ORIGINAL ARTICLE

Evaluation of in vivo and in vitro Biological Activity of a *Vibrio Cholerae* O1 Hemolysin

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Abstract

Purpose: To evaluate the hemolysin effect by ileal loop model produced by *Vibrio cholerae* O1 strains, compared with the cellular lysis or cytotoxic activity (CA) observed in cell culture.

Method: We studied nine *V. cholerae* O1 strains, obtained during the Mexican outbreak of cholera (1990-1993), which had CA in Vero and CHO cells. Hemolysin was monitored with the hemolysis test. Titers of CA were calculated by CD₅₀, and the association between CA and cholera toxin (CT) production was discarded by means of neutralization tests using an anti-CT polyclonal antibody. The CT production was measured with ELISA test. The LAL assay was performed in order to study relationships between the CA and bacterial lipopolysaccharide. Strains with CA were evaluated in rabbit and rat ileal loop models; hemorrhagic

fluid was also measured. Tissues from ileal loop were included in paraffin to detect intestinal epithelial damage.

Results: The hemolysin CA was not neutralized with the anti-CT polyclonal antibody. However, the associated factor of CA was heat labile. CA in cell cultures was not related to the bacterial lipopolysaccharide. The ileal loop test exhibited the presence of hemorrhagic tissue with inflammation.

Conclusion: The *V. cholerae* O1 strains isolated were able to secrete hemolysin which, in turn, caused CA in cell cultures and produced the hemorrhagic and inflammatory effects observed in the ileal loop of rabbit and rat models.

Cholera is a severe diarrheal disease caused by *Vibrio cholerae* O1 that uses toxin production as its principal

pathogenic mechanism. The production of this toxin is well known because the clinical manifestations of cholera disease are related to cholera toxin (CT) activity on the physiological properties of the intestinal epithelial cells.¹

Cholera toxin is considered the most important pathogenicity factor¹, although other toxins have also been described: ZOT (Zonula Occludens Toxin) or ACE toxin^{2,3,4}. Except for CT, the role of other toxins in the pathogenesis of the disease due to *V. cholerae* is not clear.⁵⁻⁸ Some cholera non-toxigenic strains are not CT producers and have demonstrated capacity to produce moderate diarrhea⁹.

Assays with cell cultures have revealed that CT generally causes a cytotoxic effect (CTN) consisting in the enlargement of cells.¹⁰ However, some *V. cholerae* O1 and non-O1 strains have produced direct cell lysis or cytotoxic activity (CA) associated to hemolysin.^{11,12} Hemolysin genes already have been cloned.^{3,13,14} CA related to CT in hyper-producer strains has not been described.

The relationship between pathogenicity and hemolysin production in *V. cholerae* O1 has been studied less than the relation between CT and pathogenicity. The possibility that *Vibrio cholerae* O1 hemolysin producer strains could play a role in CA activity and the development of cholera is unclear.

The purpose of this work was to determine the biological activity of hemolysin from *Vibrio cholerae* O1 strains isolated in the Mexican outbreak of the disease between 1990 and 1993 using the ileal loop model in rabbit and rat in *in vivo* assays and Vero and CHO cell cultures with *vitro* experiments.

Methods

Ligated Ileal loop assay in rat and rabbit

This test was performed using young New Zealand white rabbits (2.5 kg body weight) and Wistar young rats (250 g body weight). Both animal models were subjected to fasting for 18-24 h prior to performing the experiment. The animals were anesthetized and

the abdomen of each animal was opened using aseptic methods. The intestine was divided into 4 loops with string ligatures and 1 ml supernatant with CA in cell cultures, 1 ml of PBS, 1 ml of control strain supernatants and 1 ml of medium alone were inoculated in them. The abdomens of the animals were closed and the animals were sacrificed at 18 - 20 h. The volume of accumulated fluid and the length of loops were determined. The relationship between volume and length of loop was calculated.^{18,19} The hemorrhagic damage was assessed by quantifying the hemoglobin in the fluid collected into the loop using benzidine hydrochloride according to Newman.²¹ Histological tissue from the loops was included in paraffin and dyed by means of the hematoxylin and eosin technique. Animal experiments were conducted in accordance with the Mexican NOM-062Z00-1999 guidelines for production and care of laboratory animals²⁰.

