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THE ROLE OF NEWLY DISCOVERED EXOTOXIN (S TOXIN) IN PSEUDOMONAS AERUGINOSA INFECTIONS

Annual Report

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SUMMARY

A. ~~Our long term~~ goal is to determine the role of exotoxin S in humans infected with Pseudomonas aeruginosa and develop vaccines to reduce the morbidity and mortality associated with these infections. During the ~~period of the project~~ covered in this annual report (August 1, 1979 - July 31, 1980) we have:

(1) Developed a specific assay for S which allows us to distinguish between the enzymatic activities of toxins A and S ~~(Iglewski, W.J. et al. - manuscript in preparation).~~

(2) Utilized the above assay to determine the percentage of clinical isolates of P. aeruginosa which produce S ~~(Sokol, P.A., et al. - draft of manuscript is appended).~~

(3) Purified analytical amounts of S, determined its molecular weight and used the pure S to raise specific S antibodies in rabbits.

(4) Developed a defined medium for the production of S by P. aeruginosa strain 388-6 ~~(manuscript in preparation).~~

(5) Continued studies on the large scale purification of S.

(6) Purified several large batches of P. aeruginosa toxin A and diphtheria toxin, much of which has been sent to Drs. C. Alving and J. Sadoff (Walter Reed Army Institute of Research) and Dr. W. Brodsky (Mt. Sinai Medical Center) for collaborative studies supported by the Army. Several papers (#3 and #4 below) and one abstract (#5) have resulted from these collaborations this year.

B. Publications resulting from this research.

1. Thompson, M.R., Bjorn, M.J., Sokol, P.A., Lile, J.D., and Iglewski, B.H. Exoenzyme S: An ADP-ribosyl transferase produced by Pseudomonas aeruginosa in novel ADP-ribosylations of regulating enzymes and proteins. ed. by T. Sugemara and M. Smulson, Elsevier, N.Y. In press 1980 (copy appended).

2. Sokol, P.A., Sadoff, J.C., Crosa, A.S., McManus, A., Farber, B.F., and Iglewski, B.H. Exoenzyme S production by clinical isolates of Pseudomonas aeruginosa. To be submitted. Draft appended.

3. Cross, A.S., Sadoff, J.C., Iglewski, B.H., and Sokol, P.A. Evidence for the role of toxin A in the pathogenesis of human infection with Pseudomonas aeruginosa. J. Infect. Dis., 142:000-000, 1980 (In press October issue). Copy obtainable from Drs. A. Cross or J. Sadoff, Dept. Bact. Dis., Walter Reed Army Res. Inst., Washington, D.C. 20014.

4. Alving, C.R., Iglewski, B.H., Urban, K.A., Moss, J., Richard, R.L., and Sadoff, J.C. Binding of diphtheria toxin to phospholipids in liposomes. Proc. Natl. Acad. Sci. USA 77:1986, 1980. Reprints obtainable from Dr. C.R. Alving, Dept. Biochemistry, Walter Reed Army Inst. Res., Washington, D.C. 20014.

5. Sadoff, J., Reid, R., and Iglewski, B.H. Development of hybrid lipopolysaccharide-toxin A vaccines for Pseudomonas aeruginosa. I.C.A.A.C. Abstracts, 1980.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

During the course of this work the authors were greatly assisted by Dr. W. J. Iglewski, Mrs. Pamela Sokol, Mr. Jack Lile and Mrs. Joan Rittenberg. Their help is deeply appreciated. Portions of this research were done in collaboration with Drs. J. D. Sadoff, Alan Cross, and Carl Alving, WRAIR, Washington, D.C.; Dr. E. Zieger, University of California at San Diego; and Dr. J. Pennington, Harvard.

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I. Production of S by Clinical Isolates of P. aeruginosa.

A. Introduction.

Our initial studies on the production of S by clinical isolates of P. aeruginosa (see last year's progress report) involved growing each strain on trypticase soy broth dialysate (TSBD) with and without 10 mM NTA then determining the ADP-ribosyl transferase activity in a wheat germ extract of both supernatants with and without prior incubation with 4 M urea + 1% dithiothreitol (DTT). Confirmation of toxin phenotype was obtained by enzyme neutralization utilizing antisera prepared against pure toxin A or partially purified S.

While this method was reasonably accurate, it was extremely slow for screening large numbers of isolates as each strain had to be assayed in a number of ways. Also, in some strains that produced low amounts of both A and S, this procedure sometimes yielded ambiguous results depending on the relative amounts of A and S produced. Therefore a specific assay was needed to detect S. We have developed such an assay and have utilized it to determine the toxin phenotype of 124 clinical isolates of P. aeruginosa.

B. Materials and Methods.

1. Previously Described Methods. Many of the methods utilized in these studies were described in detail in past progress reports or in our publications. These include standard culture media (1); deferration, and iron determinations (2); bacterial strain characterization (3); bacterial maintenance (4); our standard ADP-ribosyl transferase assay (4) and our standard tissue culture assay for toxin A (4).

2. Development of a Specific Assay for the Enzymatic Activity of S. Toxins A and S, ADP-ribosylate different substrates (1). Toxin A, like diphtheria toxin, only ADP-ribosylates eucaryotic elongation factor 2 (EF-2) whereas S, ADP-ribosylate's a number of different eucaryotic proteins but S does not ADP-ribosylate EF-2 (1). These facts were taken advantage of to develop a specific enzymatic assay for S.

a. Isolation of Diphtheria and Pseudomonas Toxin A Resistant Cell Lines. Polyoma virus transformed baby hamster kidney cells (PyBHK) are quite sensitive to both diphtheria toxin and Pseudomonas toxin A. Diphtheria toxin at a concentration of 3×10^{-3} µgm/10^5 cells is sufficient to cause 50% inhibition of protein synthesis in our standard 5 hour assay system (5). The parent lines were exposed to increasing concentrations of diphtheria toxin for four hours. Surviving cells were grown in toxin free media then cloned in soft agar and re-exposed to still greater concentrations of diphtheria toxin. The diphtheria toxin concentrations used were: first passage (1.2×10^{-1} µgm toxin/10^5 cells); second passage (1.2 µg toxin/10^5 cells); third passage (12 µgm toxin/10^5 cells); and fourth passage (120 µgm toxin/10^5 cells).

Culture media, culture conditions, purification of diphtheria toxin, toxin A and cell toxin assays were performed as previously described (4,5). Toxin resistant survivors were cloned in soft agar as follows: 7.0 ml of 0.5% agar in culture medium (5) containing 2% fetal calf serum was placed in 60 x 15 mm tissue culture plates. After hardening, the agar was overlaid with 1.5 ml of 0.3% agar in growth medium to which was added 6.6×10^3 freshly lifted "resistant" cells/ml. The plates were incubated at 37°C in 5% CO_2 for 6 days and individual clones picked and grown; then retested for toxin sensitivity.

b. Tumor Induction and Preparation of Cell Extracts. Five week old male golden hamsters were inoculated subcutaneously and intrascapulary with 10^6 freshly lifted toxin resistant PyBHK cells (PyBHKR) or toxin sensitive (parental) PyBHK cells. Eight weeks post-inoculation, large palpable tumors were found on the animals' backs. Animals were sacrificed, tumors removed asceptically and weighed. Care was taken to remove only tissue that was within the tumor mass. The tumors were minced then homogenized in 4 volumes of 0.25 M sucrose at 4°C using a rotary homogenizer. To each ml of homogenate, 0.16 ml of diluent (4 M NaCl and 20 mM DTT) and 0.25 g of prewashed activated charcoal was added and the mixture was shaken at 5°C for 15 min to remove exogenous NAD. The extract was centifuged at 27,000 RPM (Type 30 rotor) for 75 min, the supernatant removed and the protein concentration adjusted to 7.2 mg/ml with 200 mM sodium acetate buffer pH 6.0 (S-assay buffer).

