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Altered Lethality of Murine Toxin from *Yersinia pestis* under Various Metabolic Conditions (39608)

D. E. WENNERSTROM,[1] S. D. BROWN,[1] AND T. C. MONTIE

Department of Microbiology, The University of Tennessee, Knoxville, Tennessee 37916

The etiological agent of plague is *Yersinia pestis*, a gram-negative organism that contains a protein toxin having lethal activity only against rats and mice (1). The murine toxin has been thoroughly purified (2) and characterized (3), and the chemistry of its active site has been investigated (4). Nevertheless, the mechanism of action of this toxin has remained unclear. The effects of relatively crude preparations of the toxin in mice and rats have suggested that circulatory failure was the lethal event following peripheral vascular collapse (5, 6), whereas extensive studies with isolated rat heart mitochondria have led to the hypothesis that heart malfunction was the lethal event followed by circulatory failure (7). The latter hypothesis was developed from the observation that purified toxin preparations blocked the NADH-Coenzyme Q reductase activity in the terminal respiration system (8). An important observation was the ability of the purified toxin to inhibit the respiration of heart mitochondria from the rat and mouse but not that from the rabbit, dog, chimpanzee, and monkey, which are resistant species (9). However, the amounts of toxin which were required to inhibit mitochondrial respiration (0.5–2.0 mg/ml of mitochondrial suspension) were much greater than those needed to kill susceptible animals (0.5–3.0 μg/mouse, ip). This fact raised the possibility that a primary site of lethal activity of the toxin has not been identified. Therefore, we have examined the effects of highly purified preparations of the murine toxin in mice. This investigation shows that the susceptibility of mice to the toxin is related to their metabolic state and suggests that hypothermia may be a primary determinant of lethal activity. A preliminary report has been presented (10).

Materials and methods. Mice. Male and female C_3H/HEJ mice (Jackson Memorial Laboratory, Bar Harbor, Me.) weighing 16 to 26 g were maintained on Purina chow and water *ad libitum*. Animals in every experiment were carefully matched in sex, age, and weight.

Toxin. Y. pestis murine toxin A was obtained by isolation and purification procedures previously described (11). The purified toxin was lyophilized and stored at −20°. For injection into mice, the toxin was weighed and dissolved in 0.15 *M* sodium chloride containing 0.02 *M* sodium phosphate, pH 7.5. The toxin concentration was determined according to the Lowry method as modified by Oyama and Eagle (12). A soluble, inactive toxin (toxoid) was prepared by heating the toxin for 30 min at 70° in 0.01 *M* Tris-hydrochloride, pH 7.6.

Animal treatments. The mice were treated to modify their susceptibility to subsequent challenge ip with 0.5 to 5 μg of toxin. Treated mice were compared to an untreated group which was challenged similarly with toxin, and for which the toxin doses were calculated to be either sublethal or fully lethal at 25°. Deaths were recorded through 48 hr after challenge. The treatments were as follows: (a) The mice were fasted for 12, 24, or 49 hr (Expts. I, II, III) before challenge, with fasting continuing throughout the experiment. In other experiments, the Purina chow was replaced before challenge with a fat-free diet (Nutritional Biochemical Co.) for 19 and 24 days (Expts. I and III) or for 48 hr after a 24-hr fast (Expt. II). (b) Mice were pretreated with glucagon (Eli Lilly and Co.) 40 μg sc at 20 min before challenge with toxin, and every 60 min thereafter for 8 hr (Expt. I) or 12 hr (Expt. II). The control mice received saline sc. (c) Cortisone acetate (Merck and Co.) at 5 mg per dose was injected sc 20 min before toxin (Expts. I and II) and 13 hr after challenge (Expt. II). (d) Mice were made

[1] Present address: Department of Microbiology and Immunology, The University of Arkansas for Medical Sciences, Little Rock, Arkansas 72201.

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diabetic by the injection iv of alloxan monohydrate (Sigma Chemical Co.) at 70 mg/kg in saline. They were challenged with toxin 96 hr (Expt. I) or 120 hr (Expt. II) after the alloxan injection when fasting blood glucose values exceeded 200 mg/100 ml. (e) $N^6,O^{2'}$-dibutryl cyclic AMP (db-cAMP, Sigma) 0.5 mg per dose in saline was injected ip 5 min before and 3 hr after toxin challenge. The control mice received saline. (f) Groups of mice were conditioned to a room temperature of 5, 17, 25, or 37° for 1 hr before challenge and maintained at the respective temperature for the subsequent 48 hr.

