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RELEASE OF ENDOTOXIN BY TOXIGENIC AND NON-TOXIGENIC VIBRIO CHOLERAE 01

CHOWDHURY RAFIQUL AHSAN AND IVAN ČIŽNÁR

Laboratory Sciences Division, International Centre for Diarrhoeal Disease Research, Bangladesh, GPO Box 128, Dhaka 1000, Bangladesh

Abstract

Aspects relating to the release of endotoxin into a growth medium was compared between *Vibrio cholerae* 01 strains that lack the cholera toxin gene (CT–) and fully toxigenic strains (CT+). For detection of endotoxin and/or lipopolysaccharide, tests such as double diffusion, rocket immunoelectrophoresis, and limulus amoebocyte lysate (LAL) were applied. The release of endotoxin was detected in the early log phase of growth of all strains studied. The amount released by (CT(–) strains was four times higher than that of the CT(+) strains.

Key Words: Vibrio cholerae; Cholera; Cholera toxin; Endotoxins.

Introduction

It has been established that the physiopathology of cholera is explained by the action of cholera toxin. The Ogawa and Inaba serotypes of classical *Vibrio cholerae* 01 have been found to produce variable amounts of cholera toxin (CT), but are generally believed to be more toxigenic than the El Tor biotype (1, 14, 23). The classical biotype, indeed has been observed to cause the more severe disease. Some strains of *V cholerae* produce only a small amount of CT, which led researchers to assume that additional factors may be responsible. Subsequently, it was indeed demonstrated that *V cholerae* 01 produces a toxin different from the known CT in its antigenic structure, receptor site, and mode of action (20). The cytotoxic potential in suckling mice of another non-CT factor produced by *V cholerae* 01 strains has been described recently (12, 13). In addition to these exotoxins, it was shown in the 1960's that *V cholerae* 01 releases an endotoxin during the late log phase of growth (3, 4, 24). This phenomenon has since been re-examined and found to occur in cells from an earlier phase of growth when no physical or chemical attack or autolytic processes are involved (7,15).

It is recognised that many biological consequences of an endotoxin action are due to its effect on cellular membranes (21). An endotoxin released locally, and interacting with the host's membranes at the site of the pathogens' adherence could be a leading factor in the early pathogenesis of many infections caused by Gram-negative bacteria. Moreover, some bacterial proteins can increase the host's susceptibility to the effect of an endotoxin (16). Thus, the quantity of the released endotoxin could represent a factor contributing to the pathogenic potential of an infecting agent.

The aim of this study was to compare the release of endotoxin by strains of *V cholerae* producing a high concentration of cholera toxin with strains of *V cholerae* which did not show any chromosomal homology with CT gene and were shown by standard tests not to produce cholera toxin.

Correspondence and reprint requests should be addressed to: Dr Ivan Ciznar.

Materials and methods

V. cholerae strains and culture conditions

Table I summarises the characteristics of the *Vibrio cholerae* strains used in this study. Four strains did not produce CT, as shown by negative results by tissue culture and the enzyme-linked immunosorbent assay (ELISA) (5, 11, 19), nor did they show any genetic homology with CT or *Escherichia coli* heat-labile toxin (CT-) by a DNA probe test (J. B. Kaper, Center for Vaccine Development, University of Maryland School of Medicine, U.S.A.). Four toxigenic (CT+) strains were isolated from the stools of cholera patients hospitalised at the treatment centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). All strains were cultured in Richardson's medium (17) on a reciprocal shaker (90 oscillations/min) at 30°C. To determine growth curves, side-arm Erlenmeyer flasks were used. Each 500 ml flask, containing 100 ml of the medium was inoculated with 1 ml of a bacterial suspension. The inoculum was prepared from an 8-hour culture in Richardson's medium and contained 10^4 colony forming counts, corresponding to an optical density (O.D.) of 0.1 on a Colemann spectrophotometer (Coleman, Junior II, Model 6/20). The O.D. of the culture was measured at intervals of two hours. To determine the number of cells, samples of the suspension were serially diluted and plated on a nutrient blood agar base (Difco) without blood. The number of colonies was counted, and based on the O.D. and the number of colonies a growth curve was constructed.

