E strain of *P. aeruginosa* through fusion of human tonsillar cells and mouse myeloma cells, and confirmed its protective ability (Sawada *et al.*, 1985a). However, in that work we did not study opsonic activity and examined only one human Mab, against serotype E. There are as many as 17 *P. aeruginosa* serotypes (Liu *et al.*, 1983), each determined by its O polysaccharide; thus treatment of *P. aeruginosa* infections with human Mabs would ideally require 17 kinds of human Mab. Our attempt to prepare these, however, had to be abandoned because it was time-consuming and inefficient. Instead, we decided to develop five kinds of human Mab binding to the major serotypes of LPS, i.e. A, B, E, G and I, which are frequently detected in patients in Japan (Sato *et al.*, 1981) and are thought to account for 70–80% of all *P. aeruginosa* strains. In the work described here, we developed five specificities of human Mab which bind to O antigens and evaluated their biological activities *in vitro* and *in vivo*.

METHODS

Bacterial strains. Homma standard serotype strains of *P. aeruginosa* (ATCC 27577–90) and Fisher immunotype strains (ATCC 27312–18) were purchased from the American Type Culture Collection (Bethesda, Md, USA). *P. aeruginosa* NC5 (serotype G) was a gift from Dr J. Y. Homma, Kitasato Institute, Tokyo. The other strains used for this study were from our laboratory collection. All strains were cultured in heart infusion agar or broth (Nissui Pharmaceuticals) at 37 °C. Serotypes A, B, E, G and I described here are thought to be similar to 3, 2 and 5, 11, 6 and 1, respectively, in the international typing scheme.

Preparation of human Mabs. Human peripheral blood lymphocytes (PBL) were obtained from 21 patients with *P. aeruginosa* infections and tonsillar cells were prepared from tonsils surgically removed from 76 patients with tonsillitis. The lymphocytes were separated with a Ficoll-Paque solution (Pharmacia). To determine the ability of these lymphocytes to give a high yield of hybridomas which would secrete human Mabs specific for *P. aeruginosa* LPS, 1×10^6 lymphocytes were suspended in 1 ml RPMI 1640 medium (Gibco) containing 2 mM-L-glutamine, 1 mM-sodium pyruvate, 0.2 mM-L-serine, 80 µg gentamicin sulphate (Schering) ml⁻¹, and 10% (v/v) foetal calf serum (FCS) (General Scientific Laboratories). To this cell suspension, pokeweed mitogen (PWM) (Gibco) was added to a concentration of 10 µg ml⁻¹. Samples (1 ml) of this cell suspension were transferred into flat-bottomed 24-well plates (Costar 3424) and cultured at 37 °C for 6 d in an incubator with an atmosphere of 5% CO₂ and 95% air. The 6 d culture supernatants were assayed by ELISA for the amount of human antibodies specific for *P. aeruginosa* LPS.

Human lymphocytes which produced a high antibody titre were selected for use in fusion with mouse myeloma cells. The selected human lymphocytes were cultured in the same medium for 4 d and then washed once with serum-free RPMI 1640 medium. Lymphocytes (5×10^6) were fused with 5×10^6 nonsecreting mouse myeloma P3-X63-Ag8-U1 (P3U1) (Yelton *et al.*, 1978), which was provided by Dr M. Taniguchi (Chiba University, Japan), in 1 ml fusion solution consisting of 35% (v/v) polyethylene glycol 1500 (Wako Pure Chemicals) and 7.5% (v/v) dimethyl sulphoxide in serum-free RPMI 1640 medium. The fused cells were dispensed into a 96-well tissue culture plate (Costar 3596) at a concentration of 2×10^4 cells per well and cultured in the above medium containing 1×10^{-4} M-hypoxanthine, 4×10^{-7} M-aminopterin and 1.6×10^{-5} M-thymidine. The medium was changed once a week. Hybridomas were cloned by limiting dilution to give 0.3 cells per well. The hybridoma culture supernatants were tested for antibody to *P. aeruginosa* cells by ELISA as described below. Hybridoma cells were also cultured in serum-free medium, the composition of which is described by Murakami *et al.* (1982).