Epinephrine effects in vivo. Mice were fasted for 4 hr before challenge with the toxin. The effect of the murine toxin on epinephrine-induced increases in blood glucose and plasma free fatty acids (FFA) was determined 1.5 or 6 hr after challenge. At this time each mouse was injected ip with 6 or 10 μg of epinephrine (Adrenaline chloride, Parke Davis and Co.). Heparinized heart blood was collected from mice under ether anesthesia either 10 or 30 min following epinephrine injection. The blood was kept at ice temperature until all animals had been sampled. The concentration of blood glucose in each mouse was determined in duplicate according to Nelson (13) following protein precipitation to give a final dilution of 1:20. The free fatty acids in 1 ml of plasma were extracted and determined according to Dole and Meinertz (14) with palmitic acid as the standard. The plasma was obtained following centrifugation of blood pooled from four mice.

Results. Table I shows the ability of various treatments to modify the susceptibility of mice to ip challenge with the murine toxin. In each experiment, altered mouse susceptibility was manifested by a change in the time required for the toxin to kill the mice and by the number of mice killed. The toxin was more effective when mice were exposed to ambient temperatures of 5 or 17° than to 25°, whereas exposure to 37° completely antagonized lethal activity. At 25°, the toxin was most lethal when the Purina diet was replaced by the fat-free diet. In one

TABLE I. MODIFICATION OF THE SUSCEPTIBILITY OF MICE TO THE MURINE TOXIN.

Treatment	Conditions[a]	Expt. I 12[b]	Expt. I 24	Expt. I 48	Expt. II 48	Expt. III 48
Diet	Purine chow	2/6[c]	2/6	2/6	0/8	0/4
	vs.					
	Fat-free diet	3/6	6/6	6/6	7/8	4/4
	Purina fed	5/6	6/6	6/6	5/5	4/5
	vs					
	Fasting	0/6	1/6	4/6	2/5	0/5
Hormones	Glucagon	1/5	2/5	3/5	2/7	
	vs					
	Untreated	5/5	5/5	5/5	7/7	
	Cortisone	0/5	2/5	3/5	1/4	
	vs					
	Untreated	5/5	5/5	5/5	4/4	
	Alloxan diabetes	1/6	3/6	3/6	4/7	
	vs					
	Untreated	5/6	6/6	6/6	7/8	
Cyclic AMP	Dibutryl cAMP	1/6	3/6	3/6	1/5	1/3
	vs					
	Untreated	4/6	5/6	5/6	4/5	3/3
Ambient temperature	5°			5/5		
	vs					
	17°			4/5	8/8	5/5
	vs					
	25°			2/5		2/5
	vs					
	37°			0/5	0/8	

[a] See Methods for details.
[b] Hours after challenge with the murine toxin. Control and treated samples were injected with the same toxin sample (LD_{50} units).
[c] Number of deaths/number of mice challenged.

experiment (Expt. III), a sublethal dose of toxin in Purina-fed mice was fully lethal in animals receiving a fat-free diet. In contrast, the lethal expression of the toxin was decreased in fasting and diabetic mice. Likewise, the injection of pharmacological amounts of glucagon (0.36 and 0.52 mg), cortisone (5 and 10 mg), or db-cAMP (1 mg) antagonized toxin lethal activity. However, the injection of pharmacological doses of epinephrine was consistently without effect in modifying the lethal activity of the toxin.

Further experiments were performed to determine the responsiveness of toxin-challenged mice to epinephrine because the physiological response to epinephrine is important in maintaining the metabolic and mechanical hemeostasis. The data in Table II show that the mobilization of free fatty acids in response to exogenous epinephrine was completely blocked by the murine toxin at 1.5 hr after challenge. Figure 1 shows that the toxin also blocked the ability of epinephrine to induce hyperglycemia in mice at 6 hr after challenge. The animals contained demonstrable liver glycogen and the toxin did not change plasma levels of immunoreactive insulin (D. Wennerstrom and T. Montie, unpublished). Neither response to epinephrine was affected by toxin that had been inactivated by heat (toxoid).

TABLE II. THE EFFECT OF THE MURINE TOXIN ON EPINEPHRINE-STIMULATED MOBILIZATION OF FREE FATTY ACIDS.