3. Clinical Isolates of P. aeruginosa. Clinical isolates of P. aeruginosa were obtained from Walter Reed Army Institute of Research, Fort Sam Houston Burn Center and the University of Virginia School of Medicine. These isolates were determined to be different strains on the basis of their Fisher-Devlin-Gnabasik serotype (6), colonial morphology, pigment production and protease production. Seventy-one of the clinical isolates were from (nonburn) bacteremia patients and 53 of the strains were from burn patients.

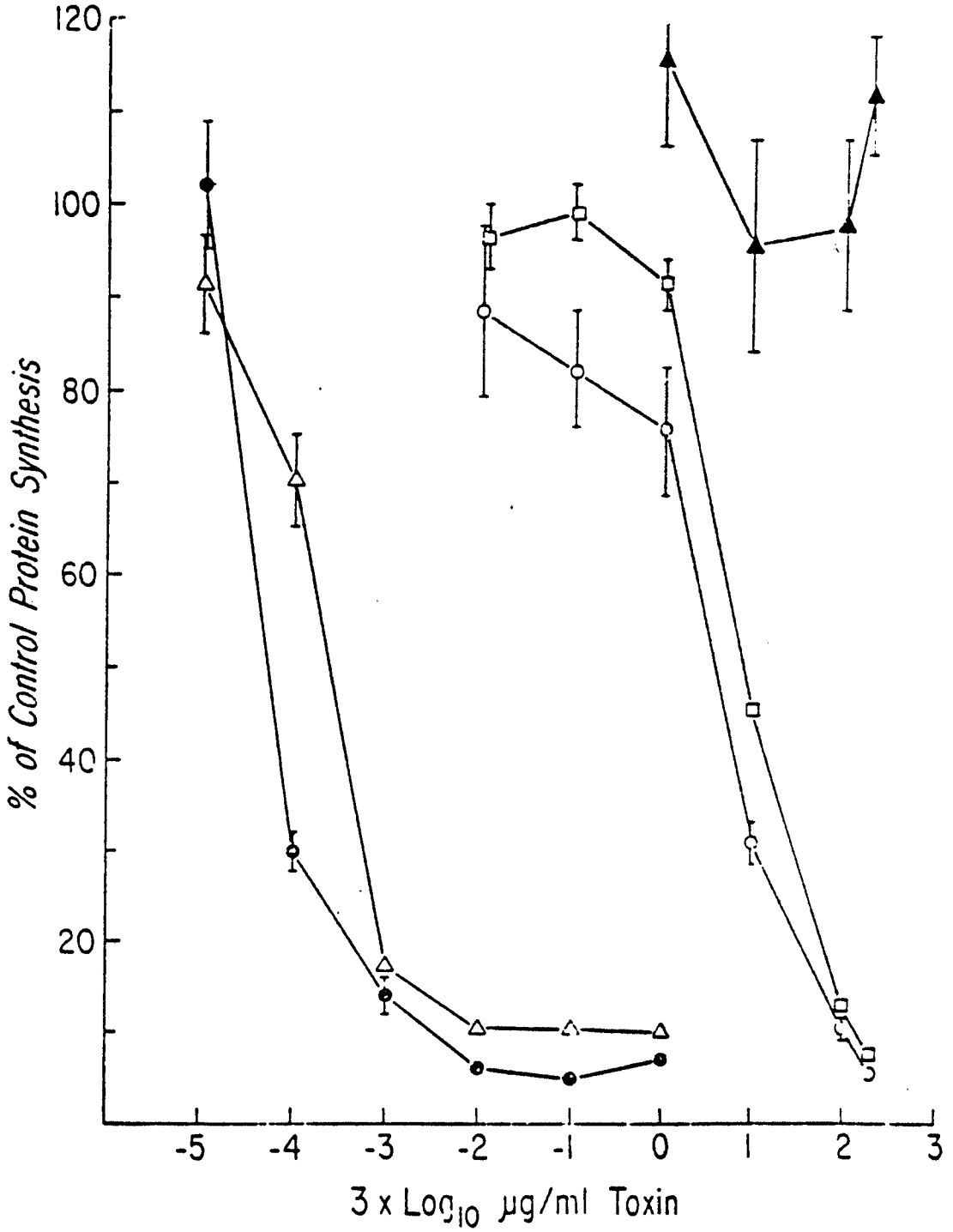
C. Results and Discussion.

1. The Isolation and Characterization of Toxin A Resistant PyBHK Cells (Iglewski, W.J. - manuscript in preparation). Cells (PyBHK) surviving prior exposure to 1.2×10^{-1} µg diphtheria toxin/10^5 cells (first passage) showed little increase in their subsequent resistance to inhibition of protein synthesis by diphtheria toxin (Fig. 1). However, exposure of these cells to 1.2 µg/ml yielded survivors with a greatly increased resistance to diphtheria toxin induced inhibition of protein synthesis. Subsequent exposure of these toxin resistant PyBHK cells to 12 and 120 µg/ml toxin further increased this resistance. Many survivors resulted from the final toxin exposure of the PyBHK resistant cells. The cells were therefore plated for isolation of single cell clones in 96-well tissue culturing trays. The resulting toxin resistant clones varied in their resistance to intoxication by diphtheria toxin. The clone with the highest resistance to toxin was termed PyBHKR (Fig. 1) and used in further experiments. Further exposure of these PyBHKR cells to toxin concentrations of up to 750 µg/ml for up to 24 hours had no discernible lethal effect on the cells.

In order to determine that the PyBHKR cells were descendents (and not contaminants) of the parental BHK cells, they were characterized. The PyBHK and PyBHKR cells had the same morphology; similar to that of other virus transformed cells. They both appeared as squamous or somewhat rounded, short fibroblast with a tendency to overlap and form piles. The cloning efficiency at various serum levels (2-10%) of the PyBHK and PyBHKR cells were indistinguishable. Both PyBHK and PyBHKR cells had similar rates of cell division (average doubling time in hours was 13.2 and 12.5, respectively). Karyotypic analysis showed that both the PyBHK and PyBHKR cells contained 84 chromosomes in a similar distribution and both cell lines induced tumors in golden hamsters. These data support the conclusion that the PyBHKR cells were derived from the parental PyBHK cells.

Perhaps of greatest significance was the observation that PyBHK cells were sensitive to both Pseudomonas toxin A and diphtheria toxin whereas PyBHKR cells were resistant to both of these toxins. It is known that the cellular receptors (7) and perhaps also the uptake (8) of these two toxins differ.

Fig. 1. Increasing resistance of PyBHK cells to diphtheria toxin. ●——●, PyBHK parent cells; △——△, first passage cells surviving 1.2×10^{-1} µg toxin/10^5 cells; o——o second passage cells surviving 1.2 µg toxin/10^5 cells, □——□ third passage cells surviving 12 µg toxin/10^5 cells, ▲——▲ , a single clone from fourth passage cells surviving 120 µg toxin/ml (PyBHKR cells).



