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Passive Protection Against *Pseudomonas aeruginosa* Infection in an Experimental Leukopenic Mouse Model

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An experimental leukopenic mouse model was used to evaluate the protective capacities of immunoglobulin G (IgG) fractions directed against toxin A (AT-IgG), elastase (AE-IgG), and lipopolysaccharide (ALPS-IgG) against fatal *Pseudomonas aeruginosa* infection. Statistically significant protection, as measured by long-term survival, was observed only when mice were treated with serotype-specific ALPS-IgG. The mean lethal dose for *P. aeruginosa* could be increased by as much as 6,600-fold for mice given ALPS-IgG as compared to mice which received only normal rabbit IgG. ALPS-IgG afforded high levels of protection, even when administered up to 6 h postchallenge. Experiments designed to monitor the growth and spread of a locally administered challenge showed that ALPS-IgG prevented bacteremia and organ colonization, which were pronounced in control animals. The effectiveness of combined antibiotic and immune therapy was tested. Gentamicin alone or in combination with AT-IgG or AE-IgG provided no detectable protection. However, its use with ALPS-IgG afforded substantially higher levels of protection than ALPS-IgG alone.

A critical factor in immunity to *Pseudomonas aeruginosa* is the presence of an intact functioning leukocyte population (32, 34). Patients who are rendered granulocytopenic due to underlying malignancies or by treatment with immunosuppressive drugs are particularly susceptible to *P. aeruginosa* infections (28). Antibiotic therapy is not highly effective in this patient population (29). Persistent problems in the management of *P. aeruginosa* infections in neutropenic patients have led to the evaluation of new therapeutic regimens, including immunotherapy (9, 10), granulocyte transfusion (5), and combined therapy (11, 18).

Recent studies indicate that *P. aeruginosa* virulence is multifactorial, involving not only somatic antigens (27) but several toxic exoproducts (13). Of these exoproducts, toxin A and elastase have been most intensively studied in regard to their potential role in virulence. Toxin A has been shown to be a potent inhibitor of eucaryotic protein synthesis (14). Mutants deficient in toxin A production are less virulent in nonlethal animal models (22, 33). Additionally, survival of patients suffering from *P. aeruginosa* bacteremia has been correlated with elevated serum antitoxin levels (2). Purified elastase causes substantial tissue necrosis upon injection (17). Elastase is produced during the course of human infections based on an increase in serum antielastase titer (3), and its production appears to be necessary for full expression of virulence (33).

To data, only active immunization with a polyvalent, lipopolysaccharide (LPS)-containing cell-extract vaccine (8) or antisera raised against certain of its components have been tested for their ability to protect against *P. aeruginosa* infections under granulocytopenic conditions (9, 10, 15, 18, 24, 35). The present study was conducted in an attempt to evaluate and compare the protective capacity of anti-exoproduct antibody (i.e., antitoxin A and antielastase) and anti-somatic antibody (anti-LPS) in an experimental leukopenic mouse model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PA220 (serotype G) was kindly provided by B. Wretlind, Karolinska Institute, Stockholm, Sweden, *P. aeruginosa* M-2 (serotype B) was a gift of I. A. Holder, Shriners Burns Institute, Cincinnati, Ohio. *P. aeruginosa* PA103 (serotype E) was provided by B. H. Iglewski, University of Oregon Health Sciences Center, Portland, Ore. Strains PA220 and M-2 produce toxin A and elastase (23, 31). Cultures were maintained lyophilized. Cultures for challenge studies were grown on the deferrated TSBD medium of Björn et al. (1). Fermentor cultures for LPS isolation were grown on TSB medium supplemented with glycerol (1% [vol/vol]).

Extracellular antigens. Toxin A was purified by a slight modification of previously described techniques

(15, 19). To 190 liters of TSBD medium in a 200-liter fermentor (Giovanola Frères S.A., Monthey, Switzerland) was added 2 liters of a log phase PA103 culture. The culture was grown at 32°C with vigorous agitation and aeration for 11 h. The cell-free supernatant was concentrated 20-fold by using an Amicon H10P10 cartridge (Amicon Corp., Lexington, Mass.), followed by dialysis against 4 volumes of distilled water. Toxin A was purified by batch binding and batch elution with DEAE, followed by chromatography on DEAE and hydroxylapatite as previously described (19). The purified product migrated as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an M_r of 66,000. The mean lethal dose (LD_{50}) upon intraperitoneal injection in 18- to 20-g Swiss-Webster white mice was 0.24 μg.

