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In Vivo Production of Exotoxin A and Its Role in Endogenous *Pseudomonas aeruginosa* Septicemia in Mice

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We have examined the production of *Pseudomonas aeruginosa* exotoxin A (ETA) and its role in endogenous bacteremia in mice. Mice given *P. aeruginosa* D4 orally died of bacteremia between days 10 and 13 following cyclophosphamide-induced leukocytopenia. In this model, serum endotoxin was detected beginning on day 7 by the *Limulus* assay and *P. aeruginosa* was cultured from blood beginning on day 9. ETA and tumor necrosis factor alpha (TNF) were also detected in serum by enzyme-linked immunosorbent assay beginning on day 9. Purified ETA did not stimulate the production of TNF in normal mice primed with a synthetic derivative of muramyl dipeptide in the absence of endotoxin. However, ETA enhanced and primed endotoxin-induced TNF production in mice. The mortality rate of mice given ETA mutant PAO-PR1 (5.0%) was significantly lower than that of mice given the parent strain (78.8%). These data indicate that ETA may be an important factor in the occurrence of *P. aeruginosa* bacteremia and/or the death of mice. Also, ETA may be responsible for enhancing the production of a lethal dose of TNF in the presence of endotoxin in *P. aeruginosa* bacteremia.

Pseudomonas aeruginosa is an opportunistic pathogen and frequently causes severe septicemia in immunocompromised hosts (2, 5). *P. aeruginosa* septicemia carries a higher fatality rate than that caused by any other gram-negative bacteria (2, 5, 31). *P. aeruginosa* is typically refractory to many commonly used antibiotics, and chemotherapy in patients infected with this pathogen is consequently often ineffective (5, 21). Since *P. aeruginosa* is a gram-negative bacillus, lipopolysaccharide (LPS) of this pathogen should be an important virulence factor (17). However, LPS isolated from the cell walls of *P. aeruginosa* is not as toxic as those isolated from enteric bacilli (17). Therefore, it is clear that additional factors are involved in the virulence of this organism. *P. aeruginosa* produces a large number of extracellular products which are much more toxic than LPS and have been shown to play a role in the pathogenesis of the infections caused by this organism (17, 22).

One of these extracellular products is exotoxin A (ETA), which is a protein toxin that inhibits polypeptide synthesis through ADP ribosylation of elongation factor 2, as does diphtheria toxin (16, 25). It is the most toxic substance produced by *P. aeruginosa* (17) and is cytotoxic for a number of mammalian cells (18). Moreover, it inhibits human granulocyte and macrophage progenitor cell proliferation (26, 30). We previously demonstrated that clinical blood isolates of *P. aeruginosa* have the ability to produce large amounts of ETA in vitro and are associated with significantly higher mortality rates in neutropenic mice with endogenous bacteremia than are strains isolated from other clinical sources such as sputum and urine (7). Recently, it was reported that ETA alters the production of tumor necrosis factor alpha (TNF) by human leukocytes (29) and of interleukin-1 by murine peritoneal macrophages (20) in vitro, both of which are principal mediators in the cascade of pathophysiologic events in bacterial septicemia (9). These data indicate that ETA may affect the occurrence and the pathophysiology of *P. aeruginosa* bacteremia. However, the conditions leading to optimal production of ETA in vitro differ from those in vivo, and the production and kinetics of ETA in vivo are not well known. Also, the role of ETA in the occurrence of *P. aeruginosa* septicemia and the production of cytokines in vivo is still unclear.

To gain a better understanding of these points, we have evaluated the changes that occur over time in the serum levels of endotoxin, ETA, and TNF in mice with endogenous *P. aeruginosa* bacteremia. The present model incorporated oral inoculation of bacteria, subsequent bacterial colonization, overgrowth in the intestinal tract, and invasion into the bloodstream. Consequently, this animal model closely mimics the pathophysiology of septicemia in humans (12).

We also examined the necessity of ETA in the production of *P. aeruginosa* endogenous septicemia by using several ETA-producing strains and their ETA-deficient mutants. We also investigated whether purified ETA affects the production of endotoxin-induced TNF and whether it induces TNF production in mice by itself independently of endotoxin.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are listed and characterized in Table 1. *P. aeruginosa* D4 was isolated from the blood of a neutropenic mouse with bacteremia (13). This strain produces relatively large amounts of ETA and proteases in vitro and consistently caused a 70 to 100% mortality rate in a mouse model of endogenous septicemia (7). *P. aeruginosa* PA103 (17), PA103-29 (24), PA103*toxA*Ω (32), PAO1 (14), and PAO-PR1 (4) were kindly provided by B. H. Iglewski, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. Strain PA103, which was clinically isolated from sputum, is well known as a hyperproducer of ETA and produces very low levels of alkaline protease and no elastase (24). PAO1 has also been well characterized and produces most of the recognized virulence factors, including ETA (22). All strains were