Experiment	Challenge[a]	Free fatty acids (μEq/liter) +Buffer	Free fatty acids (μEq/liter) +Epinephrine
I	Buffer	1184 ± 15[b]	1763 ± 15
	Toxoid	1210 ± 21	1647 ± 33
	Toxin	986 ± 13	1026 ± 15
II	Buffer	1212 ± 17	1909 ± 25
	Toxoid	1181 ± 25	1742 ± 15
	Toxin	1015 ± 15	1075 ± 15

[a] The mice were fasted for 4 hr prior to ip challenge with buffer, toxoid, or toxin (1 μg). After an additional 1.5 hr, each mouse was injected ip with buffer or buffer containing 10 μg of epinephrine. Heart blood was collected 10 min later.

[b] Each value represents the mean ± the standard error for duplicate determinations on duplicate pools of plasma from four mice.

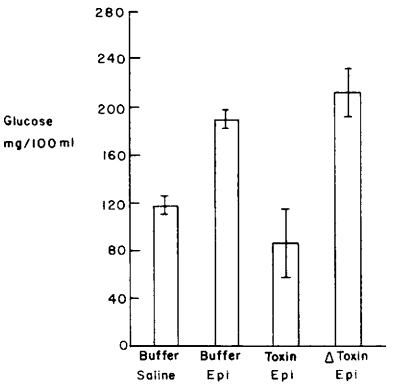

FIG. 1. The effect of toxin on epinephrine-induced hyperglycemia in fasting mice. Mice were fasted 4 hr prior to challenge ip with 0.3 ml of 10 m*M* Tris-chloride buffer (pH 7.6), 3 μg of toxin A in 0.3 ml of the buffer, or 3 μg of toxin A heat-inactivated (Δ toxin) at 70° for 30 min in the buffer. After 6 hr, each mouse was injected ip with saline or 6 μg of epinephrine (Epi) and bled after 30 min. Each bar is the mean value of individual samples determined in duplicate ± the standard error for five mice per group, except the toxin group which is from four mice.

Discussion. The mechanism of action of *Y. pestis* murine toxin *in vivo* has been controversial (15). A previous investigation has shown that crude preparations induced pooling of blood and hypothermia in rats and mice (5). The vascular system was not affected by exogenous epinephrine and the loss of blood pressure was confirmed in rats challenged with partially purified toxin preparations (6). These studies suggested a direct action of the toxin on peripheral vascular elements. In contrast, our results which show that the effectiveness of highly purified murine toxin could be modified by the nutritional and hormonal state of the challenged mice and by the ambient temperature, suggest that disruption of normal metabolism and the consequent development of hypothermia are primary determinants of lethal activity. The impairment of metabolism by the toxin is supported by the inability of challenged mice to mobilize FFA and glucose in response to exogenous epinephrine. This finding is consistent with the negative effects of this hormone in modifying toxin lethality noted previously (5), and further suggests that the toxin impaired the metabolic and calorigenic activity controlled

by the sympathetic nervous system. Because sympathetic amines increase the oxygen consumption of myocardium (16), it might be argued that the ineffectiveness of epinephrine favors the hypothesis of a direct action of the toxin on the electron transport chain of heart mitochondria (7). However, the decreased susceptibility of mice in the hypermetabolic state (increased oxygen consumption) of diabetes (17) and the increased susceptibility induced by the hypermetabolic state of the high carbohydrate fat-free diet (18) suggest that the toxin interfered with metabolism at a level other than respiration. Our results suggest that susceptibility to the toxin is related to the mobilization and metabolic use of lipid because FFA is preferentially oxidized in fasted or diabetic mice whereas glucose is preferentially oxidized in mice receiving a high carbohydrate fat-free diet (19). Additionally, the metabolic use of lipid is promoted by glucagon and cyclic AMP and by cortisone which enhances the activity of glucagon (20).

The high susceptibility of animals exposed to the cold can be attributed to an increased blockage of FFA mobilization and resulting hypothermia (S. Brown, Ph.D. dissertation, University of Tenn., manuscript in preparation). Mice placed at 37° showed no alteration in epinephrine-induced mobilization of FFA. Thus, the increased resistance of animals placed at 37° is correlated with the decreased effect of toxin in inhibiting epinephrine-induced mobilization of FFA.

Summary. The susceptibility of mice to highly purified preparations of the murine toxin of *Y. pestis* was decreased significantly when the animals were fasted, or made diabetic, or exposed to an ambient temperature of 37°, or injected with glucagon, dibutryl cyclic AMP, or cortisone. In contrast, the lethality of toxin was significantly increased in mice that received a high carbohydrate fat-free diet and that were exposed to temperatures of 5 or 17°. In contrast to controls, epinephrine was unable to elicit hyperglycemia or increase the plasma level of free fatty acid in animals challenged with the toxin. These results support a mode of action *in vivo* based on hypothermia as a primary determinant of lethality.

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