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Cytotoxic effect of diphtheria toxin in mammalian cell lines

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Abstract

Diphtheria toxin is an exotoxin secreted by *Corynebacterium diphtheriae*, the bacterial pathogen that causes diphtheria. The present study was focused to find out the toxicological effect of diphtheria toxin in guinea pigs and cell lines like Vero, MA104, HeLa, Murine neuroblastoma-2A, BHK-21, Rhabdomyosarcoma and L20B. Animals inoculated with the toxin at different concentrations showed erythema around the site of inoculation after 48 h. When susceptible cell lines inoculated with similar concentration of toxin showed similar morphological changes with certain minor differences were observed. Diphtheria toxin has the capacity to inhibit protein synthesis in mammalian cell lines and thus causing cell death. The study concluded that Vero, MA104 and Rhabdomyosarcoma cell lines could be used as an alternative to *in vivo* assay for the toxicology study of diphtheria toxin and enables us free from ethical issues.

Keywords: Diphtheria toxin, cell lines, erythema, guinea pigs.

Introduction

Diphtheria is caused by the noninvasive infection of the nasopharyngeal tissues by toxinogenic *Corynebacterium diphtheriae* strains. The toxin industrially produced by the growth in suitable medium of *C. diphtheriae* vaccine strain. Minute doses of diphtheria toxin produce visible lesions when injected intradermally in sensitive animals. The small doses of diphtheria toxin are lethal to mammalian cells, cultured from sensitive species (Placido-Sausa & Evans, 1957). Hence the investigation is an attempt to study the comparative toxicological effect of diphtheria toxin in guinea pigs and mammalian cell lines like Vero, MA104, HeLa, Murine neuroblastoma-2A, BHK-21, Rhabdomyosarcoma (RD) and L20B to search for alternatives to animal models.

Materials and methods

Diphtheria toxin

The glycerinated diphtheria toxin was obtained from quality control laboratory of PII, Coonoor.

Animals & cell lines

Albino white guinea pigs (250-350 g) maintained at PIIC was used to conduct in vivo biological assay. Cell lines (Vero, MA104, HeLa, Murine Neuroblastoma-2A, BHK-21, Rhabdomyosarcoma & L20B) maintained at PIIC were used to conduct *in vitro* toxicology assay.

Cell culture media

Cell culture media used for growth and maintenance of the cells can influence susceptibility. A defined medium which supports growth and has no negative influence on cell susceptibility is essential. Fetal bovine serum (FBS) is good for promoting cell growth because of the absence of the viral inhibitors. All sera used for cell culture should be inactivated at 56^{o}C for 30 min. Eagle's minimal essential medium (MEM) is used as a growth and maintenance medium. The primary cell culture used for the present investigation were Vero, MA 104, HeLa, Murine Neuroblastoma-2A, BHK-21, Rhabdomyosarcoma (RD) and L20B and the growth media of Vero and MA104 have 5% FBS and the growth media of HeLa, Murine Neuroblastoma-2A, BHK-21, RD and L20B have high added serum content (10% FBS). They promote rapid growth and all media used were supplemented with neomycin (50 µg/ml) and fungizone (2 µg/ml).

Microtitre plate

Sterile 96 wells, flat bottom tissue culture microtitre plates were used in the in vitro assay system.

Test for sterility

From the glycerinated toxin bottle 8 ml of toxin was drawn and 1 ml was transferred into each of 4 bottles containing alternate thioglycolate medium (ATM) and 4 bottles containing soyabean casein digest medium (SCDM). ATM bottles were incubated at 35^{o}C and SCDM bottles were incubated on 20-25^{o}C for 14 d. Bottles were observed on 2nd, 4th, 7th, 10th and 14th day. The test was passed when there was no contamination (Rao *et al.,* 1991).