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TABLE 1. Characteristics of *P. aeruginosa* strains used in this study

Strain	Reference	Description	ETA in culture supernatant (μg/ml)[a]
D4	13	Prototypic ETA producer isolated from mouse blood	2.84 ± 0.24
PA103	21	ETA hyperproducer clinically isolated from sputum	16.40 ± 6.18
PA103-29	24	Chemically derived *regA* mutant of PA103; produces no ETA	ND
PA103*toxA*Ω	32	Insertionally inactivated chromosomal *toxA* of PA103; produces no ETA	ND
PAO1	14	Prototypic ETA producer clinically isolated from a wound	1.03 ± 0.15
PAO-PR1	4	Chemically derived *toxA* mutant of PAO1; produces nontoxic immunological cross-reactive material (CRM66)	1.09 ± 0.25

[a] Data represent immunological activity measured by ELISA, not enzymatic activity. ND, not detected.

maintained frozen at −80°C in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) containing 30% glycerol.

Quantitation of ETA in culture supernatants in vitro. Bacteria were incubated in Trypticase soy broth dialysate deferrated with Chelex 100 (Bio-Rad) (TSBD-C) overnight at 32°C (16) and then diluted 1:20 in fresh medium. The iron content in Trypticase soy broth deferrated with Chelex 100 was 0.09 μg/ml, as determined with bathophenanthroline. After 24 h of growth with reciprocal shaking (180 cycles per min), the supernatants were collected by centrifugation, passed through a 0.22-μm filter, and used as samples. The assay was run in duplicate for each flask. Data are expressed as the means of five replicate flasks.

Immunological activity of ETA in samples was measured by a sandwich-type enzyme-linked immunosorbent assay (ELISA), using goat and rabbit anti-ETA antibodies as described previously (11). Alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G antibodies (E-Y Laboratories, Inc., San Mateo, Calif.) were used as a third antibody. Purified *P. aeruginosa* ETA (List Biological Laboratories, Inc.) was used to construct a standard curve; the limited sensitivity was 1.0 ng/ml.

Animals. Specific-pathogen-free male ddY mice (Japan S.L.C. Co., Ltd., Shizuoka, Japan) weighing 20 to 24 g were used in the experiments. The animals were housed in sterile cages with filter hoods and fed a sterile diet; they received sterile distilled water except during the period of oral administration of the bacteria. Fecal specimens were obtained before the study and examined to ensure the absence of *P. aeruginosa*.

Production of endogenous bacteremia with *P. aeruginosa* D4 and preparation of serum samples. Endogenous bacteremia was produced as described previously (12). Bacteria were grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 18 h, suspended in sterile 0.45% saline, and adjusted to a concentration of 10^7 CFU/ml with a spectrophotometer. The bacterial suspension was given in the drinking water between days 1 and 4. To aid in the colonization of *P. aeruginosa*, the normal intestinal flora of the mice was disturbed by administering 200 mg of sodium ampicillin (Viccillin; Meiji Seika Kaisha, Ltd., Tokyo, Japan) per kg by intraperitoneal injection daily between days 1 and 4. Mice were then given 150 mg of cyclophosphamide (Endoxan; Shionogi & Co., Ltd., Osaka, Japan) per ml by intraperitoneal injection on days 5, 7, and 9. We had determined that these doses of cyclophosphamide induced leukocytopenia (<1,000/mm^3) in mice from days 8 to 14 without lethality in the absence of oral bacterial inoculation (data not shown).

Mice were placed in two groups: for observation of death and for preparation of serum samples. The first group, consisting of 20 mice, was observed four times daily, and deaths were recorded up to day 14. To obtain cardiac blood samples, necropsies were performed within 6 h of either death or sacrifice at the end of the experiment. The blood samples were plated onto Trypticase soy agar and cultured at 37°C for 24 h for detection of the challenge *P. aeruginosa* strain.

The mice used for preparation of serum samples were anesthetized with pentobarbital (at least five mice per day) on days 0 (before the experiments), 5 (before the administration of cyclophosphamide), 7 (before the second administration of cyclophosphamide), and 9 to 12. Cardiac blood samples were then obtained aseptically, using pyrogen-free materials. The blood samples were allowed to clot at 4°C in pyrogen-free glass tubes and then centrifuged at 2,000 × *g* for 15 min. A portion of the blood samples was also tested for bacterial culture.