The culture supernatant of each hybridoma was separately pooled and precipitated with ammonium sulphate (final saturation, 50%), and after dialysis against phosphate-buffered saline (PBS) it was subjected to Protein A-Sepharose (Pharmacia) column chromatography. The eluted human Mab gave two protein bands identical to the heavy and light chains of immunoglobulin G (IgG) in SDS-PAGE (12.5%, w/v, acrylamide slab gels) (Laemmli, 1970), and no other contaminating protein bands were observed. We calculated the IgG concentration of the purified materials by measuring the absorption at 280 nm or by the single radial immunodiffusion method with an LC-Partigen-IgG plate (Behring-Werke). This purified material was used for the experiments on the biological activities *in vitro* and *in vivo*. The isotype of each human Mab was determined by immunodiffusion in human immunoglobulin RID plates (Serotec) and by ELISA with goat antibodies to human λ and κ chains (Tago).

Preparation of pooled normal human serum IgG (NHlgG). Plasma, collected from more than 1000 normal blood donors, was fractionated with ethanol at the Chemo-Sero Therapeutic Research Institute (Kumamoto, Japan).

Screening for human Mabs by ELISA. To screen for Mabs specific for *P. aeruginosa*, we performed ELISA with plates coated with a mixture of strains of the five major serotypes (Homma strains ATCC 27577, 27578, 27581, 27583, 27584, 27586 and 27589). The ELISA procedure was as described previously (Sawada *et al.*, 1985a). To determine the specificities of the Mabs, we coated Homma standard serotype strains, Fisher Immunotype strains, 48 clinical isolates of *P. aeruginosa* and other Gram-negative bacterial strains such as *Escherichia coli* or *Salmonella minnesota* onto 96-well plates, and measured the binding of the Mabs to these antigens by ELISA. For the binding of LPS to 96-well plates, 50 μ l of 1 μ g LPS ml⁻¹ in distilled water was added to 96-well flat plates, dried and fixed to the plates at 70 °C for 5 h. The wells were washed twice with PBS and filled with PBS containing 1% (w/v) bovine serum albumin, and the plates were stored at 4 °C.

Preparation of LPS. *P. aeruginosa* was cultured to the late-exponential phase in 101 L-broth (Bertani, 1951). The cells were killed by addition of formaldehyde to a concentration of 0.3%, harvested by centrifugation at 8000 *g* for 7 min, washed once with distilled water and lyophilized. LPS was extracted by the hot phenol/water method of Johnson & Perry (1976). LPS was prepared from strains N-2 (serotype E), IT1 (G), PAO-1 (B), ATCC 27577 (A) and ATCC 27586 (I). All the LPS preparations contained <0.3% (w/w) protein and <0.25% (w/w) nucleic acid.

Determination of antigen recognition site of human Mabs. LPS was hydrolysed with 1% (v/v) acetic acid at 100 °C for 2 h in a closed ampoule to prepare acetic-acid-treated LPS (A-PS) as described previously (Sawada *et al.*, 1985b). A-PS preparations from various strains of *P. aeruginosa* were subjected to column chromatography on Sephadex G-50 (Pharmacia) and eluted with 50 mM-pyridine/acetic acid buffer (pH 4.2). The contents of hexosamine and hexose in each fraction were determined as described previously (Sawada *et al.*, 1985b). Each fraction was used in competitive inhibition of the binding of each human Mab to *P. aeruginosa* in order to determine the Mab recognition site. The percentage of residual activity was calculated as described previously (Sawada *et al.*, 1985b). The fractions containing O antigen were determined by the binding inhibition test using rabbit antiserum specific for the O antigen of *P. aeruginosa* (Denka Seiken Co.).