Schick test

Schick test toxin is the preparation used in the Schick test to determine susceptibility to diphtheria. The toxin may be purified. The toxin was diluted to different concentration (1 Lf, 0.1 Lf, 0.01 Lf, 0.001 Lf, 0.0001 Lf, 0.00001 Lf, 0.000001 Lf & 0.0000001 Lf) by using sterile normal saline as a diluent. For each concentration, 0.2 ml of toxin was injected intradermally into the depilated skin of guinea pig. Measurement of the longitudinal and transverse area of the resulting lesions at the injection site, 2 d after the inoculation (Indian Pharmacopoeia, 1996), should be made. Animals inoculated with toxin should show erythema around the site of inoculation.

Determination of Limes flocculation

The Limes flocculation (Lf) of diphtheria toxin is determined by incubating together the preparations being examined and the standard preparation of diphtheria antitoxin for flocculation test in appropriate

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Table 1. Determination of Limes flocculation

Volume of toxin (ml)	Volume of normal saline (ml)	Volume of antitoxin (ml) (100 Lf/ml)	Appearance of flocculation	Kf (min.)
1.00	0.60	0.40 (40Lf/ml)		
1.00	0.55	0.45 (45Lf/ml)		
1.00	0.55	0.50 (50Lf/ml)	F_2	
1.00	0.45	0.55 (55Lf/ml)	F_1	7
1.00	0.40	0.60 (60Lf/ml)		

F_1 - 1st Flocculation; F_2 - 2nd Flocculation; Kf - Time taken to flocculate

concentrations. When the concentration of the toxin is kept constant and the concentration of the antitoxin varied in mixtures of constant volume, the mixture flocculating first is that which contains the most nearly equivalent quantities of toxin and antitoxin (Indian Pharmacopoeia, 2007).

Table 2. In vivo toxicology study.

Toxin (Lf/ml)	Zones of erythema in mm after 48 h in guinea pigs
1	Animal died
0.1	24
0.01	12.7
0.001	3.2
0.0001	0
0.00001	0
0.000001	0
0.0000001	0

Table 3. Effect of diphtheria toxin on mammalian cell lines

Cell line	Degree of cytotoxicity	Toxin (Lf/ml)							
		1	0.1	0.01	0.001	0.0001	0.00001	0.000001	0.0000001
Vero	24th h	4+	4+	3+	2+	1+	N	N	N
	48th h	4+	4+	4+	3+	3+	N	N	N
	72nd h	4+	4+	4+	4+	4+	1+	N	N
MA-104	24th h	4+	4+	3+	2+	1+	N	N	N
	48th h	4+	4+	4+	3+	2+	N	N	N
	72nd h	4+	4+	4+	4+	3+	1+	N	N
HeLa	24th h	1+	1+	1+	N	N	N	N	N
	48th h	4+	4+	1+	N	N	N	N	N
	72nd h	4+	4+	2+	N	N	N	N	N
Murine Neuroblastoma -2A	24th h	1+	N	N	N	N	N	N	N
	48th h	3+	N	N	N	N	N	N	N
	72nd h	4+	N	N	N	N	N	N	N
BHK-21	24th h	4+	3+	1+	N	N	N	N	N
	48th h	4+	4+	3+	N	N	N	N	N
	72nd h	4+	4+	4+	N	N	N	N	N
RD	24th h	3+	2+	1+	N	N	N	N	N
	48th h	4+	4+	4+	3+	N	N	N	N
	72nd h	4+	4+	4+	4+	1+	1+	N	N
L20B	24th h	N	N	N	N	N	N	N	N
	48th h	N	N	N	N	N	N	N	N
	72nd h	N	N	N	N	N	N	N	N

4+-Destruction of 75-100% of cells; Lf- Limes flocculation; 3+-Destruction of 50-75% of cells; N-Normal cells; 2+-Destruction of 25-50% of cells, 1+-Destruction of less than 25% of cells

Test procedure for cytotoxicity

Cells were added in the wells of microtitre plate (0.1 ml/well) followed by 0.1 ml of various dilutions of diphtheria toxin (1 Lf, 0.1 Lf, 0.01 Lf, 0.001 Lf, 0.0001 Lf, 0.00001 Lf, 0.000001 Lf & 0.0000001 Lf) in the appropriate wells. Triplicate cultures were prepared for each test dilutions. Then the plates were incubated at 37°C in CO_2 incubator at 4% CO_2 atmosphere and examined microscopically after 24 h, 48 h and 72 h. The degree of toxicity in individual cell cultures were evaluated using a scale based on the ratio of destroyed to intact cells. Destruction of 75-100% of cells was classified as 4+, 50-75% as 3+, 25-50% as 2+ and less than 25% as 1+.