Purified elastase was purchased from Nagase Biochemicals Ltd., Kyoto, Japan. The LD_{50} for elastase was 125 μg injected intraperitoneally into 18- to 20-g mice.

Purification of LPS. Early stationary phase cells of PA220 (serotype G) were used as starting material. The cells were washed twice in saline. Washed cells (approximately 85 g [wet weight]) in 200 ml of a Tris-sodium chloride buffer (0.05 M Tris, 0.3 M NaCl [pH 9]) were mixed with 200-g glass beads (Sigma type I, 7.5 to 150 μm; Sigma Chemical Co., St. Louis, Mo.). Cells were disrupted by blending in a Sorvall Omnimixer (Du Pont Co., Newton, Conn.) for 30 min on ice. Glass beads were removed by settling. Residual glass beads and intact cells were pelleted by centrifugation at 1,000 × *g* for 15 min. Cell wall fragments were collected by centrifugation at 24,000 × *g* for 30 min, washed in Tris-sodium chloride buffer, and pelleted. The cell wall fragments were resuspended in 120 ml of distilled water, and 154 ml of 80% (wt/vol) phenol preheated to 75°C was added. The mixture was stirred for 5 min at 68 to 70°C, cooled, and centrifuged for 20 min at 20,000 × *g*. The water phase was collected and extensively dialyzed against distilled water. LPS was sedimented by centrifugation for 3 h at 100,000 × *g*. The pelleted material was resuspended in distilled water and subjected to two more rounds of centrifugation at 100,000 × *g*. The final pellet was resuspended in distilled water and lyophilized. The final material contained less than 1% (wt/wt) protein as determined by the method of Lowry (20).

Antisera. Antisera were prepared in rabbits, For LPS, rabbits were hyperimmunized over a period of 70 days by five intramuscular injections given on day 0 (10 μg in Freunds complete adjuvant, day 14 (20 μg in Freunds complete adjuvant), and days 28, 40, and 56 (50 μg in aluminum hydroxide). Rabbits were exsanguinated on day 70, and the sera were pooled. For toxin A and elastase, the initial immunization (day 0) consisted of 50 μg of protein in Freunds complete adjuvant given intramuscularly. On days 7, 14, 21, and 28, 100 μg of protein was given intramuscularly in Freunds incomplete adjuvant. Rabbits were exsanguinated on day 35, and the sera from each group were pooled.

Isolation of IgG from antisera. Hyperimmune sera and normal rabbit serum were subjected to three rounds of ammonium sulfate fractionation (21). The final pellet was dissolved in phosphate-buffered saline (PBS) to the initial serum volume and extensively dialyzed. Immunoglobulin G (IgG) fractions from anti-toxin (AT-IgG), antielastase (AE-IgG), and normal rabbit serum (NR-IgG) were absorbed twice with 2×10^{10} NaN_3-killed cells of the challenge strain. The final preparations were then filter sterilized, lyophilized, and stored at 4°C. IgG fractions were reconstituted in sterile distilled water. The enzyme-linked immunosorbent assay (ELISA) titers (and protein contents in milligrams per milliliter) for AT-IgG, AE-IgG, and ALPS-IgG were 2,500 (38.9), 1,200 (33.2), and 1,450 (40.2), respectively, for their homologous antigens; PA220 LPS titers were <1. NR-IgG had ELISA titers of <1 (32.4) in all assay systems (see below).

