0019-9567/78/0019-0029$02.00/0
INFECTION AND IMMUNITY, Jan. 1978, p. 29-33

Vol. 19, No. 1
Printed in U.S.A.

Mechanism of Action of *Pseudomonas aeruginosa* Exotoxin A in Experimental Mouse Infections: Adenosine Diphosphate Ribosylation of Elongation Factor 2

OLGERTS R. PAVLOVSKIS,[1]* BARBARA H. IGLEWSKI,[2] AND MATTHEW POLLACK[1]

Department of Microbiology, Naval Medical Research Institute, Bethesda, Maryland 20014;[1] Department of Microbiology and Immunology, University of Oregon Health Science Center, Portland, Oregon 97201;[2] and Department of Bacteriology Diseases, Walter Reed Army Institute of Research Washington, DC 20012[2]

Received for publication 3 August 1977

The data presented indicate that one of the primary actions of *Pseudomonas aeruginosa* exotoxin during experimental infection is the inactivation of elongation factor 2 (EF-2) in various mouse organs. Organs from mice infected with the toxigenic *P. aeruginosa* strain PA103 contained considerably less EF-2 activity than did organs from uninfected controls. Whereas EF-2 activity was reduced in all organs examined from PA103-infected animals, the largest decrease was observed in the liver, where the active EF-2 levels were reduced by 70 to 90%. In addition, consistent inhibition of protein synthesis in livers but not in other organs was observed in mice infected with the toxigenic PA103 strain. Treatment of mice with antitoxin before infection with strain PA103 prevented inactivation of EF-2. When mice were infected with lethal doses of the nontoxigenic *P. aeruginosa* WR5 strain, tissue EF-2 levels were not markedly reduced below those derived from uninfected control animals.

Previous studies have documented that purified *Pseudomonas aeruginosa* exotoxin A (2, 3, 9) inhibits protein synthesis in cultured mammalian cells (10, 11) and in mouse organs (13). Iglewski and Kabat reported that toxin catalyzes the nicotinamide adenine dinucleotide (NAD)-dependent inhibition of protein synthesis in rabbit reticulocyte lysates. This was shown to be the result of the inactivation of elongation factor 2 (EF-2) (6). In addition, following the intraperitoneal injection of purified toxin into mice, EF-2 activity decreased significantly in the liver and other organs (7).

Although there is evidence that toxin is produced in animals during experimental pseudomonas infections (12, 16) and in patients (14), it is not known whether the toxin produced in vivo elicits biochemical effects similar to those of exogenously administered toxin (7, 13). Recently, Saelinger et al. (16) showed that EF-2 levels decreased significantly in the livers and spleens of burned mice infected with pseudomonas. However, whether this was due to the specific action of toxin or to some other factor as a result of the infection is not known. The purpose of this study was to examine this effect, using as controls mice treated with specific antitoxin to neutralize the toxin produced in vivo during an infection as well as mice infected with a nontoxigenic strain of pseudomonas. The data presented indicate that protein synthesis in the liver is inhibited during infection with a toxin-producing strain of *P. aeruginosa* as the result of EF-2 inactivation.

MATERIALS AND METHODS

Organisms. *P. aeruginosa* strain PA103, isolated by Liu (8), produces toxin, but only low amounts of protease (8, 18) and no detectable hemolysin or lecithinase when grown in vitro.

P. aeruginosa WR5, a nontoxigenic, protease-producing strain (1) isolated from a patient at Walter Reed Army Hospital, Washington, D.C., was kindly provided by J. C. Sadoff, Walter Reed Army Institute of Research.

Experimental burn infection model. Swiss white female mice of the NIH-NMRI CV strain, weighing 20 ± 2 g, were used. The burned-mouse model developed by Stieritz and Holder (17) was used with the modifications described previously (12). The mice were anesthetized with methoxyflurane (Penthrane, Abbott Laboratories, North Chicago, Ill.); the challenge inoculum was prepared as previously described (12). Mice were infected subcutaneously in the burn area immediately following burn trauma with 2 mean lethal doses (LD_{50}) of organisms (approximately 3.6×10^4 for PA103; 14×10^6 for WR5), which resulted in fatal infections in about 90% of the mice in about 50 ± 10 h postinfection (12). Control, burned-uninfected mice received an equivalent volume (0.5 ml) of sterile phosphate-buffered saline.

Antisera. Rabbit bovine serum albumin antiserum

(anti-BSA) (12) and antitoxin serum (3) were prepared as previously described.

