Some Nutritional Requirements for Enterotoxin Production by *Clostridium perfringens* NCTC 8239 in the Ligated Intestinal Loop of Rabbit

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Both protein and carbohydrate were necessary for sporulation and enterotoxin production of *Clostridium perfringens* NCTC 8239 in the ligated intestine of the rabbit. The administration of 10^8 vegetative cells suspended in 2 ml of skim milk was confirmed to induce positive reactions consistently. However, skim milk dialysate, which contained the same amount of protein as the 10% skim milk, had no effect. An addition of 10% lactose to the skim milk dialysate restored the effect. Proteose peptone (Difco), peptone for toxin production (Mikuni), meat extract (Wako) and purified casein (Merck) could replace the skim milk dialysate.

In addition to lactose, raffinose and fructose were effective as carbon sources whereas galactose, sucrose and glucose were less effective. Enterotoxin was not produced in the ligated intestine when the vegetative cells were administered together with any one of these carbohydrate or protein sources alone.

1. Introduction

Ingestion of food contaminated with large numbers of enterotoxigenic *Clostridium perfringens* results in food poisoning. Symptoms similar to those encountered in natural cases of food poisoning have been produced in monkeys¹⁾ and humans²⁾ by feeding vegetative cells of *C. perfringens*, and the enterotoxin plays a crucial role in the development of the main symptom, diarrhea.³⁾ Therefore, it is believed that the intestinal tract provides suitable conditions for sporulation and enterotoxin production of *C. perfringens*.

The ligated intestinal technique developed by De and Chatterje⁴⁾ has been applied for testing *in vivo* production of *C. perfringens* enterotoxin. Enterotoxigenic strains sporulate and produce enterotoxin followed by fluid accumulation in the ligated intestine. This test is markedly influenced by the method of preparation of the challenge inoculum and suspending media. Duncan *et al.*⁵⁾ showed that cultures grown for 4 hr at 37°C in skim milk consistently produced positive reactions. Gyobu

*et al.*⁶⁾ confirmed the value of skim milk as the suspending medium for these cells. Hauschild *et al.*⁷⁾ employed CP-2VS, consisting of dextrin, proteose peptone, yeast extract, cystine-HCl, sodium dibasic phosphate and potassium monobasic phosphate. To our knowledge, however, there is no other published study in which the role of the composition of the suspending media in enterotoxin production by *C. perfringens* in the ligated intestine has been further investigated.

Food and digestive products of food may play an important role in the onset of food poisoning, not only as a vehicle for the organisms but also as an energy source for sporulation and enterotoxin production of *C. perfringens* in the intestine. Thus, investigation of the nutritional requirements for *in vivo* production of enterotoxin by *C. perfringens* appears important from the food hygienic point of view and may provide useful information for the improvement of the ligated intestinal loop technique.

2. Materials and Methods

2.1 Challenge organism

C. perfringens NCTC 8239 was maintained at room temperature in a cooked meat medium (CM; Difco Laboratories, Detroit, Mich). The stock culture (0.3 ml) was inoculated into freshly prepared CM (5 ml), heat-treated for 20 min at 75°C and subcultured overnight at 37°C. The CM culture was transferred into 100 ml of fluid thioglycolate medium (FTG; Nissui Seiyaku, Tokyo) and cultured for 6 hr at 37°C. The actively growing cells were harvested by centrifugation for 10 min at $1,000 \times g$. Cell numbers were determined as reported previously⁹ and appropriate challenge doses were resuspended in 2 ml of the different suspending media listed in 2.2.

2.2 Suspending media

Sporulation media used for *in vitro* tests. Sporulation media used were those reported by Duncan and Strong,⁹ Ellner,¹⁰ Tsai *et al.*¹¹ and Oliveira-Tortora [João Carlos De Oliveira-Tórtora 1976 (Master's thesis): Thiotone (0.5%), soluble starch (0.3%), magnesium sulfate heptahydrate (0.02%), sodium dibasic phosphate dihydrate (0.9%), thiamine dihydrochloride (20 µg/12 ml)].

Skim milk. Commercially purchased skim milk powder [Snow Brand Milk Products, Tokyo; protein (35%), carbohydrate (52%), fat (1%), ash (8%), and water (4%)] was dissolved in distilled water to obtain a 10% solution. To remove low-molecular weight components, a 20% skim milk solution was dialyzed against chilled distilled water for 2 days. After dialysis, the protein content of the solution was determined and adjusted to that of 10% skim milk (SK) by the addition of distilled water (skim milk dialysate, SKD).