These results suggest that the resistance of the PyBHKR cells was mediated at a point common to both toxins; the ADP-ribosylation of EF-2. Extracts were prepared from the PyBHK tumors and the PyBHKR tumors as described in Section I.B. above. The ability of toxin A, S and culture supernatants from various strains of P. aeruginosa, whose toxin phenotype was known, to ADP-ribosylate proteins in these extracts was compared. Controls containing wheat germ extracts were included. The results of these experiments (Table 1) show that EF-2 in the PyBHKR cell extracts is not ADP-ribosylated by toxin A (either pure toxin A or culture supernatants from A^+S^- strains of P. aeruginosa. Identical results were obtained with purified fragment A of diphtheria toxin. On the other hand, proteins in the PyBHKR extracts are ADP-ribosylated by partially purified S and by culture supernatants of S^+ strains of P. aeruginosa. More recently, identical results were obtained using pure S. Thus these extracts (PyBHKR) provide us with a single, simple, specific assay for S.

2. Production of S by Clinical Isolates of Pseudomonas aeruginosa. A total of 124 clinical isolates of P. aeruginosa were analyzed for production of toxins A and S. Production of S was assayed using the PyBHKR assay described above. Toxin A production was identified by a significant increase in ADP-ribosyl transferase activity in TSBD (no NTA) culture supernatants following preincubation with 4 M urea-1% DTT (9). The results (Table 2) showed that 48 (38%) of all strains tested produced S. There was no significant difference in the percentage of S producers between the bacteremia and burn patients. The majority of the strains that produce S also produce A. The incidence of toxin A production agrees with that previously reported (3,10) although the percentage of toxin A producers among the strains from bacteremia patients is slightly lower than expected. It is interesting to note that out of 53 burn isolates, there were no A^-S^- strains suggesting that the ability of a strain to produce A or S may provide a selective advantage for the organism in this type of infection.

The relationship between S and/or A production and patient mortality is shown in Table 3. There are no significant differences between the bacteremic and the burn isolates in the mortality rates for each phenotype. Although the numbers are small, there appears to be an increased mortality rate in patients infected with strains of P. aeruginosa which produce both S and A. Using a T-test for proportions, when the mortality rate of the A^+S^+ strains is compared to the mortality rates of the other three phenotypes, a value of 1.66 is obtained. A value of 1.65 or greater is considered significant. Although more strains must be analyzed before a positive correlation can be made, these data suggest that a patient infected with a strain which produces both S and A has less of a chance of survival than a patient infected with a strain which produces only one or neither of these enzymes.

II. Production of S In Vitro: Development of a Defined Medium for the Production of S.

A. Introduction.

As described in detail in last year's annual report, we had optimized production of S in a complex medium. More recently we began to repeatedly obtain inconsistent S yields which varied with each lot of TSB. Furthermore, in the TSBD medium, S was a relatively minor component of many >50 other bacterial proteins. We thus sought to develop a defined medium which would give consistently high yields of S and if possible reduce the total yield and number of other bacterial proteins.

TABLE 1. Comparison of Toxin A and S Activities in Extracts of Wheat Germ, PyBHK and PyBHKR cells.

Strain	Toxin Phenotype	pmoles ADPR transferred / mg Protein — Source of Cell Extracts: Wheat Germ	PyBHK	PyBHKR
WR5	(A^-S^-)	0	0	0
PA103	(A^+S^-)	38.1	18.8	0
Toxin A (.05µg)		42.4	19.4	0
Ps388	(A^-S^+)	181.2	539	432
Partially pure S		309.3	757	660
PA16	(A^+S^+)	19.9	49.8	64

TABLE 2. Production of Toxins A and S by Clinical Isolates of *Pseudomonas aeruginosa*.

Toxin Phenotype	No. Positive[a] Patient Categories: Bacteremia	Burn	Total
A^+S^-	32 (45)	33 (62)	65 (53)
A^+S^+	21 (29)	13 (25)	34 (27)
A^-S^+	7 (10)	7 (13)	14 (11)
A^-S^-	11 (16)	0 (0)	11 (9)
A^+	53 (74)	46 (87)	99 (80)
S^+	28 (39)	20 (38)	48 (38)

[a] Numbers in parentheses are percentages.

TABLE 3. Association of the Production of Toxins A or S with Patient Mortality.

	Patient Category					
	Bacteremia		Burn		Total	
Toxin Phenotype	% S[a]	% D[b]	% S	% D	% S	% D
A^+S^-	59	41	60	40	60	40
A^+S^+	43	57	38	62	41	59
A^-S^+	57	43	43	57	50	50
A^-S^-	55	45	0	0	55	45

[a] S = Patients surviving Pseudomonas infection.

[b] D = Patients dying from Pseudomonas infection.

B. Methods and Materials.

The bacterial strain, 388-6, enzymatic assays, conditions and culture methods have all been described in detail in previous annual reports.

C. Results and Discussion.

. Dennis Ohman had previously developed a defined medium for the production of toxin A by strain PAO-1 (Ohman, D.E., Doctoral dissertation, University of Oregon Medical School, 1980). In this defined medium, strain 388-6 grew very well but produced less than 1% of the S compared to good lots of TSBD-NTA. In TSBD medium NTA and glycerol are required for S production. A metal analysis on virgin and spent TSBD-NTA media showed that S production was accompanied by a large uptake of Mg^{++} but virtually no consumption of Ca^{++} or Zn^{++}. With this in mind, we optimized the concentrations of glycerol (5%), NTA (10 mM) and Mg^{+2} (5 mM) before investigating other components. EDTA could replace NTA but was very inhibitory to growth.

The next components investigated were other metal ions; namely, Fe^{+2}, Cu^{+2}, Mn^{+2}, Co^{+2}, Zn^{+2}, Bo_3^{-3} and $Mo_7O_{24}^{-6}$. Fe^{+2} at up to 10 µg/ml had no inhibitory effect on growth or S production and 1 µg/ml was the minimal optimum concentration. The following concentrations of the other metal ions had no effect on growth or S production: 100 M Zn^{+2}, 10 M Cu^{+2}, 10 M Mn, 2 M Co^{+2}, 20 M BO_3^{-3} and 1 M $Mo_7O_{24}^{-6}$. Based on these results, Fe^{+2} is routinely incorporated in the media at 1 µgm/ml and the other trace metals are not added. It is particularly interesting that in the defined medium iron supplementation is required and even at relatively high iron concentrations S yields are not inhibited. Yet, in the complex medium, S yields are inversely proportional to the concentration of iron in the medium. This paradox remains unexplained.

The other components of the defined media were investigated (i.e., salt concentration). The final composition of the defined medium found to give reproducibly high yields of S is shown in Table 4 which compares the composition of the defined medium to that of the complex medium we had previously developed (last year's progress report).

Besides giving reproducibly high yields of S, the defined medium (Table 4) has a second major advantage over the complex medium; namely, the total amount of extracellular protein is about 10% of that produced in the complex medium. Furthermore, many proteins which had similar molecular weights as S (60,000 daltons) are not detectable in supernatants from defined medium culture. Thus the defined medium offers a significant first step purification of S.

III. Purification of S and Production of Specific S Antiserum.

A. Introduction.

We have continued to investigate methods of purifying S. This year we concentrated on purifying small amounts of S and using this material to raise specific S antibody in rabbits.

TABLE 4. Comparison of Complex and Defined Media for the Production of S.