Bacterial strains.

Nine clinical *Vibrio cholerae* O1 strains were isolated from patients diagnosed and treated for the cholera disease during an outbreak in Mexico between 1990 and 1993. The strains were capable of producing CA in cell cultures. Strain C6707 was CTN, isolated in a Peruvian cholera outbreak and 25922 CA hemolysin producer strains were used as controls. All strains were tested for hemolysin by hemolysis test.

Production of supernatants with CA.

All strains were grown in Craig broth medium (Casaminoacids 3%, yeast extract 4%, glucose 2% and monobasic potassium phosphate 0.05%) with shaking and in three different time periods (6, 18 and 24 h). The cultures were harvested and centrifuged at 10,000 x g and the supernatants were filtered through 0.22 µm membranes (Millipore Corporation, Bedford, MA).

Cytotoxic dosage at 50%.

96-well flat-bottom tissue culture plates (Nunc; Intermed, Roskilde, Denmark) were used to grow either CHO (Chinese hamster ovary) cells in Ham F-12 medium or Vero (kidney monkey green) cells in Eagle minimum essential medium, both supplemented with 5% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA), penicillin G and streptomycin (Gibco BRL, Carlsbad, CA, USA). The plates were incubated at 37°C and in 5% CO₂ atmosphere. After confluent growth of the cell cultures, supernatants from the bacterial strains were added to the cell cultures using a 1:10 dilution factor ($10^1 - 10^5$) with Eagle medium supplemented with 2% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA). These cultures were incubated under standard conditions described above and examined with an inverted microscope (Zeiss, Jena, Germany) to observe morphological changes. The cytotoxic dose to 50% (CD_{50%}) was defined as the amount necessary to produce 50% of the CA in a confluent cell culture.

Neutralization assay.

Polyclonal antibodies against the CT were used to determine whether CA could be inhibited. Antibodies were made using New Zealand white rabbits (2.5 Kg body weight) which were inoculated subcutaneously with 2.5 µg of CT in a water-oil emulsion in the first two weeks and intramuscularly with 2.5, 5 and 10 µg on the third, fourth and fifth week, respectively. Serum was collected and stored at -20°C. The reactivity was tested by Ouchterlony immunodiffusion test.

Bacterial strain supernatants were adjusted to CD_{50%} and added to CHO and Vero cells growing in 96-wells plates (Nunc; Intermed, Roskilde, Denmark) with MEM medium and 2% fetal bovine serum (In Vitro Corp. Masch, USA). Polyclonal antibodies were added at 10^{-1} - 10^{-4} dilutions, and the titer of neutralization was calculated as 50% (ED_{50%}). Purified CT (Sigma-Aldrich, USA) was used as control.

Heat sensitivity.

In order to determine the sensitivity to the heat of the toxin, the supernatants from cytotoxic strains were heated at 80°C for 10 min, and were assayed in CHO and Vero cells under the conditions described above using the DCT_{50%} and appropriate controls.

Detection of CT production in CA strains by ELISA.

Supernatants from strains with CA in cell cultures were added to 96-wells plaques (Nunc; Intermed, Roskilde, Denmark) with a U flat bottom adsorbed with GM1 ganglioside. The plates were washed and polyclonal anti-CT was added to them. Then, the plates were washed again, a monoclonal anti-rabbit labeled with para-nitrophenol-phosphate (Sigma-Aldrich, USA) was added and they were revealed with alkaline phosphatase. Cut-off was established with purified CT.^{15,16}

Lipopolysaccharide detection by LAL assay.

Supernatants from strains with CA in cell culture were assayed for lipopolysaccharide (LPS) using the *Limulus* Amebocytes lisate (LAL) with the Pyrogenal LAL assay® kit (Cambrex corp. USA).¹⁷

Ligated Ileal loop assay in rat and rabbit.