Agglutination test. This was done by mixing 100 μ l of a twofold serial dilution series (starting from 100 μ g ml⁻¹) of each human Mab and 50 μ l of 10⁹ formalin-killed *P. aeruginosa* ml⁻¹. The mixture was left for 3 h or overnight at room temperature, and the agglutination activity was expressed as the endpoint concentration of human Mab that gave agglutination.

Opsonophagocytic killing assay. *P. aeruginosa* strains were grown in heart infusion broth for 4 h at 37 °C. The cultures were suspended in Hanks' balanced salt solution containing 0.1% gelatin (HBSS-Gel). The concentration was adjusted spectrophotometrically to approximately 1 $\times$ 10⁷ c.f.u. ml⁻¹. Human polymorphonuclear leucocytes (PMN) were prepared from PBL obtained from a healthy donor. The concentration was adjusted to approximately 1 $\times$ 10⁷ cells per ml of HBSS-Gel. Human serum, obtained from the same donor, was absorbed for 1 h at 0 °C with each of the *P. aeruginosa* strains used for these experiments and diluted 1 in 40 with HBSS-Gel. This absorbed human serum was used as the complement source. The concentration of human Mab was adjusted to 40 μ g ml⁻¹ with HBSS-Gel. Portions (100 μ l) of each of the four components were mixed. Control tubes were run with each assay by omitting antibody, complement or PMN and substituting 100 μ l HBSS-Gel. The mixtures were held at 37 °C and constantly rotated end-over-end for 2 h. A small portion was removed, mixed with 10 vols distilled water to disrupt the PMN, serially diluted with saline and plated to determine the bacterial counts. The plates were incubated overnight at 37 °C, and the c.f.u. were counted. Mean c.f.u. and standard deviations (SD) were calculated from experiments done in triplicate. The *t*-test was applied to determine the significance of differences.

Protective activity of human Mabs against *P. aeruginosa* infection. Three experimental models were used.

(i) **Intraperitoneal (i.p.) administration of human Mab before i.p. challenge.** A group of 10 ICR mice (4 weeks old, males) was injected i.p. with 0.5 ml serially-diluted human Mab. Two hours after injection the animals were challenged i.p. with *P. aeruginosa* (2–4 LD₅₀). The LD₅₀ for strains 122, IT-2, IT-3, NC5 and IT-4 was calculated to be 1 $\times$ 10⁶, 5 $\times$ 10⁶, 2 $\times$ 10⁶, 5 $\times$ 10⁵ and 5 $\times$ 10⁶, respectively. The control mice were administered PBS. The animals were observed for 5 d after challenge.

(ii) **Administration of human Mab after subcutaneous (s.c.) challenge at a burned site.** This test was done as described by Stieritz & Holder (1975). A group of 10 ICR mice (4 weeks old, males) was anaesthetized by injecting Nembutal (Abbott Laboratories), and then a 3 cm² burn was created on the shaved back by burning for 10 s with a low flame of a gas burner. Immediately after burning, 10⁵ c.f.u. (20 LD₅₀) of *P. aeruginosa* NC5 in 0.2 ml 0.9% NaCl were injected s.c. into the burned site. Two hours after challenge, Mab P8-1 in 0.5 ml of PBS was injected intravenously. The animals were observed for 6 d after challenge.

(iii) **I.p. administration before i.p. challenge of leucopenic mice.** A group of 10 ICR mice (4 weeks old, males) was rendered leucopenic by three i.p. injections of cyclophosphamide (100–150 μ g per g weight of mouse) in 0.5 ml PBS on days 1, 3 and 5. Leucopenia ($\leq$ 1000 leucocytes per ml blood) was seen on day 5. The mice were injected i.p. with 0.5 ml of serially-diluted Mab P8-1 on day 5. One hour after injection, 10⁴ c.f.u. (10 LD₅₀) of *P. aeruginosa* NC5 in 0.5 ml 0.9% NaCl were injected i.p. The animals were observed for 5 d after challenge. The mean protective dose (PD₅₀), i.e. the dose of each Mab able to protect half the animals in the group, was calculated by probit analysis.

