J. Microbiol. Biotechnol. (2003), 13(3), 444–450

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Protection of Rabbits from Experimental *Pseudomonas* Endophthalmitis by Human Anti-*P. aeruginosa* Outer Membrane Proteins IgG

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Received: January 13, 2003
Accepted: April 10, 2003

Abstract In order to develop an effective means to treat *P. aeruginosa* infections, we have purified *P. aeruginosa* outer membrane proteins (OMPs)-specific human IgG antibody. In this study, we investigated the protective activity of the purified anti-OMPs IgG against *P. aeruginosa* infection in a rabbit endophthalmitis model. Rabbits were inoculated by an intravitreal injection with *P. aeruginosa*, and treated with a single dose of 1 mg anti-*P. aeruginosa* OMPs IgG. All the control rabbits predominantly developed edematous responses and opacity in the eyes, but the rabbits treated with the antibody showed only very limited degree of edema. Aliquots of the vitreous humor were extracted and analyzed for the number of viable bacteria and endotoxin level. The results showed that the anti-OMPs IgG significantly reduced the bacterial count compared with the control group, and that the endotoxin level of the vitreous from the IgG-treated rabbits was more than 70-fold lower 6 h after the administration than the control animals. These data suggested that the anti-*P. aeruginosa* OMPs IgG is effective in inhibiting the bacterial growth and thereby in reducing endotoxin levels in the vitreous, warranting further development of the anti-*P. aeruginosa* OMPs IgG as a therapeutic means for treating *Pseudomonas* endophthalmitis.

Key words: *P. aeruginosa*, human anti-OMPs IgG, endophthalmitis

Bacterial endophthalmitis occurs when a complication of intraocular surgery or trauma occurs [1, 2, 14], and a direct intravitreal injection of antibiotics is a general treatment to manage this disease due to poor intravitreal penetration of drugs that are administered topically or parenterally [4, 5]. Its prognosis, however, is generally very poor even with prompt treatment, often resulting in fatal vision loss, which is largely caused by tissue damage from inflammatory responses [2]. Corticosteroids are administered simultaneously with antibiotics to reduce inflammatory exudation and granular tissue formation, but the beneficial effect of its use is still controversial [6, 18, 26].

P. aeruginosa is a Gram-negative human pathogen that infects people with defective immune systems by severe burns, immunosuppressive or cancer chemotherapy, eventually leading to a fatal septic shock [10, 20, 28]. It also causes recurrent respiratory infections and subsequent lung collapse in cystic fibrosis patients, which are the primary cause of death in these patients [19]. Even with effective antibiotics, *P. aeruginosa* has remained as one of the most frequent isolates from hospital-acquired infections due to its natural resistance to most commercially available antibiotics and frequent occurrences of new resistant strains [27–29]. *P. aeruginosa* is also one of the major causative agents of bacterial endophthalmitis, which is often difficult to treat and leaves poor outcome compared to other bacterial endophthalmitis [3, 24]. In a study using a rabbit endophthalmitis model, it was shown that early administration of commonly used antibiotics such as ciprofloxacin, gentamicin, and imipenem inhibited bacterial growth; however, delayed treatment showed only a little effect [7].

A *P. aeruginosa* vaccine composed of outer membrane proteins (OMPs) has been developed by CJ Corp. (Icheon, Korea) [22] and shown to be safe and capable of eliciting antibodies with protective efficacy in humans as well as in experimental animals [11, 12, 17, 22, 23]. In an attempt to obtain therapeutic antibody for treatment of *P. aeruginosa* infection, human IgG antibody specific to *P. aeruginosa* OMPs was purified by a large-scale affinity chromatography by using the *P. aeruginosa* OMPs as affinity ligands [16]. The purified anti-OMPs IgG retained opsonophagocytic killing capacity and protective activity against experimental infections with *P. aeruginosa* in mice [16]. It was also

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shown to be cross-protective against heterologous LPS immunotype strains of *P. aeruginosa* [15].

