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Exotoxin Production by Clinical Isolates of *Pseudomonas aeruginosa*

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Seventy-five consecutive clinical *Pseudomonas aeruginosa* isolates were tested for in vitro exotoxin production. Exotoxin was demonstrated in culture filtrates biologically, by its ability to produce characteristic dermonecrotic lesions in guinea pigs, and serologically, by counterimmunoelectrophoresis (CIE) with rabbit antiserum elicited with purified exotoxin. By these two methods, exotoxin was detected in 87 and 89% of *P. aeruginosa* strains, respectively ($r = 0.48$, $P < 0.001$). Although less sensitive than CIE in detecting exotoxin, immunodiffusion demonstrated a reaction of antigenic identity in most cases. Exotoxin was produced by all seven Fisher-Devlin immunotypes and by untypable strains. In contrast, exotoxin was not detected in the culture filtrates of 16 non-*aeruginosa* pseudomonas isolates and 48 non-pseudomonas organisms. The production of biologically similar and antigenically closely related exotoxins is thus a characteristic of the majority of *P. aeruginosa* strains derived from diverse clinical sources.

An exotoxin (exotoxin A) derived from a non-protease-producing strain of *Pseudomonas aeruginosa* (PA103) has been purified (2, 3, 8) and its effects have been characterized (1, 5-7, 11, 12; O. R. Pavlovskis, L. T. Callahan III, and M. Pollack, *Microbiology-1975*, p. 252-256, American Society for Microbiology, Washington, D.C.). Antigenically similar material has been detected in cultures of other strains of *P. aeruginosa* (3), and mice, passively immunized with anti-exotoxin A serum, were protected against challenge with live heterologous *P. aeruginosa* strains (7; Pavlovskis, Pollack, and Callahan, unpublished data). In addition, exotoxin-neutralizing antibodies have been demonstrated in sera from patients recovering from infections caused by *P. aeruginosa* strains of various serotypes (13).

These data, suggesting the possible wide distribution of exotoxin among strains of *P. aeruginosa*, are confirmed in this study, which examines exotoxin production among a moderately large number of clinical pseudomonas isolates by biological and immunological tests. Exotoxin was detected in culture filtrates by its ability to produce characteristic dermonecrotic lesions in guinea pigs and by immunodiffusion and counterimmunoelectrophoresis (CIE) assays with specific rabbit antitoxin serum.

MATERIALS AND METHODS

Microorganisms. Seventy-five consecutive *P. aeruginosa* and other clinical isolates were obtained from the Bacteriology Laboratory, National Naval Medical Center, Bethesda, Md., between January and April 1976. Twelve additional strains representing various *Pseudomonas* species were obtained from the American Type Culture Collection, Rockville, Md. Standard identification procedures were used, including the API-20E system (Analytab Products, Inc., Plainview, N.Y.), for identification of the genera and species of the *Enterobacteriaceae* and tentative identification of *Pseudomonas* species (14). *Pseudomonas* strains were characterized by growth in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 42°C, oxidation of maltose and mannitol, nitrite production, motility, ornithine and arginine decarboxylation, indolphenol oxidase reaction, fluorescence on Sellers medium, and liquefaction of gelatin (4).

Growth of organisms and preparation of culture filtrates. Stock cultures were streaked on Trypticase soy agar (TSA) plates (Baltimore Biological Laboratories, Cockeysville, Md.) and incubated overnight at 30°C. Isolated colonies were picked, streaked on TSA slants, and incubated overnight. The growth was suspended in Trypticase soy broth dialysate (TSBD) which contained 15% (vol/vol) glycerol and was stored in aliquots at −70°C.

For each experiment, an aliquot was thawed and used to inoculate a TSA slant. After overnight incubation at 30°C, growth from this slant was suspended in TSBD and used to inoculate 40 ml of TSBD in a 300-ml baffled nephelometer flask. To inhibit protease production (3), nitrilotriacetic acid (NTA) was added to this culture mixture in a final concentration of 5 mM. As shown in a previous study (3), this concentration of NTA has no effect on the growth of several *Pseudomonas* strains tested and enhances rather than depresses exotoxin production

by protease-producing strains while having little or no effect on exotoxin production by PA103. Cultures were shaken at 250 rpm for 24 h at 34°C in a shaking incubator (Psycrotherm, New Brunswick Scientific, New Brunswick, N.J.) and centrifuged for 20 min at 10,000 rpm, and supernatants were filter-sterilized with prewashed 0.45-μm membrane filters (Nalge, Rochester, N.Y.).

