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Effect of Heptakis(2,6-*O*-Dimethyl)β-Cyclodextrin on the Production of Pertussis Toxin by *Bordetella pertussis*

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The effect of heptakis(2,6-*O*-dimethyl)β-cyclodextrin (MeβCD) on the production of pertussis toxin was evaluated. The addition of MeβCD to the medium stimulated cell growth and pertussis toxin production. MeβCD enhanced pertussis toxin production 100 times more in synthetic media, such as Stainer-Scholte medium (D. W. Stainer and M. J. Scholte, J. Gen. Microbiol. 63:211–220), than in MeβCD-free medium in 2-day shake cultures. Maximum production of pertussis toxin was estimated as 50 mg of protein per liter of culture broth both by in vitro and in vivo assays. Purified toxin was demonstrated to be biochemically and biologically identical to the toxin produced in MeβCD-free static cultures.

One of the biologically active proteins produced by *Bordetella pertussis* has been called by many different names, such as leukocytosis-promoting factor (LPF) (9), histamine-sensitizing factor (7), islets-activating protein (8, 20), and LPF-hemagglutinin (4), each of which reflects a particular biological reaction. This protein was also termed pertussigen by Munoz and Bergman (11). Recently, Katada and Ui (5) reported that this protein has the characteristics of a bacterial exotoxin in the catalysis of ADP-ribosylation of the guanine nucleotide-binding protein. In this paper, the name pertussis toxin (PT) is used in accordance with the proposal of Pittman (13) to avoid confusion caused by many different names. Recently, PT has been shown to be one of the main protective antigens against whooping cough infection in mice (10, 11, 17) and is also one of the components of a pertussis component vaccine developed in Japan in March 1981 (Ministry of Health and Welfare, Minimum Requirement of Bacterial Products. 1982, p. 129). A synthetic medium, such as Stainer-Scholte (SS) medium (18), has been recommended for the production of a less reactogenic vaccine; SS medium has been used for the production of a component vaccine in Japan, but production of PT in this medium is rather difficult, especially in shake cultures. The reasons for this are thought to be, first, that the virulence of *B. pertussis* phase I changes during cultivation, and many biological activities are frequently lost (12). Second, *B. pertussis* is known to be susceptible to a number of inhibitors present in liquid medium, including sulfur, peroxides, and fatty acids (15). Third, the in vivo assay of PT requires many animals and a lot of time, so that a large number of quantitative analyses of PT is rather troublesome. Recently, a convenient and accurate in vitro assay system for toxin determination which was designated the HP-ELISA system was developed (16). To overcome the growth inhibitors, as reported in our previous paper (3), the effects of cyclodextrins (CDs) were evaluated. Various CDs, such as αCD, βCD, γCD, and their dimethylated derivatives were used and compared with starch. It was found that CDs, especially heptakis(2,6-*O*-dimethyl)βCD (MeβCD), is significant growth stimulant for *B. pertussis* phase I. In this paper, we show the effect of MeβCD on PT production.

MATERIALS AND METHODS

Bacterial strain. *B. pertussis* Tohama phase I was used. This strain produced a larger amount of PT than did any other strain tested.

Culture. SS medium (18) was used as a basal medium. For maximum production of PT, we used modified SS medium, designated as CD liquid (CL) medium. The compositions of the media are listed in Table 1. Cell concentration was expressed in international opacity units (IOU). One IOU is defined as being equivalent to 10^9 organisms. Lyophilized *B. pertussis* Tohama phase I cells were suspended in a 1% Casamino Acids solution and cultured on a plate of Bordet-Gengou medium for 72 h at 35°C. Cells harvested from these plates were subcultured on Bordet-Gengou medium and incubated for 24 h. Cells were harvested into SS medium without supplements and adjusted to a turbidity of about 1,000 Klett units with a Klett-Summerson colorimeter (660 nm). Seed suspension (2 ml) was inoculated into culture medium consisting of 200 ml of medium in a 500-ml shaker flask. Under these conditions, the initial cell concentration was about 10^9 cells per ml. Incubation was carried out in a reciprocal shaker at 35-mm strokes and 125 oscilla-

tions per min at 35°C. These culture conditions were generally used, but some other experimental conditions, described below, were also used.

