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Production of Exoenzyme S During *Pseudomonas aeruginosa* Infections of Burned Mice

MICHAEL J. BJORN,[1] OLGERTS R. PAVLOVSKIS,[2] MICHAEL R. THOMPSON,[1] AND BARBARA H. IGLEWSKI[1]*

Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon 97201,[1] and Department of Microbiology, Naval Medical Research Institute, Bethesda, Maryland 20014[2]

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Antisera which distinguished between *Pseudomonas aeruginosa* exoenzyme S and toxin A neutralized the adenosine diphosphate ribosyl transferase activity of the homologous, but not the heterologous, enzyme. Skin extracts and sera from burned mice infected with the exoenzyme S-producing strain *P. aeruginosa* 388 contained adenosine diphosphate ribosyl transferase activity that was not found in skin extracts or sera from uninfected mice. On the basis of immunological reactivity and enzymatic properties, the adenosine diphosphate ribosyl transferase activity present in skin extracts and sera from *P. aeruginosa* 388-infected mice was identified as exoenzyme S. Active elongation factor 2 levels in tissues from strain 388-infected mice were normal at 24 h postinfection, indicating that strain 388 does not produce detectable amounts of toxin A in vivo. An unexpected finding in this investigation was the presence of exoenzyme S-inactivating activity in the sera from some nonimmunized animals.

Pseudomonas aeruginosa is an opportunistic pathogen that produces a wide variety of extracellular products that may contribute to its pathogenicity (17, 18). Toxin A has the potential to be a major virulence factor (1, 3, 10, 11, 17, 19, 21-25). Toxin A exerts its lethal effect by inhibiting protein synthesis in the same manner as diphtheria toxin, i.e., by catalyzing the transfer of the adenosine diphosphate (ADP) ribose moiety of nicotinamide adenine dinucleotide onto eucaryotic elongation factor 2 (EF-2) (6, 9-11).

A second extracellular protein (exoenzyme S) produced by some strains of *P. aeruginosa* has recently been shown to have ADP-ribosyl transferase activity (13). Exoenzyme S differs from toxin A in that it does not ADP-ribosylate EF-2 but, rather, modifies one or more different proteins present in eucaryotic cell extracts (13). Furthermore, exoenzyme S is not precipitated or neutralized by A antitoxin (13). The enzymatic activity of S is partially destroyed by pretreatment with urea and dithiothreitol (DTT) (13), whereas such pretreatment potentiates the enzymatic activity of toxin A (16, 28).

No studies have been done to determine if exoenzyme S plays a role in *P. aeruginosa* infections. As a first step in evaluating this possibility, the present study was undertaken to determine if exoenzyme S is produced in vivo. A second objective was to further examine the immunological relationship between exoenzyme S and toxin A.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strain 388 was kindly provided by B. Minshew, University of Washington School of Medicine, Seattle, Wash., and strain PA-103 was provided by P. V. Liu, University of Louisville School of Medicine, Louisville, Ky. Strain 388 has been shown to produce exoenzyme S, but not toxin A, in vitro (13). Strain PA-103 produces toxin A, but not exoenzyme S, in vitro. The strains were serotyped as described by Fisher et al. (7). Relevant characteristics of these strains are shown in Table 1.

Reagents. Nicotinamide adenine dinucleotide ([^{14}C]adenine) was purchased from Amersham Corp. DTT, histamine, casein, elastin-congo red, and nitrilotriacetic acid were purchased from Sigma Chemical Co. Norit A neutral-activated charcoal was obtained from Fisher Scientific Co.

Growth and exoenzyme S production by *P. aeruginosa* 388. The medium used for the growth of strain 388 was as previously described (13). An overnight culture (4 ml) inoculated into 100 ml of medium in each 2-liter flask was grown at 32°C with vigorous shaking. At 22 h, the cells were removed by centrifugation at 10,000 × *g* for 20 min at 4°C, and the supernatants were pooled.