ELISA. ELISA for detection of AT-IgG, AE-IgG, and ALPS-IgG were performed as follows. Microtiter plates (Linbro; Flow Laboratories, Hamden, Conn.) were coated with 200 μl (per well) of a toxin A solution (5 μg/ml) or an elastase solution (20 μg/ml) in 0.1 M sodium carbonate (pH 9.6). A 20 μg/ml solution of LPS in 5 mM sodium phosphate (pH 7.2) was used. Plates were incubated at 37°C for 3 h and then stored at 4°C until needed. ELISAs were performed as described elsewhere (6). The absorbance was measured at 405 nm in a Titertek Multiskan reader (Flow Laboratories). Titers were expressed as the reciprocal of the highest serum dilution which gave half-maximal absorbance.

CHO cell cytotoxicity assay. The Chinese hamster ovary (CHO) cell cytotoxicity-neutralizing assay was performed essentially as previously described (15). The minimal cytotoxic dose (MCD) per 2×10^4 CHO cells was 2 and 800 ng for toxin A and elastase, respectively. Neutralization assays were performed against 4 MCDs of toxin A or 2 MCDs of elastase. AT-IgG and AE-IgG neutralized 256 and 320 μg of their respective antigens per ml.

Passive transfer of IgG. Sterile IgG (0.2 ml) was transferred via the tail vein at the times indicated in the text. The half-life of passively transferred IgG in the serum of recipient mice was approximately 55 h.

Leukocyte quantitation. Blood samples were collected directly into a leukocyte pipette and immediately mixed with 9 volumes of Türk solution (E. Merck Co., Darmstadt, West Germany) for 15 min before counting. Values are expressed as the number of leukocytes per cubic millimeter of blood.

Experimental infection. Infection of leukopenic mice with *P. aeruginosa* was performed as described previously (4). Briefly, outbred Swiss-Webster white mice were rendered leukopenic by three intraperitoneal injections of cyclophosphamide (150 μg/g weight of mouse) in 0.25 ml of PBS on days 0, 2, and 4. Leukopenia (≤800 leukocytes per mm^3 of blood) lasted from day 4 through day 8. Mice were anesthetized and challenged on day 4 by instilling the washed bacteria, in 5 μl of PBS, into a 0.5-cm-long incision made on the shaved backs of mice. The LD_{50} for PA220 and M-2 was less than 20 organisms, and the mean time to death ranged from 48 to 56 h.

Quantitation of *P. aeruginosa* in blood and tissues. Groups of three mice were sacrificed at various times postchallenge, and the number of *P. aeruginosa* organisms in the blood, liver, and skin at the site of infection was determined. Blood samples were collected in 1-ml plastic syringes containing 100 μl of 3.8% sodium citrate. A full-thickness skin specimen (400 to 500 mg [wet weight]) was obtained from the challenge site, weighed, and homogenized in 10 ml of PBS by

using a Sorvall Omnimixer. Livers were aseptically removed and homogenized in 10 ml of PBS. Appropriate dilutions were plated on TSB plates. Results are expressed in CFU.

Serotyping. The O antigen serotype for *P. aeruginosa* strains was based on the typing scheme of Homma (12). Antisera were purchased from the Thoshiba Kagaku Co., Ltd., Tokyo, Japan.

Statistical analysis. LD_{50}s were calculated by the method of Reed and Muench (26). *P* values were calculated by the chi-square method.

RESULTS

The first series of experiments was designed to determine the ability of various IgG preparations to protect mice against a low challenge dose of *P. aeruginosa* PA220 (serotype G) (approximately 50 organisms) (Table 1). In both experiments, neither AT-IgG nor AE-IgG offered statistically significant protection against PA220 challenge compared with NR-IgG. The mean time to death was also comparable in these three groups. In contrast, ALPS-IgG conferred highly significant protection in both experiments. Furthermore, the two deaths observed in the ALPS-IgG-treated groups were greatly delayed in comparison with the other three experimental groups. The protection afforded by ALPS-IgG was found to be serotype specific. Thus, the mortality rates for mice which received ALPS-IgG (anti-serotype G) and for those treated with NR-IgG after challenge with *P. aeruginosa* M-2 (serotype B) were identical (100%) (data not shown).