CDs. MeβCD was synthesized from βCD by a methylation method. βCD was prepared in our laboratory by the standard method described briefly as follows. Crude supernatant containing 50 Tilden-Hudson units (19) of cyclodextrin glycosyltransferase prepared by the cultivation method of Lane and Pirt (6) with *B. macerans* IFO 3490 was mixed with 50% (final wt.vol) dextrin (lot NSD-210, Nihon Shiryo Co., Ltd., Tokyo, Japan). The enzymatic reaction was carried out for 2 days at 37°C with the dropwise addition of 10% trichloroethylene (final vol/vol) as a complexing agent. By repeated steam distillation, the complexing agent was removed, and repeated crystallization then resulted in purified βCD. The purity was determined to be 98% by thin-layer chromatography and gel filtration with Sephadex G-15. MeβCD was prepared basically by the method of Boger et al. (1). The purity as a 2,6-*O*-dimethylated derivative was determined to be >90% by thin-layer chromatography and nuclear magnetic resonance. The residual portions consisted of partially methylated derivatives.

In vivo assay of PT. Leukocytosis activity was measured by intravenous injection of toxin into mice. The LPF unit of a test sample was determined from the titer relative to the reference toxin titer (3,000 LPF units per ml) as reported previously (16). One unit of LPF activity of the reference toxin was defined as that amount of sample causing an increase of 10,000 leukocytes per mm^3. The toxin was diluted with phosphate-buffered saline containing 0.2% gelatin. Since the same reference toxin was used for HP-ELISA, the titer of the reference toxin was also given as 3,000 HP-ELISA units per ml for convenience.

In vitro assay of PT. To estimate the amount of PT in culture broth, we used the HP-ELISA system developed by Sato et al. (16). A standard dose-response curve for reference PT, the HP-ELISA unit of which was arbitrarily assigned as 3,000 units per ml, was drawn on a log-log graph. At the same time, the test sample was plotted at serial dilutions, and the dilution rate showing the same absorbance showed by the reference toxin at a 3,000-fold dilution was the test sample unit.

Preparation of PT from culture broth. PT was prepared essentially as described previously (2, 17). The supernatant (3.6 liters, pH 8.3) obtained from the culture broth was applied to a column (100 ml) of hydroxylapatite (BDH Chemicals Ltd., Poole, England) equilibrated with 0.01 M phosphate buffer (pH 8.0) and 4 liters of the solution passed through was adjusted to pH 6.0. This solution was further applied to a column (140 ml) of hydroxylapatite equilibrated with 0.01 M phosphate buffer (pH 6.0). Elution of PT was carried out with 0.1 M phosphate buffer (pH 7.0) containing 0.5 M sodium chloride. The protein fractions obtained were pooled (100 ml) and applied to a haptoglobin-Sepharose 4B column (30 ml) equilibrated with 0.1 M phosphate buffer containing 0.5 M sodium chloride. Fractionation was carried out with 0.1 M phosphate buffer (pH 7.0) containing 3 M potassium thiocyanate and 0.5 M sodium chloride. The protein fractions obtained were pooled and dialyzed against 0.1 M phosphate buffer (pH 7.0), and about 20 mg of protein was obtained.

TABLE 1. Compositions of SS and CL media[a]

Component	Amt (g/liter) in: SS medium	CL medium
Sodium L-glutamate	10.7	10.7
L-Proline	0.24	0.24
NaCl	2.5	2.5
KH_2PO_4	0.5	0.5
KCl	0.2	0.2
$MgCl_2 \cdot 6H_2O$	0.1	0.1
$CaCl_2$	0.02	0.02
Tris	6.1	6.1
L-Cysteine[b]	0.04	0.04
$FeSO_4 \cdot 7H_2O$[b]	0.01	0.01
Niacin[b]	0.004	0.004
Glutathione (reduced)[b]	0.10	0.15
Ascorbic acid[b]	0.02	0.40
Casamino Acids (certified)		10.0
MeβCD		1.0

[a] Distilled water was added to a total volume of 1 liter, and the pH was adjusted to 7.6 with 3.0 N HCl. Sterilization was carried out by autoclaving at 121°C for 15 min.

[b] Sterilized by filtration and then added aseptically to the autoclaved medium.

Polyacrylamide slab gel electrophoresis. The procedure for polyacrylamide slab gel electrophoresis (PAGE) at pH 4.5 was essentially the same as that described by Reisfeld et al. (14). The sample contained 30 μg of protein in 10% sucrose and malachite green as a tracking dye. Electrophoresis was carried out in 0.35 M Reisfeld β-alanine-acetic acid buffer (pH 4.3) and 7.5% polyacrylamide gels at room temperature for 3 h at 20 mA. Staining was performed with Coomassie brilliant blue.