Culture filtrates

For each of the 8 strains, six Erlenmeyer flasks were inoculated and incubated as described above. At two-hour intervals, one of the flasks

TABLE I – STRAINS OF *VIBRIO CHOLERAE* USED IN THE STUDY

Strain	Serotype Biotype	Origin of strain	Y-1/CHO*	GM_1* ELISA	CT and L** gene probe
VL-6085	El Tor Ogawa	England	–	–	–
1077-79	Inaba	U.S.A.	–	–	–
2740-80	El Tor Inaba	U.S.A.	–	–	–
VL-6007	El Tor Inaba	England	–	–	–
Y-29041	Classical Inaba	Bangladesh	+	+	ND
Y-29061	El Tor Inaba	Bangladesh	+	+	ND
X-25013	El Tor Ogawa	Bangladesh	+	+	ND
Z – 21672	Classical Ogawa	Bangladesh	+	+	ND

ND = not done.
* = Tests performed at ICDDR,B.
** = Tests performed at the Centre for Vaccine Development, University of Maryland, U.S.A.

was removed, the O.D. of the medium was measured, and the culture centrifuged at 20,000 g at 4°C for 30 minutes. The supernatant was filtered through 0.22 µm (Millipore^R) membranes. The filtrate was then either stored at – 20°C or concentrated by dialysis against polyethylene glycol (J.T. Baker, U.S.A.) and lyophilised.

Preparation of antigens and immunisation of rabbits

Cells were separated after 18 hours of culture by centrifugation at 20,000 g, then were washed with cold physiological saline (0.9% NaCl w/v), and resuspended in distilled and deionised water. The suspension was sonicated at 20 kHz, and 60 watts (Labline Instruments, U.S.A.). The sonicate was heated for 20 minutes in a boiling water-bath and finally diluted in physiological saline to give a concentration corresponding to 10^9 cells/ml. For each serotype and biotype, two New Zealand white rabbits weighing about 2 kg were immunised intravenously. A first dose of 10^8 cells was followed after one week by a series of four doses (10^7, $5x10^7$, 10^8, $5x10^8$,), each at two-day intervals. The serum was collected one week after the last dose and stored at –20°C.

Preparation of lipopolysaccharide (LPS)

LPS was extracted from *V cholerae* of both serotypes and biotypes by phenol-water extraction and further purified by ultracentrifugation at 105,000 g (25). The purified preparations were tested for antigenicity in a double-diffusion assay against rabbit antiserum. All revealed an intense precipitin band giving reaction of identity at all tested dilutions.

Immunodiffusion and rocket electrophoresis

Gel diffusion was performed in 1% agarose type 1 (Sigma, St. Louis, U.S.A.) using a barbiturate-citrate buffer, pH 8.6. Rocket electrophoresis was performed in 1% agarose (Bio-Rad Laboratories, Richmond, U.S.A.) using Tricin buffer (pH 7.2) on 5x5cm glass plates. The optimal concentration of the rabbit antiserum was found to be 20 µl for 3.5 ml of agarose gel. Electrophoresis was performed with a potential of 5 V/cm and the plates were washed, dried, and stained with Coomasie Brilliant Blue.

Limulus amoebocyte lysate test (LAL)

The test was performed using Sigma E-Toxate Multiple Test Vial (Stock No. 210–2). New glassware, free of endotoxin contamination, was used. For repeated experiments, the glassware was treated as recommended by the manufacturer. Serial dilutions of the culture filtrates were tested in triplicate and the presence of endotoxin was recorded when the hard gel was not disrupted by gently inverting the tube.

Skin permeability factor (PF)

The test was performed in duplicate as originally described by Craig (2). The samples of endotoxin were diluted 1:2, 1:10, 1:100 in gel borate buffer (pH 7.5) and injected intracutaneously in the intrascapula region of rabbits. The reaction was recorded from both sides of the rabbits' skin after intravenously administering 5% Pontamine sky blue.