This test was performed using young New Zealand white rabbits (2.5 kg body weight) and Wistar young rats (250 g body weight). Both animal models were subjected to fasting for 18-24 h prior to performing the experiment. The animals were anesthetized and the abdomen of each animal was opened using aseptic methods. The intestine was divided into 4 loops with string ligatures and 1 ml supernatant with CA in cell cultures, 1 ml of PBS, 1 ml of control strain supernatants and 1 ml of medium alone were inoculated in them. The abdomens of the animals were closed and the animals were sacrificed at 18 - 20 h. The volume of accumulated fluid and the length of loops were determined. The relationship between volume and length

TABLE 1. *Vibrio cholerae* O1 strains tested on Vero cell cultures with toxin and lipopolysaccharide production

Strain	DCT _{50%}	Toxin (ng/ml)	Lipopolysaccharide (ng/ml)
C6707	ND	20	0.4
251488	-0.3	30	0.02
22161	0.5	79	0.02
1	0.6	100	0.2
1508	-0.5	0	0.2
1137	0.5	40	0.2
5537	-0.3	65	0.2
5762	-0.3	20	0.2
9171	-0.1	20	0.4
7876	0.6	36	0.4
26361~	-0.1	158	0.4

DCT_{50%}. Cytotoxic dosage 50%
 26361~ Vacuolating strain

of loop was calculated.^{18,19} The hemorrhagic damage was assessed by quantifying the hemoglobin in the fluid collected into the loop using benzdine hydrochloride according to Newman.²¹ Histological tissue from the loops was included in paraffin and dyed by means of the hematoxilin and eosin technique.

Results

Cytotoxic dosage at 50%.

Strains 1, 22161 and 7876 appeared with a low CD_{50%} (0.5), in which the activity became cytotoxic after dilution of the supernatants. Strains 5762 and 5537 showed the highest titers (-0.3); strain 26361 (-0.1) produced vacuolating activity; 4/9 CA strains presented values from 0.6 to 0.1 and had no effect in the supernatant dilutions (Table 1).

CA thermosensitivity.

After boiling, supernatants with CA were not capable of producing cytotoxicity CA in the monolayers of CHO and Vero cells (Table 1).

TABLE 2. Ileal loop animal test rabbit or rat with *Vibrio cholerae* strains.

Strain	V/L (ml/cm) Rabbit	V/L (ml/cm) Rat	Hemoglobin Rabbit (mg/ml)	Hemoglobin Rat (mg/ml)
C6707	0.5	0.3	0.01	0
251488	0.05	0.3	0.3	0.2
22161	2	1	0.4	0
1	2	0.6	0.08	0
1508	0	0	ND	ND
1137	0.9	0.6	0.2	0.2
5537	0.09	0.06	3.8	3.2
5762	0.78	0.3	4.2	4
9171	0.08	0.05	0.2	0.1
7876	0.02	0.03	0.5	0.2
26361	2	1.6	0.2	0.3

V/L. Volume/length(ml/cm)

Relation between CT and CA production.

Cytotonic activity of supernatants in Craig's media was inhibited in the presence of anti-CT antibodies, but the CA was not.

Detection of CT production in cytotoxic strains.

Strains 22161 and 1 exhibited hyper-production of cholera toxin with values of 100 and 79 ng respectively, but strain 1508 was not a CT producer. The rest of the strains presented values between 20 and 65 ng/ml (Table 1).

Non-involvement of the Lipopolysaccharide in strains with CA.

LAL methodology revealed LPS values of 0.2- 0.4 ng/ml in the supernatants had not significant differences between cytotoxic CTN and cytotoxic CA in cell cultures (Table 1).

Damage produced in the ileal loop by cytotoxic strains.

In the intestinal ileal loop of the experimental animals, cytotoxic supernatants presented only clear fluid accumulation (traditionally associated to CT), while the

strain cytotoxic supernatants produced hemorrhagic damage with less fluid. Table 2, shows strains 1 and 22161 with low levels of hemoglobin (0.4 and 0.08 mg/ml in rabbit, and 0 and 0 in rat), but higher values was observed related with volume and length (2 and 2 ml/cm in rabbit, and 1 and 0.6 ml/cm in rat). The histological tissue of hemorrhagic ileal loops of cytotoxic strains exhibited inflammatory damage with leukocyte infiltration and erythrocytes, while cytotoxic supernatants only presented enlargement of the intestinal epithelial cells associated to fluid accumulation.