Casein, peptone and meat extract. Purified casein (Hammarsten, Merck, Darmstadt) was dissolved in 0.1 N NaOH to give a 20% solution and then diluted with distilled water to obtain the desired final concentrations. Peptone for toxin production (Mikuni Kagaku Sangyo, Tokyo), proteose peptone (Difco) and meat extract (Wako Pure Chemicals, Tokyo) were used as 5% solutions. Carbohydrates (Wako) were added to SK or SKD to obtain the indicated concentrations.

2.3 Administration procedures

For *in vivo* tests, the ligated intestinal loop test developed by De and Chatterge⁴ was employed with minor modifications. New Zealand white rabbits weighing 2.5 to 4 kg were fasted for 2 days, but allowed to drink water *ad libitum*. They were anesthetized with Somnopentyl (Pitman-Moore, Inc., Washington, N.J.). The small intestine was ligated into 6 to 10 segments, each about 8 to 10 cm in length. Between test segments, a short blank segment was left. The challenge material was injected through the blank segment just before ligation. A negative control loop injected with sterile saline and a positive one with more than 1×10^8 cells of the test organisms suspended in SK were prepared in each rabbit. Each loop received a dose of vegetative cells (1×10^8 to 5.5×10^8) suspended in 2 ml of one of the media listed in 2.2.

2.4 Postchallenge examinations

The rabbits were sacrificed 15 to 24 hr later and the ligated intestinal loops were examined for fluid accumulation and length. The secretory responses in the loops were expressed as the ratio of the volume of the contained fluid (in ml) to the length of the empty loop (in cm). The number of spores¹³ and the amount of enterotoxin¹² in the fluid were determined as reported previously.

2.5 Protein contents

Protein content was determined by the method of Lowry *et al.*¹⁴ with bovine serum albumin (Armour and Co., Chicago, Ill.) as a standard.

3. Results

The effects of different suspending media on sporulation and enterotoxin production of NCTC 8239 in ligated intestinal loops of the rabbit were as follows:

3.1 *In vitro* sporulation media

Neither of the two rabbits responded by fluid accumulation in the intestine when inoculated with 1×10^8 cells suspended in 2 ml of any of the 4 different *in vitro* sporulation media. Four control loops inoculated with cells in SK showed fluid accumulation in ratios of 0.3 to 1.0. The test organisms were confirmed to sporulate well in tubes containing these media.

Table 1. Effect of Skim Milk on Enterotoxin Production by *Clostridium perfringens* NCTC 8239 in Ligated Intestinal Loops of the Rabbit

Inoculum			Fluid accumulation (ml/cm) in ligated intestine	
Cell number	Suspending medium (2 ml)			
	Protein (10%)	Carbohydrate (10%)	Rabbit no. 3	Rabbit no. 4
0	Skim milk	NA ^{a)}	0 ^{b)}	0
	Skim milk dialysate	NA	0	0
		Lactose	0	0
5.4 × 10 ⁹	Skim milk	NA	0.99	0.61
	Skim milk	NA	0	0
	Skim milk dialysate	Lactose	0.90	0.95
		Glucose	0	0

^{a)} Not added^{b)} Less than 0.10**3.2 Skim milk (Table 1)**

A 10% solution of skim milk used as the suspending medium consistently gave positive reactions. Plain SKD had no such effect, but the addition of 10% lactose restored the effect. Glucose was not a satisfactory substituent for lactose. More than 1 µg/ml of enterotoxin was found in all fluid samples from the positive loops.

3.3 Skim milk dialysate containing various amounts of lactose (Table 2)

An addition of 10% lactose to SKD played an important role in enterotoxin production of NCTC 8239 in the intestinal loops of the rabbit (3.2). Lactose still showed a positive effect at 5%, but was less effective at 3%, or less.

3.4 Casein (Table 3)

Pure casein alone did not permit enterotoxin production of NCTC 8239 in the ligated intestinal loops, but was effective when supplemented with 10% lactose.

Table 2. Effect of Concentration of Lactose on Enterotoxin Production by *Clostridium perfringens* NCTC 8239 in Ligated Intestinal Loops of the Rabbit

Rabbit No.	% lactose in suspending medium (2 ml) ^{a)}	Fluid accumulation (ml/cm)	Enterotoxin (µg/cm)	Spores /cm
R7	10	0.38	30	24 × 10 ⁷
	7	0.47	38	40
	5	0.21	17	4.8
	3	0.15	6.1	3.6
	1	0.37	15	19
	0	0 ^{b)}	0 ^{c)}	0.96
R9	10	0.82	33	25
	7	0.72	7.2	7.9
	5	0.42	4.2	3.4
	3	0	0	0.024
	1	0	0	0 ^{d)}
	0	0	0	0

