Acta Veterinaria (Beograd), Vol. 43, No. 4, 191—198, 1993

UDK 619:579.852.13

IN VITRO EFFECT OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN ON VERO CELLS AND *IN VIVO* EFFECTS IN ANIMALS

LJILJANA MARKOVIĆ*, RUŽICA AŠANIN** and B. DIMITRIJEVIĆ*

** Laboratory for Radiobiology and Molecular Genetic Institute of Nuclear Sciences — Vinča, P. O. Box 522, 11000 Belgrade, Yugoslavia*

*** Faculty of Veterinary Medicine, University of Belgrade, Bul. JNA 18, 11000 Belgrade, Yugoslavia.*

(Received, 9 May 1993)

Clostridium perfringens type B was investigated for enterotoxin production. Enterotoxin was detected in the strain B L 361. The cell extract was purified by ammonium sulfate fractionation and by gel filtration of the extract from sonically disruped cells. This, the two precipitation were followed by gel filtration on Sephadex G-100. This enterotoxin preparation was tested in vitro on Vero cells and in vivo on experimental animals. The enterotoxin induced erythema when injected intradermally into depilated guinea pigs and produced chronic alterations in the intestinal system of mice. The cumulative effects of Clostridium perfringens enterotoxin on Vero cells inhibited cellular metabolism and induced morphological changes in the cultured cells. These effects were more rapidly detected in the Vero cell system (within 30-60 minutes after inoculation).

Key words: Enterotoxin, Clostridium perfringens type B, Vero cells, experimental mice and guinea pigs.

INTRODUCTION

Clostridium perfringens type A produces a sporulation specific enterotoxin protein. An early observation was that the partially purified enterotoxin from strain A can cause typical disease symptoms such as erythema, increased permeability of capillaries, diarrhea, and cramp. Moreover it has been accepted that the enterotoxin can alter intestinal transport of fluid, ions, and glucose, thereby causing tissue damage in the gut and inhibition of metabolic processes in intestinal tissue (McDonel, 1979, 1980). The enterotoxin acts very rapidly and affects the basic function and structural integrity of the cell membrane, thus damaging individual cells.

This enterotoxin is produced only during sporulation (Duncan and Strong, 1968). This is important because sporulation can serve as a relatively simple system for the study of different processes. McClain and McDonel (1979), studied the metabolic effect of *Clostridium perfringens* in a cell culture system.

Cell cultures have two advantages for studying the action of the enterotoxin. First, the homogeneous cell population has a lower variability than that found in animals sistems. Secondly, cell culture is essentially adapted to various microanalytical tehniques. Vero cells in culture have proved to be very sensitive to *Clostridium perfringens* enterotoxin (McDonel and McClain, 1981) and have been employed in studies of the effects of the enterotoxin on cell morphology, adhesion, viability, plating efficiency and the synthesis of macromolecules. Here we present experiments used to test *Clostridium perfringens* type B L 361 and a partially purified extract. Tests were done on Vero cells and experimental guinea pigs and mice.

MATERIALS AND METHODS

Bacterial cell culture. The strains of C. the perfringens type B L 361 used in this study were obtained from the Faculty of Veterinary Science, Belgrade. Working stock cultures were maintained in cooked meat medium and kept at room temperature.

Growth of bacteria and sporulation conditions. Stock culture (0.5 ml) was inoculated into 10 ml of the thioglycolate medium. This stock culture was heat shocked in a water bath at 75° C for 20 to 60 minutes. Incubation at 37° C for 4 to 5 hour followed. A 1 ml aliquot of this culture was inoculated into 10 ml of fresh thioglycolate medium and incubated under the same conditions for 15 hours. The resulting 10 ml culture was transferred to new tubes with 90 ml of DS (Duncan-Strong) sporulation medium (Duncan and Strong , 1968). The resulting spores, after 7 to 10 hours of incubation at 37° C, were colected by centrifugation, DS filtrate refers to sonically disrupted spores as described under Purification of the toxin.

