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In Vitro Production of *Clostridium perfringens* Enterotoxin and Its Detection by Reversed Passive Hemagglutination

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The reversed passive hemagglutination (RPHA) test yielded a positive reaction in 2 h with as little as 0.5 ng of purified *Clostridium perfringens* enterotoxin (CPE) per ml as well as with cultures of some *C. perfringens* grown in Duncan-Strong (DS) medium. This method is the most sensitive, the simplest, and the fastest among all reported. The time course of CPE production of *Clostridium perfringens* NCTC 8798 in DS was investigated by RPHA. CPE in culture was detectable at 4 h, increased gradually, reached a maximum at 12 to 14 h, and remained at a high level of 20 μg/ml through 48 h of incubation. CPE synthesized within cells is released easily by sonic disruption of young cultures and by aging the cultures 20 h or more. Heat shock of the cell inoculum was essential for CPE production by *C. perfringens* in DS.

In vivo assay of *Clostridium perfringens* type A enterotoxin (CPE) can be carried out by mouse intravenous injection (12), by the guinea pig skin test (11), and in ligated rabbit ileal loops (5, 6). The two first tests are not specific, since other toxins such as the α toxin, which is also produced by *C. perfringens*, give positive reaction (16, 19). The ileal loop test may be specific but is not very sensitive (9). In vivo assays, therefore, require adequate controls and also a high proficiency in injection to achieve sufficient accuracy.

For in vitro assay, electroimmunodiffusion (3) is a very sensitive method for detecting CPE. Reversed passive hemagglutination (RPHA) is another sensitive and quite simple immunological method, which has been applied for the quantitative assay of *C. botulinum* type A, type B (13), and type E toxins (T. Uemura, S. Sakaguchi, and G. Sakaguchi, unpublished data) as well as type B staphylococcal enterotoxin (15). RPHA has also been found to compare favorably with other assay methods for CPE (9).

In this paper we describe the RPHA quantitative assay of CPE in detail and discuss some problems which concern mass screening for CPE-producing *C. perfringens*.

MATERIALS AND METHODS

Strains. *C. perfringens* type A, NCTC 8798, NCTC 8239, and 10239 were obtained from C. L. Duncan, Food Research Institute, University of Wisconsin; 80335 and S-79 were obtained through the courtesy of A. H. W. Hauschild, Research Laboratory, Food and Drug Directorate, Department National Health and Welfare, Ottawa, Canada. Eighteen strains, consisting of eight strains of type A, three of type B, two of type C, four of type D, and one of type E, were kindly provided by L. D. S. Smith, Virginia Polytechnic Institute and State University, Blacksburg, Va. ATCC 3624 and five other strains were available from our laboratory stock culture collection.

Enterotoxin and anti-enterotoxin. CPE was purified from the extract of sonically treated *C. perfringens* NCTC 8792 sporulating cells from an 8-h culture in Duncan-Strong (DS) medium (4). The method consisted of precipitation of CPE at 40% saturation of ammonium sulfate at pH 7, differential solubilization in 0.02 M phosphate buffer, pH 6.7, and repeated gel filtration on Sephadex G-200. The details of the procedure will be published in a future article (G. Sakaguchi, T. Uemura, and H. P. Riemann). The purified enterotoxin was at least 98% pure in ultracentrifugation, polyacrylamide gel electrophoresis, and agar gel double diffusion.

Rabbit anti-CPE sera were prepared by the method described elsewhere (9), and the anti-CPE potency

was monitored by passive hemagglutination (PHA). One to 0.01 dilution of the antiserum yielded a distinct precipitation line against 0.7 μg of CPE per ml in the microslide gel diffusion test (18), and the PHA titer was 640,000. The concentration of CPE was based on protein determination by the method of Lowry et al (14) with crystalline bovine serum albumin (Armour and Co., Chicago, Ill.) as standard.

Specific immunoglobulin G anti-enterotoxins (AT) were obtained by two gel filtrations on Sephadex G-200 (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 8.0, containing 0.2 M NaCl and used to sensitize Formalin-treated sheep erythrocytes (FSRBC). The specific activity of the purified AT was increased seven times on the basis of PHA titer per milligram of protein.