Both antisera were heated at 56°C for 30 min and absorbed with Formalin-killed whole cells of the challenge pseudomonas strain, as previously reported (12). Following absorption, the cell-free antisera were found to be negative for hemagglutinating antibodies to the type-specific lipopolysaccharides of the challenge strains. These latter tests were kindly performed by G. C. Cole and M. W. Fisher (Parke-Davis Co., Detroit, Mich.).

In vivo protein synthesis. Food was withheld from mice 24 h prior to the start of each experiment. Mice were traumatized and injected with PA103 or sterile phosphate-buffered saline as described above. At 48 h postinfection, 2.0 μCi (0.10 ml) of a ^{14}C-labeled amino acid mixture (New England Nuclear Corp., Boston, Mass.) was injected into the mice intraperitoneally. Two hours later the mice were anesthetized with methoxyflurane and perfused through the heart with heparinized saline until the perfusate was clear. Various organs were excised, rinsed with cold saline, blotted, and stored at −20°C until tested. ^{14}C-labeled amino acid incorporation by the organs was determined as previously described (13).

Extractions and quantitation of mouse organ EF-2. The organs were obtained as described above, except that the organs from six mice were pooled. EF-2 was extracted from homogenates of the pooled mouse tissues by a previously described modification (7) of the procedure of Gill and Dinius (4). A 0.1-ml sample of the NAD+ free, clear supernatant which contained EF-2 was incubated in the presence or absence of 6 μg of diphtheria toxin fragment A (kindly provided by R. J. Collier, University of California at Los Angeles) in a 0.3-ml-total reaction mixture containing 0.25 μCi of [^{14}C]NAD (specific radioactivity, 1.2×10^6 cpm/mol), 100 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 8.2), 0.15 mM ethylenediaminetetramine, 55 mM dithiothreitol, and 250 mM histamine. After incubation for 10 min at 37°C, the reaction was stopped by precipitation with cold trichloroacetic acid (5% final concentration), and the precipitate was collected and counted as previously reported (7). All assays were done in duplicate and averaged. The difference between the counts with and without diphtheria toxin fragment A represented the adenosine diphosphate-ribosyl EF-2 formed. The average active EF-2 content of each tissue was calculated as described by Gill and Dinius (4).

RESULTS

Protein synthesis. Past studies (13) have shown that in mice injected with purified exotoxin, shortly before the expected time of death, protein synthesis is inhibited almost completely in the liver (>85%) and to a lesser extent (20 to 50%) in other organs. In this study, however, when burned mice were infected with PA103 (Table 1), the liver was the only organ in which reduction in amino acid incorporation could be demonstrated in most cases. Differences in amino acid incorporations by spleen, kidney, lung, heart, pancreas, intestine, muscle, or brain between burned-infected and uninfected mice were not significant (not shown).

TABLE 1. *Protein synthesis in the livers of burned-infected and uninfected mice*[a]

Expt no.	Treatment	No. of mice	Amino acid incorporation		
			cpm/mg of protein ±SEM[b]	Percent decrease	*P*[c]
1	Uninfected	16	443 ± 34		
	Infected	12	259 ± 42	41.5	<0.001
2	Uninfected	20	953 ± 48		
	Infected	17	645 ± 66	32.3	<0.001
3	Uninfected	10	762 ± 30		
	Infected	9	682 ± 67	10.5	0.3

[a] Protein synthesis was between 50 and 52 h postinfection. All mice in the control group (burned-infected, nonpulsed) died, indicating that the mice were infected.

[b] SEM, Standard error of the mean.

[c] Probability of random distribution.

Since the infecting organisms may incorporate exogenous ^{14}C-labeled amino acids and possibly add to the total incorporation, one series of experiments was done in which both infected and uninfected mice were injected intravenously with 400 μg of gentamicin 2 h prior to pulse labeling in order to inhibit the metabolism of the bacteria. No significant changes in amino acid incorporation were observed (data not shown).

EF-2 levels in infected mice. The active EF-2 levels of burned-noninfected mice from 19 separate experiments, each involving tissue from pools of six mice, are shown in Fig. 1. The results are similar to earlier values obtained with normal, nontraumatized mice (7). Thus the burn injury did not affect the active EF-2 content of the mouse organs.

The EF-2 activity in various tissues during the first 36 h of infection with PA103 as compared with uninfected mice is shown in Fig. 2. As in the case of inhibition of protein synthesis, the most significant decrease was seen in the liver. At 36 h the liver contained only 12% of the initial EF-2 activity, while more modest decreases were noted in the kidney, heart, and lung. The EF-2 activity of the spleen remained approximately constant at least through the first 24 h of infection. Since during these initial experiments variation in active EF-2 content in the organs of similarly treated animals was considerable (Fig. 1), all subsequent experiments (Fig. 3) were repeated three separate times and the EF-2 values were then averaged. As with previous experiments, each homogenate was prepared with organs pooled from six mice.