RESULTS

Production of human Mabs

Human lymphocytes (21 preparations of PBL, and 76 of tonsillar cells) were cultured *in vitro* in the presence of PWM for 6 d, and then the presence of antibody to *P. aeruginosa* LPS was examined by ELISA. No antibody response to *P. aeruginosa* LPS was obtained when PBL were cultured with PWM. Although most tonsillar cell cultures, such as nos 83 and 91, failed to produce specific antibody to *P. aeruginosa*, five tonsil samples did secrete specific IgG antibody to *P. aeruginosa* LPS (Table 1). They were nos 31 (specific for serotype E and G), 63 (specific for B), 66 (specific for A), 77 (specific for B and G) and 105 (specific for I). We used these five kinds of tonsillar cells for fusion. After 4 d culture of the selected lymphocytes in RPMI 1640 medium containing 10% FCS, the lymphocytes were washed with serum-free RPMI 1640 medium and then fused with the nonsecreting mouse myeloma P3U1. Fusion was performed 10 times in total throughout the experiment. Hybridomas grew in 2–3 weeks. About 5000 wells containing hybridomas were obtained. The efficiency of appearance of hybridomas was calculated to be 1×10^{-4} per lymphocyte.

The supernatant of each well was transferred to 96-well plates coated with a mixture of strains of the five major serotypes (see Methods) and assay was performed by ELISA using alkaline phosphatase-labelled anti-human IgG as the second antibody. Each hybridoma whose supernatant gave a positive score was cloned by limiting dilution. The supernatant of each cloned hybridoma was assayed again, and 25 clones were found to secrete antibody reactive with the mixture of strains. Their binding specificity to 14 different Homma standard serotype strains was next determined by ELISA. From among the 25 clones, we selected five human Mabs, P1-1, P5-1, P7-1, P8-1 and P10-1 (derived from tonsillar lymphocyte cultures 66, 31, 63, 77 and 105, respectively), because they were stably produced and their hybridomas grew well. The ELISA binding specificities of these five human Mabs are shown in Table 2. P1-1, P5-1, P7-1, P8-1 and P10-1 reacted specifically with serotypes A, E, B, G and I, respectively, and bound completely to the respective serotype strains tested. In total, more than 70 strains of different serotypes were examined. The same results as shown in Table 2 were obtained. The Mabs did not bind to other serotypes or to other Gram-negative bacterial strains (data not shown). Karyotypic analysis of the hybridoma cells demonstrated a polyploid chromosome number with chromosomes of both human and murine origin; for example, the mean chromosome number of P5-1 was 98, whereas for the parent P3U1 it was 58. Antisera directed against mouse heavy or light chains, which detect 1–10 ng murine immunoglobulin ml⁻¹ in ELISA, failed to react with the supernatants of these hybridomas. The concentration of each human Mab in 2 d cultures ranged from 10 to 20 µg ml⁻¹.

Recognition site

Each human Mab was transferred to an LPS-coated plate to examine its reactivity with LPS. All the human Mabs reacted with the corresponding serotypes. To identify further the recognition site, PAO-1 LPS (serotype B) was hydrolysed with acetic acid to yield A-PS, which was fractionated by Sephadex G-50 column chromatography (Fig. 1). Three peaks were detected by assay for hexose and hexosamine. Fraction I contained a large amount of hexosamine and a small amount of hexose, and O-antigen was eluted in this fraction as revealed by the inhibition test using rabbit IgG specific for the O-antigen of serotype B. Fraction II was composed of hexose, hexosamine and phosphorus (Sawada *et al.*, 1985*b*), and the core antigen was eluted in this fraction (Wilkinson & Galbraith, 1975). Fraction III consisted mainly of phosphorus and 2-keto-3-deoxyoctonate (Sawada *et al.*, 1985*b*). Activity blocking the binding of P7-1 to strain PAO-1 cells was found to be in fraction I (Fig. 1). Blocking activity was also observed in fraction I for each of P1-1, P5-1, P8-1 and P10-1 when the same experiment was done using the corresponding serotype LPS.