Assay of endotoxin, ETA, and TNF in serum samples. The endotoxin levels in the serum samples were assayed by the chromogenic *Limulus* test (Endospecy; Seikagaku Co., Tokyo, Japan). A standard curve was prepared with purified endotoxin of *Escherichia coli* O111:B4 (Seikagaku Co.); the limited sensitivity for serum was 17.9 pg/ml.

ETA in the serum was measured by ELISA as described above.

Murine TNF in samples was quantified by a sandwich-type ELISA (murine TNF ELISA kit; Endogen Inc., Boston, Mass.). A reference curve was obtained with recombinant mouse TNF; the limited sensitivity was 25 pg/ml.

Production of endogenous bacteremia with *P. aeruginosa* PA103 and PAO1 and their mutants. Endogenous bacteremia was induced as described above with minor modifications. Mice were given 200 mg of cyclophosphamide per kg on the indicated days. At least 16 mice for each challenge *P. aeruginosa* strain were observed four times daily, and deaths were recorded up to day 20. Cardiac blood samples were taken at necropsy as described above.

Influence of purified ETA on in vivo production of TNF in mice. Each mouse was given 100 μg of a synthetic derivative of muramyl dipeptide (MDP) [MDP-Lys(L18); a kind gift of Daiichi Seiyaku Co., Tokyo, Japan] in 0.2 ml of sterile phosphate-buffered saline intravenously as a priming agent 4 h before the administration of LPS, which was used as a triggering agent. Each mouse was injected intraperitoneally with 10 μg of *E. coli* O55:B5 (Difco) LPS at time zero. Control groups received an equal volume of pyrogen-free saline for injection instead of the priming and triggering agents. Blood was obtained from mice by cardiac puncture at 2 h postinoculation. After centrifugation of the blood samples, the serum was removed.

To examine whether ETA affects MDP-endotoxin-induced

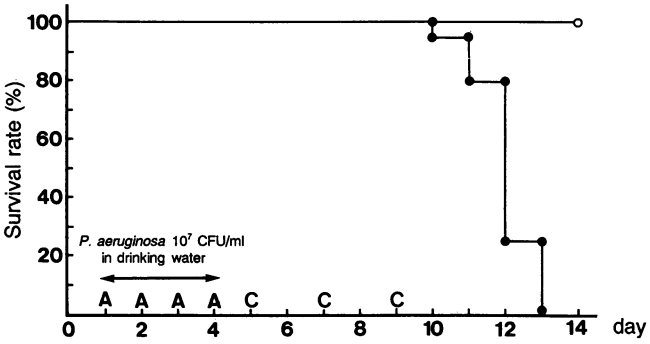

FIG. 1. Survival kinetics of mice with endogenous bacteremia due to *P. aeruginosa* D4. Mice were administered 200 mg of ampicillin (A) and 150 mg of cyclophosphamide (C) per kg on the indicated days (20 mice per group). All of the mice given *P. aeruginosa* D4 orally died of bacteremia due to challenge strain D4 by day 13. Control mice without bacterial challenge experienced no deaths ($P < 0.01$). Symbols: ●, mice with oral bacterial challenge; ○, mice without bacterial challenge.

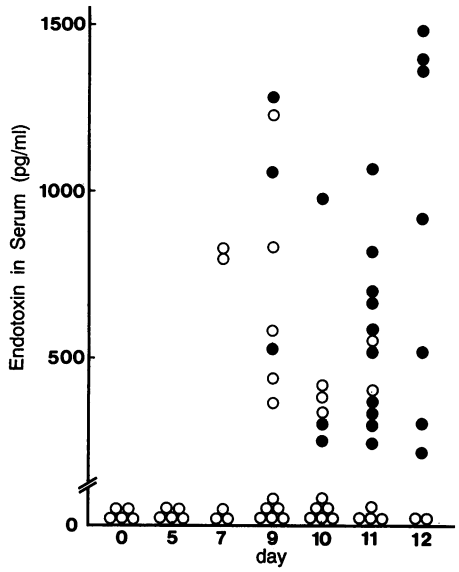

FIG. 2. Serum endotoxin in mice with endogenous bacteremia due to *P. aeruginosa* D4. Symbols: ●, mice with cardiac blood samples that were culture positive for challenge strain D4; ○, mice with cardiac blood samples that were culture negative for strain D4.

TNF production, 0.03 to 3.0 μg of purified ETA in 0.2 ml of pyrogen-free water per mouse was administered by intraperitoneal injection before or after endotoxin challenge.

In some experiments, ETA was introduced instead of LPS following MDP priming to examine whether ETA triggers TNF production independently of endotoxin. Also, to evaluate the possibility of ETA as a priming agent, each mouse was given 0.03 to 3.0 μg of purified ETA either 4 or 24 h before the administration of 10 μg of LPS. Blood samples were obtained as described above.