In the present study, we investigated the protective efficacy of the human anti-*P. aeruginosa* OMPs IgG against *Pseudomonas* endophthalmitis in an animal model. It was found that the endotoxin level as well as bacterial count in vitreous was greatly reduced when human anti-*P. aeruginosa* OMPs IgG was administered, suggesting that the human IgG provides protection against *Pseudomonas* endophthalmitis.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

P. aeruginosa strains used to prepare affinity ligands were CFCPA10142, CFCPA20215, CFCPA30720, and CFCPA60534 (Fisher-Devlin Immunotypes 1, 2, 3, and 6, respectively) [22]. *P. aeruginosa* strain 170043 used for an endophthalmitis model was a clinical isolate from E. Stanislavsky (Mechinikov Institute, Russia) and belongs to Fisher-Devlin Immunotype 4. *Acinetobacter calcoaceticus* Ac-54, *Enterobacter cloacae* C2161, *Proteus vulgaris* ATCC13315, *Escherichia coli* C4002, *Klebsiella pneumoniae* C1040, and *Serratia marcescens* T55 were obtained from the Institute of Science and Technology, CJ Corp.

All bacterial strains were grown at 37°C on tryptic soy broth (TSB; Becton Dickinson Co., Sparks, MD, U.S.A.) agar plates or liquid media. For assays, *P. aeruginosa* strains were prepared by inoculating an overnight culture into fresh TSB liquid medium and growing on a rotary shaker at 200 rpm for 6 h. Bacterial cells were harvested from the culture by centrifugation, washed with phosphate-buffered saline (PBS, pH7.2), and resuspended in 0.9% NaCl solution. Cell preparations were adjusted to optical density of 0.6 at 600 nm, which yields approximately 6.8×10^8 colony forming units (CFU)/ml. Cells were further diluted to an appropriate concentration with saline before use. Actual numbers of viable bacteria cells were determined each time by spreading diluted cell preparations on agar plates and counting colonies grown overnight at 37°C.

Preparation of Bacterial OMPs

Bacterial OMPs were isolated as described previously [22]. Briefly, bacteria cells cultured in liquid media were harvested by using the Sartocon II system (Sartorius, Goettingen, Germany) or centrifugation at 8,000 ×g for 20 min at 4°C. The cell pellet was treated with 3 volumes of acetone three times, and resuspended in 10 mM PBS, pH 7.2. OMPs were extracted by stirring at 50-200 rpm with a homogenizer for 10 min at 4°C, and proteins ranging from 10 to 100 kDa were fractionated by ultrafiltration, followed by ultracentrifugation at 180,000 ×g for 3 h at 4°C. The supernatant was sterilized by filtration through a 0.22 μm membrane.

Preparation of Human IgG Antibodies

Anti-*P. aeruginosa* OMPs IgG was prepared from a normal human plasma pool by a large-scale purification process [16]. An affinity column was prepared by covalently coupling CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) with a mixture of OMPs isolated from the four *P. aeruginosa* strains. A plasma pool of normal blood donors supplied by the Blood Center of the Korea Red Cross (Suwon, Korea) was defibrinated and loaded onto the affinity column. After removing unbound materials by extensively washing with PBS, the bound proteins were eluted with 0.2 M acetate buffer (pH 3.5) containing 0.15 M NaCl and subjected to further purification by using a protein A column. The purified IgG was concentrated by ultrafiltration on a Prep/Scale Tangential Filtration Flow membrane (MW cut-off 30 kDa, Millipore, U.S.A.). Normal human serum IgG was purified from the human plasma pool using a protein A column. Protein content in antibody preparations was determined using a Bradford protein assay kit (BioRad, Hercules, CA, U.S.A.).

Enzyme-Linked Immunosorbent Assay (ELISA)

Reactivity of human antibodies to various bacterial OMPs was determined by ELISA. Wells of 96-well polystyrene microtiter plates were coated overnight at 4°C with 100 μl of 20 μg/ml OMPs solution and washed three times with PBS containing 0.05% Tween 20. After blocking with 1% bovine serum albumin, the plate was incubated with 100 μl of two-fold serial dilutions of purified IgG for 2 h at 37°C. After five rinses, 50 μl of goat anti-human IgG antibody conjugated with HRP was added and incubated for 1 h, followed by development with *o*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO, U.S.A.) as a chromogenic substrate. The reaction was stopped by adding 50 μl of 0.2 N H_2SO_4 and absorbance was measured spectrophotometrically at 490 nm by using an ELISA reader.

