The Effect of *Clostridium perfringens* Type D Culture Filtrate on the Mouse Body Weight

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Summary

The effect of freeze-dried Clostridium perfringens *Type D culture filtrate on the mouse body weight was studied. After preparation of filtrate and freeze dried crude prototoxin, three series of experiments were set up. At first the minimum lethal dose/ml (MLD/ml) was determined and according to MLD, fifty percent lethal dose* (LD_{50}) *was calculated. Finally, mice with 18 to 20g body weight were injected with different concentrations of freeze-dried prototoxin activated prior to injection. Decrease in body weight was observed for two days after injection. The results indicate that the activated* Clostridium perfringens *culture filtrate temporarily inhibits mouse general metabolism.*

Introduction

Clostridium perfringens Type D is the causal agent of enterotoxaemia of the sheep. This microorganism produces 4 major and 8 minor toxins. Epsilon(ε) is one of its major toxins and is lethal for animals (Batty and Glenny, 1947; Bullen and Batty, 1956). In the small intestine of infected animals, the microorganism produces the prototoxin which is activated and turned into a toxin by trypsin (Bosworth and Glover, 1935). This can well be done, *in vitro*, by other proteolytic enzymes (Turner and Rodwell, 1943). Prototoxin activation by trypsin is due to the cleavage and removal of a small 14 amino acids peptide from the amino terminal (Bhown and Habeeb, 1977). There is a tryptophane residue and a histidyl residue in the structure of epsilon toxin which are respectively important and essential for its lethal activity (Sakurai and Nagahama, 1985). The effect of *C. perfringens* Type D culture filtrate on the mouse small intestine permeability (Bullen and Batty, 1956) and also *in vitro* effects of *C. perfringens* Type D epsilon toxin on the mouse, the

guinea-pig and the rabbit cells have been described (Buxton, 1978). Electron microscopy and I^{125} labelling have revealed epsilon binding sites on different cells and its distribution in various organs of the mouse (Buxton, 1978). *C. perfringens* epsilon toxin induces contraction of the isolated rat ileum due to an indirect action mediated through the nervous system (Sakurai *et al.*, 1989). It also possesses presser activity on cardiovascular system of rats (Sakurai *et al.*, 1983). In Iran toxigenic strains of *C. perfringens* Type D have been isolated from the soil (Ardehalli *et al.*, 1991) and standard *C. perfringens* Type D antitoxin has been prepared (Moosawi *et al.*, 1994). In the present work the effect of *C. perfringens* Type D culture filtrate on the mouse body weight was investigated.

Materials and methods

Medium and culture procedures: A fermenter was used for bacterial growth and toxin production. Medium consisting of peptone (2.5%), Na_2HPO_4 (1%), ClNa (O.25%), vitamin and trace elements, as trace vitamin solution, (0.7%) and dextrin (1%) was prepared according to Ardehali and Darakshan (1977), sterilised at 121°C for 30 min and , after cooling to 37°C, was inoculated with 2% of *C. perfringens* Type D (C.N Type 407 Razi Institute) culture. Dextrin and trace vitamin solution was added at the time of inoculation. Incubation period was 6 h under controlled conditions such as fixed temperature (37°C) and at pH=7.1. Immediately after the growth period, 2 litres of culture suspension was removed from the fermenter and used for further studies.

Filtrate: Ten ml of The culture suspension was centrifuged for 1 h. at 3000 RPM. To the supernatant (filtrate) 1% trypsin was added and incubated at 37°C for 1h.

Freeze dried activated toxin: To one litre of the filtrate 70% ammonium sulphate was slowly added and mixed. The mixture was transferred to a 4°C refrigerator and left over night. Next day, toxin was extracted as described by Thompson (1963), dialysed against tap water and concentrated with polyethylene glycol (B.D.L. Carbowax M.W. 6000) up to the final volume of 20ml and freeze-dried.

Freeze dried inactive toxin (prototoxin): Culture filtrate without treatment with trypsin was freeze-dried. To turn prototoxin into toxin, directly before inoculations, to 100 mg of prototoxin 1 mg trypsin was added and incubated at 37°C for 1h.