Hemagglutination (RPHA) test. The FSRBC were prepared as described by Hanaki et al. (10), and sensitization with CPE or AT was carried out in the following way. The antitoxins or CPE preparations were diluted to a concentration of 500 and 20 μg/ml, respectively, in 0.15 M phosphate buffer, pH 7.3 (PB), and 3 ml was mixed with 0.1 ml of FSRBC suspension. One and one-half milliliters of a 1:10 or 1:15 dilution of bis-diazotized benzidine in PB was added, and the whole preparation was gently mixed for 10 to 20 min at room temperature. The sensitized FSRBC were then washed twice with PB and suspended in 5 ml of 0.15 M phosphate-buffered saline (PBS) containing 0.25% bovine serum albumin (Albumin Bovine Fractions V, Nutritional Biochem Co., Cleveland, Ohio).

The RPHA test was carried out by using a "U"-bottom microtiter plate. Each sample was diluted in a test tube with PBS containing bovine serum albumin at 0.25% PBS (wt/vol). Then 0.075 ml of the diluted sample was mixed with 0.025 ml of 1% suspension of the sensitized cells in a well, and the result was read after 2 h of incubation at room temperature and then confirmed after overnight incubation.

Agar gel diffusion. Generally, a 1:50 dilution of the crude antiserum was used; it gave distinct precipitation lines and the sensitivity was about 1 μg of CPE per ml.

The microslide double gel diffusion method used for detecting CPE in the culture supernatant was carried out by Untermann's modification (18) of Casman's method (1). The titers obtained by RPHA were compared with another immunological method, the single gel immunodiffusion test (SGI). SGI tubes (3.8 mm internal diameter and 10 cm long) were prepared as described elsewhere (8, 9). The concentration of anti-CPE sera in the 0.3% agar was 1:50. A standard curve was constructed by adding 0.3 ml of the known quantities of CPE diluted with DS; the tubes were incubated at 30 C for 24 h, and the migration distances of the precipitin bands were plotted against the log of the CPE concentration.

Cultures. Stock cultures in cooked meat medium (Difco) were kept refrigerated. Liquid thioglycolate medium (TM; Difco) and DS medium were employed throughout the study to grow inocula and for sporulating cultures, respectively.

Except for 500 ml of DS in a glass bottle (8 by 15 cm) used in the sonic treatment studies, 10-ml amounts of each medium in screw-cap tubes (1.5 by 12.5 cm) were used. Stock culture (0.3 ml) was inoculated into TM, followed by heat treatment at 75 C for 20 min in a water bath and incubation for 15 to 18 h. Two subsequent transfers in fresh TM followed; each transfer consisted of 1 ml of culture after incubation for 4 h. In later studies the last transfer in TM was omitted because it had no measurable effect on CPE production. All incubation temperatures were 37 C.

Cells and spores were observed and enumerated in a Petroff-Hausser chamber with a phase-contrast microscope. The ratio of number of spores per field to the total number of cells plus spores was used to calculate the percentage of sporulation in test media. Growth of the organisms was estimated also by reading optical density in a Coleman Junior Spectrophotometer at 630 nm.

"Whole culture," which was a well-stirred culture containing cells, and "culture supernatant," which was the supernatant of a "whole culture" centrifuged at 10,600 rpm for 20 min, were used in the RPHA test.

Sonic disruption. Twenty milliliters of a culture in DS was transferred into a 50-ml plastic centrifuge tube (10 by 3 cm) and sonically treated for different time periods by means of a Sonifier model S-75 (Bronson Instruments, Inc., Stamford, Conn.). The culture was chilled in an ice-water bath during sonic disruption. The sonically treated suspensions were centrifuged at 12,000 × *g* for 20 min, and the supernatant fluid was saved for the RPHA.

RESULTS AND DISCUSSION

Sensitivity of RPHA. To determine the sensitivity of RPHA, the RPHA test was carried out with serial twofold dilutions of CPE. An amount of 0.0375 ng in 0.075 ml of PBS (0.5 ng/ml) yielded a weak but positive reaction (plus 1); 1 ng of CPE per ml gave a stronger reaction (plus 3). One RPHA titer corresponds to 1 ng of CPE per ml.

These data confirm that the RPHA test is the most sensitive available for detection of CPE method (9).

Time course of CPE production by C. perfringens NCTC 8798 in DS. CPE production is known to be closely related to spore formation (3), but only one report (2) has been published on the quantitative relationship between sporulation, CPE production, and release of CPE from cells.