A modified Schwartzman test and tolerance to endotoxin

A Schwartzman reaction (19) was induced in rabbits as follows: at the start (0 hour) 0.2 ml of various dilutions of culture filtrates were injected intradermally in adult rabbits. At 16 hours, the homologous purified LPS was administered intravenously. The skin reaction was evaluated 6 hours later following standard procedure (2). To induce tolerance (16), rabbits received a dose of 0.1 mg of homologous endotoxin given intravenously. Eighteen hours later the culture filtrates were applied intradermally, and the skin reaction recorded as described above.

Results

Growth of Vibrios

In the culture environment of this study, all strains showed the same rate of growth. The growth curve for two strains is shown in Fig. 1. No difference was observed between CT(+) and CT(–) strains in terms of the number of cells at a given phase of growth.

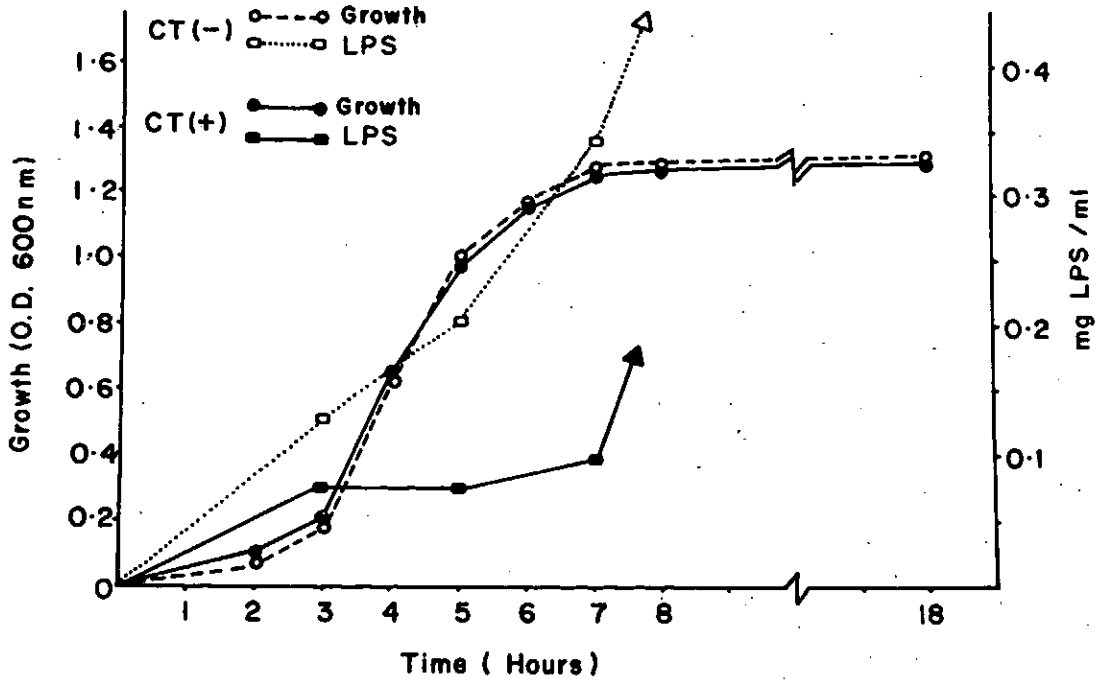

Fig. 1 — Growth curve and endotoxin releasing by the Vibrio cholerae El Tor (Ogawa) VL-8065, CT(−) and Vibrio cholerae El Tor (Inaba) Y-29061, CT(+) strains.

Immunodiffusion and rocket immunoelectrophoresis

Samples of the culture filtrates at different time intervals were serially diluted and assayed in a double-diffusion test against antiserum raised in rabbits (Fig. 2). The lowest dilution of filtrate which gave a reaction was considered to correspond to a known concentration of reference homologous LPS which was recorded. The distance of the band from the well or canal also indicated the concentration corresponding to the homologous LPS. The identity of the bands formed by LPS from the culture filtrate and by homologous LPS extracted from the cell by the phenol-water extraction was verified by the continuity of the spur, beginning with the highest concentrations of both LPS and the culture filtrate.