Rabbit Endophthalmitis Model

Experimental endophthalmitis was induced in New Zealand white rabbits purchased from Samyook Laboratory Animals (Seoul, Korea) weighing 2.0-2.5 kg. Rabbits were examined by slit-lamp and indirect ophthalmoscopy before undergoing experiments. The rabbits were anesthetized by initial intramuscular injection of ketamine (5 mg/kg body weight) and xylazine (2 mg/kg body weight), and given half doses at every hour throughout the experiments. Topical 1% tropicamide and 2.5% phenylephrine hydrochloride were applied before intravitreal inoculation of bacteria, antibody treatment, and sampling. Each eye of six rabbits was

inoculated into vitreous cavity 2 mm posterior to the limbus with an 100 μl aliquot containing 1×10^4 CFU of *P. aeruginosa* 170043. At 3 h post-infection, 1 mg of anti-OMPs IgG in 100 μl saline was administered to each eye of three rabbits by a direct intravitreal injection. For the other three rabbits, 100 μl saline was given to each eye. One hundred microliters of the vitreous humor was extracted from the alternate eyes of each rabbit using a 28-guage needle inserted through the pars plana before bacterial inoculation, before and after the antibody treatment, and at 0.5, 1, 3, and 6 h after antibody treatment.

Bacterial Enumeration

For enumeration of viable bacteria, 20 μl of rabbit vitreous was diluted five times with PBS, and 50 μl was spread on TSB agar plates. After the overnight incubation at 37°C, the number of colonies grown was counted, and the result was used to determine the number of bacteria per ml of the vitreous humor. All samples were plated in duplicate, and the average of the results was used for calculations.

Endotoxin Assay

Endotoxin level of the vitreous humor was measured by the chromogenic Limulus Amebocyte Lysate Test using the QCL-1000 kit (Biowhittaker Inc., Walkersville, MD U.S.A.). A standard curve was drawn using the standard endotoxin supplied by the manufacturer. Vitreous samples (20 μl) taken from rabbits were mixed with 80 μl of PBS. Fifty microliters of the mixture were further diluted 10 times with LPS-free water in duplicate and assayed for the endotoxin level. The results were converted to the concentration of endotoxin per ml of the vitreous humor.

RESULTS

Alleviation of *Pseudomonas* Endophthalmitis Symptoms by Human Anti-*P. aeruginosa* OMPs IgG in Rabbits

To investigate the effect of the anti-*P. aeruginosa* OMPs IgG on the *Pseudomonas* endophthalmitis, a rabbit endophthalmitis model was used in which rabbits were infected intravitreally with a virulent strain *P. aeruginosa* 170043. The time for antibody treatment and bacterial dose used in the study were chosen based on our preliminary results to obtain significant growth of bacteria in the vitreous. Intravitreal inoculation of rabbits with *P. aeruginosa* did not cause any apparent symptoms until 3 h after infection. Five hours later, i.e., at 8 h after infection, however, all of the control rabbits infected with *P. aeruginosa* and given saline predominantly developed edematous responses (Figs. 1A and 1B), and the eyes showed chemosis and vitreous opacity. In contrast, the eyes of the rabbits treated with the antibody showed only very limited degree of edema (Figs. 1C and 1D). These

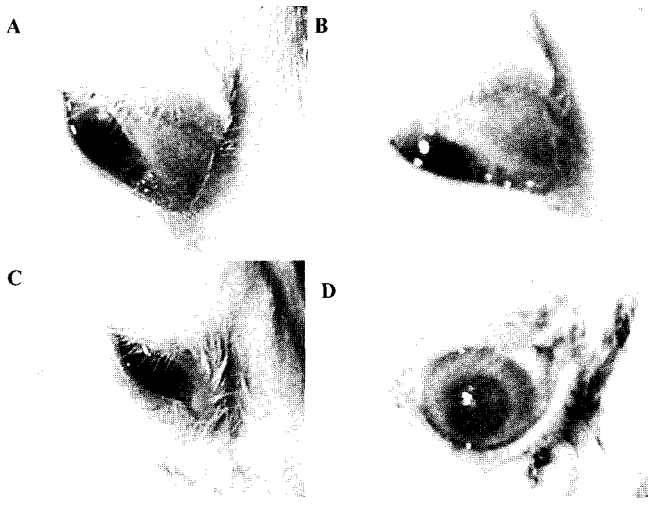

Fig. 1. Effect of human anti-*P. aeruginosa* OMPs IgG on rabbit *Pseudomonas* endophthalmitis.
Rabbits were inoculated into vitreous cavity 2 mm posterior to the limbus with *P. aeruginosa* (1×10^4 CFU), and 3 h later received 1 mg of anti-OMPs IgG or saline. The photograph was taken at 8 h post-infection. A and B, rabbit eyes inoculated with *P. aeruginosa* and given saline; C and D, rabbit eyes inoculated with *P. aeruginosa* and treated with anti-OMPs IgG.

results indicated that administration of anti-*P. aeruginosa* OMPs IgG greatly relieved the inflammatory responses of the eyes infected with *P. aeruginosa*.