A more accurate assessment of the degree of protection each of the various IgG preparations could offer was obtained by determining the LD_{50} values for recipient mice (Table 2). For both determinations, the LD_{50} for mice which received NR-IgG, AT-IgG, or AE-IgG was less than the lowest challenge dose tested. Passively transferred ALPS-IgG greatly increased the LD_{50} in both experiments. In experiment 1, 84% of the mice survived the highest challenge dose tested (equivalent to at least 100 LD_{50} for NR-IgG-treated mice). The LD_{50} for the second determination was 6×10^4, corresponding to greater than 6,600-fold protection. The above experiments demonstrate that neither AT-IgG nor AE-IgG offers an appreciable degree of protection against *P. aeruginosa* infection, whereas serotype-specific ALPS-IgG can provide high levels of protection.

Next, the ability of ALPS-IgG to protect against fatal infection when administered at various times relative to the time of challenge was determined (Table 3). ALPS-IgG given 24 h before or up to 6 h after challenge was highly effective (mortality rates $\leq$ 20%; $P > 0.999$). When administered 24 h postchallenge, ALPS-IgG provided little protection. However, it should be noted that death was delayed in all

TABLE 1. Passive protection of mice against *P. aeruginosa* PA220 infection by various IgG preparations[a]

IgG transferred[b]	No. of survivors/ total no.	Mean time to death (h)	*P*
Expt 1			
NR-IgG	1/10	62.3	
AT-IgG	1/10	66.3	<0.5
AE-IgG	1/9	54.3	<0.5
ALPS-IgG	9/10	141	>0.999
Expt 2			
NR-IgG	1/10	62.8	
AT-IgG	3/10	57.8	<0.5
AE-IgG	0/10	53.7	<0.5
ALPS-IgG	9/10	124	>0.999

[a] The challenge dose for both experiments was approximately 50 viable *P. aeruginosa* PA220 organisms.

[b] IgG was transferred 24 h before infection. Anti-LPS was raised against purified PA220 LPS.

TABLE 2. LD_{50} values for mice receiving various IgG preparations before challenge with *P. aeruginosa* PA220

IgG transferred[a]	LD_{50}[b]	
	Expt 1	Expt 2
NR-IgG	<0.92	<0.9
AT-IgG	<0.92	<0.9
AE-IgG	<0.92	<0.9
ALPS-IgG	$>0.92 \times 10^3$[c]	6×10^4

[a] IgG was transferred 24 h before infection. Groups of six mice were used.

[b] Expressed as number of viable *P. aeruginosa* PA220 organisms.

[c] Highest challenge dose tested; 84% of animals survived.

TABLE 3. Passive protection of mice against *P. aeruginosa* PA220 infection by anti-LPS administered pre- and post challenge[a]

IgG transferred	Time of transfer (h)[b]	No. of survivors/ total no.	Mean time to death (h)	*P*
LPS-IgG	−24	8/10	145.5	>0.999
	+4	9/10	126	>0.999
	+6	8/10	84	>0.999
	+24	2/10	84	<0.5
NR-IgG	−24	0/10	70	

[a] The challenge dose was approximately 50 viable *P. aeruginosa* PA220 organisms.

[b] Times given are relative to the time of challenge (0 h).

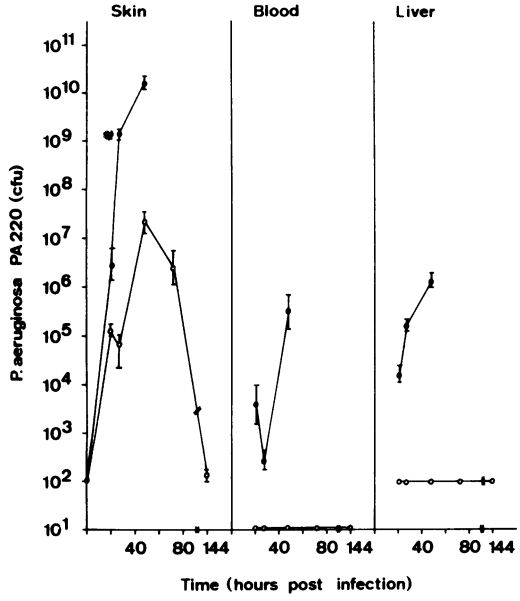

FIG. 1. Quantitation of *P. aeruginosa* PA220 in the skin (at the site of infection), blood, and liver ± standard error of the mean at various times after challenge with 50 *P. aeruginosa* PA220 organisms (time 0). Each mouse received 0.2 ml of either NR-IgG (●) or ALPS-IgG (○) 24 h before challenge. Each time point represents data obtained from three mice. The limit of detection for bacteria was 10^2/g of skin, 10/ml of blood, and 10^2/liver.

groups which received ALPS-IgG and that the extent of the delay was dependent on the time of ALPS-IgG administration relative to the challenge.