TNF levels in the samples were measured by ELISA as described above.

Statistics. The chi-square test and Student's *t* test were used to compare survival rates and means, respectively. A level of 5% was accepted as statistically significant.

RESULTS

Survival kinetics of mice with endogenous bacteremia due to *P. aeruginosa* D4. The mortality rate of mice given *P. aeruginosa* D4 orally was 100% (Fig. 1). These mice died between days 10 and 13 of systemic bacteremia due to the challenge strain. In the absence of an oral bacterial challenge, no deaths were observed through day 16 in mice treated with cyclophosphamide and ampicillin. Bacterial culture was negative for all control mice sacrificed at the end of the experiment.

Time course of levels of endotoxin, ETA, and TNF in mice with endogenous bacteremia due to *P. aeruginosa* D4. Endotoxin was detected in the sera of two of five mice on day 7 without concomitant detection of *P. aeruginosa* D4. On day 9, endotoxin was detected in 8 of 14 mice (57.1%), and the challenge strain D4 was also detected in 3 of these mice. On day 12, seven of nine mice (77.8%) were positive for strain D4, and individual endotoxin levels ranged from 222.7 to 1,484.8 pg/ml. The mean endotoxin levels in mice positive for endotoxin were 790.24 ± 360.95 and 886.24 ± 544.31 pg/ml (mean ± standard deviation) on days 9 and 12, respectively (Fig. 2).

ETA was detected on day 9 in 2 of 14 mice. One of these two mice was negative for both endotoxin and the challenge *P. aeruginosa* strain, D4. On day 12, ETA was detected in all but one mouse which was culture positive; the levels ranged from 19.1 to 300.3 (116.0 ± 111.4) ng/ml (Fig. 3).

Figure 4 shows serum TNF levels in endotoxin-positive mice. Serum TNF was detected in mice beginning on day 9. The mean serum TNF level in mice positive for TNF on day 12 (2.782 ± 0.426 ng/ml) was significantly higher than that on day 9 (1.699 ± 0.644 ng/ml) ($P < 0.01$; Fig. 4).

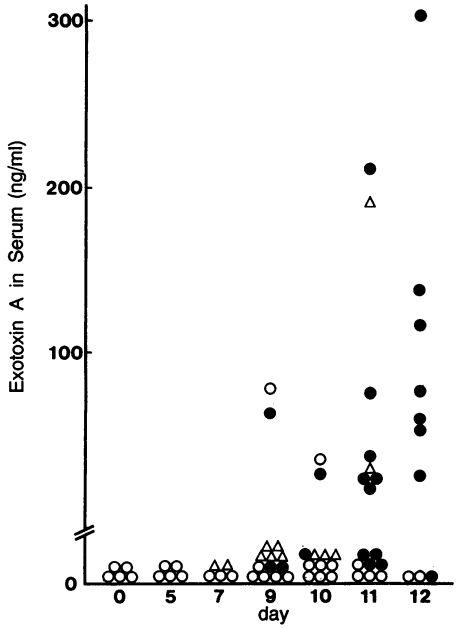

FIG. 3. Serum exotoxin A in mice with endogenous bacteremia due to *P. aeruginosa* D4. Symbols: ●, mice positive for culture of challenge strain D4 from cardiac blood; △, mice positive for endotoxin but negative for culture; ○, mice negative for both endotoxin and culture.

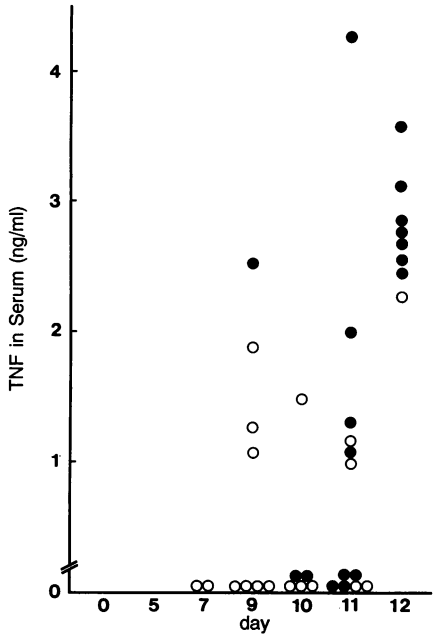

FIG. 4. Serum TNF in mice positive for endotoxin with endogenous bacteremia due to *P. aeruginosa* D4. Symbols: ●, mice positive for exotoxin A; ○, mice negative for exotoxin A.