Animals: White male and female N.M.R.I. mice weighing 18-20 g.(bred at Razi Vaccine and Serum Research Institute) were used. Animals were kept at normal period of light and fed with standard pellet.

Minimum lethal dose (MLD) determination: MLD/ml was determined by dissolving 10 mg of freeze dried toxin in 1ml normal saline and thereafter dilutions were prepared. Of each dilution (10,000 up to 40,000 with 5,000 intervals), 0.5ml was injected IV into a mouse, using 2 mice for each dilution.

Fifty percent endpoints (LD_{50}) determination: LD_{50} was determined according to Reed and Muench (1938).

Body weight measurements: Four groups of mice, each consisting of six mice weighing 18-20g (mean 19g) were used. Animals in each group were injected with single subdoses of LD_{50} and were observed for two days. Body weight measurements were carried out from the second day after injection and repeated daily for six days. A group of six mice, kept as control, each was injected with 0.5ml of normal saline.

Statistical analysis: Body weight means ±SD was determined for each group, Student's (t) test was performed and the probability (P) was calculated.

Results

M.L.D: MLD/ml for filtrate was found to be 19000. While it was 12500 for freeze-dried toxin, it was 40000 for freeze-dried prototoxin that was activated at the time of injection .

LD_{50}: The data for determination of LD_{50} are shown in Table 1. This was determined to be 0.000215 mg for mice with 18-20 g body weight.

Table 2 indicates that a single dose injection of toxin can cause body weight loss, relative to the size of the dose applied, which follows by the resumption of body weight gain within the next few days. Figure 1 shows that the group which was once injected with 0.000195 mg/ml dose underwent severe loss of body weight whereas this in the other groups was less. The data were statistically analysed by Student's t test.

Toxin effect on the body weight: Table 2 shows summarised results of body weight measurements for four groups of mice which were injected once with one of the following dilutions: 0.0001 mg/ml (0.46% of LD_{50}), 0.000125 mg/ml (58% of LD_{50}), 0.000156 mg/ml (72.5% of LD_{50}) and 0.000195 mg/ml (90% of LD_{50}) of the prototoxin, activated after reconstitution by addition of 1mg trypsin to 100mg of freeze-dried prototoxin. The probability (P) was determined for body weight differences between groups. Tables 3,4,5,6 show these results.

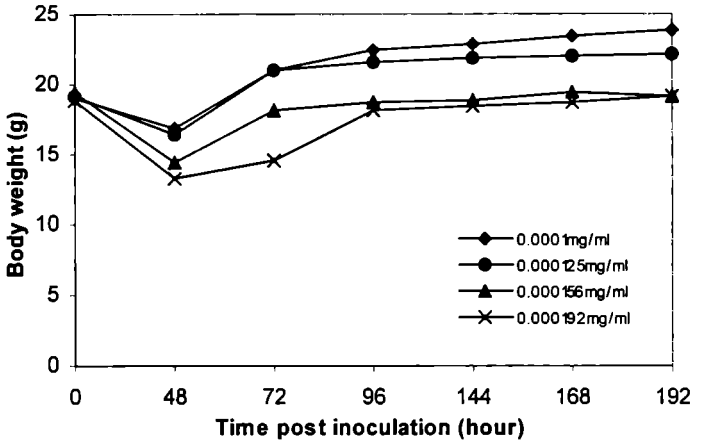

Fig.1 Mean (6) body weight changes in 4 groups of mice inoculated different amounts of *Clostridium perfringens* epsilon toxin

Table 1. *LD_{50} test for the activate freeze dried prototoxin that was activated at the injection time*

Toxin dose mg/ml	Inoculated/dead	Morality %	Time to death h
0.0001	6/0	0	---
0.000125	6/0	0	---
0.000156	6/0	0	---
0.000195	6/1	20	24
0.000244	6/3	50	24
0.000305	6/6	100	24
0.000381	6/6	100	24
0.000476	6/6	100	24