Bacterial Growth Inhibition by Human Anti-*P. aeruginosa* OMPs IgG in Rabbit Vitreous

The number of viable bacteria in the rabbit vitreous was determined. The vitreous was confirmed to be sterile before bacterial inoculation (Table 1). All the six rabbits were given the same size of inoculum, but for some unknown reason, one of the control rabbits had a much higher bacteria number 3 h after inoculation, which resulted in a higher count for the control group. However, there was no statistically significant difference between the two groups ($p=0.08$). The discrepancy seemed to be due to idiosyncrasy of the rabbit No. 2, for the vitreous samples were taken from the alternate eyes of each rabbit and the rabbit No. 2 showed a higher count at all time points compared to the other two control rabbits.

The bacterial count of the control group continued to increase up to 9 h post-infection when the animals were sacrificed. In the IgG-treated group, meanwhile, there was a sharp decline in bacterial numbers following the intravitreal injection of anti-*Pseudomonas* OMPs IgG, and the bacterial count was reduced by 85% at 30 min after the antibody was administered. Bacterial regrowth was observed at 6 h after the administration, most likely due to a decline in the vitreal IgG concentration. The bacterial count reached the level similar to that observed before the antibody-treatment, and was still significantly lower than the control group.

Table 1. Inhibition of *P. aeruginosa* growth in rabbit vitreous by human anti-*P. aeruginosa* OMPs IgG.

Time	Number of viable bacteria in rabbit vitreous ($\times 10^2$ CFU/ml)[a]								p-value[b]
	Control rabbits				IgG-treated rabbits				
	#1	#2	#3	Mean±SD	#1	#2	#3	Mean±SD	
-3 h[c]	0	0	0	0	0	0	0	0	
0 h[d]	192	480	99	257±199	211	184	93	163±62	0.08
0.5 h	156	500	82	246±223	29	33	12	25±11	<0.05
1 h	191	460	94	248±189	15	62	36	38±23	<0.05
3 h	359	676	458	498±162	48	72	47	56±14	<0.001
6 h	>800	>800	>800	>800	139	267	89	165±92	<0.001

[a]The number of bacteria in vitreous samples was counted by a direct plating method and converted to colony forming unit per 0.1 ml of vitreous.
[b]IgG-treated group *vs.* control group.
[c]Vitreous samples were taken before *P. aeruginosa* inoculation.
[d]Vitreous samples were taken and then IgG or saline was administered to the eyes.

Effect of the Anti-OMPs IgG on Vitreous Endotoxin Level

Next, the endotoxin level of the rabbit vitreous was assessed. No endotoxin was detected before the bacterial inoculation (Table 2). At 3 h after infection, the vitreal endotoxin level was found to be similar between the two groups. In control rabbits, the level began to rise and doubled by 6 h post-infection. But, as the bacteria began to grow exponentially by 9 h post-infection, it dramatically increased, reaching over 1 mg ml^{-1} vitreous. In contrast, in the anti-*P. aeruginosa* OMPs IgG-treated group, the endotoxin level significantly decreased to the concentration of 2.2 μg/ml at 30 min after the IgG-treatment and remained low until 3 h after the treatment. At 6 h post-treatment, as bacterial growth resumed, the endotoxin level also began to rise, still being 70-fold lower than the control group at the same time point. These results indicated that the human anti-*P. aeruginosa* OMPs IgG is capable of reducing the endotoxin level as well as inhibiting bacterial growth.

Cross-Reactivity of the Anti-OMPs IgG to Gram-Negative Pathogens

Reactivity of the anti-*P. aeruginosa* OMPs IgG to various Gram-negative pathogenic bacteria was evaluated by measuring ELISA titers against OMPs isolated from each microorganism. As seen in Fig. 2, the anti-OMPs IgG showed various degrees of reactivity to all Gram-negative bacteria tested. *P. vulgaris* and *E. coli* were the highest, and *S. marcescens* were the lowest in binding activity among the bacteria. For making a comparison, human total serum IgG purified from normal human plasma was also analyzed. The total serum IgG reacted significantly with none of the pathogens tested, and the titer of the anti-OMPs IgG was found to be at least 16-fold higher than the total serum IgG, indicating that the anti-*P. aeruginosa* OMPs IgG is highly cross-reactive with the OMPs from the Gram-negative pathogens. ELISA titers of the anti-OMPs IgG against whole cells of the pathogens also showed similar patterns but the difference between the anti-OMPs IgG and the total serum IgG was not as high, probably due to LPS-reactive antibody present in the total serum IgG (data not shown).