The time course of CPE production in *C. perfringens* NCTC 8798 in DS is shown in Fig. 1. The RPHA titer of culture supernatant was as low as 40 in a 4-h culture; it increased gradually and reached maximum between 16 and 24 h. Sporulation reached a maximum of 2×10^8 spores per ml between 6 and 8 h, and the number of spores remained constant for 24 to 48

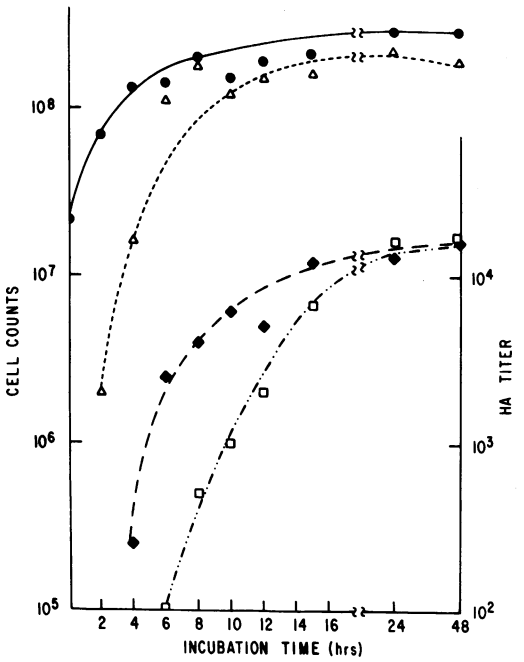

FIG. 1. *Production of cells, spores, and enterotoxin by C. perfringens type A in DS medium. Symbols: ●, total numbers of cells per ml; △, numbers of spores per ml; ■, enterotoxin in "whole culture"; □, enterotoxin in supernatant fluid.*

h. The RPHA test using whole culture was more closely correlated to spore formation, and the apparent lag was shortened. With whole culture the ratio of RPHA titer to spore counts reached a plateau at 12 h, but with culture supernatant as test material it took 16 h. As cell particles might affect the RPHA reaction, the mixture of culture supernatant and cell suspension of a non-CPE-producing strain was compared with culture supernatant alone. No effect of cell particles was demonstrated.

It seemed possible that some CPE might remain in cells even when the culture is incubated for 16 h or more and thus escape detection by RPHA. To resolve this question, CPE extraction was carried out by sonic treatment. Six-hour, twenty-h, and 48-h cultures were used for this purpose. The RPHA titer of the supernatant fluid of a sonically treated 6-h culture was twice that of the whole culture of the same age and eight times higher than the culture supernatant (Table 1). This shows that, in a 6-h culture, CPE remained within the cells but was easily released into the fluid by sonic disruption.

The degree of CPE release by the sonic disruption depends on sonicating power and time and some destruction of CPE occurs. CPE was not destroyed by heat alone, since we found that it takes 4 min at 60 C to destroy 90% of the CPE, but probably by the combined action of heat and physical impact.

The RPHA titer of whole culture (supernatant and cells combined) of a 6-h culture was not much changed by sonic disruption. This might suggest that not only soluble CPE but also CPE attached to bacterial cell surfaces is involved in the RPHA reaction.

No increase of RPHA titer of 20- or 48-h culture was observed after sonic disruption.

These data show that, for determining the potential toxicity of *C. perfringens* in DS, young cultures should be sonically treated. It is, how-

TABLE 1. *Release of enterotoxin from cells spontaneously and by sonic disruption of a DS culture of C. perfringens NCTC 8798*

Sonic disruption			RPHA titer[b]
Power (Amp)	Duration (min)	Terminal temperature[a] (C)	
6-h culture			
4 (4.2)	1	10	400
(4.2)	5	27	1,600
(4.2)	10	29	3,200
	20	30	800
6 (6.2)	1	18	1,600
	3	27	1,600
	5	34	400
	10	40	400
Before sonic disruption[c, d]			
Whole culture			4,000
Culture supernatant			400
8 (8.2)	1	23[e]	1,600
	3	46[e]	400
	5	54[e]	100
Before sonic disruption[d]			
Whole culture			4,000
Culture supernatant			400
20-h culture			
4 (4.0)	10	29	4,000
Before sonic disruption			
Whole culture			8,000
Culture supernatant			8,000
48-h culture			
8 (8.2)	1	NC[f]	8,000
(8.2)	3	NC	2,000
(8.2)	5	NC	2,000
Before sonic disruption			
Whole culture			8,000
Culture supernatant			8,000

[a] Initial temperature, 3 C.