LD_{50} = 0.000215 mg/ml for 18-20 grams mouse or 0.0107 mg/kg of mouse body weight

Table 2. *Body weight means in four groups of mice in six measurements* ($\bar{X}$)

Treatment mg/ml (0.5ml)	Weight after 48 h. (g)	Weigh after 72 h. (g)	Weight after 96 h. (g)	Weight after 144h. (g)	Weight after 168h. (g)	Weight after 192h. (g)
1st. 0.0001	16.91	21	22.5	22.8	23.4	23.9
2nd. 0.000125	16.5	21	21.58	21.83	22	22.16
3rd. 0.000156	14.5	18.083	18.75	18.91	19.41	19.16
4th. 0.000195	13.3	14.6	18.1	18.5	18.75	19.125

Table 3. *Results of body weight analyses of two groups of mice which were exposed to 0.0001mg/ml and 0.000195mg/ml toxin* ($\bar{X} \pm$ SD)

Experiments (0.5ml)	Weight after 48 h. (g)	Weigh after 72 h. (g)	Weight after 96 h. (g)	Weight after 144h. (g)	Weight after 168h. (g)	Weight after 192h. (g)
1st. treatment 0.0001mg/ml)	16.91 ± 1.655	21 ±1.264	22.5 ± 1.036	22.8 ±1.183	23.41 ±1.083	23.9 ±1.244
4th treatment 0.000195mg/ml	13.3 ± 1.350	14.6 ± 2.183	18.1 ± 1.474	18.5 ± 1.732	18.75 ± 1.707	19.125 ± 1.701
P	P<0.01	P<0.001	P<0.001	P<0.01	P<0.01	P<0.01

Table 4. *Results of body weight analysis of two groups of mice which were exposed to 0.000125mg/ml and 0.000195mg/ml*($\bar{X} \pm$ SD)

Experiments	Weight after 48 h. (g)	Weigh after 72 h. (g)	Weight after 96 h. (g)	Weight after 144h. (g)	Weight after 168h. (g)	Weight after 192h. (g)
2nd treatment 0.000125mg/ml (0.5ml)	16.5 ±2.236	21 ± 1.095	21.58 ±1.393	21.8 ±2.359	22 ± 2.569	22.16 ± 2.359
4th treatment 0.000195mg/ml (0.5ml)	13.3 ±1.350	14.6 ± 2.183	18.1 ± 1.474	18.5 ± 1.732	18.75 ± 1.707	19.125 ± 1.701
P	P<0.05	P<0.001	P<0.01	P<0.05	0.05<P<.01	0.05<P<0.1

Table 5. *Results of body weight analysis of two groups of mice which were exposed to 0.0001mg/ml and 0.000156mg/ml*($\bar{X}$ ± SD)

Treatments (0.5ml)	Weight after 48 h. (g)	Weigh after 72 h. (g)	Weight after 96 h. (g)	Weight after 144h. (g)	Weight after 168h. (g)	Weight after 192h. (g)
1st. 0.0001mg/ml	16.91 ± 1.655	21 ±1.264	22.5 ± 1.036	22.8 ± 1.183	23.41 ±1.083	23.9 ±1.244
3rd. 0.000156mg/ml	14.5 ±1.183	18.083 ± 1.068	18.75 ± 0.880	18.91 ± 2.396	19.41 ± 2.498	19.16 ± 2.695
P	P<0.02	P<0.01	P<0.001	P<0.01	P<0.01	P<0.01

Table 6. *Results of body weight analysis of two groups of mice which were exposed to 0.000125mg/ml and 0.000156mg/ml*($\bar{X}$ ± SD)

Experiments	Weight after 48 h. (g)	Weigh after 72 h. (g)	Weight after 96 h. (g)	Weight after 144h. (g)	Weight after 168h. (g)	Weight after 192h. (g)
2nd. 0.000125mg/ml	16.5 ± 2.236	21 ± 1.095	21.58 ± 1.393	21.8 ± 2.359	22 ±2.569	22.16 ± 2.359
3rd. 0.000156mg/ml	14.5 ±1.183	18.083 ±1.068	18.75 ± 0.880	18.91 ± 2.396	19.41 ±2.498	19.16 ± 2.695
P	P<0.05	P<0.001	P<0.01	0.05<P<0.1	0.01<P	0.05<P,0.1