Toxin purification. Strain 388 supernatant was diluted with 3 volumes of ice-cold water, 50 g of equilibrated diethylaminoethyl (DE-52)-cellulose (Whatman, Inc., Clifton, N.J.) was added, and the mixture was then stirred for 1 h. The DE-52 was

removed by filtration onto Whatman no. 1 filter paper and washed with 2 liters of 50 mM NaCl–10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.0, and then exoenzyme S was eluted with 300 mM NaCl–10 mM Tris-hydrochloride, pH 8.0. The eluate was filter sterilized (Nalge Co., Rochester, N.Y.) and concentrated by ultrafiltration, using a PM-10 membrane (Amicon Corp., Lexington, Mass.), and the buffer was reequilibrated to 50 mM NaCl–10 mM Tris-hydrochloride, pH 8.0. Approximately 15 ml (1.1 mg/ml) of this material was applied to a diethylaminoethyl-Sephadex A-25 column (2.5 by 8.0 cm). A linear gradient from 50 to 400 mM NaCl was applied in 10 mM Tris-hydrochloride, pH 8.0. The major active peak at 200 mM NaCl was pooled and concentrated. These procedures resulted in a 30-fold purification of exoenzyme S which contained 0.48 mg of protein per ml with a ratio of optical density at 280 nm to that at 260 nm 1.4. Aliquots were frozen at −70°C.

P. aeruginosa PA-103 was used as a source of toxin A, which was produced and purified as previously described (28).

Preparation of specific antisera. A 1-ml mixture consisting of equal parts of Freund complete adjuvant (Difco) and 200 μg of partially purified exoenzyme S per ml in phosphate-buffered saline was injected into each adult male New Zealand rabbit as follows: 0.1 ml subcutaneously in each hind foot, 0.4 ml subcutaneously in the back, and 0.4 ml intramuscularly. The animals were then injected three times every 2 weeks, using the same sites and doses, in Freund incomplete adjuvant (Difco). Ten days after the last injection, the rabbits were bled, and the separated serum (rabbit S antiserum) was stored in small aliquots at −20°C. Purified toxin A was used to immunize rabbits and a sheep as previously described (12).

Purification of toxin A antibody. Toxin A was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) as described by March et al. (19). The toxin A-Sepharose column buffer consisted of 50 mM Tris-hydrochloride, pH 8.0, 200 mM NaCl, and 1 mM ethylenediaminetetraacetate. Immunoglobulin was precipitated from sheep antitoxin A with ammonium sulfate (12) and then dialyzed against column buffer. Ten milliliters was applied to the toxin A-Sepharose column at 4°C. Material unbound after 20 min was washed out with column buffer, and then bound material was eluted with 50 mM glycine·hydrochloride, pH 3.2. The eluates (antitoxin A immunoglobulin) from three to four columns were pooled, concentrated to 1 mg/ml on an Amicon PM-30 membrane, and then reequilibrated with column buffer by ultrafiltration and stored at 4°C.

ADP-ribosyl transferase activity. Ten microliters of each sample was incubated at 25°C with 25 μl of wheat germ extract (150 μg) (12), 25 μl of reaction buffer (5 mM Tris-hydrochloride, pH 8.2, 0.1 mM ethylenediaminetetraacetate, 40 mM DTT), and 5 μl of nicotinamide adenine dinucleotide ([^{14}C]adenine) (280 mCi/mmol; 12.5 μCi/ml). Reaction mixtures containing mouse skin extract or serum were incubated for 30 min, whereas those with toxin A or exoenzyme S were incubated for 5 min. All reactions were stopped by the addition of 0.1 μl of 10% trichloroacetic acid and processed, and radioactivity was measured as previously described (12). Where indicated, samples were preincubated with an equal volume of 8 M urea and 2% DTT for 15 min at 25°C (12) and then assayed for ADP-ribosyl transferase activity.

Enzyme neutralization by sera. All sera were heat inactivated (56°C for 15 min) before their ability to neutralize ADP-ribosyl transferase activity was tested. Culture supernatants of *P. aeruginosa* strains 388 and PA-103 were used as a source of crude exoenzyme S and toxin A, respectively. Rate-limiting concentrations of these enzymes were obtained by diluting crude exoenzyme S 1:30 and crude toxin A 1:3 before use. Crude toxin A was then activated with urea and DTT (12). Skin extracts and serum samples from mice were used undiluted. Neutralization was examined by preincubating equal volumes of the appropriate serum and sample for 15 min at 37°C and then assaying ADP-ribosyl transferase activity as described above.