Agglutination activity

Agglutination activity was examined in 96-U-well plates. P1-1, P5-1, P7-1, P8-1 and P10-1 agglutinated the respective homologous strains: 122 (serotype A), IT-2 (E), IT-3 (B), NC5 (G)

Table 1. *P. aeruginosa*-specific IgG production by PWM-stimulated tonsillar cells

Tonsillar cell culture no.	Specific IgG production* to serotype				
	A	E	B	G	I
31	0.1	1.4	0.0	1.3	0.0
63	0.0	0.1	1.2	0.1	0.0
66	1.2	0.2	0.2	0.3	0.1
77	0.1	0.1	1.2	1.1	0.0
83	0.2	0.0	0.0	0.1	0.0
91	0.1	0.0	0.0	0.0	0.1
105	0.0	0.1	0.1	0.0	1.3

* ELISA score, expressed as A_{405} after 30 min incubation.Table 2. Binding of Mabs to *P. aeruginosa* strains of different serotype

In all assays, 5×10^6 formalin-killed whole cells were used as antigen and 1 μ g Mab as antibody. Results are recorded as A_{405} after 60 min. The background reading (0.1) was subtracted from each reading. A blank space indicates a zero reading.

Strain	ELISA reading					Strain	ELISA reading				
	P1-1	P5-1	P7-1	P8-1	P10-1		P1-1	P5-1	P7-1	P8-1	P10-1
<i>Serotype A</i>						<i>Serotype F</i>					
ATCC 27577	1.5					ATCC 27582					
C-8	1.7					64					
122	1.8					<i>Serotype G</i>					
N-1	1.8					ATCC 27584				1.5	
<i>Serotype B</i>						IT-1				1.9	
ATCC 27578			1.3			NC5				1.7	
ATCC 27583			1.5			O7				1.8	
ATCC 27589			1.2			<i>Serotype H</i>					
PAO-1			1.9			ATCC 27585					
97			1.9			IT-5					
IT-3			1.9			1258					
IT-7			2.0			<i>Serotype I</i>					
<i>Serotype C</i>						ATCC 27586					1.6
ATCC 27579						IT-4					1.6
IT-6						38					1.7
<i>Serotype E</i>											
ATCC 27581		1.5									
IT-2		1.9									
N-2		1.8									
O101		1.9									
C-12		1.8									

and IT-4 (I). The endpoint antibody concentrations of P1-1, P5-1, P8-1 and P10-1 causing agglutination were calculated to be 1.5, 3.1, 6.2 and 6.2 μ g ml⁻¹, respectively, but a higher concentration (25.0 μ g ml⁻¹) of P7-1 was necessary to cause agglutination.

Opsonophagocytic killing activity

Fig. 2 shows the viable counts after 2 h phagocytosis, with Mabs P1-1, P7-1, P8-1 and P5-1. When all the components (PMN, Mab, complement) were incubated, all four Mabs showed more than 90% reduction of the c.f.u., to values significantly lower than those of control experiments without Mab ($P < 0.01$). Mabs P1-1 and P7-1 showed killing activity against strains 122 and IT-3, respectively without complement ($P < 0.01$). When P7-1 (serotype B specific) was added instead of P8-1 (G specific), no opsonophagocytic killing activity of the serotype G strain IT-1 was observed (Fig. 2 c). The other serotype-specific human Mab, P10-1, similarly showed opsonophagocytic killing activity against strain IT-4 (serotype I) (data not shown).