Discussion

Although CT is the principal virulence factor of *Vibrio cholerae* O1 to produce disease in humans, more pathogenic factors could also be involved, because some *V. cholerae* O1 and other non-O1 non-toxigenic strains have been found to cause diarrhea and gastroenteritis due to the production of other toxins.^{9,22} Also, several experiments *in vitro* have shown that hemolysin could be involved in the pathogenicity of cholera through cell lysis, although some studies with human granulocytes have suggested that they are able to activate inflammation, but the possibility of taking part is not clear.²³ Thus, studies on accessory pathogenic factors are of great value as they permit possible mechanisms to be established. These incidentally take part in cholera pathogenicity, either alone or through a synergistic relationship between such factors and CT.

CTN produced by cholera CT-positive strains presents enlargement of cells due to changes in the ionic efflux across the membrane of the enterocyte²⁴⁻²⁶. In our study, two strains (22161 and 1) with CA revealed CTN after dilution. In other *in vitro* studies *V. cholerae* non-O1 is able to induce vacuolation.^{3,27,28} In this study, one strain (26361) showed a vacuolation effect once the supernatant obtained was diluted. Thus, at low doses, the effect induced by the hemolysin is vacuolation but, at higher doses, CA is induced as the effect of hemolysin (CA and vacuolating activity) which masks the CTN effect produced by CT.

There is no evidence of CA by CT. Whereas toxin production by our strains was different (table 1) to exclude participation of CT in the development of CA, we tested whether the effect of CA was inhibited by a polyclonal antibody anti CT, despite differences in the production of toxin from one strain to another. We found that CA was not inhibited by treatment even in a non CT producer strain (1508) had a high cytotoxicity titer. These results show that CT was not involved in the development of CA. Furthermore, given that a heat stable enterotoxin was found in a strain of *V. cholerae* positive CT⁵, we tested whether the factor related with CA could be thermostable. The hemolysin related with CA was not thermostable.

A mechanism related to lipopolysaccharide (LPS) is involved in the activation of some cellular pathways that lead to cellular lysis. Thus, we evaluated whether the lipopolysaccharide released in the medium during bacterial growth could be involved in CA. In addition, the LPS released from the bacteria to the medium induced CA in cell cultures.²⁹ However, since our results showed no significant difference between the LPS produced in the CA and CTN strains, we did not find a relationship between CA and LPS (table 1).

We studied the effect of strains with CA in animal models *in vivo* using the ligated ileal loop in rabbit and rat. We used both models because the rabbit is more sensitive to CT than the rat and because we anticipated that the hemolysin effect would be more visible in the rat. In the intestinal ileal loop of the animal model, cytotoxic supernatants only presented clear fluid accumulation traditionally associated with CT. Cholera toxin has been shown to induce fluid accumulation without hemorrhagic damage.³⁰ However, we showed that, in the animal models tested, the strains with CA also developed hemorrhagic damage with inflammation and abundant erythrocytes and leukocyte infiltration, all of which was manifested by the release of hemoglobin into the lumen together with the fluid. The effect was more visible in the rat than in the rabbit because the fluid accumulation in the rabbit masked the hemorrhagic effect. This is consistent with

other studies *in vivo* in which findings were similar but in *V. cholerae* non-O1 non 0139.³¹ This suggests that *V. cholerae* O1 hemolysin is able to develop tissue destruction and that could be a trigger of other pathways either apoptosis, inflammation or including necrosis observed by other groups in *V. cholerae* non-O1.^{32,33} If hemolysin can trigger those mechanisms that lead to the lysis and inflammation, then it is possible that these same mechanisms could be involved in the development of gastroenteritis in which *Vibrio cholerae* O1 El Tor non-cholerae CT negative has been involved.³⁴ Despite the low frequency of reports of strains of hemolysin producers with CA and other accessory factors of *V. cholerae* O1 (ZOT, ACE) further studies are required to understand how they could be taking part in cholera pathogenesis.