Experimental burn infection model. A burned mouse model, previously described (22, 26), was used. Female Swiss white mice (strain NIH-NMRI CV) weighing 20 ± 2 g were anesthetized with methoxyflurane (Penthrane; Abbott Laboratories, North Chicago, Ill.) and subjected to a 10-s alcohol flame burn involving 15% of the total body surface. Mice were injected subcutaneously in the burn area immediately after burn trauma with two 50% lethal doses of the appropriate strain, which resulted in fatal infections in about 90% of the mice 50 ± 10 h postinfection. Control animals consisted of anesthetized, nontraumatized, or burned mice injected subcutaneously with 0.5 ml of sterile phosphate-buffered saline. At appropriate intervals postinfection, mice were sacrificed by cervical dislocation and blood was obtained by cardiac puncture. Full-thickness specimens of burned skin (or unburned skin from appropriate control animals) were removed, and skin extracts were prepared as described by Saelinger et al. (24).

Extraction and quantitation of mouse organ EF-2. Livers, kidneys, and spleens were removed from mice immediately after they were sacrificed, and the tissues were frozen at −70°C. EF-2 was extracted from and quantitated in tissue homogenates by the method of Gill and Dinius (8) as modified by Iglewski et al. (11).

Other methods. Protein concentrations were determined by the method of Bradford (4), modified by using a commercial reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) purchased from Bio-Rad Laboratories, Richmond, Calif. Bovine gamma globulin (Bio-Rad) was used as the standard. Proteolytic activity in crude supernatants of *P. aeruginosa* strains PA-103 and 388 was determined by the method of Kunitz (14) as modified by Wretlind and Wadstrom (29), using casein as the substrate. Elastase activity was quantified with elastin-congo red as a substrate as previously described (29).

RESULTS

Specific neutralization of exoenzyme S activity. To determine if exoenzyme S was produced in vivo, we used an immunological method to specifically identify this enzyme and distin-

guish it from toxin A. Antisera obtained from rabbits immunized with exoenzyme S neutralized the enzymatic activity of exoenzyme S but not that of toxin A. The enzymatic activity of exoenzyme S was not neutralized by rabbit A antitoxin, which completely neutralized the toxin A enzymatic activity. Since these antisera specifically neutralized the enzymatic activity of the homologous, but not the heterologous, enzyme, they could be utilized to identify the enzymatic activity in an unknown sample.

We also tested the neutralizing ability of A antitoxin which had been raised in sheep by immunization with pure toxin A. Surprisingly, this sheep antitoxin A neutralized the enzymatic activity of both toxin A and S exoenzyme. However, when examined, it was found that the preimmunization serum from this sheep neutralized S enzymatic activity, but not toxin A enzymatic activity. The anti-S titer of the pre- and post-toxin A immune sheep sera were identical. Anti-S activity copurified with gamma globulin during ammonium sulfate precipitation but did not copurify with specific antitoxin A immunoglobulin when it was purified on a toxin A-Sepharose 4B affinity column. Anti-S activity was also found in other (four of six) normal sheep sera, one of five normal rabbit sera, and two of six normal mouse sera (data not shown). It is interesting that none of the normal sera tested neutralized the enzymatic activity of toxin A.

In vivo production of exoenzyme S. The 50% lethal dose of strain 388 was markedly reduced when mice were burned (Table 1). The 50% lethal dose of strain 388 in normal (unburned) mice was 2.0×10^6 organisms, in contrast to a 50% lethal dose of 1.1×10^2 organisms in a burned mouse.