The concentration of the LPS in the culture filtrates showed a difference between CT(+) and CT(−) strains from the third hour of inoculation and onwards. CT(−) strains released 135 µg/ml and CT(+) strains 75 µg/ml. This

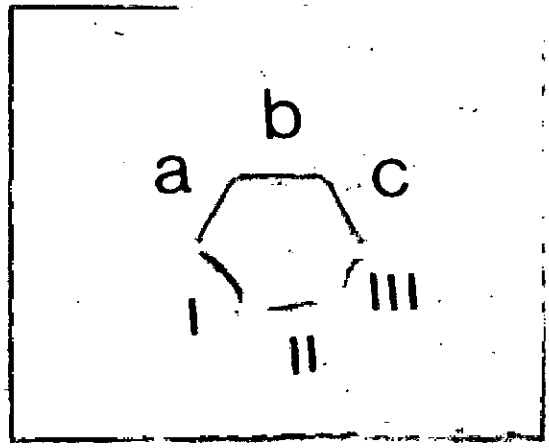

Fig. 2— Double diffusion test with purified LPS and 7–hour culture filtrate of Vibrio cholerae El Tor (Ogawa) VL-6085, CT(−). a, b, c=LPS in decreasing concentration 2, 1, 0.5 mg/ml. I, II, III = culture filtrate and its dilutions 1:2 and 1:4. Central well contained homologous rabbit antiserum.

difference increased, and turned four-fold after 18 hours with 2500 µg/ml for CT(–) and 600 µg/ml for CT(+). It should be noted that the filtrates obtained after culturing for 3 and 5 hours were assayed after being concentrated 10-fold by dialysis against polyethyleneglycol.

The results of the double-diffusion test were confirmed by rocket electrophoresis as shown in Fig. 3. A culture filtrate sample was electrophoresed simultaneously with known concentrations of the reference LPS, and from the height of the peak the concentration of LPS in the filtrate was calculated. The results of this analysis are summarised in Fig. 1.

Detection of endotoxin by Limulus amoebocyte lysate (LAL)

The results of this test are summarised in Table II. The sensitivity of the LAL test was checked for endotoxin produced by each *V. cholerae* strain and corresponded to 0.1 ng/ml. The LAL test of sterile medium showed that it contained less than 100 ng/ml of an endotoxin. The results of the LAL test confirmed the kinetics of endotoxin release as estimated by immunodiffusion. The quantity and the concentration of endotoxin released during the growth phases were approximately the same for both tests. The highest concentration of endotoxin after an 18-hour culture of CT(–) *V. cholerae* was 2.5 mg/ml while it was 0.64 mg/ml for CT(+) *V. cholerae*. Thus, the LAL test confirmed the four-fold higher release of endotoxin by CT(–) as compared to CT(+) strains.

Skin permeability factor (PF)

The test confirmed previous findings described by Sanyal *et al.* (20). The culture filtrates of CT(–) strains always produced a reaction around site of the injection in varying degrees of haemorrhage, necrosis and induration which almost obscured the blueing zone. This reaction was not noted with the culture filtrates of CT(+) strains which showed a clear blueing zone only.

The local Schwartzman test and tolerance to endotoxin

To determine whether or not the endotoxin contributes to the haemorrhage and necrosis, a local Schwartzman-type of reaction was applied with the culture filtrates. All the culture filtrates of CT(–) strains induced substantially more intense haemorrhage and necrosis, than the CT(+) strains after intravenous injection of homologous endotoxin. The reaction of culture filtrates from CT(+) and CT(–) strains is shown in Table III. The effect of endotoxin on the total