^{a)} Basal medium was skim milk dialysate containing the same amount of protein as 10% skim milk solution. The number of inoculated cells was 4.2 × 10⁹.^{b)} Less than 0.10^{c)} Less than 0.1^{d)} Less than 1 × 10⁵**Table 3.** Effect of Casein on Enterotoxin Production by *Clostridium perfringens* NCTC 8239 in Ligated Intestinal Loops of the Rabbit

Rabbit No.	Suspending medium (2 ml) ^{a)}		Fluid accumulation (ml/cm)	Enterotoxin (µg/cm)	Spores /cm
	Casein (%)	Lactose (%)			
R26	2	0	0 ^{b)}	0 ^{c)}	0.025 × 10 ⁷
		10	1.2	9.5	7.0
	4	0	0	0	0 ^{d)}
		10	1.6	50	11
	6	0	0	0	0.12
		10	1.3	21	12
R27	2	0	0	0	ND ^{e)}
		10	0.42	0.14	ND
	4	0	0	0	ND
		10	0.57	18	ND
	6	0	0	0	ND
		10	0.52	18	ND

^{a)} Contained 3.0 × 10⁹ vegetative cells^{b)} Less than 0.10^{c)} Less than 0.1^{d)} Less than 1 × 10⁵^{e)} Not determined

3.5 Protein sources (Table 4)

Proteose peptone, peptone for toxin production and meat extract at 5% could replace SKD. The addition of 10% lactose to each of these protein sources was necessary. The protein contents of 5% solutions of proteose peptone, peptone for toxin production and meat extract after dialysis against distilled water for 2 days were 21, 9 and 13 µg/ml, respectively.

3.6 Carbohydrates (Table 5)

Test carbohydrates other than glucose were effective when added to SKD at 10%. Glucose was less effective; enterotoxin production of

Table 4. Effect of Nitrogen Source on Enterotoxin Production by *Clostridium perfringens* NCTC 8239 in Ligated Intestinal Loops of the Rabbit

Rabbit No.	Suspending medium (2ml) ^{a)}		Fluid accumulation (ml/cm)	Enterotoxin (μg/cm)	Spores /cm
	Protein	Lactose (%)			
R 12	5% Peptone	0	0 ^{b)}	0 ^{c)}	×10 ⁷ ND ^{d)}
		5	1.1	4.6	3.7
	5% Proteose peptone	0	0	0	ND
		5	0	0	ND
	5% Meat extract	0	0	0	ND
		5	0.98	20	2.5
	10% Skim milk dialysate	5	0.32	3.2	0.69
R 14 ^{e)}	5% Peptone	0	0	0	ND
		5	0.96	77	20
	5% Proteose peptone	0	0	0	ND
		5	1.4	57	11
	5% Meat extract	0	0.16	0	ND
		5	1.2	94	15
	10% Skim milk dialysate	5	1.0	82	29

^{a)} Contained 5.5×10⁸ vegetative cells

^{b)} Less than 0.10

^{c)} Less than 0.1

^{d)} Not determined

^{e)} Rabbit No. 14 was dead (within 17 hr after administration) when postchallenge examinations were carried out.

Table 5. Effect of Carbohydrate on Enterotoxin Production by *Clostridium perfringens* NCTC 8239 in Ligated Intestinal Loops of the Rabbit

Suspending medium (2 ml) ^{a)}			Result ^{b)}
Protein	Carbohydrate		
5% Proteose	Raffinose	5%	2/4
		10%	2/2
	Lactose	5%	3/4
		10%	2/2
	Sucrose	5%	0/4
		10%	0/2
	Fructose	5%	3/4
		10%	1/2
	Galactose	5%	1/4
		10%	0/2
	Soluble starch	5%	1/2
		10%	0/2
10% Skim milk dialysate	Glucose	5%	0/2
		7%	0/2
		10%	2/4
	Raffinose, Lactose, Sucrose, Fructose, Galactose or Soluble starch	10%	2/2

^{a)} Contained 1×10⁸ cells

^{b)} No. of positive loops/no. of test loops. The positive loops gave a fluid accumulation ratio (ml/cm) of more than 0.1, and the fluid contained more than 0.1 µg/ml of enterotoxin.

NCTC 8239 in the loops of 4 rabbits was reduced when glucose was employed at 7%, or less. No significant increase in liquid content was seen in either of the two rabbits which received 10⁸ cells plus 2 ml of 10% carbohydrate, nor was any sporulation or enterotoxin production noted. Table 5 also shows the results in loops that received 10⁸ cells suspended in 2 ml of 5% proteose peptone containing 5 or 10% carbohydrate. Sucrose and glucose (not shown in Table 5) had no effect. Raffinose, lactose and fructose were effective, but galactose was less effective.

Neither sporulation nor enterotoxin production was noted when vegetative cells of NCTC

8239 were incubated in tubes containing SK, SKD plus 10% lactose, 5% casein plus 10% lactose or 10% proteose peptone plus 10% lactose.