Preparation of cell filtrates. Stock culture (0.5 ml) was inoculated into 10 ml of thioglycolate medium. This stock culture was heat shocked in a water bath at 75° C for 20 to 60 minutes and then incubated for 4 to 5 hours at 37° C. One hundred μl of the culture was transferred into a new tube with 10 ml of thioglycolate medium and incubated for 15 hours under the same conditions. Following the incubation, the culture was centrifuged at 4° C, at 5000 g, for 30 minutes. The supernatant was filter sterilized (pore size 0.22 μm). The filtrate was concentrated by dialysis against polyethylene glycol (PEG — 6000) at 4° C. Filtrates were reconstituted with saline 33 fold and stored at 4° C.

Preparation of the extract from spores. After 7 to 10 hours incubation in DS medium (Duncan and Strong, 1968) spores were harvested by centrifugation at 5000 x g for 30 minutes. The pellet was suspended in ice-could water and washed 3 times. The final suspension was stored in distilled water at 4° C. Spores were sonicated until all cell walls of all spores had been disrupted. The sonication was done at 65 W in an "MSE" ultrasonicator.

Purification of the toxin. The sonicated suspension was centrifuged at 5000 x g after adding an equal volume of 80% saturated ammonium sulfate in 0.02 M phosphate buffer, ph 6.8 (Granum and Whitker, 1980). The pellet was suspeneded in 15% saturated ammonium sulfate and recentrifuged after 30

min. The precipitated protein was dissolved in 0.02 M phosphate buffer, pH 6.8 and chromatographed on a Sephadex G-100 column at room temperature (23 to 25° C) in the same buffer (Starc and Duncan, 1972).

Enumeration of spores. Before harvesting spores from the sporulation medium, a 10 ml aliqout was heat shocked as described. The viable count of these heat treated spores was determined from duplicate plates using SPS (sulphite polymyxin sulfadiazine) agar. Dilutions were made in 0.1% peptone water. Incubation was in anaerobic jars at 37° C for 24 hours.

Vero cell cultures. Vero (African green monkey kidney) cells were grown in Medium 199 (Gibco) supplemented with 5% fetal calf serum (FCS) and 0. 75% sodium bicarbonate (McClain et al., 1979). When monolayers are confluent they were removed by trypsinization, 0.25% trypsin in Ca and Mg free Hanks balanced salt solution (Ca-Mg free HBSS). Trypsinized cells were inoculated into 16 mm tissues culture dishes. These dishes were incubated at 37°C in a humidified atmosphere containing 5% CO2 in air. After 3 days fresh medium was added. After 6 days each well contained a confluent monolayer with $6\text{-}10^6$ cells.

Experimental animals. In this experiment we used male and female mice of 20 to 25 g in weight. The strains were BALBcAnHCR and BALB/c. Enterotoxin was intraperitoneally inoculated as a 0.5 ml portion of either bacterial extract or partially purified toxin. Each test was performed on two mice. The animals were under observation for 72 hours and then sacrificed for pathological examination. Control animals received a placebo (saline). We also used guinea pigs weighing 300 to 400 g. The animals were depilated over on area of 2.5 cm. in diameter and 0.5 ml aliquots of toxin preparation injected intradermally. Samples were applied in duplicate on the same animals. The placebo controls were on the same animal but using a remote depilated area. The animals were examined for erythema of the skin after 18 to 24 hours (Starc, 1971).

RESULTS AND DISCUSSION

The results obtained demonstrated the dominant features of *Clostridium perfringens* type B L 361 which is commonly used as the strain of choice for vaccine production. We attempted to analyze the antigenic properties of this cause of enteritis in lambs.

The cell extract of C. perfringens type B L 361 produced erythema activity in the skin of guinea pigs that was observable after 18 to 24 hours (table 1). Areas of erythema between 0.5 and 1.2 cm in diameter directly depended on the amount of enterotoxin in the injected sample (Figure 1). All samples were injected in duplicate on the same guinea pigs. The placebo injection produced no erythema.

Filtrates of viable cells grown in the thioglycolate medium and the partially purfied cell extract of enterotoxin from the strain under investigation were heat shocked at 75° C for 20 to 60 minutes. Samples prepared in this way were intraperitoneally inoculated into experimental mice. Upon pathological examina-

tion it was found that all animals that received partially purfied toxin had necrotic changes in the intestinal system (McDonel, 1980, 1981). These changes were observed in sacrificed animals since lethal effects were absent. Filtrates of bacteria grown in thioglycolate medium did not have any toxic activity.