Immunotyping. *P. aeruginosa* isolates were typed by a slide agglutination test (10) using seven immunotype sera kindly supplied by M. Fisher and H. Devlin (Parke-Davis Co., Detroit, Mich.).

Antitoxin serum. Exotoxin isolated from the prototype *P. aeruginosa* strain (PA103) was purified (2, 3). This preparation had one major component (exotoxin) and a second minor band (copurifying protein) by analytical polyacrylamide gel electrophoresis, and was used to immunize rabbits. The resulting antitoxin serum used in CIE and immunodiffusion tests had a cytotoxicity neutralization titer (13) of 1:4,000.

CIE. Unconcentrated culture filtrates were reacted with antitoxin serum by CIE (Austigen II CIE system, Hyland, Costa Mesa, Calif.) for 1 h at a cell current of 30 mA. Plates were refrigerated overnight, and unstained precipitin lines were scored from 0 to 4+.

Immunodiffusion. Culture filtrates prepared as above were concentrated 100-fold by means of a Minicon B15 concentrator (Amicon Corp., Lexington, Mass.). This procedure was performed at 4°C in order to limit proteolytic digestion of exotoxin. Slides measuring 50 by 75 mm were coated on one side with 6 ml of 1% refined agar (Oxoid Ionagar no. 2, Consolidated Laboratories, Inc., Chicago Heights, Ill.) in 0.75 M barbital buffer, pH 8.2. Antitoxin serum and concentrated culture filtrates were placed in appropriate agar wells, the slides were incubated for 24 h at room temperature and placed at 4°C overnight, and precipitin reactions were read the following day, without staining.

Guinea pig skin reactions. The backs of 600-g female guinea pigs were shaved and depilated with 5% calcium thioglycolate trihydrate cream (Tilden-Yates Laboratories, Wayne, N.J.). A checkerboard pattern of 2-cm squares was drawn on each guinea pig's back, and 0.2 ml of each culture filtrate was injected intradermally in duplicate squares. Skin reactions were read qualitatively (see Results) and scored form 0 to 4+ at 1, 12, 24, 48, and 72 h.

Protease assay. Nonspecific protease activity of culture filtrates was measured using a casein substrate (9).

Statistical analysis. Linear regressions were performed by the method of least squares, and validity of fit was expressed as the correlation coefficient, r. The significance of r was determined by a two-tailed Student t test.

RESULTS

Guinea pig skin reactions. Culture filtrates from 93% of 75 clinical *P. aeruginosa* isolates produced guinea pig skin lesions. These reactions were of two, often superimposed types: (1) an early hemorrhagic lesion that appeared within minutes of injection and evolved fully into a necrotic reaction by 4 h, and (2) a late, bland lesion composed of a pale white center and surrounding ring of intense erythema that first appeared 12 to 24 h after injection. This reaction evolved over 72 to 96 h, with the central portion becoming gradually necrotic and the surrounding ring of erythema first intensifying and then fading.

The early or type 1 lesion, produced by 67% of the 75 strains tested, correlated in intensity with protease activity of culture filtrates as measured by the casein assay ($r = 0.38$, $P < 0.05$) and was partially or entirely inhibited by the inclusion of NTA in the culture media. The late or type 2 lesion, produced by 87% of the 75 strains tested (Table 1), was identical to that produced by injection of highly purified exotoxin. The intensity of this lesion correlated with the amount of exotoxin present, as quantified by CIE (see below), and was totally abolished by heating filtrates at 56°C for 30 min or by incubating them with antitoxin serum before injection.