Fig. 1. Elution profile of A-PS released from *P. aeruginosa* strain PAO-1 (serotype B) after hydrolysis with 1% acetic acid at 100 °C for 2 h. The material was eluted from a Sephadex G-50 column (33 × 1.5 cm) with 50 mM-pyridine/acetate buffer, pH 4.2. Fractions of 1 ml were collected, and portions were assayed for hexose (○), hexosamine (●) and percentage residual activity of human Mab P7-1 (△). O-antigen was eluted in the void volume (as revealed by the inhibition test using rabbit antiserum to O-antigen of *P. aeruginosa* serotype B).

Fig. 2. Opsonophagocytic killing of *P. aeruginosa* by human Mabs. Control tubes were run with each assay by omitting Mab, complement (C) or PMN as indicated, and substituting 100 µl HBSS-Gel. The plates were incubated overnight at 37 °C, and c.f.u. were counted the next day. Results are the mean of three independent observations ± SD. The following human Mabs and *P. aeruginosa* strains were used: (a) P1-1, 122; (b) P7-1, IT-3; (c) P8-1, IT-1 (*P7-1 added instead of P8-1); (d) P5-1, IT-2.

In vivo protection

Four-week-old male ICR mice were injected i.p. with human Mabs, and 2 h later they were challenged by i.p. injection of virulent strains which secreted exotoxin A, elastase and/or protease (Table 3). On the basis of their PD₅₀ values, Mabs P1-1, P5-1, P8-1 and P10-1 were highly active, while P7-1 was moderately active. P5-1 and P8-1 did not protect the mice from challenge with mismatched serotype strains IT-4 and 122, respectively; thus these Mabs conferred serotype-specific immunoprotection. Table 3 also shows that protection was achieved even when exoprotein-producing strains were used for the challenge.

We estimated the protective activity of P8-1 against infection in burned and leukopenic mice. In the burned mouse model, the PD₅₀ value of P8-1 against infection was calculated to be 0.6 µg

Table 3. *Protective activity of human Mabs against i.p. infection by P. aeruginosa*

Mab	Serotype specificity	Challenge strain (serotype)	Mab PD ₅₀ (µg per mouse)*	Exoproduct synthesis†		
				ExA	Ela	Pro
P1-1	A	122 (A)	0.3 (0.2–0.8)	NT	NT	NT
P5-1	E	IT-2 (E)	0.5 (0.3–0.8)	+	+	–
P5-1	E	IT-4 (I)	>100	+	+	+
P8-1	G	NC5 (G)	0.8 (0.4–2.0)	+	+	+
P8-1	G	122 (A)	>100	NT	NT	NT
P7-1	B	IT-3 (B)	4.2 (1.8–9.8)	+	+	+
P10-1	I	IT-4 (I)	0.5 (0.2–1.2)	+	+	+

NT, Not tested.

* The corresponding 95% confidence limits are given in parenthesis below each PD₅₀.

† ExA, exotoxin A; Ela, elastase; Pro, protease.

Table 4. *Summary of characteristics of anti-P. aeruginosa human Mabs*

	P1-1	P5-1	P7-1	P8-1	P10-1
Isotype	IgG2, κ	IgG2, λ	IgG2, λ	IgG2, λ	IgG2, κ
Antigen	O	O	O	O	O
Binding specificity	A	E	B	G	I
Agglutination activity	+	+	+	+	+
Opsonophagocytic killing	+	+	+	+	+
<i>In vivo</i> (mouse) protection	++	++	+	++	++
Productivity (µg per 10 ⁶ cells in 24 h)	10	12	15	20	10
Stability (months)	>18	>20	>15	>16	>14
Growth in serum-free culture	Yes	Yes	Yes	Yes	Yes

(95% confidence limits 0.4–0.9 µg) per mouse, whereas that of NHIgG was 1270 µg (683–2390 µg) per mouse. In the leukopenic mouse model, the PD₅₀ values of P8-1 and NHIgG were 0.5 µg (0.3–1.3 µg) and 1320 µg (497–3500 µg) per mouse, respectively.