Discussion

By studying the determined MLD values, one can realise that the highest value (40000) belonged to the freeze-dried prototoxin activated immediately prior to injection. Conversely, the lower value (12500) was seen in active freeze-dried toxin. Therefore, one may infer that activation, concentration and freeze-drying of prototoxin had led to reduction of its activity. This loss of activity could be interpreted in different ways. Possibly, the proteolytic enzyme trypsin may have continued to further degrade the toxin. Another interpretation is that the toxin after activation, before and during freeze-drying process, had been denatured. The latter interpretation is more probable as epsilon toxin, a protein, has a definite half life and naturally undergoes degradation. Whatever the reason, it was obvious that the prototoxin activated shortly before injection had a much higher activity and, for this reason, this method was chosen for preparation of the toxin.

There is a threshold for teratogenic and carcinogenic materials (Brent, 1986). When this threshold is passed the pathological effects have direct relationship with the size of the dose. Therefore, amounts of toxin injected were selected on a decreasing scale of a subdose of LD_{50}.

Table 2 and Figure 1 show that the group (4th treatment) which had received only one injection of 0.000195 mg/ml toxin (9/10th of LD_{50}) suffered a severe loss in body weight whereas this was less markedly noticeable in other groups. Maximum body loss, 48 h after injections, compared against the body weight mean (19 g), for the first treatment (0.0001 mg/ml), the second treatment (0.000125 mg/ml), the third treatment (0.000156 mg/ml) and the fourth treatment (0.000195 mg/ml) were 11%, 13.5%, 23.68% and 30%, respectively. This showed the direct effect of the size of the dose. Furthermore, 72h after injections body weight means were 21g in the first and the second groups whereas these were 18.75 and 18.1 in the third and the fourth group, respectively. From this time point, all animals showed weight gains which were much slower in the third and the fourth groups. From Table 3, by comparative analysis of the data one can deduce that, at 48th and 96th h post-inoculation, the differences between the first and the fourth treatments are significant. Table 4 indicates that the results up to 144 h after injection are significant between the second and the fourth groups but, thereafter, the significancy is doubtful; the standard error differences of each mean is high and the overlapping of parameters is obvious. Table 5 shows that differences between the first and the third treatments on all occasions are significant, whereas, the differences between the second and the third treatments was significant only up to 96 h after injection (Table 6). From this study one can infer that although sub-LD_{50} doses of freeze-dried prototoxin activated at the time of injection cannot kill mice it can severely affect and disrupt, temporarily, the natural metabolism. The closer the

applied dose to LD_{50} the longer and more severe the effects would be. According to Buxton (1978) epsilon toxin increases cAMP concentration in mouse plasma. Also, *E. Coli* heat resistant enterotoxin, *V. Cholera* enterotoxin, *S. aureos* deltatoxin, increase cAMP concentration *in vitro* in the guinea-pig ileal mucosal cells. Thus, according to Buxton (1978) studies, *C. perfringens* epsilon toxin is an enterotoxin which can bind to specific cell surface receptors of certain cells in blood vessels, kidney and liver and finally can cause wide damages mediated by adenyl cyclase-cAMP system. In another study of Buxton (1978a) it is indicated that toxin may bind to mouse brain blood vessels, endothelium luminal surface of thalamus, cortex, cerebrum white nucleus, pons, and meninges. According to the present work and data discussed above, we suggest that wide damages mediated by adenyl cyclase-cAMP system can cause severe and temporary body weight decrease. The mechanisms of these effects remain to be elucidate in future investigations.

Acknowledgements

The authors wish to express their sincere thanks to Dr.J. Akbarzadeh and his colleagues for their kind cooperation in supplying the animals and also to Dr.A. Akbari for his guidance in LD_{50} determination.