Complex	Defined	
Trypticase Soy Broth Dialysate 30 g/l H_2O	Potassium Phosphate	15 mM
Nitrilotriacetic Acid 10 mM	Sodium Succinate	110 mM
Monosodium Glutamate 100 mM	Ammonium Chloride	90 mM
Glycerol 1%	Magnesium Sulfate	5 mM
pH to 7.0	Nitrilotriacetic Acid	10 mM
	Monosodium Glutamate	50 mM
	Ferrous Sulfate	18 μM
	Glycerol 5% (by volume)	
	pH to 7.0 with 1 N HCl	

B. Methods and Materials.

1. Purification of S. The defined medium described above (II.C. and Table 4) was used to grow strain 388-6. Strain 388-6 was grown for 16-19 hr at 32°C with shaking. We have found it crucial to monitor yields of S hourly (starting at about 15 hr) in order to identify the best time to harvest the culture. This assures a high yield of S with minimal amounts of contaminating proteins. Binding to DE52 and various other methods of purification we used have all been detailed in previous progress reports or are described under Results (III.C.).

2. Immunization of Rabbits. Antisera was raised in rabbits against purified S by methods similar to those used to prepare specific toxin A antiserum (4). Rabbits (New Zealand White, approximately 2 kg) were immunized with 50 µg of S suspended in an equal volume of Freund's Complete Adjuvant with equal volumes of antigen injected subcutaneously, intramuscularly and into the rear footpads. At 2 and 4 weeks the rabbits were boosted with 50 µg of S suspended in Freund's Incomplete Adjuvant by a similar series of injections. Serum was collected at weekly intervals and both precipitating and enzyme neutralizing titers determined. We realize that greater quantities of antisera could have been obtained by using larger animals. However, we wanted to have the option of using immunoprecipitation involving Staph A for future experiments (i.e., characterizing CRM proteins); therefore, we decided to use rabbits.

C. Results and Discussion.

1. Purification of S. While the defined medium (Table 4) has many advantages, a new problem arose with its use; namely, the high salt concentration and the very low protein concentration in the supernatant (30 µgm/ml) made concentrating S very difficult. Binding to DE-52 (previously done with A [11]) requires a 20-fold dilution (to reduce the salt concentration) and ammonium sulfate (up to 75% saturation) did not precipitate S from the defined medium culture supernatants. If the supernatant was diluted five-fold and the pH lowered to pH 6, cation exchanges did bind most of the S activity. However, after one round of binding and elution, only 60-70% of the activity was recovered and only a 3- to 5-fold decrease in the original volume was obtained. Other methods such as vacuum dialysis, ultrafiltration, ultracentrifugation and affinity chromatography also give poor results.

It was noticed last year that a small amount of the activity in the TSB supernatant was lost upon filtering the centrifuged supernatant through a cellulose acetate-cellulose nitrate filter. This was also seen with the defined media. When (by chance) pure nitrocellulose filters were used, all of the S activity was observed to bind to the filter. Subsequent experiments have shown the following:

a) Essentially all of the S activity in 100 ml of defined media supernatant is bound to a 47 mm nitrocellulose filter of 0.2 µm pore size.

b) Binding capacity increases as the pore size decreases but due to decreasing flow rates, the 0.2 µm pore is the most practical.

c) After binding S, the filter can be washed with a variety of salts and buffers from pH 6 to 9 without eluting S activity.

d) NP-40 is required to elute the S and glycerol at 20% improves the recovery. To date, the best eluting buffer is 200 mM Tris-200 mM NaCl - 20% glycerol - 0.2% NP-40. A 20-fold concentration and 100% recovery of activity can be achieved using this method.

Upon electrophoresis in SDS-polyacrylamide (7.5%) gels, the major band that elutes from the nitrocellulose filter is S. It was possible to slice the gels and obtain S from eluates of specific slices. The S obtained in this manner gave a single band of protein and co-migrating enzymatic activity on both denaturing and nondenaturing polyacrylamide gels run at several pH's (6-8) and several acrylamide concentrations (7-12%). A single band is also seen on isoelectricfocusing. Thus, this material appears to be homogenous. By scaling up this procedure and using slab gels, it has been possible to purify 200 µg S per gel slab. This purified S was used to immunize rabbits.

2. Characterization of S Antisera. The antisera obtained had enzyme neutralizing titers ranging from 1/256 - 1/1024. While quantitative precipitin titers were not determined, the amount of precipitating antibody estimated from immunodiffusion analysis ranged from 20 µgm/ml to 500 µgm/ml. Sera with approximately equivalent amounts of antibody (highest titers) were pooled (S antiserum) and further characterized.

This S antiserum neutralized the enzymatic activity of crude and pure S but not crude or pure A. Furthermore, this antiserum gave a single line of identity between crude and purified S. Utilizing this antiserum, elek assays (3) were done with 35 strains of P. aeruginosa whose toxin phenotype had previously been determined (see Section I.C.2.). Ten of these strains were A^-S^+, 10 were A^+S^-, 10 were A^+S^+ and 5 were A^-S^-. A positive reaction was obtained with all of the S^+ strains and a negative reaction was obtained with all of the S^- strains. Thus this antiserum appears to be highly specific for S.

The availability of specific S antiserum will make it possible to begin to determine the relative contribution of S to virulence of P. aeruginosa. Furthermore, our results using the defined medium and nitrocellulose filters suggest we should now be able to purify large amounts of S for further characterization (i.e., toxicity studies).

IV. Literature Cited.

1. Iglewski, B.H., Sadoff, J., Bjorn, M.J. and Maxwell, E.S. Pseudomonas aeruginosa exoenzymes S: an adenosine diphosphate ribosyl-transferase distinct from toxin A. Proc. Nat. Acad. Sci. USA 75:3211, 1978.

2. Bjorn, M.J., Sokol, P.A., Iglewski, B.H. Influence of iron on yields of extracellular products in Pseudomonas aeruginosa cultures. J. Bact. 138:193, 1979.

3. Bjorn, M.J., Vasil, M.L., Sadoff, J.C. and Iglewski, B.H. Incidence of exotoxin production by Pseudomonas species. Infect. Immun. 16: 362, 1977.

4. Iglewski, B.H. and Sadoff, J.C. Toxin inhibitors of protein synthesis: Production, purification and assay of Pseudomonas aeruginosa toxin A. In Methods of Enzymology, ed. by K. Moldave and L. Grossman, LX; 780, 1979.

5. Iglewski, B.H., Rittenberg, M.B. and Iglewski, W.J. Preferential inhibition of growth and protein synthesis in Rous sarcoma virus transformed cells by diphtheria toxin. Virology, 65:272, 1975.

6. Fisher, M.W., Devlin, H.B., and Gnabasik, F.J. New immunotype schema for Pseudomonas aeruginosa based on protective antigens. J. Bact. 98:835, 1969.

7. Vasil, M.L. and Iglewski, B.H. Comparative toxicities of diphtheria toxin and Pseudomonas aeruginosa exotoxin A: Evidence for different cell receptors. J. Gen. Microbiol. 108:333, 1978.

8. Middlebrook, J.L. and Dorland, R.B. Differential chemical protection of mammalian cells from the exotoxin of Corynebacterium diphtheriae and Pseudomonas aeruginosa. Infect. Immun. 16:232, 1977.

9. Vasil, M.L., Kabat, D. and Iglewski, B.H. Structure-activity relationships of an exotoxin of Pseudomonas aeruginosa. Infect. Immun. 16: 353, 1977.

10. Pollack, M., Taylor, N.S. and Callahan, L.T. III. Exotoxin production by clinical isolates of Pseudomonas aeruginosa. Infect. Immun. 15:776, 1977.