4. Discussion

The intestinal tracts of humans and animals provide a suitable environment for the sporulation and enterotoxin production of *C. perfringens*. The results of *in vitro* experiments indicate that the temperature¹⁴⁾ and pH¹⁴⁾ in the small intestine are within a range suitable for enterotoxin production. Clarification of the nutritional requirements for *in vivo* production of enterotoxin is difficult because of the many unknown variables. The composition of materials in the intestine changes constantly as a result of the processes of digestion and absorption, even if a simple substance is introduced. Ligated intestinal loop tests in animals conducted by Duncan *et al.*,⁵⁾ Hauschild *et al.*⁷⁾ and Gyobu *et al.*⁶⁾ showed that *C. perfringens* required certain nutrients for *in vivo* production of enterotoxin.

The effectiveness of skim milk, reported by Duncan *et al.*⁵⁾ and Gyobu *et al.*,⁶⁾ was helpful as a starting point for this study. The administration of 10⁸ vegetative cells of NCTC 8239 suspended in 2 ml of 10% skim milk consistently produced a positive reaction. Neither SKD nor lactose alone was effective, but a positive reaction was restored when SKD was supplemented with 10% lactose (Table 1). The effectiveness of pure casein was similar to that of SKD: casein alone, even at a concentration of 6%, was ineffective, as was SKD alone (Table 3). These findings suggest that the effectiveness of skim milk is a result of the cooperative effect of lactose and casein. Minor components of skim milk, such as vitamins and minerals, were not essential.

Proteose peptone, peptone for toxin production or meat extract could replace casein or SKD, but were less effective than SKD. This might be due to the lower content of non-dialyzable protein in these media as compared to SKD. These protein sources also required supplementation with lactose (Table 4). Failure of enterotoxin production when vegetative cells suspended in one of the 4 *in vitro* sporulation media were inoculated into the ligated intestine might be due to the low contents of both

protein and carbohydrate. Various carbohydrates were tested instead of lactose for the production of *C. perfringens* enterotoxin. As shown in Table 5, all the test carbohydrates (10%), except for glucose, were effective as SKD supplements. However, differences in the effects of the carbohydrates were noted when the protein source was changed from SKD to 5% proteose peptone: raffinose, lactose and fructose were more effective than sucrose and galactose. In the previous *in vitro* study,¹²⁾ it was found that in food poisoning strains other than NCTC 8239 the degrees of sporulation and enterotoxin production varied markedly according to the particular carbohydrate used. The other strains may be more restrictive as regards the environment for *in vivo* sporulation and enterotoxin production. Based on the present data, we tentatively conclude that *C. perfringens* requires both protein and carbohydrates in relatively high concentrations for enterotoxin production in the intestinal tract. Further work is necessary to study the variation in the nutritional requirements of different strains.

References

- 1) Duncan, C.L., Strong, D.H.: *Infect. Immun.*, **3**, 167 (1971).
- 2) Strong, D.H., Duncan, C.L., Perna, G.: *Infect. Immun.*, **3**, 171 (1971).
- 3) Uemura, T., Sakaguchi, G., Itoh, T., Okazawa, K., Sakai, S.: *J. Med. Sci. Biol.*, **28**, 165 (1975).
- 4) De, S. N., Chatterje, D. N.: *J. Pathol. Microbiol.*, **66**, 559 (1953).
- 5) Duncan, C. L., Sugiyama, H., Strong, D. H.: *J. Bacteriol.*, **95**, 1560 (1968).
- 6) Gyobu, Y., Yamazaki, M., Arai, M., Kodama, H.: *Japan J. Bacteriol.*, **33**, 242 (1978).
- 7) Hauschild, A.H.W., Niilo, L., Dorward, W.J.: *Appl. Microbiol.*, **16**, 1235 (1968).
- 8) Uemura, T., Sakaguchi, G., Riemann, H. P.: *Appl. Microbiol.*, **26**, 381 (1973).
- 9) Duncan, C.L., Strong, D.H.: *Appl. Microbiol.*, **16**, 82 (1968).
- 10) Ellner, P.D.: *J. Bacteriol.*, **71**, 495 (1956).
- 11) Tsai, C., Torres-Anjel, M.J., Riemann, H. P.: *J. Formosan Med. Assoc.*, **73**, 404 (1974).
- 12) Uemura, T., Hamazaki, Y.: *J. Food Hyg. Soc. Japan*, **20**, 33 (1979).
- 13) Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: *J. Biol. Chem.*, **193**, 265 (1951).
- 14) Labbe, R.G., Duncan, C.L.: *Can. J. Microbiol.*, **20**, 1493 (1974).