Table 1. Toxic effects in Clostridium perfringens type B.

MODEL SYSTEM	GUINEA PIG SKIN TEST	LETHALITY IN MICE	EFECT ON VERO CELLS
FILTRATE OF CELLS GROWN IN DS MEDIUM	+	—	+
PARTIALLY PURIFIED EXTRACT OF ENTEROTOXIN	+	—	+
FILTRATE OF CELLS GROWN IN THIOGLYCOLATE MEDIUM	—	—	—
POSITIVE CONTROL	+	+	+
NEGATIVE CONTROL	—	—	—

Table 1. Preparation of the partially purified enterotoxin and filtrates of cells grown in either DS or thioglycolate medium were described in Materials and Methods. The same holds for the skin test in guinea pigs, lethality of mice, and the Vero cell system. The positive control was analogues preparations from the well studies Clostridium strain NCTC 8239 (National Collection of Type Cultures).The negative control was saline applied in the same manner as the various preparations.

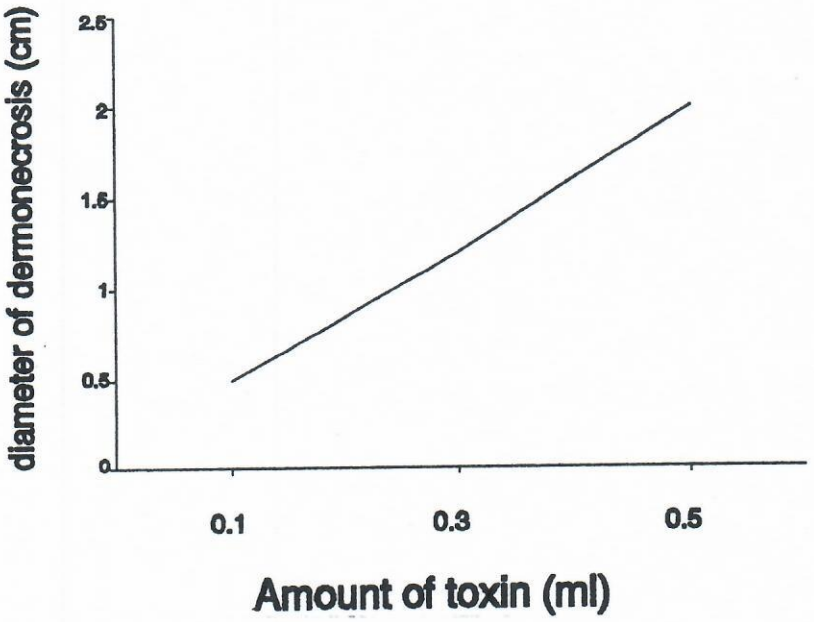

Figure 1. Analysis of dermonecrosis of the skin. Diferent amounts of the enterotoxin were applied and the area of affected skin was recorded as described in Materials and Methods.

Vero cells can be used in an in vitro system for the study of the mechanism of action of enterotoxins. This system is advantageous since it has a lower

variability than most animal model systems. Also, this system is suitable for an investigation of the primary biochemical effects of enterotoxin. We showed that enterotoxin is highly active on VERO cells which confirmed the results of Mc-Donel et al., (1981) and McClain et al., (1979, 1988). Within 30 minutes of exsposure of monolayers to the enterotoxin, gross morphological damage was observed (Figure 2a and 2b). After 3 to 4 hours degenerative alterations of the cell surface disappeared.