11. Leppla, S. Large-scale purification and characterization of the exotoxin of Pseudomonas aeruginosa. Infect. Immun. 14:1077, 1976.

Production of Toxins A and S by Clinical Isolates of Pseudomonas aeruginosa

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ABSTRACT

Exoenzyme S production was detected in 38% of 124 clinical isolates of P. aeruginosa. An increased mortality rate was associated with strains producing both exoenzyme S and toxin A.

Pseudomonas aeruginosa is an opportunistic pathogen which can cause serious and lethal infections in compromised patients (1,3). P. aeruginosa produces a variety of extracellular products that may contribute its pathogenicity (2). Toxin A has been shown to be the most toxic extracellular product of P. aeruginosa on a weight basis (3). The mechanism of action of toxin A is identical to that of diphtheria toxin in that it inhibits mammalian protein synthesis by catalyzing the transfer of the adenosine 5'-diphosphate moiety of nicotinamide adenine dinucleotide onto elongation factor 2 (4,5,6,7). The resultant ADPR-elongation factor 2 is inactive in protein synthesis (5,6).

Some strains of P. aeruginosa produce a second extracellular protein (exoenzyme S) that has been shown to have ADP-ribosyl transferase activity (8). Exoenzyme S differs from toxin A in that it does not ADP-ribosylate EF-2, but rather catalyzes the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide to a number of substrate proteins in crude extracts of eukaryotic cells (8). Exoenzyme S also differs from toxin A in its heat stability and its destruction, rather than potentiation by pretreatment with urea and dithiothreitol (DTT) (8). Furthermore, exoenzyme S is not precipitated or neutralized by antitoxin A (8).

Exoenzyme S has been shown to be produced in vivo in a burned mouse model (9). The role of exoenzyme S in human P. aeruginosa infections has not yet been determined. This study was undertaken primarily to determine the incidence of exoenzyme S production by clinical isolates of P. aeruginosa. A second objective was to evaluate the possibility of a correlation between exoenzyme S and/or toxin A production and patient mortality.

Clinical isolates of P. aeruginosa were obtained from two groups of patients, those with Pseudomonas burn infections or bacteremia. These types of infections were chosen because of their severity. The mortality rate associated with Pseudomonas bacteremia is reported between 70 and 80%

(10,11,12). Pseudomonas is the bacterial species most often associated with the fatal infection of the burn wound (13). Clinical isolates were obtained from Walter Reed Army Institute of Research, Fort Sam Houston Burn Center, and the University of Virginia School of Medicine. These isolates were determined to be different strains on the basis of their Fisher-Devlin-Gnabasik serotype (14), colonial morphology, pigment and protease production.

The Pseudomonas isolates were grown in a dialysate of trypticase soy broth (TSBD) with and without nitriloacetic acid (NTA) at 32°C for 20 hr (8). Supernatants from the cultures grown without NTA were tested for toxin A production in the ADP-ribosyl transferase assay using crude wheat germ extracts. This assay was performed with and without prior incubation with 4M urea and 1% DTT ( ). The enzymatic activity of toxin A increases with preincubation in urea and DTT whereas exoenzyme S activity is reduced by this treatment (8). Supernatants from cultures grown in the presence of 10 nM NTA were tested for exoenzyme S activity in the py-BHK-r tumor extract assay ( ) which is specific for exoenzyme S. These methods make it possible to distinguish between strains that produce toxin A but not exoenzyme S (A^+S^-); those that produce both toxin A and exoenzyme S (A^+S^+); those that produce S but not A (A^-S^+); and those that do not produce either A or S (A^-S^-).

A total of 124 clinical isolates of P. aeruginosa were analyzed for exoenzyme S and toxin A production including 71 strains from bacteremia patients and 53 strains from burn patients. These results are shown in Table 1. Of the 124 strains tested, 38% produced exoenzyme S. There was no significant difference in the percentage of S producers between the bacteremic and the burn patients. The majority of the strains that produce exoenzyme S also produce toxin A. The incidence of toxin A production agrees with that previously reported (15,17,18), although the percentage of toxin A

producers among the strains from bacteremia patients is slightly lower than expected. It is interesting to note that out of 53 burn isolates, there were no A^-S^- strains suggesting that the ability of a strain to produce toxin A or exoenzyme S may provide a selective advantage for the organism in this type of infection.

The relationship between exoenzyme S and/or toxin A production and patient mortality is shown in Table 2. There are no significant differences between the bacteremic and the burn isolates in the mortality rates for each phenotype.

Although the numbers are small there appears to be an increased mortality rate in patients infected with strains of P. aeruginosa which produce both exoenzyme S and toxin A. Using a T-test for proportions, when the mortality rate of the A^+S^+ strains is compared to the mortality rates of the other three phenotypes a value of 1.66 is obtained. A value of 1.65 or greater is considered significant. Although more strains must be analyzed before a positive correlation can be made, these data suggest that a patient infected with a strain which produces both exoenzyme S and toxin A has less of a chance of survival than a patient infected with a strain which produces only one or neither of these enzymes.

The incidence of exoenzyme S production by P. aeruginosa strains may actually be higher than these results indicate. It is possible that the media used may not be suitable for in vitro exoenzyme S production by all strains. Also, some strains may only produce small quantitites of exoenzyme S that may not be detected by present assay methods. This study, however, indicates that the ability to produce exoenzyme S is widely distributed among clinical strains of P. aeruginosa. Additionally, there may be an increased mortality rate associated with patients infected with strains having the ability to produce both exoenzyme S and toxin A.

TABLE 1. Production of Toxins A and S by Clinical Isolates of Pseudomonas aeruginosa.

Toxin Phenotype	No. Positive[a] Patient Categories: Bacteremia	Burn	Total
A^+S^-	32 (45)	33 (62)	65 (53)
A^+S^+	21 (29)	13 (25)	34 (27)
A^-S^+	7 (10)	7 (13)	14 (11)
A^-S^-	11 (16)	0 (0)	11 (9)
A^+	53 (74)	46 (87)	99 (80)
S^+	28 (39)	20 (38)	48 (38)

[a] Numbers in parentheses are percentages.

TABLE 2. Association of the Production of Toxins A or S with Patient Mortality.

	Patient Category					
	Bacteremia		Burn		Total	
Toxin Phenotype	% S[a]	% D[b]	% S	% D	% S	% D
A^+S^-	59	41	60	40	60	40
A^+S^+	43	57	38	62	41	59
A^-S^+	57	43	43	57	50	50
A^-S^-	55	45	0	0	55	45

[a] S = Patients surviving Pseudomonas infection.

[b] D = Patients dying from Pseudomonas infection.