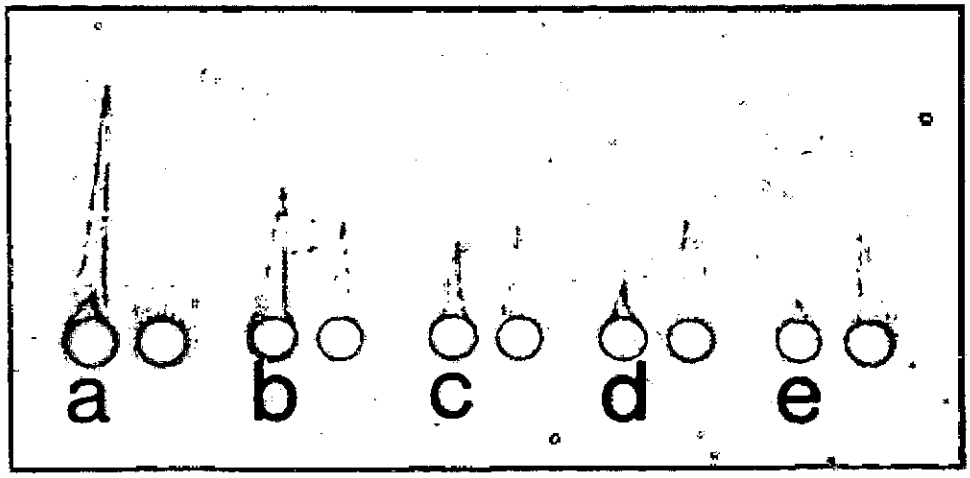

Fig. 3 – Rocket electrophoresis. a, b, c, d, e = 1, 0.5, 0.25, 0.125 0.0625 mg LPS/ml. In the right wells was placed 7-hour culture filtrate of Vibrio cholerae El Tor (Ogawa), VL-6085 CT(–) strain.

TABLE II – CONCENTRATION OF ENDOTOXIN MEASURED IN FILTRATES OF CT(+) AND CT(–) *VIBRIO CHOLERAE* BY THE LAL TEST

Time (hours)	*V. cholerae* CT(+)		*V. cholerae* CT(–)	
	Strain	mg endotoxin/ml culture medium	Strain	mg endotoxin/ml culture medium
3	Y–29041	0.12	VL–6085	0.20
	Y–29061	0.12	1077–79	0.20
	X–25013	0.06	2740–80	0.20
	Z-21672	0.06	VL–6007	0.20
5	Y–29041	0.12	VL–6085	0.40
	Y–29061	0.12	1077–79	0.80
	X–25013	0.12	2740–80	0.40
	Z–21672	0.12	VL–6007	0.40
7	Y–29041	0.16	VL–6085	0.64
	Y–29061	0.08	1077–79	1.28
	X–25013	0.16	2740–80	0.64
	Z–21672	0.16	VL–6007	0.64
18	Y–29041	0.64	VL–6085	2.50
	Y–29061	0.32	1077–79	2.50
	X–25013	0.64	2740–80	2.50
	Z–21672	0.64	VL–6007	2.50

TABLE III – LOCAL SCHWARTZMAN REACTION OF THE CULTURE FILTRATES OF *VIBRIO CHOLERAE* CT(+) AND CT(–) STRAINS

Strain	Dilution of 18h culture filtrate	Endotoxin dose (g)	Haemorrhage and necrosis
Vibrio cholerae	Undiluted	100	++++
El Tor Ogawa	1:10	100	++++
VL–6085, CT(–)	1:100	100	++
	Undiluted	0	+++
	1:10	0	++
	1:100	0	+
Vibrio cholerae	Undiluted	100	+
El Tor Inaba	1:10	100	–
Y–29061, CT(+)	1:100	100	–
	Undiluted	0	–
	1:10	0	–
	1:100	0	–

reaction in the skin was apparent. However, when we tried to induce a tolerance to endotoxin in experimental rabbits and to test the culture filtrates on these animals, no significant difference was obtained between the pre-treated and the control rabbits.

Discussion

Although cholera toxin is the most important toxic factor produced by *V. cholerae* 01 evidence of other extracellular products possessing cytotoxic, enterotoxic and other biological characteristics have also been found. This is an important point particularly in relation to identifying and selecting a strain for use as a live cholera vaccine. Some strains developed by recombinant DNA techniques which were expected to be non-toxigenic, had in fact, exhibited some features of enterotoxigenicity (9, 10). Contrary to this, some *Vibrio* isolates from cholera patients failed to produce a positive reaction for cholera toxin by standard laboratory tests. There appears to be an inverse relation between producing cholera enterotoxin and producing other extracellular cytotoxic substances.