[b] One HA titer corresponds to 0.001 μg of *C. perfringens* enterotoxin per ml.

[c] "Whole culture" and "culture supernatant" as described in Materials and Methods.

[d] These two 6-h cultures are different.

[e] In these cases, the cultures were not cooled.

[f] NC, Not checked.

ever, preferable to use a whole culture of 16- to 24-h age, or the supernatant fluid of such cultures, since practically all detectable CPE is released.

The data discussed above are in good agreement with results published by Duncan in a recent report (2) on quantitative relationships between sporulation, CPE production, and release of CPE from cells of *C. perfringens*. Duncan used the same strain as we did but the analytical procedures were different.

CPE production by different types of C. perfringens. CPE production by 29 strains of different types of *C. perfringens* was tested by RPHA (Table 2); strains of types B, C, D, and E were negative. Ten type A strains did not grow after the heat treatment of TM and 6 of the 11 strains which did grow produced no CPE.

Toxicity and growth rates of percent CPE-positive strains are illustrated in Table 3. Three of these strains have been isolated from food poisoning outbreaks and a fourth causes food poisoning symptoms in human volunteers (17).

The SGI test was carried out as comparison for the toxicity indicated by the RPHA test.

The toxicity levels detected by RPHA and SGI are in fairly good agreement. However, the toxin titer obtained by SGI was slightly higher than that obtained by the RPHA test. This is not very significant, because it must be considered that we carried out serial twofold dilution of the sample material for the RPHA test, which means that a range rather than a precise end point is determined. Within a toxin range of 5 μg/ml to about 50 μg/ml, the SGI test yields more precise data than RPHA. With amounts smaller than 5 μg/ml, the SGI test might show a little higher toxicity, but such toxin concentrations are close to or below the sensitivity limit of SGI.

Strains NCTC 8239, NCTC 10239, and ATCC 3624 produced low toxicity, and this finding is in agreement with the small numbers of spores obtained. The results may thus reflect degree of sporulation rather than characteristics of the strains used. With these strains we sometimes obtained higher toxicity (10 μg/ml). DS seems to be suitable for mass screening for CPE production, but, as shown in Table 4, heat treatment (heat selection for spore formers) is essential for CPE production. Some strains show poor sporulation and do not survive the heat treatment. Such strains might still be potential CPE producers. However, when they were grown in DS without the heat treatment of the TM inoculum, sporulation was very poor in spite of good vegetative growth, and none produced detectable amounts of CPE.

As shown in Table 4, even NCTC 8798 and S-79, which are good CPE-producers, did not produce much CPE without heat shock; the amount of growth was much higher, and the spore count was around one-third of that of the heat-treated culture, but the amount of CPE produced was 256 times smaller.

The mechanism of the heat-shock effect on

TABLE 2. *Growth and toxin production of different types of C. perfringens, 24-h DS cultures*

Type	No. of strains tested	No. of strains grown	Toxin production[a]	
			>50 ng/ml culture	<50 ng/ml culture
A	14	11	5	6
B	5	4	0	4
C	4	1	0	1
D	5	2	0	2
E	2	1	0	1

[a] One to one hundred dilution of culture supernatant was subjected to RPHA test.

TABLE 3. *Growth and enterotoxin in 16-h DS cultures of five C. perfringens type A strains*

Strain	OD at 630 nm	Percent spores	Toxin produced		
			RPHA titer[a]		Single gel[b] immunodiffusion (μg/ml)
			Whole culture	Culture supernatant	
NCTC 8798	0.350	50	8,000	8,000	13
NCTC 8239	0.330	10	2,000	2,000	3
NCTC 10239	0.251	5	1,000	1,000	2
S-79	0.311	60	8,000	8,000	13
ATCC 3624	0.344	10	500	100	0

[a] One thousand HA titer corresponds to approximately 1 μg of enterotoxin per ml.

[b] Toxicity of culture supernatant of each strain calculated according to the standard curve in Fig. 2.