Survival kinetics of mice with endogenous bacteremia due to *P. aeruginosa* PA103 and PAO1 and their mutants. Mice given strain PA103 instead of strain D4 showed no or a very low mortality rate when they were given three doses of cyclophosphamide, 150 mg/kg, as described above (data not shown). Mice inoculated with strain PA103 and given five doses of cyclophosphamide, 200 mg/kg, had a 50% mortality rate. The mortality rate of mice given the ETA-deficient mutants PA103-29 and PA103*toxA*Ω was also 50% (data not shown).

The mortality rate of mice given strain PAO1 and two doses of cyclophosphamide, 200 mg/kg, was 78.8%. The mortality rate of mice given the cross-reactive material-producing mutant PAO-PR1 was 5.0%, which was significantly lower than that of the parent strain ($P < 0.01$; Fig. 5).

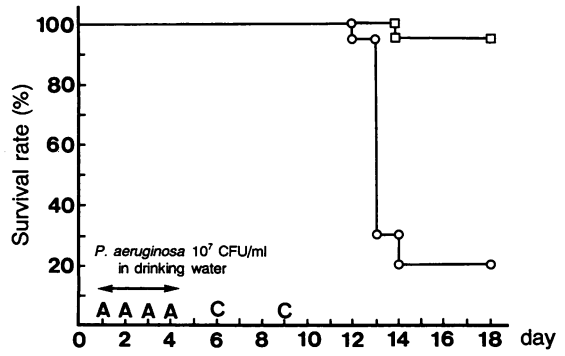

FIG. 5. Survival kinetics of leukopenic mice given *P. aeruginosa* PAO1 and its ETA mutant. Mice were given 200 mg of ampicillin (A) and 200 mg of cyclophosphamide (C) per kg on the indicated days. The survival rate of mice given the ETA mutant strain PAO1-PR1 was significantly higher than that of mice given the parent strain ($P < 0.01$). Symbols: ○, mice given strain PAO1 (parent) (33 mice); □, mice given strain PAO-PR1 (20 mice).

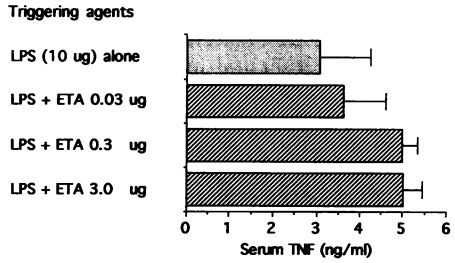

FIG. 6. Synergistic activity of ETA in MDP-endotoxin-induced TNF production in mice. Mice were each given 10 μg of LPS with or without the indicated dose of ETA 4 h after intravenous injection of MDP (100 μg per mouse). The serum TNF level was significantly enhanced when 0.3 or 3.0 μg of ETA per ml was inoculated concomitantly with endotoxin ($P < 0.02$).

Influence of purified ETA on in vivo production of TNF in mice. The serum TNF level in mice treated with LPS after priming with MDP was 3.304 ± 0.051 ng/ml. The administration of ETA did not affect MDP-endotoxin-induced TNF production when it was given 1 or 5 h before or 1 h after endotoxin challenge (data not shown). However, the serum TNF level was significantly enhanced when 0.3 or 3.0 μg of ETA per mouse was given concomitantly with endotoxin ($P < 0.02$; Fig. 6). The serum TNF concentrations in mice given LPS alone and LPS with 3.0 μg of ETA were 3.016 ± 1.249 and 5.006 ± 0.449 ng/ml, respectively. ETA did not stimulate the production of TNF in MDP-primed mice independently of endotoxin (data not shown). Figure 7 shows, however, that ETA possessed a dose-dependent priming effect when it was administered 4 or 24 h before challenge with endotoxin. The serum TNF concentrations in mice primed with MDP and 3.0 μg of ETA 4 h before LPS injection were 3.076 ± 1.088 and 1.239 ± 0.397 ng/ml, respectively. The priming effect of ETA given 4 h before LPS injection was not as strong as that of MDP. However, the priming effect of ETA was equivalent to that of MDP when mice were primed 24 h before LPS injection. The serum TNF levels in mice primed with MDP and 3.0 μg of ETA 24 h before LPS injection were 1.582 ± 0.253 and 1.591 ± 1.137 ng/ml, respectively.