In an attempt to gain some knowledge about how lethal infection is prevented by ALPS-IgG, the growth and spread of the bacterial challenge was studied in mice which received ALPS-IgG or NR-IgG (Fig. 1). Rapid bacterial multiplication occurred at the site of infection, reaching greater than 10^{10} CFU/g of skin 48 h postinfection in NR-IgG-treated mice. Bacteremia, together with colonization of the liver, was first noted 21 h postchallenge and persisted at high levels throughout the course of the experiment. ALPS-IgG treatment not only delayed bacterial growth in the skin but also reduced the bacterial load. Maximal bacterial numbers were observed 48 h postinfection. At subsequent times, the bacterial load was found to progressively decline, so that by 144 h the skin was all but cleared of *P. aeruginosa*. At no time was *P. aeruginosa* detected in the blood or liver of ALPS-IgG-treated mice.

Finally, we investigated the effect of combined antibiotic (gentamicin) and passive immunotherapy on lethal *P. aeruginosa* infection (Table 4). The LD_{50} values for gentamicin used in combination with NR-IgG, AT-IgG, or AE-IgG were not significantly different from those of paired groups receiving only passive therapy. Therefore, gentamicin in combination with these IgG preparations was ineffective in controlling infection. However, when gentamicin was used in combination with ALPS-IgG, a substantially higher level of protection was obtained in comparison to ALPS-IgG therapy alone. It should be noted that, although most animals which received only ALPS-IgG survived, they were listless for a period of time (usually between 24 and 72 h postchallenge). Animals which received the combined therapy were more active and appeared in markedly better overall condition throughout the course of the experiment.

TABLE 4. Protective effect of combined gentamicin and passive antibody therapy against *P. aeruginosa* PA220 infection

IgG transferred[a]	Gentamicin administered[b]	LD_{50}	
		Expt 1	Expt 2
NR-IgG	No	<0.9	3
	Yes	<0.9	<0.9
AT-IgG	No	<0.9	ND[c]
	Yes	<0.9	ND
AE-IgG	No	<0.9	ND
	Yes	<0.9	ND
ALPS-IgG	No	6.2×10^4	7.1×10^4
	Yes	5×10^5	$>0.9 \times 10^6$[d]

[a] Each mouse received 0.2 ml of IgG intravenously 24 h before challenge.
[b] When indicated, each mouse received 2 mg of gentamicin intraperitoneally 24 h after challenge.
[c] ND, Not determined.
[d] Highest challenge dose tested; greater than 50% of animals survived.

DISCUSSION

In the present study, we evaluated, in an experimental leukopenic mouse model, the protective capacity of several IgG preparations directed against *P. aeruginosa* somatic (LPS) and toxic extracellular (toxin A and elastase) antigens. Although prior studies have investigated the use of passively administered anti-LPS for the prevention of fatal experimental *P. aeruginosa* infections under immunosuppressed conditions (10, 18), no study to date has examined the protective capacity of anti-exoproduct antibody under similar conditions. Our findings indicate that, although AT-IgG and AE-IgG afforded no significant protection, serotype-specific ALPS-IgG was highly effective in preventing fatal *P. aeruginosa* sepsis.