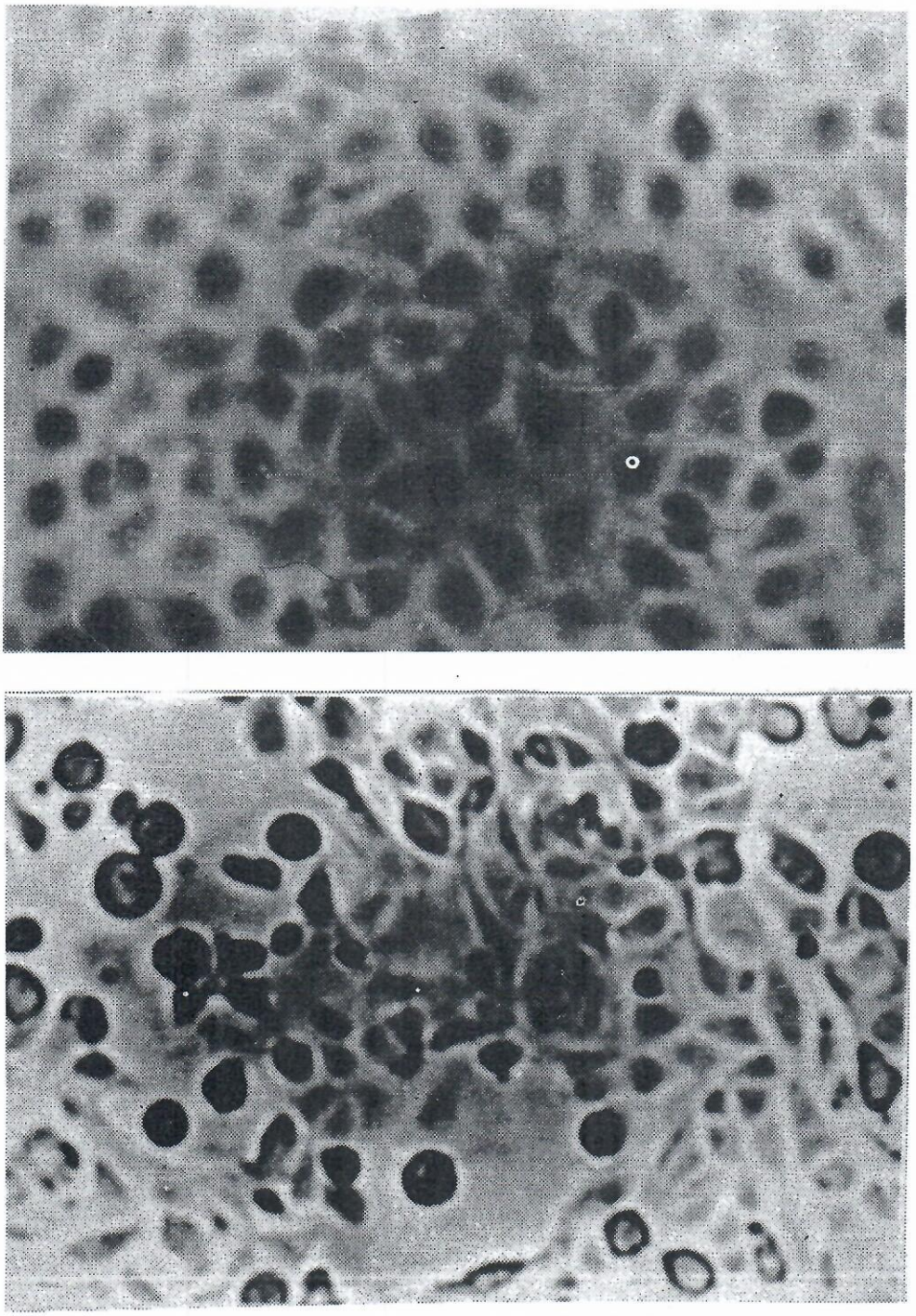

Figure 2. Effects of enterotoxin on morphology of Vero cells. (2a) Control culture showing typical Vero cell morphology. (2b) Vero cells after 30 minutes exposure to enterotoxin.

Our preliminary results on the analyses of *Clostridium perfrngens* type B L 361 indicate that the observed changes are the result of the dominant antigenic properties of this strain. This we have confirmed the results previously reported by Skjelkvale et al., (1975) The changes were observed as dermonecrotic alterations in guinea pigs and necrotic changes in mice in accordance with the findings of Stark et al., (1971, 1072). A toxic effect was rapidly detected in the Vero cell culture system (McDonel, 1981, McClain, 1979, 1988). Microorganisms from *Clostridium perfringes* species have several important antigens. Our investigation of type B L 361 demonstrated the presence of α and β antigens while antigen ε was not detected.

Further research will be directed towards a more detailed analysis of the components that constitute the antigenic structure of this strain.

REFERENCES

1. *Dancan, C. L., Strong, D. H.* 1968. Improved medium for sporulation of Clostridium perfringens. *Appl. Microb. 16. 1,* 82—89.