Sodium dodecyl sulfate-PAGE. A sample was added to a mixture of 1% sodium dodecyl sulfate (SDS)–1% 2-mercaptoethanol containing 50% sucrose and malachite green as the tracking dye. The sample (5 μl) was layered on a 10% polyacrylamide gel. Electrophoresis was performed at 20 mA for 3 h with electrode buffer containing 0.1 M sodium phosphate (pH 7.2) and 0.1% SDS.

RESULTS

Effect of various doses of MeβCD on PT production. A cell suspension was inoculated into 10 ml of SS medium containing various levels of MeβCD and allowed to grow at 35°C in a Monod test tube with reciprocal shaking. Cell growth and PT production of the culture supernatant 18 h after the start of culturing were measured. Experiments were carried out five times, and the results are shown in Fig. 1. When MeβCD was added to the medium at 50 to 1,000 μg/ml, PT production was remarkably enhanced. The enhancing effect of MeβCD on PT production as well as on cell growth was not as strong in static cultures as it was in shake cultures. In addition, pertussis cells frequently tended to clump in the late stage of culturing under MeβCD-free conditions, but this phenomenon was not observed

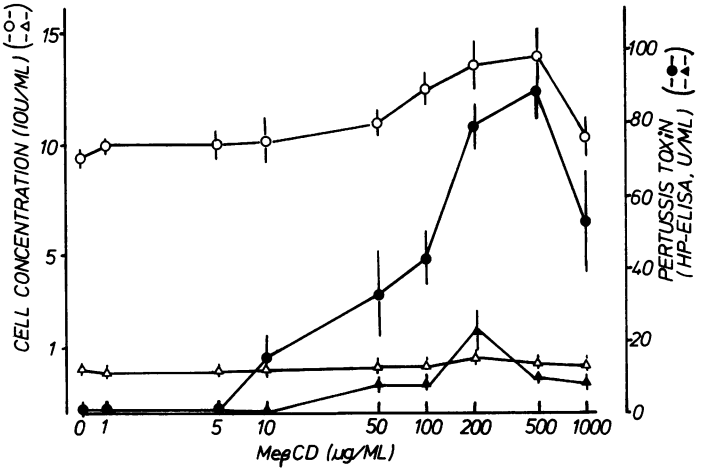

FIG. 1. Effect of MeβCD on cell growth and PT production in *B. pertussis* Tohama phase I. Cell growth and PT production after 18 h of incubation are shown. The initial cell concentration was 10^8 cells per ml, and incubation was carried out under static (△, ▲) or reciprocal (○, ●) conditions. The results are the means of five experiments. The bars indicate the standard errors of the means.

when MeβCD was added at the start of culturing.

Time course of PT production. Incubation was carried out in a 500-ml shaker flask containing 150 ml of SS medium seeded with 10^9 *B. pertussis* cells per ml. MeβCD was added at a concentration of 50 μg/ml to SS medium before autoclaving, and subsequently, cultivation was started on the reciprocal shaker at 35°C. Cell growth and PT production of the culture supernatant were monitored periodically. Experiments were carried out five times, and typical results are shown in Fig. 2. When the culture time exceeded 20 h, cell growth was not greatly enhanced by the addition of MeβCD, but the yield of PT was greatly enhanced. In MeβCD-free medium, PT was produced on day 1 of cultivation (5 HP-ELISA units per ml), but the levels soon decreased (1 HP-ELISA units per ml). On the other hand, medium containing MeβCD promoted PT production about 100 times more than did MeβCD-free medium after 2 days of culturing.

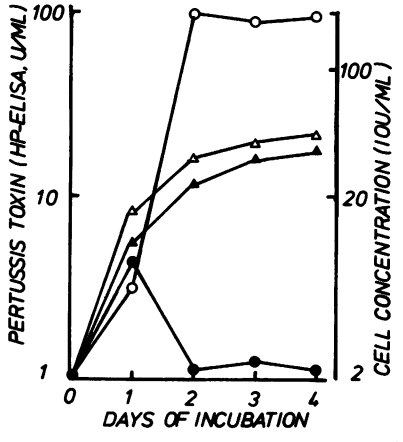

FIG. 2. Time course of PT production. Incubation was carried out on a reciprocal shaker. Cell growth (△) and PT production (○) when MeβCD was added to SS medium; cell growth (▲) and PT production (●) in MeβCD-free medium.