REFERENCES

1. *Liu, P.V. 1976. Biology of Pseudomonas aeruginosa. Hosp. Pract. 11:139-147.*

2. *Liu, P.V. 1966. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. III Identity of the lethal toxin produced in vitro and in vivo. J. Infect. Dis. 116:481-489.*

3. *Liu, P.V. 1974. Extracellular toxins of Pseudomonas aeruginosa. J. Infect. Dis. 130:594-599.*

4. *Collier, R.J. 1975. Diphtheria toxin: mode of action and structure. Bacteriol. Rev. 39:54-85.*

5. *Iglewski, B.H., L.P. Elwell, P.V. Liu, and D. Kabat. 1976. ADP-ribosylation of elongation factor 2 by Pseudomonas aeruginosa exotoxin A and by diphtheria toxin. p. 150-155. In S. Shaltiel (ed.), Proceedings of the 4th International Symposium on the Metabolism Interconversions of Enzymes. Srpinger-Verlag, New York.*

6. *Iglewski, B.H. and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. USA 72:2284-2288.*

7. *Iglewski, B.H., P.V. Liu and D. Kabat. 1977. Mechainsm of action of Pseudomonas aeruginosa exotoxin A, adenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138-144.*

8. *Iglewski, B.H., J. Sadoff, M.J. Bjorn and E.S. Maxwell. 1978. Pseudomonas aeruginosa exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc. Natl. Acad. Sci. USA 75:3211-3215.*

9. *Bjorn, M.J., O.R. Pavlovskis, M.R. Thompson and B.H. Iglewski. 1979. Production of exoenzyme S during Pseudomonas aeruginosa infections of burned mice. Infect. Immun. 24:837-842.*

10. *McCabe, W.R. and Jackson, G.G. 1962. Gram negative bacteremia. I. Etiology and ecology. Arch. Intern. Med. 110:847-855.*

11. *Tapper, M.L. and Armstrong, D. 1974. Bacteremia due to Pseudomonas aeruginosa complicating neoplastic disease: A progress report. J. Infect. Dis. 130:514-523.*

12. *Whiticar, J.D., Jr., Lung, M. and Bodey, G.P. 1970. Pseudomonas bacteremia in patient, with malignant disease. Am. J. Med. Sci. 260:216-223.*

13. *Pruitt, B.A., Jr. 1974. Infections caused by Pseudomonas species in patients with burn and in other surgical patients. J. Infect. Dis. 130:S-8 - S-13.*

14. *Fisher, M.W., H.B. Devlin and F.J. Gnabasik. 1963. New immunotype schena for Pseudomonas aeruginosa based on protective antigens. J. Bacteriol. 98:835-836.*

15. *Vasil, M.L., D. Kabat and B.H. Iglewski. 1977. Structure-activity relationships of an exotoxin of Pseudomonas aeruginosa. Infect. Immun. 16:353-361.*

16. *Bjorn, M.J., M.L. Vasil, J.C. Sadoff and B.H. Iglewski. 1977. Incidence of exotoxin production by Pseudomonas species. Infect. Immun. 16:362-366.*

17. *Pollack, M., Taylor, N.S. and Callahan, L.T. III. 1977. Exotoxin production by clinical isolates of Pseudomonas aeruginosa. Infect. Immun. 15:776-780.*

18. *Sanai, Y., K. Takeshi, J. Homma and H. Kamata. 1978. Production of exotoxin, protease and elastase of Pseudomonas aeruginosa strains isolated from patients and environmental specimens. Japan J. Exp. Med. 48:553-556.*

In Novel ADP-ribosylations of Regulatory Enzymes and Proteins ed. by Sugimara, T. and Smulson, M. Elsevier, N.Y. in press 1980.

EXOENZYME S: AN ADP-RIBOSYL TRANSFERASE PRODUCED BY PSEUDOMONAS AERUGINOSA

(Appendix #2)

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INTRODUCTION

Exoenzyme S is an adenosine diphosphate (ADP) ribosyl transferase that is produced by some strains of Pseudomonas aeruginosa (1). Unlike activated pseudomonas toxin A, which catalyses the transfer of the ADP ribose moiety of nicotinamide adenine dinucleotide (NAD^+) specifically to elongation factor 2 (EF-2) (2), exoenzyme S catalyses the transfer of the ADP ribose moiety of NAD^+ to a number of substrate proteins in crude extracts of eukaryotic cells (1). Exoenzyme S activity also has been shown to differ from pseudomonas toxin A in heat stability, in its destruction, rather than potentiation by pretreatment with urea and dithiothreitol (DTT), and in its inability to be neutralized by toxin A antibody (1).

In this paper we shall discuss several aspects of exoenzyme S, including factors influencing its production, characterization of the enzymatic reaction and a preliminary characterization of the enzyme. An initial study of the incidence of S production by clinical isolates of P. aeruginosa will also be described.

PRODUCTION OF EXOENZYME S

We have developed a medium and defined culture conditions which optimize the yield of exoenzyme S in liquid culture. Exoenzyme S was quantitated by an ADP ribosyl transferase assay as previously described (1). Pseudomonas aeruginosa strain 388 (kindly provided by B. Minshew, Seattle, WA and previously described (3)), was used as a source of exoenzyme S. The medium developed for optimum exoenzyme S yields was found to be similar to that previously developed for toxin A production (4,5). Common elements include the use of dialysed trypticase soy broth (TSB) as the base medium, a requirement for monosodium glutamate, glycerol and low iron concentrations. Similar culture conditions such as temperature (optimum 32°-35°) and vigorous aeration were required. However, exoenzyme S production required the presence of a chelating agent, whereas

toxin A production does not. Not all chelators tested supported exoenzyme S production (Table I). Citrate or tricine, when added to TSB_D, resulted in negligible S yields. The addition of 10 mM Nitrilotriacetic acid (NTA) or 10 mM Ethylenediaminetetracetate (EDTA) markedly enhanced exoenzyme S yields. However, at this concentration (10 mM) EDTA inhibited the growth of strain 388, whereas NTA did not.

TABLE I

EFFECT OF METAL CHELATORS ON BACTERIAL GROWTH AND EXOENZYME S YIELDS IN CULTURES OF P. AERUGINOSA 388[a]

	Tricine		Citrate		EDTA		NTA	
Conc of chelator mM	Bact growth (OD_{540})	ADPR incorp (pmoles)	Bact growth (OD_{540})	ADPR incorp (pmoles)	Bact growth (OD_{540})	ADPR incorp (pmoles)	Bact growth (OD_{540})	ADPR incorp (pmoles)
0.1	8.4	1.0	9.0	0.6	8.4	0.5	9.5	3
1.0	8.7	0.6	9.0	0.4	8.4	0.6	10	15
10.0	8.4	0.7	8.7	0.6	6.0	667	10	550
20.0	8.4	0.7	9.0	0.4	5.7	620	10	500

[a]Media were supplemented with 100 mM MSG and 1% glycerol. Cultures were incubated for 22 h with shaking at 32°C. S was quantitated by ADP ribosyl transferase assay as previously described (1).

Yields of exoenzyme S in media which have been used to produce other bacterial toxins were compared to S yields in TSB_D. The media tested included syncase (6) without iron, protease peptone (7) and pantothenate, glutamic acid and tryptophan medium (8). While the yields of S were maximal in TSB_D, the chelators NTA or EDTA were required for S production regardless of the growth medium tested. In view of a previous report that NTA and EDTA inhibit pseudomonas proteases (9), the effect of various concentrations of NTA in the medium on S yields and protease activity was examined. Protease activity was found to decrease while S yields increased as the concentration of NTA in the medium was increased (Table II). However, addition of NTA to 5 mM resulted in a complete loss of detectable protease activity while S yields were found to markedly increase from 1 mM through 10 mM NTA. This suggests that inhibition of protease activity is not the only mechanism by which NTA enhances S yields. This suggestion is supported by the observation that another protease inhibitor, ammonium sulfate (12), in the absence of NTA, will not support detectable production of exoenzyme S.

TABLE II

THE EFFECT OF NTA ON THE YIELDS OF EXOENZYME S AND PROTEASE ACTIVITY IN CULTURES OF P. AERUGINOSA 388

Conc of NTA mM	Protease[a] Activity %	ADPR[b] Incorporated pM
0	100	0
0.1	87	10
1.0	2	30
5.0	0	240
10.0	0	560

[a]Protease activity was determined by a modification (10) of the methods of Kunitz (11).

