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Measurement of Biological Activities of Purified and Crude Enterotoxin of *Clostridium perfringens*

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Enterotoxin of *Clostridium perfringens* was assayed and compared with toxicity in mice and erythemal activity in guinea pigs. Conversion factors were used to express these biological activities of crude enterotoxin in terms of weight of pure enterotoxin protein. One microgram of enterotoxin was equivalent to 3.41 erythema units and to 0.68 mouse median lethal dose.

Various biological and serological tests have been used for detection and assay of enterotoxin of *Clostridium perfringens*. With recent success in purification and biochemical characterization of this enterotoxin (5, 6, 16, 18), it is now possible to express the activity of pure preparations in terms of weight of enterotoxin protein. When the biological activity-weight relationships under particular test conditions have been established, these test methods can be applied for weight assay of enterotoxin in crude preparations and biological materials. Results based on weight units can then be usefully compared between different laboratories. This paper describes an attempt to reassess the quantitative biological effects of erythemal activity in the skin of guinea pigs and toxicity for mice by using pure and crude preparations of enterotoxin of *C. perfringens*.

Crude enterotoxin was produced from *C. perfringens* type A strain NCTC 8239, as previously described (9). This preparation was purified by the procedure of Stark and Duncan (18) to a degree of 98% as determined by electrophoresis in polyacrylamide gel (1). Protein was assayed by the method of Lowry et al. (8).

Erythemal activity of the enterotoxin was assayed in the skin of albino guinea pigs (4, 17) by using 0.05-ml volumes of finely graded dilutions of crude or purified enterotoxin in saline. Also tested in parallel with the purified preparations were mixtures of known amounts of pure enterotoxin and crude nonenterotoxic cell extract. In these mixtures the enterotoxin constituted 1 to 10% of total protein, which was adjusted to 4.0 mg/ml. The reactions were read 16 to 24 h after inoculation. One erythema unit (EU) was defined as that amount of enterotoxin injected which produced an area of erythema 8 mm in diameter under the conditions of the test. The tests were replicated five times.

For toxicity test in mice, serial dilutions of purified, crude, or mixed enterotoxin were prepared as for the erythema test. The mice were weighed and enterotoxin preparations were injected on basis of body weight in 20% protein increments in volumes of 0.15 to 0.3 ml intravenously (i.v.) and up to 0.5 ml intraperitoneally (i.p.). Each dose was inoculated into five mice, which weighed from 9 to 21 g (mean, 16 g), and the mice were observed for up to 24 h. Usually, four groups of mice were sufficient to determine the median lethal dose (LD_{50}), which was calculated by the method of Reed and Muench (15). The LD_{50} of pure enterotoxin was expressed as micrograms per kilogram of body weight; for mouse LD_{50}, the value was converted to a mouse weight of 18 g to allow direct comparison with data reported by other workers.

The results of the biological tests on the purified enterotoxin are listed in Table 1 and are compared with previously published data of other workers. The addition of crude cell extract to pure preparation did not alter the toxicity of the enterotoxin for mice or influence the endpoint titer in the skin test. However, in skin sites which received 50 or more EU, the reactions of crude inocula were slightly more intense, although not larger in diameter, than the sites injected with the same amount of pure enterotoxin. All reactions were prevented by neutralization of the inocula with enterotoxin-specific antiserum. The erythemal activity of 3,410 EU of purified enterotoxin per ml differs significantly from an earlier reported value of 400 EU/mg (5). This is due to change in criteria used for calculation of the EU. The old unit, first proposed by Hauschild (4), was based on an arbitrary standard which at 1:160 dilution produced an area of erythema 12 mm in diameter under the conditions of the test. By comparing the erythemal reactions of the two methods,

the old standard was found to differ from the present method by a factor of 8.97.

Toxicity of the pure enterotoxin for mice when injected i.v. and corrected for body weight was 684 LD_{50}/mg of protein, which compares with 556 LD_{50}/mg of protein obtained by Genigeorgis et al. (3). Since the latter value was not weight-corrected certain differences can be expected, since the response of the animals to i.v.-injected enterotoxin is dependent on the body weight (10). Other published values on toxicity of pure enterotoxin for mice, ranging from 336 to 750 minimal lethal doses (MLD)/mg (5, 16, 18) are somewhat more difficult to compare because they were expressed in terms of MLD. There are no published values available for comparison on i.p. technique, but a value lower than that obtained by i.v. inoculation would be expected because of slower or incomplete absorption through the peritoneum. Also, this method had greater variation in response than the i.v. route of injection.

Since it is not always practical to purify preparations, the findings reported here allow an assay of crude enterotoxin preparations or enterotoxin-containing tissue extracts and body fluids to be carried out by the biological methods and the results to be converted into weight of pure enterotoxin protein for comparative studies. Table 2 shows such conversion factors. Both the erythemal activity as well as the toxicity for mice appear to be reliable criteria, but the intravenous toxicity test in mice appears to be the most accurate method, particularly for confirmation of the results obtained by the former test.

Table 3 compares the response of various animal species to *C. perfringens* enterotoxin, previously published, now expressed in terms of weight of pure enterotoxin (revised EU applied). The response of various animal species to i.v.-injected enterotoxin varies; it appears to be inversely related to the body weight of the

TABLE 1. *Erythemal activity and mouse toxicity of purified C. perfringens enterotoxin compared with previously published data*

Characteristic	Value	Published data	
		Value	Reference
EU/mg of protein	3,410		
		400	5
		5,000	18
		3,230	2
		4,000	3
Mouse LD_{50}, i.v.			
μg/kg	81.16		
μg/18-g mouse	1.46		
No./mg of protein	684		
		556	3
		336 (MLD)	5
		356 (MLD)	18
		750 (MLD)	16
Mouse LD_{50}, i.p.			
μg/kg	112.56		
μg/18-g mouse	2.03		
No./mg of protein	494		

TABLE 2. *Conversion factors of C. perfringens enterotoxin in terms of weight, EU, and mouse LD_{50}*

Protein (μg)	EU	No. of mouse LD_{50} (i.v.)
1	3.41	0.68
0.29	1	0.20
1.46	4.98	1

TABLE 3. *Biological effects of crude C. perfringens enterotoxin on various animal species expressed in terms of weight of pure enterotoxin*[a]

Animals	Method	Effective dose[b], (μg/kg)	Lethal dose[c], (μg/kg)	Reference
Calves	i.v.	5.93 (MED)	12.90 (MLD)	10
Sheep	i.v.	4.63 (ED_{50})	13.42 (LD_{50})	10
Rabbits	i.v.	6.24 (MED)	14.56 (MLD)	10
Guinea pigs	i.v.	16.64 (ED_{50})	29.13 (LD_{50})	10
Mice	i.v.	69.19 (ED_{50})	80.12(LD_{50})	10
Mice	i.v.		81.16 (LD_{50})	This study
Mice	i.p.		112.56 (LD_{50})	This study
Sheep	Intestinal loops	26-80 μg/loop		7, 14
Calves	Intestinal loops	42-80 μg/loop		11, 13
Chickens	Intestinal loops	20-30 μg/loop		12
Rabbits	Intestinal loops	28-40 μg/loop[d]		18
Rabbits	Intestinal loops	29 μg/loop[d]		3

[a] Original data from previous publications was converted.
[b] Minimum, or mean effective, dose (MED; ED_{50}) for clinical or pathological effect.
[c] Minimum, or mean lethal, dose (MLD; LD_{50}).
[d] Purified enterotoxin used.

species. Conversely, local response in the ligated intestinal loops shows less interspecies variation than the systemic response.

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