References

Ardehali, M and Darakshan, H. (1977). Production and standardization of polyvalent *Clostridium perfringens* vaccine in Iran. Archives de l'Institut Razi, **29**: 41-45

Ardehali, M. ; Moosawi, M.; and Pilehchian, R. (1991). Isolation of toxigenic strains of *Clostridium perfringens* from soil. XXIV World Veterinary Congress August 18 to 23 Brazil, Abstracts, 7.8.2.

Batty, I. and Glenny, A.T. (1947). Titration of *C. welchii* epsilon toxins and antitoxin. The British Journal of Experimental Pathology, **28**: 110-126

Bhown, A.S. and Habeeb, A.F. (1977). Structural studies on prototoxin of *Clostridium perfringens* Type D. localisation of the site of tryptic scission necessary for activation to toxin. Biochemical and Biophysical Research Communications, **78**: 889-896

Bosworth, T.J. and Glover, R.E. (1935). The effects on *C. welchii* toxins of the substanse present in the normal intestine. University of Cambridge Insstitute of Animal Pathology. 4th Report, 79-93

Brent, R.L. (1986). Definition of tratogen and relationship of tratogenicity to carcinogenicity. Teratology, **34**: 359-360

Bullen, J.J. and Batty, I. (1956). The effect of *Clostridium welchii* type D culture filtrates on the permeability of the mouse intestine. The Journal of Pathology and Bacteriology, **71**: 311-323

Buxton, D. (1978). In vitro effects of *Clostridium welchii* type D epsilon toxin on guinea-pig, mouse, rabbit and sheep cells. Journal of Medical Microbiology, **11**: 299-302

Buxton, D. (1978a). The use of an immunoperoxidase technique to investigate by light and electron microscopy the sites of binding of *Clostridium welchii* Type D epsilon toxin in mice. Journal of Medical Microbiology, **11**: 282-292

Habeeb, A. (1963). Some studies on the chemical modification of toxin of *Clostridium perfringens* Type D Biochemica et Biophysica Acta, **74**: 113-121

Moosawi, M.; Ardehali, M. and Pilehchian, R. (1994). Preparation of standard *Clostidium perfringens* antitoxin in the sheep. Archives de l'Institut Razi, **44/45**: 101-105

Nagahama, M and Sakurai, J. (1991). Distribution of labeled *Clostridium perfringens* epsilon toxin in mice. Toxicon, **29**: 211-217

Reed, L.J. and Muench, H. (1938). A simple method of estimating fifty percent endpoints. The American Journal of Hygiene, **27**: 493-497

Sakurai, J. and Nagahama, M. (1985). Role of one tryptophan residue in lethal activity of *Clostridium perfringens* epsilon toxin. Biochemical and Biophysical Research communications, **128**: 760-766

Sakurai, J. and Nagahama, M. (1987) Histidin residues in *Clostridium perfringens* epsilon toxin. FEMS Microbiology letters, **41**: 317-319

Sakurai, J.; Nagahama, M. and Fujii, Y. (1983). Effect of *Clostridium perfringens* epsilon toxin on the cardiovascular system of rats. Infection and Immunity, **42**: 1183-1186

Sakurai, J.; Nagahama, M. and Takahashi, T. (1989). Contraction induced by *Clostridium perfringens* epsilon toxin in isolated rat ileum. FEMS Microbiology letters, **58**: 269-272

Thomson, R.O. (1963). The fractionation of *Clostridium welchii* antigen on cellulose Ion exchangers. Journal of General Microbiology, **31**: 79-90

Turner, A.W. and Rodwell, A.W. (1943). The epsilon toxin of *C. welchii* type D 1-Proteolytic conversion of prototoxin into toxin by trypsin and other proteases. 2-Mechanism of its development in cultures through the action of extracellular proteinases upon prototoxin. Australian Journal of Experimental Biological and Medical Sciences, **21**: 17-27

Wilsdon, A.J. (1931). Observation on the classification of *Bacillus welchii*. Univ. of cambridge. Institute of Animal Pathology, 2nd Report, PP.53-58