DISCUSSION

Ocular infection by Gram-negative bacterial pathogens has a very poor prognosis, and even with prompt antibiotic

Table 2. Measurement of endotoxin levels of the rabbit vitreous.

Time	Endotoxin level of the vitreous (μg/ml)								p-value[a]
	Control rabbits				IgG-treated rabbits				
	#1	#2	#3	Mean±SD	#1	#2	#3	Mean ± SD	
-3 h[b]	0	0	0	0	0	0	0	0	
0 h[c]	13.5	21.5	11.0	15.3±5.5	18.5	18.5	13.5	16.8±2.9	0.7
0.5 h	10.5	19.0	7.5	12.3±6.0	3.5	1.8	1.3	2.2±1.2	<0.05
1 h	15.5	25.0	30.5	23.7±7.6	0.8	6.8	2.3	3.3±3.1	<0.001
3 h	33.5	44.0	31.5	36.3±6.7	0.7	5.0	2.5	2.7±2.2	<0.001
6 h	>1,000	>1,000	>1,000	>1,000	9.0	17.0	16.0	14.0±4.4	<0.001

[a]IgG-treated group *vs.* control group.
[b]Vitreous samples were taken before *P. aeruginosa* inoculation.
[c]Vitreous humor samples were taken and then IgG or saline was administered to the eyes.

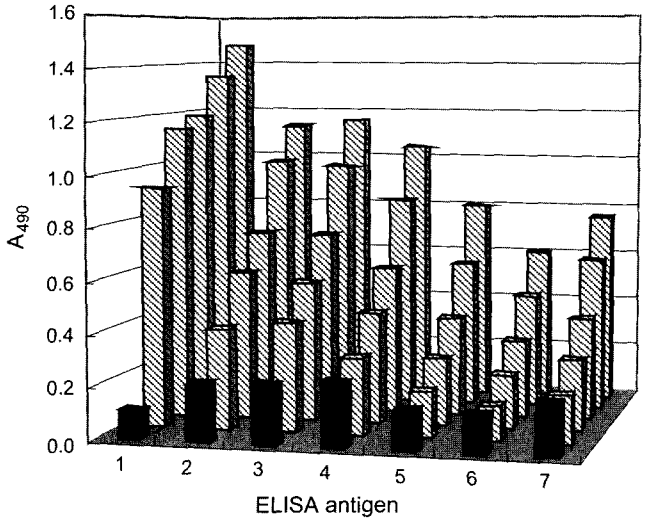

Fig. 2. Reactivity of human anti-*P. aeruginosa* OMPs IgG to pathogenic Gram-negative bacteria determined by ELISA.
Wells of microtiter plates were coated with 2 μg of OMPs isolated from various Gram-negative bacteria, and two-fold serial dilutions of anti-*P. aeruginosa* OMPs IgG at an initial concentration of 100 μg/ml were added to each well from back to front (▨). Binding activity of human total serum IgG at 100 μg/ml is shown for comparison (■). In this representative experiment, each ELISA value indicates the mean of two determinations. 1, Mixture of OMPs from the four *P. aeruginosa* strains; 2, *P. vulgaris* ATCC13315; 3, *E. coli* C4002; 4, *E. cloacae* C2161; 5, *K. pneumoniae* C1040; 6, *S. marcescens* T55; 7, *A. calcoaceticus* Ac-54.

treatment, fatal visual loss is not rare [24]. Such events are mediated by host inflammatory responses, which are up-regulated by the bacterial products. Intravitreal administration of antibiotics reduces the number of viable bacteria by bactericidal activity, but even after the bacterial death, toxic materials released from the microorganisms often cause damage to the eyes by stimulating secretion of various cellular immune mediators, which, in turn, recruit inflammatory cells, leading to devastating outcome. Therefore, successful eradication of infecting bacteria may not be enough to obtain satisfactory clinical results. To suppress the host-inflammatory responses and preserve ocular structure, corticosteroid is often co-administered with antibiotics [6, 18, 26]. In a study by Kim *et al.* [13] using a rabbit *P. aeruginosa* endophthalmitis model, however, it was found that co-treatment of dexamethasone with ciprofloxacine resulted in a higher bacterial load in vitreous than ciprofloxacine alone, possibly by interfering with immunologic responses. It was also found that intraocular administration of dexamethasone with antibiotics was associated with an increase in inflammatory scores, opaque cornea development, and retinal necrosis when compared with antibiotic injection alone [21].