[b]ADP ribosyl transferase activity was determined as previously described (1).

CHARACTERIZATION OF THE REACTION

The optimum conditions for the enzymatic activity of P. aeruginosa toxin A are similar to those developed a number of years ago for diphtheria toxin fragment A (13,5). When exoenzyme S was discovered, the same assay was employed for it as for toxin A (1). However, some exploratory work showed these conditions were not ideal for quantitating S enzymatic activity. Hence we have examined a number of parameters to determine the optimum conditions for assaying the enzymatic activity of exoenzyme S.

Optimum conditions for assay of exoenzyme S were developed using a rat liver extract as a source of substrate protein(s). Rat liver was extracted using 0.25 M sucrose containing a protease inhibitor (14), Phenylmethylsulfonyl-fluoride (PMSF), but no added reducing agents. The 100,000 x g supernatant of the liver extract was desalted by gel filtration into 10 mM Tris-HCl, pH 7.0 and frozen in aliquots at -70°. There was little difference seen between S activity in the wheat germ and rat liver extracts under a variety of assay conditions.

The standard assay with rat liver extract for exoenzyme S consisted of 50 µl of buffer, 10 µl of desalted liver extract, a variable amount of diluted crude exoenzyme S (i.e. culture supernatant from strain 388), 5 µl of [^{14}C-adenine]-NAD^+ and incubation at 25°C for a set period of time. Crude and desalted crude exoenzyme S gave the same result whenever compared. A variety of buffers were tested at 50 mM concentrations over a pH range of 4.0-10.0. In the buffers tested, the maximum enzymatic activity with exoenzyme S occurred at pH 6.0 (Fig 1).

A pH optimum of 6 for exoenzyme S differs from the reported pH optimum of 8 for diphtheria toxin fragment A and pseudomonas toxin A (15).

Using desalted liver extract and desalted crude exoenzyme S, we investigated the effect of ionic strength on enzymatic activity. The activity in the region from pH 5-7 was rather insensitive to ionic strength but in the region above pH 7.0 was very sensitive to added salt. In 50 mM imidazole, pH 8.0, increasing the concentration of NaCl or KCl from 0 to 100 mM markedly increased the enzymatic activity. At concentrations above 100 mM, NaCl or KCl were slightly inhibitory.

The buffers tested gave nearly the same activity when the pH and ionic strength were accounted for, except N-[2-Acetamido]-Iminodiacetic acid (ADA), in which case the activity was about 20% higher (Fig 1). Sodium borate almost completely inhibited toxin S activity, possibly due to the well known ability of alkaline borate to complex with cis-hydroxyl groups such as those found in NAD^+.

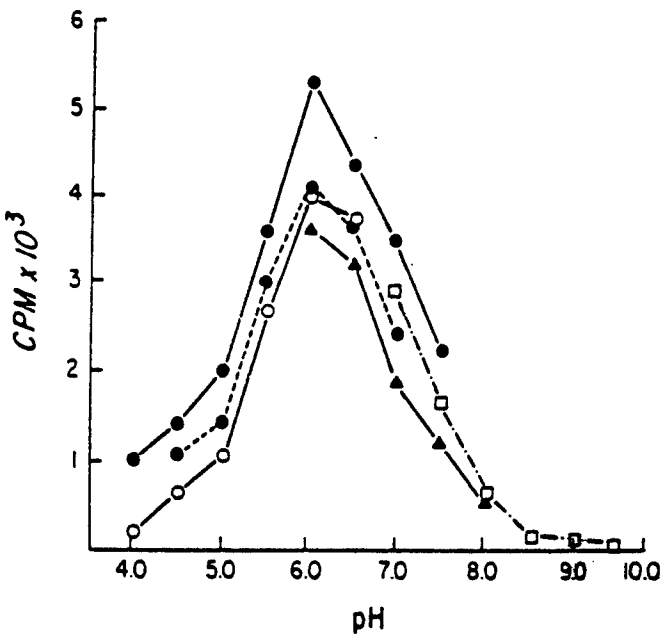

Fig. 1. Effect of pH on the enzymatic activity of toxin S in rat liver extracts. Buffers used include 50 mM ADA (●—●), 50 mM histidine (●--●), 50 mM acetate (○—○), 50 mM $NaHPO_4$ (▲—▲) and 50 mM tris (□--□).

In order to investigate possible metal ion requirements for S enzymatic activity, an assay buffer of 200 mM Na acetate, pH 6.0 was used. The following metals were added to the enzyme assay at a final concentration of 0, 10 or 100 µM: Fe^{+3}, Cu^{+2}, Ca^{+2}, Mn^{+2}, Mg^{+2} or Zn^{+2}. The addition of these metals did not increase enzymatic activity; therefore there is no apparent requirement for their addition. However, Fe^{+3} and Cu^{+2} actually had an inhibitory effect on the enzymatic acitvity of S (Table III). ADP ribosyl transferase activity was inhibited when as little as 1 µM $FeCl_3$ or $CuSO_4$ was added to the assay. The inhibition was greatest at a concentration of 30 µM $FeCl_3$ of $CuSO_4$. When the concentration of these metals was increased to 100 mM this inhibition decreased. Adding NTA, EDTA, sodium azide, or DTT to the assay at a final concentration of 0.7 mM or 7 mM had no significant effect on the enzymatic activity of S.

TABLE III

EFFECT OF IRON AND COPPER ON THE ENZYMATIC ACTIVITY OF EXOENZYME S

Metal Conc	% Relative Enzymatic activity	
	$FeCl_3$	$CuSO_4$
--	100	100
1 µM	97	95
3 µM	84	79
10 µM	46	50
30 µM	11	15
100 µM	81	93

PURIFICATION AND CHARACTERIZATION OF EXOENZYME S

Partial purification of exoenzyme S was achieved with conventional ion exchange and gel filtration procedures. We have developed a purification scheme based on 1) binding exoenzyme S directly from dilute culture supernatant to diethylaminoethyl (DEAE) cellulose (Whatman DE-52, Reeve Angel, Clifton, New Jersey), 2) binding exoenzyme S to hydroxyapatite (Bio-Rad Laboratories, Richmond, CA) and selective elution with phosphate, and finally 3) gel filtration. Purification was followed by enzyme assay (1) with wheat germ extract as a source of substrate protein. Enzymatic activity of desalted three fold concentrated culture supernatant was found to migrate as a single band upon electrophoresis in SDS polyacrylamide gels (SDS-PAGE) (16). ADP ribosyl transferase activity eluted from sliced analytical SDS-PAGE gels was found

at 60 K daltons. Upon binding and elution of S from DEAE cellulose, a second component of activity was found on subsequent SDS-PAGE at 28-30 K daltons. However, the partially purified (post DEAE-cellulose) exoenzyme S activity is found only in the void volume of a gel filtration column with a molecular exclusion of 500,000 daltons (Biogel A.5M, Bio-Rad Laboratories, Richmond, CA). This activity will barely penetrate a nondenaturing 6% polyacrylamide gel under electrophoresis. However, the post DEAE cellulose exoenzyme S does migrate in 6% polyacrylamide analytical isoelectric focus gels (17) and demonstrates a single major symmetrical peak of enzymatic activity at pH 4.4-4.5.