Several studies have described *V. cholerae* non-O1 hemolysin, that has been cloned and the supernatants from those clones were able to produce CA and vacuolation in cell cultures.³ Moreover, studies *in vivo* are essential along with molecular analysis to determine whether the hemolysin in *V. cholerae* O1 detected in the present study is the same as that found in non-O1 strains.

In conclusion, these data suggest that the *V. cholerae* O1 strains isolated were able to secrete hemolysin which, in turn, caused CA in cell cultures and produced the hemorrhagic and inflammatory effect observed in ileal loop in rabbit and rat models.

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References

1. Achensohn D. Enterotoxins in acute diarrhea. *J Infect Dis* 1992; **24**:225-45
2. Fasano A, B Baudry, DW Pumpkin, et al. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proc Natl Acad Sci USA* 1991; **88**:5242-46.
3. Figueroa-Arredondo P, Heuser J, Akopyants N, et al. Cell Vacuolation Caused by *Vibrio cholerae* Hemolysin. *Infect Immun* 2001; **69**: 1613-24.
4. Galen J, Trucksis M, Michalson J, et al. Accessory cholera enterotoxin (Ace), a new enterotoxin of *Vibrio cholerae* in: "Abstracts of 92nd General Meeting of the American Society for Microbiology. New Orleans, Louisiana. USA: 1992; 74.
5. Takeda Y, Ogawa A, Dohi S, Abe H, et al. Detection of heat-stable enterotoxin in a cholera toxin gene-positive strain of *Vibrio cholerae* O1. *FEMS Microbiol. Lett* 1991; **80**:23-8.
6. Trucksis M, G. Michalski, Fasano A, J. Kaper. Accessory cholera enterotoxin (Ace), the third toxin of *Vibrio cholerae* virulence cassette. *Proc Natl Acad Sci USA* 1993; **90**:5267-71.
7. Walia, K, Ghosh S, Singh H, et al. Purification and characterization of novel toxin produced by *Vibrio cholerae* O1. *Infect. Immun* 1999; **67**:5215-22.
8. Zitzer, A., Wassenar T, Walev I and Bhakdi S. Potent membrane-permeabilizing and cytotoxic action of *Vibrio cholerae* cytolysin of human intestinal cells. *Infect Immun* 1997; **65**:1293-98.
9. Saha K, Koley H, Mukhopadhyay A, et al. Non toxigenic *Vibrio cholerae* O1 serotype Inaba biotype El Tor associated with a cluster of cases of cholera. *J Clin Microbiol* 1996; **34**:114-17.
10. Rodriguez-Angeles M, Giono-Cerezo S, Valdespino J. Efecto citotónico y citotóxico de la toxina colérica en células Vero y su relación con PCR. *Rev.Lat -Amer. Microbiol* 1994; **36**: 263-72.
11. Alm M, Manning P. Characterization of the hly 6 gene and its role in the production of the El Tor hemolysin of *Vibrio cholerae* O1. *Mol Microbiol* 1990; **4**:413-25.
12. Ichinose Y, Yamamoto K, Nakasome N, et al. Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae*. *Infect. Immun* 1987; **55**:1090-3.
13. Manning P, Brown M, Heuzenroeder M. Cloning of the structural gene (hly) for the hemolysin of *Vibrio cholerae* El Tor strain 017. *Gene* 1987; **31**:711-20.
14. Honda T, Finkelstein R. Purification and characterization of alfa hemolysin produced by *Vibrio cholerae* biotype El Tor: Another toxic substance produced by Cholera vibrios. *Infect Immun* 1979; **26**: 1020-7.