In this study, we demonstrated that the human IgG antibodies specific to *P. aeruginosa* OMPs was effective not only in killing *P. aeruginosa* but also in reducing the level of endotoxin, which is a known major factor of Gram-negative bacteria causing inflammatory reactions [9]. In our previous study, it was found that upon intravenous inoculation with *P. aeruginosa*, the blood endotoxin level was significantly lower in the rats immunized with the *P. aeruginosa* OMPs compared to the antibiotic-treated rats, whose endotoxin levels were similar to those of untreated animals (unpublished data). We believe that the opsonophagocytic activity of antibody facilitates bacterial phagocytosis and that no LPS is released into the blood stream upon bacterial death, unlike the direct killing process by antibiotics. The data presented in this study suggest a great advantage of using opsonic antibody to treat bacterial endophthalmitis where inflammatory responses lead to fatal results. Beneficial effects of co-administration of antibody with antibiotics have been shown previously in the study by Felts *et al.* [8] where the co-treatment of burn wound infections with antibiotics and immunoglobulins improved the protective effect of the antibiotics against the antibiotic-resistant *P. aeruginosa*.

In this study, bacterial regrowth was observed 6 h after the antibody treatment. The bacterial count returned to the initial level, which was followed by a rise in vitreous endotoxin level. The half-life of the human IgG administered to the rabbit vitreous has not been determined, but our previous study showed that the human anti-*P. aeruginosa* OMPs IgG was quite stable *in vivo*, with the half-life of 40 h, when administered to rabbits by means of intravenous infusion, and that the antibody was detected in the rabbit sera until 7 days after the infusion, which is similar to the results reported by others [25]. It is likely that the half-life of the human IgG would be longer in humans than in rabbits. Based on these data, we suggest that an initial treatment with anti-*P. aeruginosa* OMPs IgG to keep the viable bacteria and endotoxin levels low, followed by antibiotic treatment to keep the bacteria from regrowing, would give the best outcome.

Unlike LPS, which is highly type-specific, OMPs are antigenically cross-reactive among all heterologous serotype *P. aeruginosa* strains. The anti-*P. aeruginosa* OMPs IgG exhibited opsonophagocytic-killing activity for different Fisher-Devlin IT strains of *P. aeruginosa* [15]. It also had a capability to protect mice from various strains of *P. aeruginosa* in passive protection assays [15]. These data clearly indicate that the human IgG could afford protection from infections with heterologous IT strains of *P. aeruginosa*. The present study demonstrated that the anti-*P. aeruginosa* OMPs IgG is also cross-reactive with OMPs of various Gram-negative pathogens, suggesting that it may have a cross-protective effect on these pathogens. Since Gram-negative pathogenic bacteria account for approximately 15–18% of endophthalmitis cases whose prognosis is notably poorer than other pathogenic microorganisms [3, 24], it is believed that the cross-protectivity against a broad spectrum of bacterial pathogens should be greatly beneficial in clinical situations.

Outer membrane protein fractions prepared from Gram-negative bacteria are often contaminated with LPS that is highly immunogenic, and LPS-specific antibodies have been demonstrated to afford protection against Gram-negative bacteria. Therefore, there could be a possibility that contaminating anti-LPS antibody is responsible for the protective effect of the human IgG preparation used in this study. Considering that LPS content of the OMPs preparation is less than 20 ng per mg protein, it seems to be too low to produce anti-LPS antibody with significant protective activity [11]. In fact, the anti-OMPs IgG has been tested for protective efficacy against heterologous *P. aeruginosa* strains. It showed opsonophagocytic killing activity against 7 different immunotype strains and was able to protect mice against the bacterial strains in passive protection assays [15]. Since LPS-specific antibody does not provide protection against heterologous strains, these data should serve as evidence that the anti-OMPs antibody is responsible for the protection.

In summary, the human anti-*P. aeruginosa* OMPs IgG relieved the symptoms of rabbit *P. aeruginosa* endophthalmitis. It had a killing activity of *P. aeruginosa* but also reduced the level of endotoxin that was released from the infecting microorganisms. These data warrant further development of the antibody as a means to treat endophthalmitis caused by *P. aeruginosa* infection.

Acknowledgment

We thank Dr. W. J. Park of CJ Corp. for providing the *P. aeruginosa* OMP preparation.

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