Skin extracts from burned mice that were infected with *P. aeruginosa* strain 388 contained ADP-ribosyl transferase activity that was not found in skin extracts from uninfected control mice (Fig. 1). The enzyme activity was present in the burned infected mouse skin extracts at the earliest time postinfection (18 h) that we tested and remained relatively constant from 18 to 48 h postinfection. Whereas there was a wide range of ADP-ribosyl transferase levels in the skin extracts of individual burned infected mice, 37 of 39 samples from infected mice had enzyme levels higher than those from all 49 control animals (Fig. 1).

The average level of ADP-ribosyl transferase activity in the sera from burned infected mice at 18 h postinfection was equal to that in the sera from control noninfected mice (Fig. 2). Levels of ADP-ribosyl transferase activity in the sera from infected mice increased markedly at 24 h postinfection and continued to increase linearly through 48 h. The average enzyme levels in sera from noninfected control mice did not change significantly over the 48-h period (Fig. 2). Enzyme levels similar to those of noninfected control mice were found in sera and skin extracts from mice infected with the toxin A- and exoenzyme S-negative strain WR-5 (data not shown).

Identification of the ADP-ribosyl transferase activity in samples from burned infected mice. The ADP-ribosyl transferase activity in skin extracts and sera from burned, strain 388-infected mice was further character-

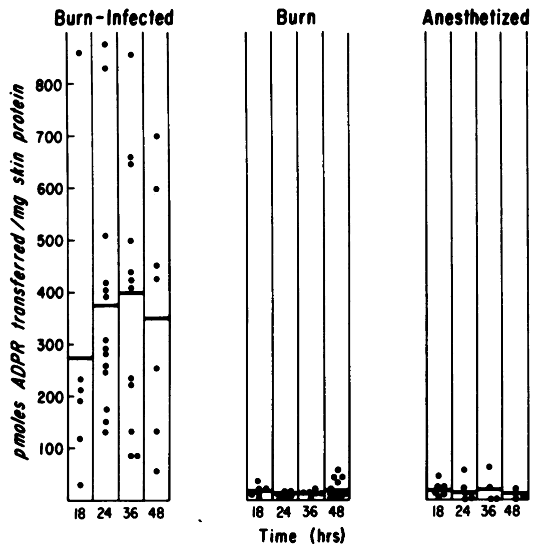

FIG. 1. *ADP-ribosyl transferase activity in skin extracts of burned mice infected with P. aeruginosa strain 388 and in skin extracts of control noninfected mice that were anesthetized and burned or anesthetized only. The horizontal lines represent the mean ADP-ribosyl transferase activity of skin extracts for each group of mice.*

TABLE 1. *Characterization of P. aeruginosa strains 388 and PA-103*

Strain	Source	Serotype	Toxin A	Exoenzyme S	Protease	Elastase	LD_{50} (CFU)[a]	
							Normal mice	Burned mice
388	Burn wound	1	−	+	+	+	2.0×10^6	1.1×10^2
PA-103	Sputum	2	+	−	+	−	1.8×10^6	1.2×10^3

[a] LD_{50}, 50% lethal dose; CFU, colony-forming units.

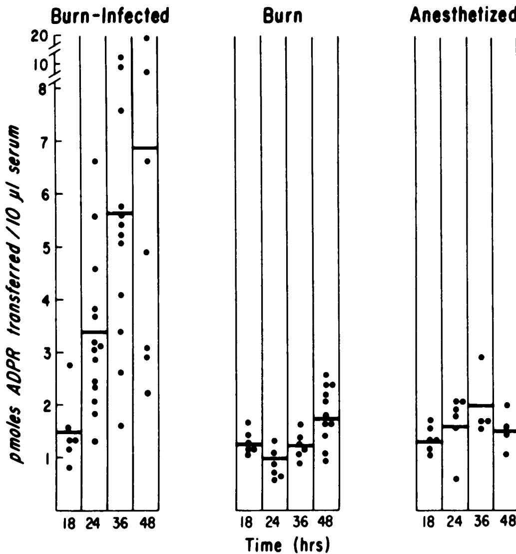

FIG. 2. *ADP-ribosyl transferase activity in sera from burned mice infected with P. aeruginosa strain 388 and in sera from control noninfected mice that were anesthetized and burned or anesthetized only. The horizontal lines represent the mean ADP-ribosyl transferase activity of sera for each group of mice.*

ized. Most of this enzyme activity was neutralized by exoenzyme S antiserum but not by A antitoxin (Table 2). The ADP-ribosyl transferase activity in sera from control (noninfected) mice was not neutralized by either A antitoxin or S antiserum (Table 2). These data indicate that most of the ADP-ribosyl transferase present in skin extracts and sera of strain 388-infected mice was due to exoenzyme S.