Maximum production of PT. To obtain high PT production, we had to determine the optimum medium composition. This composition was improved by altering the amount of one component and fixing the others. For cell growth, Casamino Acids (Difco Laboratories, Detroit, Mich.) and MeβCD were beneficial, and glutathione and ascorbic Acid were not, but for PT production, Casamino Acids, glutathione, ascorbic acid, and MeβCD played important roles (data not shown). The most important component of the medium was shown to be MeβCD, and the case in which concentration of MeβCD was changed is presented. Modified SS medium was prepared by adding Casamino Acids at a concentration of 10 mg/ml, and a modified supplemental solution was also prepared to contain 1.5 times the amount of glutathione and 20 times the amount of ascorbic acid contained in standard SS medium (Table 1). MeβCD at concentrations of 50, 500, 2,000, and 5,000 μg/ml was added to 200 ml of modified SS medium previously placed in 500-ml shaker flasks. Each flask was inoculated with *B. pertussis* phase I at a cell level of 10^9 cells per ml, and cultivation was conducted at 35°C. The results are shown in Fig. 3A and 3B. Figure 3A shows the relationship between cell concentration (IOU per ml) and cultivation time, and Fig.

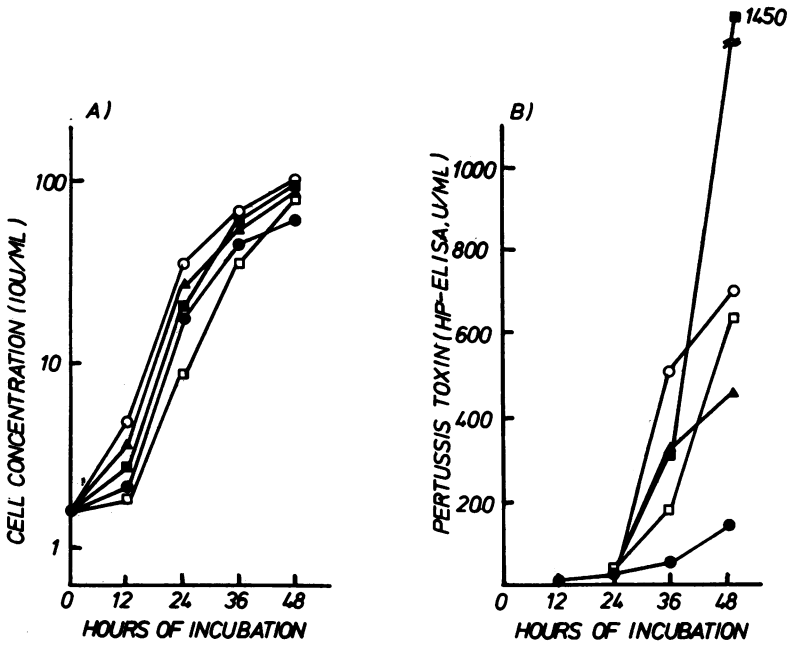

FIG. 3. Effect of MeβCD on cell growth and PT production in *B. pertussis* Tohama phase I in CL medium. (A) Cell growth; (B) PT production. The concentrations (micrograms per milliliter) of MeβCD in the medium were as follows: ●, 0; ▲, 50; ○, 500; ■, 2,000; and □, 5,000.

3B shows the time course of PT activity determined by the HP-ELISA system. It is clear from Fig. 3 that the addition of MeβCD to the culture medium at concentrations ranging from 50 to 2,000 μg/ml enhanced both cell growth and PT production. The optimum level of MeβCD may be 1 to 2 mg of MeβCD per ml. It was estimated that 50 mg of PT per liter of culture broth can be obtained by the addition of MeβCD. To evaluate the correlation between in vitro activity and in vivo activity, we examined leukocytosis-promoting activity with culture supernatant. Culture broth (48 h) was centrifuged at 10,000 rpm for 30 min, culture supernatants were heated at 56°C for 5 min, and then 0.2 ml was injected intravenously into one group of five mice. After 3 days leukocytes were counted. The results are shown in Table 2. The values were expressed as the mean of two different experiments. As shown in Table 2, good correlation was observed between the in vitro HP-ELISA and the in vivo leukocytosis assay.

Characterization of PT produced by the addition of MeβCD to the medium. To characterize the PT which was produced by the addition of MeβCD to the medium, we purified PT from the culture broth and compared it with the purified reference PT prepared from an MeβCD-free static culture. Using acid PAGE and SDS-PAGE, we compared the properties of these two preparations. The results are shown in Fig. 4. No differences in subunit profiles of SDS-PAGE and mobility in acid PAGE were demonstrated between the two preparations. The leukocytosis-promoting activities of these two preparations before and after gel electrophoresis were also examined, and the results showed that they were identical.