DISCUSSION

A number of *P. aeruginosa* strains can produce various exoenzymes in vitro, including ETA, exoenzyme S, proteases (alkaline protease and elastase), and phospholipase C

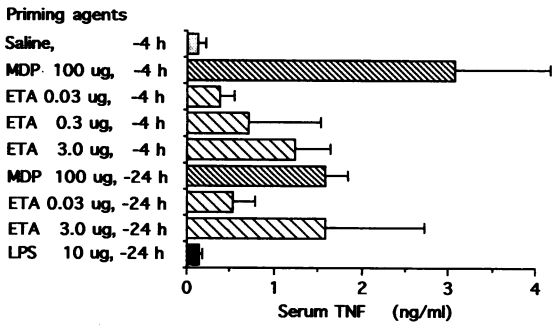

FIG. 7. Priming effect of ETA on endotoxin-induced TNF production in mice. Each mouse was given either 100 μg of MDP, the indicated dose of ETA, or 10 μg of LPS 4 or 24 h before triggering with LPS (10 μg). ETA exhibited a dose-dependent priming effect.

(22). These exoenzymes seem to be important virulence factors in several *P. aeruginosa* infections. Ohman et al. (23) reported that the experimental infections of mouse cornea produced by ETA mutants were less severe than those produced by their parent strains. The virulence of an ETA mutant was also found to be slightly lower than that of its parent strain in a burned mouse model (22). However, the role of ETA in bacteremia is not well understood. Because animals that are injected directly with a large amount of gram-negative bacteria rapidly die of endotoxin shock, the assessment of other virulence factors in conventional systemic infection models may be difficult.

Woods et al. (33) reported that clinical blood isolates of *P. aeruginosa* produce a large amount of total protease, ETA, and phospholipase C in vitro compared with isolates obtained from other infection sites. In addition, we showed that clinical bacteremic isolates of *P. aeruginosa* caused higher mortality rates in mice with endogenous bacteremia than did strains obtained from other infection sites (7). We also found that the blood isolates persisted in the intestines of mice in the bacteremia model (7). The results of our assay (7) of exoenzymes produced in the culture supernatants by the *P. aeruginosa* strains generally agreed with those of Woods et al. (33). Interestingly, erythromycin, which has no direct antimicrobial activity against *P. aeruginosa*, enhanced the survival rate of mice in the same model (12). Suppression of ETA and alkaline protease by erythromycin seemed to be one of the mechanisms in the protection of mice against *P. aeruginosa* bacteremia (11). Pollack and Young (27) and Cross et al. (3) reported that patients with *P. aeruginosa* septicemia had a better prognosis when their antibody titers to ETA were high. All of the above data suggest that ETA may play an important role in the occurrence of *P. aeruginosa* bacteremia and its lethality.

In the study presented here, the kinetics of serum ETA may be speculative, since the blood samples were not taken from a single mouse and the assay system for ETA was not as sensitive as that for endotoxin. However, we were able to detect ETA in murine serum by day 9 (Fig. 3). Other methods such as the use of immunohistochemistry to detect ETA in organs such as the intestine and liver may prove helpful in the future.

When we evaluated ETA-producing strains and their mutants, strain PA103 exhibited very low in vivo pathogenesis despite its ability to hyperproduce ETA in vitro. The possible reason for this occurrence is that this strain was isolated from sputum (1) and is serum sensitive (28). Because our previous data suggest that nonblood isolates could not adhere effectively to the intestine (7), this strain may also have a low affinity for the intestine. The fact that this strain produces a very low level of alkaline protease and no elastase may also be involved. In contrast, mice given strain PAO1 had a high mortality rate (Fig. 5). Strain PAO-PR1, which was chemically derived from strain PAO1, produces a nontoxic cross-reactive material called CRM66 which has immunological antigenicity (8). The mortality rate of mice given this mutant was significantly lower than that of mice given the parent strain (Fig. 5). These data indicate that ETA may be an important factor in the occurrence of *P. aeruginosa* bacteremia or the death of mice or both.

In the present study, no remarkable change in endotoxin levels was observed between days 9 and 12 of the experiments in the mice with endogenous bacteremia due to strain D4 (Fig. 2). In contrast, ETA and TNF levels were elevated with elapsed time (Fig. 3 and 4). Therefore, it is possible that the ETA produced in mice affected TNF production. Recently, many reports have shown the immunological activities of ETA in addition to its direct cytotoxicity against mammalian cells. Holt and Misfeldt (15) demonstrated that ETA modulates the in vitro and in vivo antibody response in mice to T-cell-dependent and -independent antigens. ETA has also been reported to stimulate in vitro production of interleukin-1 in murine peritoneal macrophages (20) and to induce murine cytotoxic T lymphocytes (34). More recently, Staugas et al. (29) reported that ETA depresses lymphoproliferation, TNF, lymphotoxin gamma interferon, and interleukin-1 production in human leukocytes in vitro. Because ETA is a protein synthesis inhibitor, depression of cytokine production may be a reasonable function of ETA. However, our data showed that ETA did not depress MDP-endotoxin-induced TNF production in vivo. In contrast, we found that ETA actually enhanced TNF production when it was introduced with endotoxin. It is possible that the different study conditions used by us versus those used by the other investigators might have caused the different results. Because we assessed the effect of ETA on TNF production in mice in vivo, our data may mimic the actual clinical pathophysiology of ETA more closely. We also found that ETA possessed a priming capacity for endotoxin-induced TNF production. Recent studies have shown that several bacterial exotoxins (e.g., toxic shock syndrome toxin 1 and enterotoxin B produced by *Staphylococcus aureus* and exotoxin produced by streptococci) induce TNF production (6, 10, 19). This induction of TNF production by these exotoxins is thought to be an important mechanism of shock production in hosts with bacteremia due to gram-positive organisms that carry no endotoxin (19). Although ETA alone did not induce TNF following MDP priming in the absence of endotoxin in our study, our results suggest that ETA may enhance the production of a lethal dose of TNF in the presence of endotoxin. Although there may be a possibility that ETA activates macrophages and subsequently primes TNF production by these cells, further study is necessary to clarify the mechanism of the priming effect of ETA in TNF production.