Although passive immunization with ALPS-IgG did not afford complete protection against

P. aeruginosa infections (mortality rate, 10 to 20%, compared to 90 to 100% in NR-IgG-immunized mice), the protection observed was highly significant ($P > 0.999$). Serotype-specific ALPS-IgG administered 24 h before challenge increased the LD_{50} value by as much as 6,600-fold over that obtained when NR-IgG was used. Furthermore, ALPS-IgG was found to be highly effective, even when administered up to 6 h after infection. Even though protection afforded by ALPS-IgG given 24 h postchallenge was not statistically significant, the long-term survival rate was higher and the mean time to death delayed when compared to the NR-IgG group. The decreased protection noted can probably be attributed to the fact that animals were moribund and presented with bacteremia and liver colonization when they received the ALPS-IgG at this late time. Neutralization of toxin A or elastase by their respective antisera appeared to be insufficient in providing protection against fatal *P. aeruginosa* sepsis under immunosuppressed conditions.

Experiments designed to monitor the growth and spread of the bacterial challenge showed that ALPS-IgG decreased both the bacterial growth rate and load at the site of infection. Perhaps of more significance was the fact that bacteremia and colonization of the liver were prevented. Therefore, although the challenge may multiply locally to relatively high numbers ($>10^7$/g of skin), prevention of bacteremia is critical to survival. Anti-LPS has been shown to promote phagocytosis and killing of *P. aeruginosa* (34). Its protective effect observed in the present study is probably related to this activity, since *P. aeruginosa* PA220 is resistant to the killing effect of whole serum, even in the presence of specific ALPS-IgG. Even though leukopenia persisted at least through day 4 postinfection, the decreased numbers of remaining leukocytes appeared to be capable (in the presence of ALPS-IgG) of eliminating sufficient numbers of *P. aeruginosa* to prevent fatal sepsis. Although residual leukocytes (postcyclophosphamide treatment) were not subjected to functional tests, studies by Jones et al. (16) have demonstrated that abnormal neutrophils can phagocytize and kill *P. aeruginosa* when specific immune globulin is present.

Antibiotic therapy often proves to be ineffective in controlling *P. aeruginosa* infections in patients who are immunosuppressed (29). Similarly, we found that gentamicin offered no protection against fatal infection when used in combination with NR-IgG, AT-IgG, or AE-IgG in our experimental system. In contrast, its use with ALPS-IgG provided maximal levels of protection, substantially greater than those seen when ALPS-IgG was used alone. These results suggest that gentamicin by itself is incapable of clearing a sufficient portion of the bacterial challenge to provide absolute protection. When used in combination with ALPS-IgG, it most probably acts to augment the bacterial clearing capacity of ALPS-IgG, hence the better protection observed.

Although prior studies in neutropenic animals have demonstrated that passively administered anti-*P. aeruginosa* LPS will significantly delay the time to death, protection, as measured by long-term survival, has not been noted (10, 18). This difference in relation to the present findings is most likely due to the design of the animal models used in these studies. Prior studies have used comparatively large challenge doses (5×10^8 intrabronchially or 10^7 intravenously administered), whereas the model used in this study employed low challenge doses (approximately 50 organisms) instilled in a localized area. Therefore, although ALPS-IgG and low numbers of residual leukocytes are capable of handling a localized infection initiated with a small challenge dose, a much larger challenge inoculum, especially given systemically, might easily overwhelm any such defense. Although little is known about the manner in which neutropenic patients are infected, minor trauma to a colonized surface appears to be a major route (36). In this case, one would expect the initial numbers of invading bacteria to be relatively small. Therefore, the protection observed in the present study may prove to be clinically relevant.

Protection against *P. aeruginosa* infections in neutropenic patients has been attempted by active immunization with a polyvalent LPS-containing extract vaccine (7, 24, 35). Results obtained from these studies, although in some cases promising, are not conclusive. Two major complicating problems were the high incidence of adverse reactions to vaccination (24, 35) and the short duration of elevated antibody titers (7). A possible way to circumvent these difficulties is the use of hyperimmune globulin, an approach shown to be effective in treatment of *P. aeruginosa* infections in burn wound patients (16). Based on the results obtained in this and other studies (10, 18, 24, 25), the use of hyperimmune globulin directed against LPS or antigens immunologically related to LPS (25, 30) warrants clinical evaluation for the prevention of fatal *P. aeruginosa* infections in neutropenic patients.

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