2. *Duncan, C. L., Label, R. G., Reich, R. R.* 1972. Germination of heat and alkali altered spores of Clostridium perfringens type A by lysozyme and an initiation protein. *J. Bact. 9, 2,* 550—559.

3. *Granum, P. E., Whitker, J. R.* 1980. Improved method for purification of Enterotoxin from Clostridium perfrengens type A. Appl. Environ. *Microb. 39, 6,* 1120—1122.

4. *McDonel, J. L. Duncan, C. L.* 1975. Histopathological effect of Clostridium perfringens enterotoxin in the rabbit ileum. I*nfect. Immun. 112,* 1214—1218.

5. *McDonel, J. L.* 1979. The molecular mode of action of Clostridium perfrengens. Enterotoxin. *Amer. J. Clinic. Nutrit. 32,* 210—218.

6. *McDonel, J. L.* 1980. Mechanism of action of Clostridium perfringens enterotoxin. *Food Technol. 4,* 91—95.

7. *McDonel, J. L. and McClane, B. A.* 1981. Highly sensitive assay for Clostridium perfringens enterotoxin that uses inhibition of plating efficiency of Vero cells grown in culture. *J. Clinic. Microb., 13, 5,* 940—946.

8. *McClain, B. A. and McDonel, J.L.* 1979. The effect of Clostridium perfringens enterotoxin on morphology, viability and macromolecular synthesis in Vero cells. *J. Cell Physiol., 99, 2,* 191—199.

9. *McClain, B. A., Hanna, P. C. and Wnek, A. P.* 1988. Clostridium perfringens enterotoxin. *Microb. Pathol. 4,* 317—323.

10. *Skjelkvale, R. and Duncan, C.* 1975. Enterotoxin formation by diferent toxigenic types of *Clostridium perfringens. 11, 3,* 563—575.

11. *Smith, R. L. and McDonel, J. L.,* 1980. Clostridium perfrigens type A: in vitro system for sporulation and enterotoxin synthesis. *J. Bacteriol, 144,* 306—311.

12. *Stark, R. L., Duncan, C. L.* 1971. Biological characteristics of Clostridium perfringens type A enterotoxin. *Infec. Immun. 4, 2,* 89—96.

13. *Stark, R. L. Duncan, C. L.* 1972. Purification and biochemical properties of Clostridium perfringens type A enterotoxin. *Infec. Immun. 6, 5,* 662—673.

Acta Veterinaria (Beograd), Vol. 43, No. 4, 191—198, 1993.
Ljiljana Marković et al.: In vitro effect of clostridium perfringens enterotoxin on vero cells and in vivo effect in animals

EFEKAT ENTEROTOKSINA CLOSTRIDIUM PERFRINGENS IN VITRO NA VERO ĆELIJAMA I IN VIVO NA ŽIVOTINJAMA

LJILJANA MARKOVIĆ, RUŽICA AŠANIN i B. DIMITRIJEVIĆ

SADRŽAJ

Ispitano je dobijanje toksina iz *Clostridium perfrigens* tipa B. Enterotoksin je otkriven u soju B L 361. Ćelije su razbijene sonikacijom, a dobijeni ekstrakt je prečišćen amonijum sulfatom i gel filtracijom. Nakon dve precipitacije usledila je gel filtracija na Sefadeksu G-100. Tako pripremljeni enterotoksin ispitan je in vitro na Vero ćelijama i in vivo na eksperimentalnim životinjama. Aplikovan intradermalno u depiliranoj koži zamoraca izazvao je crvenilo, a na crevima miševa hronična oštećenja. Nakupljeni toksin navedenog tipa *Clostridium perfringens* inhibirao je metabolizam Vero ćelija dovodeći do morfoloških promena u njima. Ta pojava je za vrlo kratko vreme bila uočena na sistemu Vero ćelija (u periodu 30 do 60 minuta posle inokuacije), nakon čega su se ćelije oporavile.

UDK 577 — 619 (05)

YU SSN 0567—8315

FACULTY OF VETERINARY MEDICINE BEOGRAD, YUGOSLAVIA

ACTA VETERINARIA

This Journal is covered in:

Current Contents
Science Citation Index
FO: VM

ACTA VETERINARIA VOL. 43 NO. 4 Pp. 189 — 250 BEOGRAD, 1993