In a previous study, we reported that *P. aeruginosa* resists blood clearance in mice in vivo and Kupffer cell association in vitro and that systemic bacteremia consequently occurs (13). In the infection model used in this study, each step involved in the production of systemic bacteremia mimics the clinical pathophysiology of primary bacteremia that originates in the intestinal microflora. We therefore speculate that ETA affects one or more steps in the bacterial colonization of the intestine, with subsequent invasion of the bloodstream and resistance against phagocytosis by Kupffer cells.

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REFERENCES

1. **Bjorn, M. J., O. R. Pavlovskis, M. R. Thompson, and B. H. Iglewski.** 1979. Protection of exoenzyme S during *Pseudomonas aeruginosa* infections of burned mice. Infect. Immun. **24**:837–842.
2. **Bryan, C. S., K. L. Reynolds, and E. R. Brenner.** 1983. Analysis of 1,186 episodes of gram-negative bacteremia in non-university hospitals: the effects of antimicrobial therapy. Rev. Infect. Dis. **5**:629–638.
3. **Cross, A. S., J. C. Sadoff, B. H. Iglewski, and P. A. Sokol.** 1980. Evidence for the role of toxin A in the pathogenesis of infection

with *Pseudomonas aeruginosa*. J. Infect. Dis. **142**:538–545.
4. **Cryz, S. J., Jr., R. L. Friedman, and B. H. Iglewski.** 1980. Isolation and characterization of a *Pseudomonas aeruginosa* mutant producing nontoxic, immunologically crossreactive toxin A protein. Proc. Natl. Acad. Sci. USA **77**:7199–7203.
5. **Dick, J. D., V. Shull, J. E. Karp, and J. Valentine.** 1988. Bacterial and host factors affecting *Pseudomonas aeruginosa* colonization versus bacteremia in granulocytopenic patients. Eur. J. Cancer Clin. Oncol. **25**(Suppl. 1):S47–54.
6. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. Infect. Immun. **57**:291–294.
7. **Furuya, N., Y. Hirakata, K. Tomono, T. Matsumoto, K. Tateda, M. Kaku, and K. Yamaguchi.** Comparison of mortality rates in mice with endogenous septicaemia due to *Pseudomonas aeruginosa* isolates from different clinical sources. J. Med. Microbiol., in press.
8. **Galloway, D. R., R. C. Hedstrom, J. L. McGowan, S. P. Kessler, and D. J. Wozniak.** 1989. Biochemical analysis of CRM 66. J. Biol. Chem. **264**:14869–14873.
9. **Grauser, M. P., G. Zanetti, J.-D. Baumgartner, and J. Cohen.** 1991. Septic shock: pathogenesis. Lancet **338**:732–739.
10. **Henne, E., W. H. Campbell, and E. Carlson.** 1991. Toxic shock syndrome toxin 1 enhances synthesis of endotoxin-induced tumor necrosis factor in mice. Infect. Immun. **59**:2929–2933.
11. **Hirakata, Y., M. Kaku, R. Mizukane, K. Ishida, N. Furuya, T. Matsumoto, K. Tateda, and K. Yamaguchi.** 1992. Potential effects of erythromycin on host defense systems and virulence of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36**:1922–1927.
12. **Hirakata, Y., M. Kaku, K. Tomono, K. Tateda, N. Furuya, T. Matsumoto, R. Araki, and K. Yamaguchi.** 1992. Efficacy of erythromycin lactobionate for treating *Pseudomonas aeruginosa* bacteremia in mice. Antimicrob. Agents Chemother. **36**: 1198–1203.
13. **Hirakata, Y., K. Tomono, K. Tateda, T. Matsumoto, N. Furuya, K. Shimoguchi, M. Kaku, and K. Yamaguchi.** 1991. Role of bacterial association with Kupffer cells in occurrence of endogenous systemic bacteremia. Infect. Immun. **59**:289–294.
14. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas aeruginosa*. Microbiol. Rev. **43**:73–102.
15. **Holt, P. S., and M. L. Misfeldt.** 1984. Alteration of murine immune response by *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. **45**:227–233.