To determine whether the reduced EF-2 ac-

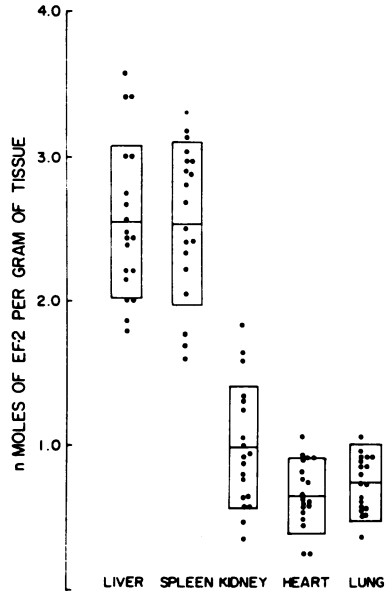

FIG. 1. *EF-2 values (nanomoles/gram of tissue ± standard error of mean) in burned-uninfected mice 24 h postburn. The results represent 19 separate experiments, with each point representing the average of two determinations on a pool of organs from six mice.*

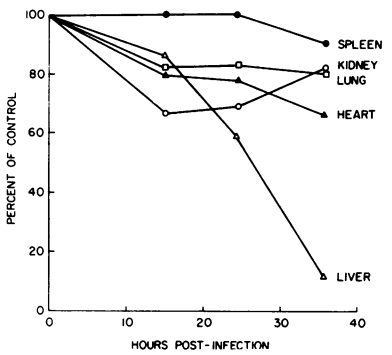

FIG. 2. *Active EF-2 content in organs of mice during pseudomonas infection (PA103) as compared with burned-uninfected mice. Each point represents an average of two determinations with homogenates prepared from a pool of organs from six mice. The values are expressed as the percentage of active EF-2 in organs obtained from burned-uninfected control mice.*

tivity was due to the exotoxin or some other bacterial product produced in vivo during the infection, mice were administered intravenously 0.2 ml of rabbit antitoxin 24 h prior to infection with PA103. Previous work had shown that this amount of antitoxin completely protected mice against the lethal effects of 2 LD_{50} of PA103 challenge (12). A second group of mice received equivalent volumes of rabbit anti-BSA. The EF-2 activities in various organs of infected, anti-BSA-treated and antitoxin-treated mice at 24 and 48 h postinfection are shown in Fig. 3A. In the anti-BSA-treated mice, the active EF-2 levels at 24 h were reduced approximately to the same extent as in the earlier experiments (Fig. 2). Thus at 24 h the only organ to show a marked decrease in active EF-2 content was the liver. By 48 h, however, the EF-2 activity was significantly reduced in all the organs of anti-BSA-treated mice as compared with values obtained with burned-uninfected control animals. In contrast, in organs from the antitoxin-treated animals, EF-2 levels were similar to those of burned-uninfected mice (Fig. 3A). The only organ from the antitoxin-treated infected animals

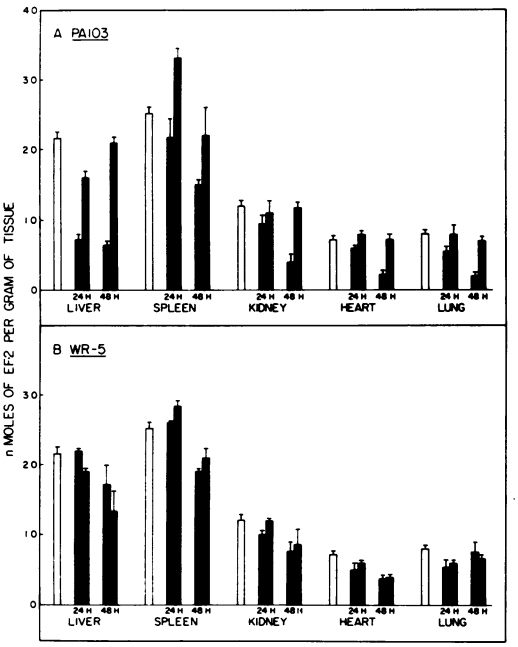

FIG. 3. *EF-2 values in organs of antiserum-pretreated mice infected with either (A) toxigenic PA103 or (B) nontoxigenic WR5. Controls: Burned-uninfected (□); burned-infected mice, pretreated with anti-BSA (▨); burned-infected mice pretreated with antitoxin (■). The bars represent the means, and the segments above the bar represent mean + standard error. For the infected animals, the standard error was determined from three separate experiments. No differences in EF-2 values were seen in controls tested at 24 and 48 h postburn. Thus these values were averaged and the standard error was determined on the basis of n = 19. The antitoxin titer by passive hemagglutination assay (15) was 1:32,000, and by mouse neutralization 0.1 ml diluted to 1:256 neutralized 5 LD_{50} of purified toxin (14).*