Most culture filtrates contained varying amounts of both exotoxin and protease, even in the presence of 5 mM NTA, and produced char-

TABLE 1. *Detection of exotoxin in crude culture filtrates of clinical P. aeruginosa isolates by CIE and guinea pig skin reaction*

Fisher serotype	No. of strains	CIE		Skin reaction	
		% Positive	Avg score[a]	% Positive	Avg score[a]
1	18	94	1.2	89	1.7
2	16	94	1.6	94	2.1
3	4	100	2.5	75	1.5
4	9	67	0.8	78	1.2
5	5	100	1.4	100	2.6
6	7	86	1.1	86	0.9
7	7	86	1.3	86	2.1
UT[b]	9	89	1.3	78	1.6

[a] Scale of 0 to 4 (negative results included).
[b] Untypable strains.

acteristics of both type 1 and type 2 skin lesions, i.e., a central zone of early hemorrhagic necrosis surrounded by later developing concentric zones of bland necrosis and erythema. In these instances the presence of NTA usually markedly reduced the size and intensity of the central hemorrhagic area, leaving a more "pure" type 2 lesion. And, conversely, if the culture filtrate was heated at 56°C for 30 min or incubated with antitoxin serum, the early hemorrhagic lesion was still produced, but without the later developing concentric zones of bland necrosis and erythema.

CIE and immunodiffusion. Exotoxin was detected by CIE in filter-sterilized culture supernatants in 89% of 75 clinical *P. aeruginosa* strains (Table 1). The majority of strains of each of the seven Fisher immunotypes as well as untypable strains reacted with antitoxin. Typical type 2 exotoxin skin lesions were produced in guinea pigs by filtrates from 87% of these same 75 *P. aeruginosa* strains, and among these strains the correlation between the intensity of precipitin lines obtained by CIE and skin lesion scores was excellent ($r = 0.48, P < 0.001$). Only 2 of 75 isolates showed no evidence of exotoxin production by skin reaction or CIE, whereas 3 organisms negative by CIE produced skin reactions and 8 eliciting no skin lesions were positive by CIE. Of 25 additional strains tested by CIE alone, 21 (84%) gave positive tests, paralleling the results obtained with the original 75 organisms tested.

No culture filtrates from 16 isolates tested, representing 9 non-*aeruginosa Pseudomonas* species, reacted with antitoxin serum or produced lesions in guinea pigs (Table 2). Likewise, there were no positive CIE tests or guinea pig skin reactions among 47 non-pseudomonas isolates (Table 3).

Culture filtrates from 53 clinical *P. aeruginosa* isolates were tested against antitoxin by immunodiffusion. Of these, 70% produced an identity reaction and 28% produced a line of non-identity in addition to (21%) or in lieu of (7%) an identity reaction (Table 4). In most cases, reactions of identity were much stronger than those of non-identity, which were often barely visible (Fig. 1). All 37 strains positive for exotoxin by immunodiffusion (i.e., producing a line of identity with purified exotoxin) were also positive by CIE, whereas 9 additional strains negative by immunodiffusion showed precipitin lines by CIE.

TABLE 2. *Crude culture filtrates of non-aeruginosa pseudomonas isolates that lacked exotoxin as determined by CIE and guinea pig skin reactions*

Pseudomonas species	No. of strains examined
fluorescens (including ATCC 13525)	5
cepacia (including ATCC 25416)	2
maltophilia (including ATCC 13637)	3
stutzeri (ATCC 17588)	1
putrefaciens (ATCC 8071)	1
alcaligenes (ATCC 14909)	1
pseudoalcaligenes (ATCC 17440)	1
pickettii (ATCC 27511)	1
diminuta (ATCC 11568)	1

DISCUSSION

The lethality of pseudomonas exotoxin for experimental animals (1, 6; Pavlovskis et al.,

TABLE 3. *Crude culture filtrates of non-pseudomonas isolates that lacked exotoxin as determined by CIE and guinea pig skin reaction*

Organism	No. of strains examined
Proteus mirabilis	3
Proteus vulgaris	1
Klebsiella pneumoniae	4
Citrobacter freundii	3
Proteus morganii	1
Proteus stuartii	1
Serratia marcescens	2
Enterobacter agglomerans	1
Enterobacter aerogenes	3
Enterobacter cloacae	3
Aeromonas hydrophilia	1
Escherichia coli	5
Streptococcus liquefaciens	1
Group D streptococci	4
Group A β-hemolytic streptococci	3
α-Hemolytic streptococci	1
Staphylococcus epidermidis	4
Staphylococcus aureus	4
Comamonas terrigena	1
Acinetobacter calcoaceticus	1

TABLE 4. *Detection of exotoxin in crude cultures filtrates of clinical P. aeruginosa isolates by immunodiffusion*

Fisher serotype	No. of strains	Precipitin reaction (%)			
		Identity	Non-identity	Both	Neither
1	16	38	12	31	19
2	8	50	0	25	25
3	4	75	0	25	0
4	7	14	0	43	43
5	3	100	0	0	0
6	3	33	33	0	33
7	7	71	0	0	29
UT[a]	5	60	20	0	20

[a] Untypable strains.