Results and discussion

Purified diphtheria toxoid is the immunizing antigen that gives protection against the adverse effects of diphtheria infection. It is prepared from the toxin produced during the growth of a standard strain of *Cornyebacterium diphtheriae* in a suitable medium. The test for sterility was intended for detecting the presence of viable form of microorganisms in or on pharmacopoeial preparations. The media used for the sterility test were ATM for detecting aerobic and anaerobic bacteria and SCDM for detecting yeast and fungi. The present study showed that the glycerinated diphtheria toxin used for the study was sterile. The Limes flocculation unit for the diphtheria toxin was 55 Lf/ml and the Kf was found to be 7 min (Table 1).

The estimation of protein-nitrogen in the toxin sample is one of the important in-process tests. The antigenic purity of the toxin is calculated on the amount of protein-nitrogen present in the sample. With a given quantity of Lf/ml, the antigenic purity is better with a low protein-nitrogen and vice-versa. The protein-nitrogen content of the sample was 0.02968 mg/ml and the purity of the toxin was 1853 Lf/mg of protein-nitrogen. The present study focused to evaluate the cytotoxic effects of diphtheria toxin in mammalian cell lines comparable to the erythematous reaction in guinea pigs at the acceptable criteria of less than 10 mm (Stainer & Scholte, 1973). Diphtheria toxin at different concentrations (1 Lf, 0.1 Lf, 0.01 Lf, 0.001 Lf, 0.0001 Lf, 0.00001 Lf, 0.000001 Lf &

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0.0000001 Lf) inoculated on the depilated skin of the guinea pig and the animal observation after 48 h was shown in the Table 2. Animal inoculated with the toxin at different concentrations (1 Lf, 0.1 Lf, 0.01 Lf & 0.001 Lf) showed erythema around the site of inoculation after 48 h.

The cytotoxic effects of diphtheria toxin in 24, 48 and 72 h cultures of Vero, MA104, HeLa, Murine Neuroblastoma-2A, BHK-21, Rhabdomyosarcoma (RD) and L20B were shown in Table 3. Although susceptible cell lines showed similar morphological changes when exposed to toxin, certain minor differences were observed. The cytopathogenic effect of diphtheria toxin on HeLa cell culture is due to the inhibitory action of the toxin on incorporation of methionine in protein synthesis (Strauss & Hendee, 1959). Cell lines like rabbit kidney, HeLa, HEp-2 and monkey kidney cells were susceptible to the diphtheria toxin (Lennox & Kaplan, 1957). The cytotoxic dose depends on the number of toxin binding surface receptors per cell (Middlebrook & Dorland, 1977). Among the cell lines Vero, MA104, Rhabdomyosarcoma, BHK-21 and HeLa were highly susceptible to diphtheria toxin even at low concentration (0.00001 Lf/ml), whereas Murine Neuroblastoma-2A, L20B did not show any cytotoxic effects with the same concentration of toxin.

Diphtheria toxin has the capacity to block protein synthesis in cultured mammalian cells, and thus causing cell death. From the present study it was concluded that Vero, MA104, Rhabdomyosarcoma (RD), BHK-21 and HeLa cell lines may be used as an alternative to *in vivo* assay for the toxicology study of diphtheria toxin and suggesting further studies are required for the standardization of the in vitro assay. *In vivo* assay requires guinea pigs, maintenance of animal house, animal attendants, animal feed, involving high costs and hence the *in vitro* assay could reduce the above costs and ethical issues.

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