to show any significant decrease in active EF-2 content was the liver. This effect appeared to be transitory, since the active EF-2 content of the livers from the antitoxin-treated animals was reduced at 24 h but was normal at 48 h postinfection. These results indicate that the antitoxin prevented progressive EF-2 inactivation in organs of mice infected with the toxigenic PA103 strain of *P. aeruginosa*.

The specificity of the action of the exotoxin was tested in mice infected with a non-toxin-producing strain of *P. aeruginosa*, WR5 (Fig. 3B). This strain is considerably less virulent for burned mice ($LD_{50} = 2 \times 10^6$) compared with the toxigenic strain, PA103 ($LD_{50} = 1.8 \times 10^4$) (12). In addition, antitoxin does not protect mice against lethal WR5 infections (12). Mice were pretreated with antitoxin or anti-BSA 24 h prior to the burn and infected as in the previous experiment. The results are shown in Fig. 3B. Only a slight decrease in EF-2 activity was seen in tissues from mice infected with this nontoxigenic strain as compared with mice infected with the toxigenic PA103. For example, in the liver of WR5-infected mice, the EF-2 activity at 24 h was normal and only reduced to 80% of the control at 48 h, while in mice infected with the toxigenic strain, the EF-2 activity decreased to 33 and 30%, respectively (Fig. 3). Similarly, only a slight decrease in active EF-2 content was seen in the other organs examined from mice infected with the nontoxigenic WR5 strain, and this decrease was seen primarily at 48 h postinfection in moribund animals. The small reductions of EF-2 activity observed in tissues from WR5-infected animals were almost identical in antitoxin- and anti-BSA-treated mice, indicating that this inactivation of EF-2 by WR5 was not due to the action of exotoxin A. An unexpected observation was that the active EF-2 content of spleens obtained at 24 h from infected animals pretreated with antitoxin was higher than the values obtained for spleens from uninfected control animals (Fig. 3). This transitory increase in EF-2 activity was not due to the antitoxin, since values for active EF-2 content of spleens from antitoxin-treated, burned-uninfected animals (antitoxin controls) was equal to that obtained with uninfected animals not given antitoxin (data not shown). Furthermore, this increase was seen at 24 h in spleens from animals infected with either strain PA103 or WR5 (Fig. 3), suggesting that this increased EF-2 activity in the spleen was due to some factor other than toxin which is elaborated during these infections.

DISCUSSION

A decrease of active EF-2 levels in the liver, kidney, spleen, heart, and lung similar to that seen by Saelinger et al. (16) was observed in mice infected with the toxigenic PA103 strain of *P. aeruginosa* (Fig. 2 and 3). Although it might be expected that inactivation of EF-2 would result in a corresponding decrease in protein synthesis, the liver was the only organ in which a significant decrease in protein synthesis was seen (Table 1).

In our previous studies (13) on protein synthesis in exotoxin-treated mice, similar results were noted. That is, in the liver the decrease in amino acid incorporation was almost immediate, while in other organs there was a lag of about 18 h. One possible explanation for the variation in the inhibition of amino acid incorporation in the liver as well as the apparent failure of inhibition of amino acid incorporation to parallel decreases in EF-2 levels is a possible excess of EF-2 in these organs. It has been shown that HeLa cells contain excess amounts of EF-2 (5) and that a considerable portion of the total EF-2 must be inactivated before protein synthesis can be inhibited. It has also been shown that cells from mammalian organs contain 20 to 40% more EF-2 molecules than ribosomes (4). It is plausible, therefore, that during experimental pseudomonas infection no decrease in protein synthesis occurs until the excess EF-2 is inactivated and EF-2 becomes rate limiting.

Since pretreatment of PA103-infected mice with antitoxin prevented EF-2 inactivation (Fig. 3A), it appears that exotoxin was produced during these experimental infections and that it acted similarly to purified exotoxin (7, 13). This conclusion is also supported by the data from mice infected with the nontoxigenic WR5 strain. Unlike the rapid and significant reduction in EF-2 levels seen in livers of mice infected with the toxigenic strain PA103, no decrease in active EF-2 content was seen in tissues from mice infected with WR5 at 24 h postinfection, and only a slight decrease occurred during the terminal stages of the infection at 48 h (Fig. 3B). Another significant difference between mice infected with the toxigenic and nontoxigenic strains was that in the case of the former, the considerable decrease in EF-2 values seen in various organs at 48 h could be prevented by antitoxin treatment (Fig. 3A), while in the latter case (Fig. 3B), the slight decrease in EF-2 activity observed was not affected by antitoxin.