16. **Iglewski, B. H., and J. C. Sadoff.** 1979. Toxin inhibitors of protein synthesis: production, purification, and assay of *Pseudomonas aeruginosa* toxin A. Methods Enzymol. **60**:780–793.
17. **Liu, P. V.** 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130**(Suppl.):S94–99.
18. **Middlebrook, J. L., and R. B. Dorland.** 1977. Response of cultured mammalian cells to the exotoxins of *Pseudomonas aeruginosa* and *Corynebacterium diphtheriae*: differential cytotoxicity. Can. J. Microbiol. **23**:183–189.
19. **Miethke, T., C. Wahl, K. Heeg, B. Echtenacher, P. H. Krammer, and H. Wagner.** 1992. T cell-mediated lethal shock triggered in mice by the superantigen Staphylococcal enterotoxin B: critical role of tumor necrosis factor. J. Exp. Med. **175**:91–98.
20. **Misfeldt, M. L., P. K. Legaard, S. E. Howell, M. H. Fornella, and R. D. LeGrand.** 1990. Induction of interleukin-1 from murine peritoneal macrophages by *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. **58**:978–982.
21. **Neu, H. C.** 1983. The role of *Pseudomonas aeruginosa* in infections. J. Antimicrob. Chemother. **11**(Suppl. B):1–13.
22. **Nicas, T. I., and B. H. Iglewski.** 1985. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. Can. J. Microbiol. **31**:387–392.
23. **Ohman, D. E., R. P. Burns, and B. H. Iglewski.** 1980. Corneal infections in mice with toxin A and elastase mutants of *Pseudomonas aeruginosa*. J. Infect. Dis. **142**:547–555.
24. **Ohman, D. E., J. C. Sadoff, and B. H. Iglewski.** 1980. Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. Infect. Immun. **28**:899–908.
25. **Pavlovskis, O. R., B. H. Iglewski, and M. Pollack.** 1978. Mechanism of action of *Pseudomonas aeruginosa* exotoxin A in experimental mouse infections: adenosine diphosphate ribosylation of elongation factor 2. Infect. Immun. **19**:29–33.
26. **Pollack, M., and S. E. Anderson, Jr.** 1978. Toxicity of *Pseudomonas aeruginosa* exotoxin A for human macrophages. Infect. Immun. **19**:1092–1096.
27. **Pollack, M., and L. S. Young.** 1979. Protective activity of antibodies to exotoxin A and lipopolysaccharide at the onset of *Pseudomonas aeruginosa* septicemia in man. J. Clin. Invest. **63**:276–286.
28. **Snell, K., I. A. Holder, S. A. Leppla, and C. B. Saelinger.** 1978. Role of exotoxin and protease as possible virulence factors in experimental infections with *Pseudomonas aeruginosa*. Infect. Immun. **19**:839–845.
29. **Staugas, R. E. M., D. P. Harvey, A. Ferrante, M. Nandoskar, and A. C. Allison.** 1992. Induction of tumor necrosis factor (TNF) and interleukin-1 (IL-1) by *Pseudomonas aeruginosa* and exotoxin A-induced suppression of lymphoproliferation and TNF, lymphotoxin, gamma interferon, and IL-1 production in human leukocytes. Infect. Immun. **60**:3162–3168.
30. **Stuart, R. K., and M. Pollack.** 1982. *Pseudomonas aeruginosa* exotoxin A inhibits proliferation of human bone marrow progenitor cells in vitro. Infect. Immun. **38**:206–211.
31. **Young, L. S., P. Stevens, and B. Kaijser.** 1982. Gram-negative pathogens in septicemic infections. Scand. J. Infect. Dis. **31**:78–94.
32. **Wick, M. J., J. M. Cook, and B. H. Iglewski.** 1992. Structure-function analysis of exotoxin A proteins with mutations at histidine 426. Infect. Immun. **60**:1128–1139.
33. **Woods, D. E., M. S. Schaffer, H. R. Rabin, G. D. Campbell, and P. A. Sokol.** 1986. Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. J. Clin. Microbiol. **24**:260–264.
34. **Zehavi-Willner, T.** 1988. Induction of murine cytotoxic T lymphocytes by *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. **56**:213–218.