Mab productivity and stability

Because human × mouse hybridomas tend to stop secreting human Mabs, some hybridomas that seemed to be promising were further cloned to establish stable clones. Four or five additional cloning steps were usually needed. These established hybridomas have been producing human Mabs for more than 14 months. Their characteristics are summarized in Table 4. All of them have been adapted to grow in serum-free medium and have been producing 10–20 µg human Mab per 10⁶ cells in 24 h for more than 3 months.

DISCUSSION

Formation of hybridomas (Croce *et al.*, 1980a; Schwaber, 1975) and transformation with Epstein–Barr virus (EBV) (Kozbor *et al.*, 1979; Boylston *et al.*, 1980) are well-known methods for production of human Mabs. Since most EBV-transformed cells from normal adults produce IgM (Brown & Miller, 1982), we preferred the hybridoma method to obtain IgG producers because IgG is expected to diffuse better in tissues and is much easier to purify than IgM. The lack of any suitable human myeloma cell line having a high fusion efficiency required us to use a mouse myeloma instead.

We obtained human Mabs which bound with LPS by fusing nonsecreting mouse myeloma cells and human lymphocytes. We thought it important to select a human lymphocyte source capable of yielding antigen-specific hybridomas through fusion with mouse myeloma cells. For this purpose, we examined the supernatants of PWM-stimulated cultures of PBL and tonsillar cells from 21 and 76 patients, respectively. No antibody response was observed with any of the PBL sources. One reason for this may be the small number of B lymphocytes in PBL. Only five of the 76 tonsillar cell cultures produced IgG antibody specific for *P. aeruginosa* LPS. Twenty-five hybridomas producing antibody against *P. aeruginosa* were obtained from these five tonsillar cell cultures. The positive response in ELISA of PWM-stimulated lymphocytes could be a reflection of the increased number of antigen-specific B cells in the lymphocyte source. We think that this method can probably be applied to obtain human Mabs specific for other antigens. Regarding the IgG isotype, the H chains of each of the five human Mabs were IgG2, and three of them had λ L chains. Besides the five human Mabs described in this report, we have developed more than 20 human Mabs specific for *P. aeruginosa* LPS. All of them are IgG2, and 80% of them contain λ chains. Since earlier studies suggested that human antibodies to bacterial polysaccharide are largely restricted to the IgG2 subclass (Yount *et al.*, 1968; Siber *et al.*, 1980), the fact that all the H chains were IgG2 was thought to be a reflection of human immunity. The high frequency of λ type seems to arise from the human $\times$ mouse hybridoma method itself: because hybridomas derived from human lymphocytes and mouse myeloma cells easily segregate human chromosome 2 (Croce *et al.*, 1980b), which carries the DNA coding for the κ chain, κ -chain-producing hybridomas are difficult to establish as stable clones. It has been said that the crucial problem with human $\times$ mouse hybridomas lies in their instability in terms of long-term secretion of antibody (Croce *et al.*, 1980b; Ruddle, 1973). We solved this problem by repeated cloning. Stable clones secreting 10–20 μ g human Mab per 10^6 cells in 24 h for more than 3 months in serum-free medium can be established, as demonstrated in this report. Our colleagues have also reported the successful establishment of stable human $\times$ mouse hybridomas showing long-term secretion of human Mabs against Herpes simplex virus (Masuho *et al.*, 1986) and cytomegalovirus (Matsumoto *et al.*, 1986). These results suggest that human $\times$ mouse hybridomas are suitable for stable production of human Mabs.