V. cholerae mutant strains which do not show any homology with the cholera toxin gene and thus do not synthesise choleragen have been found to produce other toxic factors (17). During the analysis of the culture filtrates from the same strains, we found that they contain a relatively high concentration of carbohydrate antigen which reacts with anti-LPS antibodies. Using antisera prepared in rabbits against homologous serotypes of *V. cholerae*, a double diffusion test and rocket electrophoresis detected a four-fold higher concentration of LPS in culture filtrates of CT(–) strains after 18 hours when compared with CT(+) strains. Because these reactions detect only carbohydrate antigen of LPS, the LAL test was used to estimate endotoxin activity. This test showed a similar ratio of the concentration of endotoxin in filtrates of CT(–) and CT(+) cultures.

After only 3 hours of incubation, the quantity of the endotoxin released by CT(–) strains was already greater and with further incubation, a four-fold difference was reached after 18 hours. This confirms findings of previous studies (3, 4, 7, 15, 23); but the quantitative difference between CT(+) and CT(–) strains had not been demonstrated.

Another obvious difference between the culture filtrates of CT(–) and CT(+) strains was seen in the rabbit skin test. The permeability factor, represented by a distinct blueing zone around the site of injection, was demonstrated only with the culture filtrates of CT(+) strains. The culture filtrates of CT(–) strains always revealed a haemorrhagic reaction accompanied by an induration, and in many cases by necrosis; the blueing zone was hardly visible. When we applied the culture filtrate in the modified Schwartzman test, the haemorrhage and necrosis elicited by the culture filtrates of CT(–) strains was substantially potentiated by injecting purified LPS. This reaction was not observed with the culture filtrates of CT(+) strains. Contrary to this, the experiments focussing on endotoxin tolerance did not show this difference. Rabbits made tolerant to endotoxin reacted similarly by the skin test as non-tolerant rabbits. This, perhaps, indicates that endotoxin tolerance cannot be induced for this particular type of reaction or that the experimental method would require a different approach to detect a relation between endotoxin and the permeability.

The release of endotoxin by strains of *V. cholerae* is not a new observation and has been described by Pfeifer in 1885 and also by Robert Koch as pointed out by van Heyningen and Seal (6). Earlier it was believed that endotoxin was the only responsible factor in the pathogenesis of cholera. This hypothesis, formulated and strongly supported by Koch, had delayed the discovery of an effective cholera toxin, according to some authors(6).

It is not our intention to revive an old controversy on the role of endotoxin in the pathogenesis of cholera. However, the observation that strains of *V. cholerae* which do not produce CT appear to release large quantities of endotoxin has raised questions about both the physiopathology of cholera and the immunity to this disease. These considerations may likewise be significant in the construction of a cholera vaccine. There is no doubt that the endotoxin released by *V. cholerae* cannot diffuse across the normal intestinal membrane, though it is clear that the endotoxin has a strong affinity to bind to the membranes. If released in the form of "blebs," (18) it may also function as a vehicle to deliver other biologically active products in a concentrated form directly to the cell membrane

where the *Vibrio* cell adheres. Thus, a combined local effect of endotoxin and other protein cell products could evoke effects not observed when the individual components are examined experimentally. This effect could be more pronounced in strains not synthesising the cholera toxin and releasing a high amount of endotoxin.

In a previous study, it was shown that the release of LPS is significantly increased when protein synthesis is inhibited by amino acid deprivation or by chloramphenicol treatment. It is also known that the release of LPS in *E. coli* and *S. typhimurium* is controlled by the *rel* A gene (8). We are still not aware of a similar relation for *V cholerae*. From the point of view of constructing a live cholera vaccine, the possibility that the release of endotoxin is genetically related to the cholera toxin synthesis is a question which calls for further investigation.

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