Enzymatic activity eluted from hydroxyapatite can likewise be separated by SDS-PAGE into 60 K dalton and/or 30 K dalton activity. The enzymatic activity demonstrating primarily a 60 K component behaves like crude S upon gel filtration; i.e. it is excluded in the void volume of a Biogel A.5M column. However, the material at 30 K daltons (after hydroxyapatite elution) will enter a 100,000 dalton exclusion gel filtration column (Pharmacia Fine Chemicals, Uppsala, Sweden) and migrate under nondenaturing conditions, at 45-50 K daltons. Antibodies raised in rabbits (5) against partially purified enzyme (post DEAE cellulose) will neutralize the enzymatic activity of crude S and any enzyme preparation that upon SDS electrophoresis demonstrates enzymatic activity at 60 K dalton. Anti-S antibodies will not neutralize the 30 K dalton enzyme. Anti-pseudomonas toxin A antibodies (5) also will not neutralize enzymatic activity eluting at 45-50 K dalton on gel filtration (i.e. 30 K dalton after SDS-PAGE) (Table IV). However, in a radioimmunoassay developed for pseudomonas toxin A and performed by Dr. S. Cryz in our laboratory, partially purified 30 K dalton material demonstrates cross reactivity with toxin A, whereas crude exoenzyme S and partially purified 60 K dalton enzyme demonstrated no cross reactivity in this assay.

TABLE IV

THE EFFECT OF ANTI TOXIN A AND ANTI S IMMUNE SERA ON ENZYME ACTIVITY[1]

	% neutralization	
Material	Anti A[2]	Anti S[2]
Toxin A	71	0
Toxin S culture supernatant	0	86
Toxin S G-100 void peak	7	71
Toxin S G-100 50 K dalton peak	7	0

[1]Samples of diluted toxin in 10 µl were preincubated with 10 µl serum for 15 min at 37°C then immediately assayed for ADP-ribosyl transferase activity (1).

[2]Anti A, rabbit toxin A antiserum; anti S, rabbit S antisera.

Labeling profiles of wheat germ substrates have been observed for the ADP-ribosylation reaction with autoradiography of SDS-PAGE (16,18). Preliminary evidence demonstrates multiple substrate labeling by crude exoenzyme S and by the 60 K dalton exoenzyme S eluted from SDS-PAGE. Labeling profiles of substrates modified by crude S, partially purified 60 K dalton S and by S activity extracted from the skin of burned, strain 388 infected mice (3) appeared very similar, if not identical. Reaction conditions appeared to affect the specificity of exoenzyme S. The 30 K dalton material appeared to label a single major wheat germ extract protein which had a molecular weight of 90 K daltons. It is not known at this time if the 30 K dalton enzyme is a fragment of S generated during purification, or if the purification procedures have selectively purified a fragment of toxin A that existed at a very low concentration in culture supernatant. However, the latter explanation seems more likely.

During the course of these studies we have experienced losses in S enzymatic activity either due to inactivation or actual loss of S protein. Furthermore our results suggest that in culture supernatants, S aggregates or associates with other proteins or lipoproteins so that it is stable but is excluded from molecular filtration resins. Detergents were studied as possible aids in the purification and/or stabilization of exoenzyme S. Solubilization with detergents was monitored by chromatography of S on a Bio-Gel A.5M column. Incubation (37°C for 30 min) of S with 0.5% SDS or Triton X-100 with or without reducing agents failed to alter elution in the void volume even when the test decergent was included in the chromatographic buffer. However, when the detergent NP-40 was incorporated in the chromatographic buffer (final concentration 0.1%) exoenzyme S eluted as a peak between the void volume and hemaglobin. Thus NP-40 appears to solubilize toxin S. Furthermore NP-40 also seems to stabilize the enzymatic activity of S.

INCIDENCE OF EXOENZYME S PRODUCTION BY CLINICAL ISOLATED OF P. AERUGINOSA

Strains isolated from patients with P. aeruginosa bacteremia were provided by Drs. J. Sadoff and A. Cross (Walter Reed Army Institute of Research, Wash, D.C.). These isolates were grown in TSB_D with and without NTA (final concentration 10 mM) at 32°C for 22 hr. (1). Culture supernatants were tested in the ADP-ribosyl transferase assay (1) with and without prior incubation with 4 M urea and 1% DTT (19). Toxin A is produced in equal amounts in TSB_D and in TSB_D + NTA whereas S production increases when the bacteria are grown in the presence of NTA. Furthermore, the enzymatic activity of A increases with preincubation in 4 M urea and 1% DTT whereas S activity is reduced by such

treatment (1). The phenotype of potential S producers was then verified by preincubating culture supernatants in specific anti A or anti S serum.

Utilizing the methods described above we have initiated a study to determine the incidence of S production by strains of P. aeruginosa isolated from patients having bacteremia. These methods permit us to distinguish between strains that produce toxin A but not S (A^+S^-); those that produce both toxin A and exoenzyme S (A^+S^+); those that produce S but not A (A^-S^+) and those that do not produce either A or S (A^-S^-). A total of 39 strains have thus far been examined. Twenty five (64%) were A^+S^-; 4 (10%) were A^+S^+, 3 (8%) were A^-S^+ and 7 (18%) produced no detectable enzymatic activity (A^-S^-). Of the 7 strains identified as producing S, 5 were from patients that died. Thus while the overall mortality rate of these bacteremic patients was 43% the mortality rate of those individuals infected with S producing strains was 71%. This increased mortality rate was not associated with the toxin A phenotype. Additional studies are required to evaluate the relative importance of exoenzyme S in P. aeruginosa infections.

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REFERENCES

1. Iglewski, B.H., Sadoff, J., Bjorn, M.J. and Maxwell, E.S. (1978) Proc. Nat. Acad. Sci. 75, 3211-3215.
2. Iglewski, B.H. and Kabat, D. (1975) Proc. Natl. Acad. Sci. 72, 2284-2288.
3. Bjorn, M.J., Pavlovskis, O.R., Thompson, M.R., and Iglewski, B.H. (1979) Infect. Immun. 24, 837-842.
4. Liu, P.V. (1973) A. J. Infect. Dis. 128, 506-513.
5. Iglewski, B.H. and Sadoff, J.C. (1978) Methods in Enzymology eds. Grossman, L, and Moldave, K. (Academic, New York) 60, 780-793.
6. Finkelstein, R.A., Atthasampunna, P., Chulasamaya, M. and Charunmethee, P. (1966) J. Immunol. 96, 440-447.
7. Pc , C.G. (1932) Brit, J. Exp. Path. 13, 218-223.
8. Barksdale, W.L. and Pappenheimer, A.M., Jr. (1954) J. Bacterol. 67, 220-232.
9. Callahan, L.T. (1976) Infect. Immun. 14, 55-61.
10. Wtetlind, B. and Wadstrom, T. (1977) J. Gen. Microbiol. 103, 319-327.
11. Kunitz, M. (1946/1947) J. Gen. Physiol. 30, 291-310.
12. Liu, P.V. and Hsieh, H. (1969) J. Bacteriol. 77, 406-413.
13. Collier, R.J. (1975) Bacteriol. Rev. 39, 54-85.
14. Ballal, N.R., Goldberg, D.A. and Bush, H. (1975) Biochem. Biophys. Res. Commun. 62, 972-982.
15. Chung, D.W. and Collier, R.J. (1977) Infect. Immun. 16, 832-841.
16. Laemmli, U.K. (1970) Nature 227, 680-685.
17. Vesterberg, O. (1972) Biochim. Biophys. Acta. 257, 11-19.
18. Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88.
19. Vasil, M.L., Kabat, D. and Iglewski, B.H. (1977) Infect. Immun. 16, 353-361.