In conclusion, we have shown that sufficient liver EF-2 is inactivated in animals infected with the toxigenic strain of *P. aeruginosa* PA103 to reduce significantly protein synthesis in this organ. The data here reported add to the growing evidence that exotoxin A acts in vitro and in

vivo in a highly specific manner and contributes to the severity and lethality of pseudomonas infection.

ACKNOWLEDGMENTS

We would like to thank Emilio Weiss for his suggestions and comments throughout this study. We are grateful also to G. Hare and S. C. Fegan for their expert technical assistance.

This study was supported by the Naval Medical Research and Development Command, Research Work Unit no. M0095-PN001.0009 and MR00001-01.1166, and by a grant from the National Cystic Fibrosis Society and a senior fellowship from the National Research Foundation to B.H.I.

LITERATURE CITED

1. **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.
2. **Callahan, L. T.** 1974. Purification and characterization of *Pseudomonas aeruginosa* exotoxin. Infect. Immun. **9**:113-118.
3. **Callahan, L. T., III.** 1976. *Pseudomonas aeruginosa* exotoxin: purification by preparative polyacrylamide gel electrophoresis and the development of a highly specific antitoxin serum. Infect. Immun. **14**:55-61.
4. **Gill, D. M., and L. L. Dinius.** 1973. The elongation factor 2 content of mammalian cells. Assay method and relation to ribosome number. J. Biol. Chem. **248**:654-658.
5. **Gill, D. M., A. M. Pappenheimer, Jr., and J. B. Baseman.** 1969. Studies on transferase II using diphtheria toxin. Cold Spring Harbor Symp. Quant. Biol. **34**:595-602.
6. **Iglewski, B. H., and D. Kabat.** 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. U.S.A. **72**:2284-2288.
7. **Iglewski, B. H., P. V. Liu, and D. Kabat.** 1977. Mechanism 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. **Liu, P. V.** 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of lethal toxins produced *in vitro* and *in vivo*. J. Infect. Dis. **116**:481-489.
9. **Liu, P. V., S. Yoshii, and H. Hsieh.** 1973. Exotoxins of *Pseudomonas aeruginosa*. II. Concentration, purification, and characterization of exotoxin A. J. Infect. Dis. **128**:514-519.
10. **Middlebrook, J. L., and R. B. Dorland.** 1977. Response of cultured mammalian cells to exotoxins of *Pseudomonas aeruginosa* and *Corynebacterium diphtheriae*: differential cytotoxicity. Can. J. Microbiol. **23**:183-189.
11. **Pavlovskis, O. R., and F. B. Gordon.** 1972. *Pseudomonas aeruginosa* exotoxin: effect on cell culture. J. Infect. Dis. **125**:631-636.
12. **Pavlovskis, O. R., M. Pollack, L. T. Callahan III, and B. H. Iglewski.** 1977. Passive protection by antitoxin in experimental *Pseudomonas aeruginosa* burn infections. Infect. Immun. **18**:596-602.
13. **Pavlovskis, O. R., and A. H. Shackelford.** 1974. *Pseudomonas aeruginosa* exotoxin in mice: localization and effects on protein synthesis. Infect. Immun. **9**:540-546.
14. **Pollack, M., L. T. Callahan III, and N. S. Taylor.** 1976. Neutralizing antibody to *Pseudomonas aeruginosa* exotoxin in human sera: evidence for in vivo toxin production during infections. Infect. Immun. **14**:942-947.
15. **Pollack, M., and N. S. Taylor.** 1977. Serum antibody to *Pseudomonas aeruginosa* exotoxin measured by a passive hemagglutination assay. J. Clin. Microbiol. **6**:58-61.
16. **Saelinger, C. B., K. Snell, and I. A. Holder.** 1977. Experimental studies on the pathogenesis of infections due to *Pseudomonas aeruginosa*: direct evidence for toxin production during pseudomonas infection of burned skin tissues. J. Infect. Dis. **136**:555-561.
17. **Stieritz, D. D., and I. A. Holder.** 1975. Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: description of a burned mouse model. J. Infect. Dis. **131**:688-691.
18. **Vasil, M. L., P. V. Liu, and B. H. Iglewski.** 1976. Temperature-dependent inactivating factor of *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. **13**:1467-1472.