TABLE 4. *Effect of heat treatment of inoculum on the enterotoxin production of C. perfringens*

Strain	Heat treatment (75 C for 20 min before incubation)	Total cells per ml	Spore counts per ml	RPHA titer[a]
NCTC 8798	Yes	2.7×10^8	16×10^7	25,600
S-79	Yes	1.6×10^8	12×10^7	25,600
NCTC 8798	No	7.8×10^8	5.5×10^7	100
S-79	No	6.0×10^8	2.5×10^7	100

[a] RPHA test carried out with supernatant fluid of 20-h DS cultures.

CPE production is not known; the procedure presumably selects for sporogenesis, but whether or not 75 C for 20 min is optimal is not clear; neither is it known whether all sporulating cells produce CPE. It seems clear, however, that we cannot omit the heat treatment when we test cultures for CPE.

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LITERATURE CITED

1. Casman, E. P., R. W. Bennett, A. E. Dorsey, and J. E. Stone. 1969. The microslide gel double diffusion test for the detection and assay of staphylococcal enterotoxins. Health Lab. Sci. **6**:185-198.
2. Duncan, C. L. 1973. Time of enterotoxin formation and release during sporulation of *Clostridium perfringens* type A. J. Bacteriol. **113**:932-936.
3. Duncan, C. L., and E. B. Somers. 1972. Quantitation of *Clostridium perfringens* type A enterotoxin by electroimmunodiffusion. Appl. Microbiol. **24**:801-804.
4. Duncan, C. L., and D. H. Strong. 1968. Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. **16**:82-89.
5. Duncan, C. L., and D. H. Strong. 1969. Ileal loop fluid accumulation and production of diarrhea in rabbits by cell-free products of *Clostridium perfringens*. J. Bacteriol. **100**:86-94.
6. Duncan, C. L., and D. H. Strong. 1971. *Clostridium perfringens* type A food poisoning. I. Response of the rabbit ileum as an indication of enteropathogenicity of strains of *Clostridium perfringens* in monkeys. Infect. Immunity **3**:167-170.
7. Duncan, C. L., D. H. Strong, and M. Sebald. 1972. Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. J. Bacteriol. **110**:378-391.
8. Genigeorgis, C., and W. W. Sadler. 1966. Immunofluorescent detection of staphylococcal enterotoxin B. I. Detection in culture media. J. Food Sci. **31**:441-449.
9. Genigeorgis, C., G. Sakaguchi, and H. Riemann. 1973. Assay methods for *Clostridium perfringens* type A. enterotoxin. Appl. Microbiol. **26**:111-115.
10. Hanaki, R., K. Nobuto, U. Sato, and T. Matsumoto. 1969. Studies on a toxoplasma hemagglutination test with erythrocytes fixed in alcohol-formalin and sensitized in the presence of bis-diazo-benzidine. Annu. Rep. Nat. Vet. Assay Lab. Jap. **6**:91-100.
11. Hauschild, A. H. W. 1970. Erythemal activity of the cellular enteropathogenic factor of *Clostridium perfringens* type A. Can. J. Microbiol. **16**:651-654.
12. Hauschild, A. H. W., and R. Hilsheimer. 1971. Purification and characteristics of the enterotoxin of *Clostridium perfringens* type A. Can. J. Microbiol. **17**:1425-1433.
13. Johnson, H. M., K. Brenner, R. Angelotti, and H. E. Hall. 1966. Serological studies of type A, B, and E botulinal toxins by passive hemagglutination and bentonite flocculation. J. Bacteriol. **91**:967-974.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
15. Silverman, S. J., A. R. Knott, and M. Howard. 1968. Rapid sensitive assay for staphylococcal enterotoxin and a comparison of serological methods. Appl. Microbiol. **16**:1019-1023.
16. Stark, R. L., and C. L. Duncan. 1972. Transient increase in capillary permeability induced by *Clostridium perfringens* type A enterotoxin. Infect. Immunity **5**:147-150.
17. Strong, D. H., C. L. Duncan, and G. Perna. 1971. *Clostridium perfringens* type A food poisoning. II. Response of the rabbit ileum as an indication of enteropathogenicity of strains of *Clostridium perfringens* in human beings. Infect. Immunity **3**:171-178.
18. Untermann, F. 1972. Diagnostik der Staphylokokken-lebensmittelvergiftung. Die Fleischwirtsen **52**:500-503.
19. Yamamoto, A., A. Ito, S. Soda, H. Sato, S. Sadahiro, and R. Murata. 1972. Bioassay for α-toxin of *Clostridium perfringens* using survival time in mice. Jap. J. Med. Sci. Biol. **25**:15-23.