DISCUSSION

PT has many biological activities and has been of interest as a tool for immunological and pharmacological studies. Interest has also been shown in PT for pathological and physiological studies of whooping cough infection. For progress to be made in these studies, highly purified PT is required. Recently, a convenient and accurate in vitro assay system for toxin determination was developed (16), and this method has enabled us to make progress in our experiments. As reported previously (3), CDs were growth stimulants for *B. pertussis* phase I on solid medium and made it possible for the first time to obtain a uniform and synthetic medium in place of Bordet-Gengou medium. This finding also prompted us to study the growth of *B. pertussis* in liquid cultures with respect to the method of vaccine production.

A synthetic medium is recommended for the production of a less reactogenic vaccine, but as shown in Fig. 2, the level of PT production in SS medium was very low in spite of good cell

TABLE 2. Correlation between in vitro activity and in vivo activity of culture supernatants[a]

MeβCD (μg/ml)	HP-ELISA (U/ml)	Leukocytosis assay (U/ml)
0	158 (1.0)	183 (1.0)
20	240 (1.5)	291 (1.6)
100	610 (3.9)	692 (3.8)
500	675 (4.3)	828 (4.5)
1,000	1,176 (7.4)	894 (4.9)
2,000	1,450 (9.2)	1,291 (7.1)
5,000	628 (4.0)	791 (4.3)

[a] MeβCD was added to the modified SS (CL) medium at each concentration. Cultivation was carried out on a reciprocal shaker, and 48-h culture supernatants were used for HP-ELISA and leukocytosis-promoting activity assay. Units of HP-ELISA and leukocytosis assay were determined as described in the text with reference toxin. The numbers in parentheses represent relative activities.

growth under shaking conditions. This result suggests one of the reasons that shake cultures have been unsuccessful in producing a component vaccine. On the other hand, Fig. 1 and 2 indicate that the addition of MeβCD to the medium enhanced PT production.

To produce large amounts of PT, we had to modify the composition of the medium. In a comparison of the results with SS medium and CL medium (Fig. 2 and 3), maximum cell growth in CL medium appeared to be only three times greater than that on SS medium, whereas the level of PT production was about 1,000 times higher than that on SS medium. This enhanced PT production led us to determine whether the toxin produced by the addition of MeβCD differs from the purified reference toxin produced by MeβCD-free static cultures. From the results of acid PAGE, SDS-PAGE, and in vivo assays of the toxin, the two preparations were characterized as identical. These phenomena prove that MeβCD is an excellent additive for PT production in shake cultures with jars and tanks.

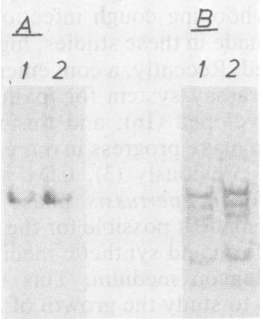

FIG. 4. Acid PAGE (A) and SDS-PAGE (B) of two preparations. Lanes 1, Purified reference toxin produced on a static culture for 5 days with MeβCD-free medium; lanes 2, purified toxin produced on a shake culture for 48 h with CL medium containing 1 mg of MeβCD per ml.

On the other hand, why MeβCD enhances PT production remains unclear. Some possible mechanisms can be assumed. First, MeβCD absorbed some growth inhibitors derived from medium components or secondary metabolites of *B. pertussis* or both, full cell growth was achieved, and PT production ultimately was enhanced. This possibility was basically confirmed by our experiment to regain MeβCD from culture supernatants and detect the trapped substances. Some fatty acids were detected in the MeβCD, and their identity is now under investigation. Second, MeβCD acted as a stabilizer to assist glutathione, an essential component for PT production, in the medium. In our preliminary experiment mutual interaction between MeβCD and glutathione was observed at least in cell growth on solid medium, and this possibility cannot be ruled out. Third, MeβCD acted as a stabilizer of the toxin itself because acid PAGE of both 48- and 72-h culture supernatants showed almost the same position and density. As indicated in Fig. 3, cell growth after 48 h occurred in the late exponential phase, and after 72 h, partial cell lysis was observed. Therefore, the apparent enhancement of PT production should be due to protection against degradation of the toxin. Actual mechanisms are now under further study.

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