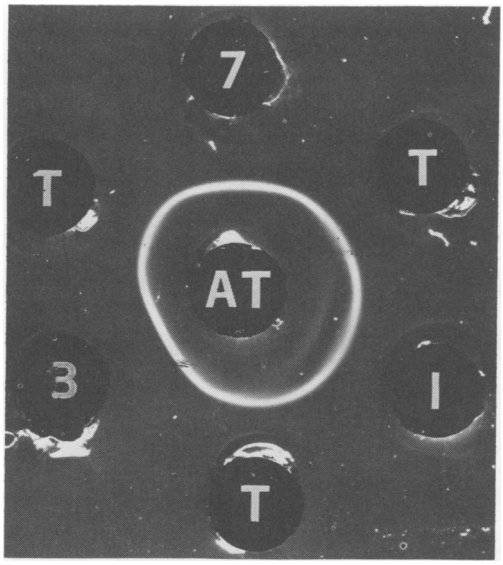

FIG. 1. *Immunodiffusion slide demonstrating antigenically homogeneous exotoxin in crude culture filtrates from three clinical P. aeruginosa isolates representing three different Fisher immunotypes. AT, Rabbit antiserum against purified PA103 exotoxin; T, purified exotoxin derived from PA103. Numbered wells contain 100× concentrated crude culture filtrates from clinical P. aeruginosa isolates, types 1, 3, and 7. Note second precipitin line showing non-identity with exotoxin in the case of the type 1 isolate.*

Microbiology–1975), its similarity of action compared with diphtheria toxin (5), and its apparent release during human infections (13) make it a candidate for a pathogenic role. The data presented underscore the significance of this pathogenic potential by showing that immunologically and biologically closely related exotoxins are widely produced by clinical *P. aeruginosa* strains of diverse immunotypes.

The antigenic relatedness of exotoxin derived from different strains of *P. aeruginosa* is suggested by the immunodiffusion tests and the close correlation between dermonecrosis produced in guinea pigs and intensity of precipitin reactions obtained by CIE. These findings do not rule out other biologically similar but antigenically distinct pseudomonas exotoxins, which Liu and Hsieh suggest on the basis of unpublished data (7). Possibly the three organisms we encountered that were negative for exotoxin by CIE but produced typical skin lesions represented the production of such a substance(s). It seems unlikely, however, on the basis of our data that if serologically heterologous exotoxins do exist they are as prevalent as exotoxin A.

Our ability to detect exotoxin in *P. aeruginosa* cultures was limited by the presence of varying amounts of protease, even when the protease inhibitor, NTA, was used, as well as by the sensitivity of the assays themselves. Our failure to demonstrate toxin by either immunoligical detection or skin reaction in a small percentage of the *P. aeruginosa* cultures tested may reflect these limitations rather than the actual inability of certain strains to produce toxin. In a related manner, both CIE and guinea pig skin reactivity were semiquantitative at best, especially in light of the presence of protease in most cultures. For these reasons, we did not attempt a quantitative comparison of exotoxin production by different strains, except to correlate CIE results with guinea pig skin reactions.

The ubiquity of immunologically closely related exotoxins with similar biological activity among many clinical strains of *P. aeruginosa*, coupled with the pathogenic potential as well as immunogenicity of exotoxin demonstrated in previous animal experiments, suggests the possibility of immunoprophylaxis or treatment of pseudomonas disease by active or passive immunization. The fact that many *P. aeruginosa* organisms contain exotoxin also underscores the possible diagnostic and epidemiological usefulness of measuring serum antitoxin that is present in high titer in patients recovering from recent pseudomonas infections and in low titer or not at all in uninfected subjects (13).

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