The treatment of skin extracts or sera from burned infected mice with urea and DTT partially destroyed the enzymatic activity (Table 3). To determine if the skin extracts or sera contained a factor which might alter these enzymes, crude exoenzyme S or crude toxin A was preincubated in skin extracts or sera from burned noninfected mice at 25°C for 15 min before assaying their enzymatic activities. The preincubation of these enzymes in uninfected mouse sera or skin extracts did not alter their enzymatic properties (Table 3, controls).

Active EF-2 levels in organs from *P. aeruginosa* 388-infected, burned mice. In agreement with previous reports (21, 22, 25), the levels of active EF-2 in tissues from burned mice infected with strain PA-103 were markedly decreased at 24 h postinfection (Table 4). In contrast, active EF-2 levels in burned mice infected with strain 388 were normal in the livers, kidneys, and spleens at 24 h postinfection (Table 4). At 48 h postinfection, only small decreases in the active EF-2 levels in the livers were found in strain 388-infected moribund animals (data not shown).

DISCUSSION

While investigating possible immunological cross-reactivity between exoenzyme S and toxin A, we found that sera from some nonimmunized animals inactivated S enzyme activity. The sera from one of five rabbits, five of seven sheep, and two of six mice partially neutralized S enzymatic activity (data not shown). Furthermore, a sheep immunized with purified toxin A had equal titers of anti-S activity in the pre-bleed and in the immune (antitoxin A) serum. The exoenzyme S-inactivating factor in this antitoxin A serum

TABLE 2. *Neutralization of ADP-ribosyl transferase activity in skin extracts and sera from mice*

Skin extracts/sera	Neutralization[a]	
	A antitoxin	S antiserum
Skin extracts: 388-infected burned mice		
Skin no.		
3	–	+ (87)
22	–	+ (84)
23	–	+ (82)
39	–	+ (86)
56	–	+ (87)
Sera: 388-infected burned mice		
Serum no.		
21	–	+ (53)
37	–	+ (80)
38	–	+ (78)
40	–	+ (58)
41	–	+ (73)
Sera: control (burned noninfected mice)		
Serum no.		
45	–	–
47	–	–
Sera: control (anesthesized only) mice		
Serum no.		
31	–	–
50	–	–

[a] Numbers in parentheses represent percentage of skin extract or serum ADP-ribosyl transferase activity that was neutralized by A antitoxin or S antiserum as compared to the ADP-ribosyl transferase activity of a sample treated with an equal volume of 0.9% saline containing 0.1 mg of bovine serum albumin per ml for 15 min at 37°C. Samples showing <10% reduction over controls were considered negative (–), and those with >10% reduction were scored positive (+).

TABLE 3. *Effect of urea and DTT on the ADP-ribosyl transferase activity of mouse skin extracts and sera*

Skin extracts/sera	ADP ribose incorporated (pmol)[a]	
	+ Water	+ Urea, DTT
Control skin extracts or sera + toxin A or exoenzyme S[b]		
Skin extract + toxin A	0.8	12.4
Skin extract + exoenzyme S	8.5	4.9
Serum + toxin A	1.7	13.4
Serum + exoenzyme S	11.9	5.5
Skin extracts from burned infected mice		
Skin no.		
3	28.0	15.4
23	48.1	32.1
37	60.0	45.9
39	50.9	29.0
56	25.9	19.6
Sera of burned infected mice		
Serum no.		
19	14.3	8.6
21	13.3	8.2
23	9.5	6.5
37	26.7	17.0
41	12.1	4.5

[a] Per 10 μl of skin extract or serum.