15. González-Bonilla C, Gutiérrez-Cogco L, Moguel-Pech L, Villanueva-Zamudio A. Evaluación del método de ELISA para la determinación de toxina colérica en cultivos de *Vibrio cholerae*. *Rev. Lat.-Amer. Microbiol* 1994; **36**: 273-6.
16. Giono-Cerezo S, Rodríguez-Angeles G, Gutiérrez-Cogco L, Valdespino-Gómez J. Caracterización fenotípica y genotípica de *Vibrio cholerae* O1. *Rev. Lat.-Amer. Microbiol* 1994; **36**: 243-51.
17. Cooper J, Hochstein H, Seligmann E. The *Limulus* test of endotoxin (pyrogen) in radiopharmaceutical and biologicals. *Bull parenters Drug assoc* 1972; **26**:253.
18. Spira M, Bradley S, Frochlich J. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *Escherichia coli* diarrhea. *Infect Immun* 1981; **23**:739-47.
19. Burrows W, Musteikis G. Cholera infection and toxin in the rabbit ileal loop. *J Infect Dis* 1966; **116**:183-90.
20. DOF. NOM-062-Z00-1999 Especificaciones para la producción, cuidado y uso de los animales de laboratorio. 22 de agosto. pp. 20-74.
21. Naumann H. The measurement of hemoglobin in plasma 1-20 In: Hemoglobin its precursors and metabolites: 1960; F. Sunderman, Jr. Eds, Philadelphia , J.B. Lippincott Co.
22. Morris G, Wilson J, Davis B, Waschmuth I, et al. Non-O1 group 1 *Vibrio cholerae* gastroenteritis in the United States. *Intern. Med* 1981;**94**:656-8.
23. Valeva A, Walev I, Weis S, Boukhallouk F, Wassenar T, Bhakdi S. Pro-inflammatory feedback activation cycle evoked by attack of *Vibrio cholerae* cytolysin on human neutrophil granulocytes. *Med Microbiol Immunol* 2007 In press.
24. Arellano-Galindo J, Giono-Cerezo S, Díaz-Silva F, Rodríguez-Angeles G, López-Saucedo C. *Vibrio cholerae* en cultivo de células Vero. *Anales Esc Nal Ciencias Biológicas* 2001; **47**:147-51
25. Kaper J, Levine M, Morris G. Cholera. *Clin Microbiol Rev* 1995; **8**:48-86.
26. Men'shikova E, Podosinnikova L. Testing the biological activity of *Vibrio cholerae* on cell subcultures. *Zh Mikrobiol Epidemiol Immunobiol* 2005; **4**:73-5.
27. Rupak M, Figueroa P, Mukhopadhyay A, et al. Cell Vacuolation, a manifestation of the El Tor Hemolysin of *Vibrio cholerae*. *Infect Immun* 2000;**68**:1928-33.
28. Coello A, Andrade J, Vicente A, Di Rita V. Cytotoxic cell vacuolating activity from *Vibrio cholerae* hemolysin. *Infect Immun* 2000; **68**:1700-5.
29. Ongradi J, Kulcsar G, Bertok L. Toxicity of microbial products in cell culture. *Folia Microbiol (praha)* 1984; **29**:450-4.
30. Sears C, Kaper J. Enteric bacterial toxins: Mechanisms of action and linkage to intestinal secretion. *Clin Microbiol Rev* 1996; **60**:167-215.
31. Saha D, Pal A, Rajendran K, Dutta P. Histopathological changes in experimental cholera with a non toxigenic non- O1 non-O139 *Vibrio cholerae* strain isolated from Kolkata, India. *Indian J. Exp Biol* 2006;**44**(3):221-7.
32. Gayatri M, Amlan B, Kalyan K, Tapas B. *Vibrio cholerae* hemolysin is apoptogenic to peritoneal B-1a cells but its oligomer shepherd the cells for IgA response. *Mol Immunol* 2008; **45**:266-70
33. Saka H, Bidinost C, Sola C, carranza P, Ortiz S, Echenique J, Bocco J. *Vibrio cholerae* cytolysin is essential for high enterotoxicity and apoptosis induction produced by a cholera toxin gene-negative *V. cholerae* non-O1, non-O139 strain. *Microb Pathog* 2007 In press.
34. Nair G, Safa A, Bhuiyan N, et al. Isolation of *Vibrio cholerae* O1 strains similar to pre-seventh pandemic El Tor strains during an outbreak of gastrointestinal disease in an island resort in Fiji. *J. Med Microbiol* 2006;**55**:1559-62.

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