Antibodies reactive with the O-antigen of LPS act as opsonins, increasing the efficiency of phagocytosis and killing of *P. aeruginosa* by host phagocytes (Young, 1972), and they have the ability to protect mice against lethal *Pseudomonas* infection (Cryz *et al.*, 1983). These results were obtained by using polyclonal antibodies. Since an Mab recognizes only one epitope on the antigen molecule, formation of a dense antigen-antibody lattice complex is thought to be difficult. Hence, there might be some doubt whether an Mab could augment bacterial agglutination, opsonization and *in vivo* protective activity. We prepared murine Mabs specific for Homma serotype 7, and found them to have protective activity against infection with *P. aeruginosa* (Sawada *et al.*, 1984). Two recent reports have documented the preparation of serotype-specific Mabs against *P. aeruginosa* LPS. Stoll *et al.* (1986) reported a murine Mab (IgG1) specific for Fisher IT-1 strain that has opsonic activity and protects burned mice against s.c. challenge with homologous strains. Barclay *et al.* (1986) described 18 murine Mabs of isotype IgG1, IgG2b, IgG3 or IgM that protected mice at a very low dose. Especially in the latter report, the amount of antibody necessary to show protective activity was calculated to be less than 10 ng per mouse, which represents 10- to 100-fold more potent protective activity than that of our murine Mabs. We think, however, that the protective activities of their Mabs are extraordinarily high, and we wonder why the protective titre of the Mabs exceeded their ELISA titre. As discussed above, murine Mabs specific for O-antigen have been proved to be protective in mice, but they can not be administered to man because of their immunogenicity. For this reason, we attempted to develop human Mabs.

Human Mabs P1-1, P5-1, P7-1, P8-1 and P10-1 recognized O-antigens of the five major serotypes of *P. aeruginosa* because they bound serotype-specifically with LPS, and their binding to *P. aeruginosa* was inhibited by Sephadex G-50 fraction I (see Fig. 1), which is considered to contain O-antigen. Fraction I contained a large amount of hexosamine and a small amount of

hexose, findings in accordance with the nature of the O-antigen (Wilkinson, 1983). Young (1972) reported an important role for the opsonic activity of human antibody, which was serotype-specific. All of our human Mabs showed opsonic activity. High agglutinating activity was also observed for all the Mabs except P7-1, which had moderate activity. All the Mabs had high protective activity against peritoneal infection in normal mice, again except for P7-1, which was moderately active. These results suggest that, besides opsonic activity, human Mabs having high agglutinating activity appear to be more protective than those with moderate agglutinating activity. One possible explanation could be that the binding sites of the moderately agglutinating Mab P7-1 are deeper in the cell wall structure than those of the highly agglutinating Mabs, i.e. the highly agglutinating Mabs bind to the exposed end of the O side chain of LPS, whereas the moderately agglutinating P7-1 binds to the base of the O side chain. In fact, since P7-1 reacted with three sub-serotype strains (ATCC 27578, 27583 and 27589) which belong to serotype B, it is possible that P7-1 recognizes the common antigen of serotype B, which is at the base of the O side chain. Because the O side chain is long, an antibody molecule bound to the deep site would not protrude enough to bind to a corresponding antigenic site on another bacterial cell, and thus sufficient 'bridges' for agglutination would not be formed between the bacterial cells. An alternative explanation for the moderate protective activity of P7-1 might lie in the binding affinity. We previously reported that a human Mab (P3D9) specific for Homma serotype 5 was nonagglutinating and protective (Sawada *et al.*, 1985*a*). In the work described in that report, P3D9 was diluted from 1 µg antibody ml⁻¹ to measure the agglutination activity because we expected it to show high agglutination activity. After publication, when we reexamined the agglutination activity, P3D9 alone agglutinated strain N-2 at a concentration of 6.2–12.4 µg ml⁻¹. In contrast to the original report, P3D9 was thus both agglutinating and protective.

Hyperimmune intravenous immunoglobulin preparations which are prepared from high-titre plasma have now been developed. The protective activity of a high-titre preparation against *P. aeruginosa* was reported to be up to five times higher than that of conventional intravenous IgG (Collins & Roby, 1984). Since the human Mabs described in this report show ~2000 times higher protective activity than conventional NHlgG, passive immunization with human Mabs is surmised to be capable of achieving prophylaxis and treatment of *Pseudomonas* diseases.

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