[b] Crude toxin A (10 μl) or crude exoenzyme S (10 μl of a 1:10 dilution) were preincubated with 90 μl of skin extract or sera for 1 h at 37°C before being tested for ADP-ribosyl transferase activity.

TABLE 4. *Comparison of the active EF-2 levels in tissue extracts from burned mice infected with P. aeruginosa PA-103 or 388*[a]

Organ[c]	% Control active EF-2 levels[b]	
	PA-103 infection	388 infection
Liver	35	102
Kidney	83	95
Spleen	82	101

[a] Mice were sacrificed 24 h after being burned and infected.

[b] Control values were obtained using the appropriate tissue from anethestized and burned uninfected mice.

[c] Organs from six similarly treated mice were pooled.

copurified with gamma globulin during ammonium sulfate precipitation but did not copurify with specific antitoxin A immunoglobulin. Whether this anti-S activity is due to antibody remains to be determined.

By immunizing only rabbits whose preimmune sera contained no detectable anti-S or anti-A activity, we were able to develop a suitable exoenzyme S antiserum. In a previous report (13), the enzymatic activity of exoenzyme S was not neutralized by A antitoxin. This observation is confirmed in this report, and it is also demonstrated that the enzymatic activity of toxin A is not neutralized by exoenzyme S antibody. Thus, these specific antisera (anti-S or anti-A) can be used to identify the enzymatic activity in an unknown sample providing the preimmune sera are first examined to ascertain that they do not have anti-S activity.

Most extracellular bacterial products known to be virulence factors have been shown to be produced in vivo. We attempted to detect the in vivo production of exoenzyme S by *P. aeruginosa* strain 388, a strain that produces the enzyme in vitro (13). Exoenzyme S was produced in vivo in burned mice infected with *P. aeruginosa* 388 (Fig. 1 and 2). ADP-ribosyl transferase activity was detected in extracts of skin obtained 18, 24, 36, and 48 h postinfection (Fig. 1). This enzymatic activity was also detected in sera from burned mice infected with strain 388 at 24 h postinfection, and the mean levels increased approximately linearly through 48 h (Fig. 2). That the ADP-ribosyl transferase activity detected in the skin extracts and sera of burned infected mice was indeed due to exoenzyme S was shown by its specific neutralization by S antiserum but not by A antitoxin (Table 2). In addition, this enzymatic activity present in skin extracts and sera from strain 388-infected animals was decreased by pretreatment with urea and DTT (Table 3), which is characteristic of exoenzyme S but not of toxin A (13). Finally, in contrast to the reduction of active EF-2 levels in tissues from burned mice infected with toxin A-producing strains of *P. aeruginosa* (21, 25), levels of EF-2 in the livers, kidneys, and spleens of burned mice 24 h after infection with strain 388 were not altered in comparison to the levels of EF-2 in noninfected control mice (Table 4). These data (Tables 2–4) indicate that strain 388 does not produce detectable amounts of toxin A in vivo. Small decreases in active EF-2 levels were observed in tissues from animals infected 48 h previously with strain 388. These slight decreases seen with strain 388 were similar to decreases previously reported using the toxin A- and exoenzyme S-negative strain, WR-5, and presumably reflect nonspecific tissue degeneration in moribund animals (21).

Exoenzyme S levels in skin extracts and sera of the burned infected mice varied over a wide range (Fig. 1 and 2). When the sera of six normal mice were tested for exoenzyme S-neutralizing activity, two of six were capable of partially neutralizing the ADP-ribosyl transferase activ-

ity of exoenzyme S (data not shown). One explanation for the wide range of responses of individual animals could be the presence of preexisting antibodies.

In conclusion, we have shown that exoenzyme S is produced in vivo in animals infected with *P. aeruginosa* strain 388. It was further demonstrated that strain 388 was virulent for burned mice and that this was not due to production of detectable levels of toxin A. Thus, exoenzyme S may be a virulence factor of *P. aeruginosa*. However, information concerning its toxicity, its production by clinical isolates, and the protective capabilities of specific S antibodies in *P. aeruginosa* infections is required to evaluate the relative importance of exoenzyme S.

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