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Acute toxicity and cytotoxicity of *Bacillus thuringiensis* and *Bacillus sphaericus* strains on fish and mouse bone marrow

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Abstract The insecticidal properties of delta-endotoxins from *Bacillus thuringiensis* (Bt) serotypes *kurstaki* and *israelensis* and crystal proteins of *Bacillus sphaericus* (Bs) serotype H5 have been used in insect control for decades. The availability of microbial toxins in biopesticides as well as in plants with incorporated protection has been increasing the concerns about biosafety. Acute toxicity to *Danio rerio* and cytotoxicity on mouse bone marrow cells and peripheral erythrocytes of *Oreochromis niloticus* were tested with Bt *israelensis*, Bt *kurstaki* and Bs H5 strains. The concentration and dose tested were 10^6 and 10^8 spores/ml, respectively. Neither lethality nor effects on mouse bone marrow were promoted by any strain. In necrosis–apoptosis study on peripheral erythrocytes of *O. niloticus* an increased frequency of necrotic cells caused by exposure to strains of *B. thuringiensis* was found. Exposure to *B. sphaericus* did not show cytotoxic effects in either tested system. None of the strains studied induced apoptosis in contrast with the chemical controls.

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Introduction

The insecticidal properties of toxins from *Bacillus* have been used in insect control for a long time. The first commercial *Bacillus thuringiensis* product was produced in France in 1938 (Kumar et al. 1996). Advances in genetic engineering in recent years have led to the development of plants that present resistance to some insects through incorporation and expression of genes encoding δ-endotoxins from the bacterium *B. thuringiensis.* A number of plant species, particularly crops, such as cotton, corn, potatoes, tobacco, tomato, and sugarcane have been modified to produce δ-endotoxin proteins from *B. thuringiensis* (Prieto-Samsonov et al. 1997; Mendelsohn et al. 2003; Romeis et al. 2006; OECD 2007). Thus, the concern about exposure to microbial toxins from plants with incorporated protection as well as to biopesticides has been raised, due to potential adverse effects on non-target species in the environment, including aquatic ecosystems.

The toxicological database on *B. thuringiensis* shows no mammalian health effects attributable to δ-endotoxins (McClintock et al. 1995a, b). Short term feeding/toxicity studies on poultry, pigs, calves, and cattle also provided additional information on the behavior of Cry1Ab protein in the gastro intestinal tract. Cry1Ab was not completely degraded in the gastro-intestinal tract and fragments of the gene and/or immunoreactive protein fragments were still present in the intestinal content and in the feces, but no residual DNA/protein could be found in animal tissues nor in the peripheral blood, nor was any risk associated with these findings (Jennings et al. 2003; Chowdhury et al.

2003; Einspanier et al. 2004; Lutz et al. 2005; OECD 2007).

Bacillus sphaericus is a naturally occurring soil bacterium and that can effectively kill mosquito larvae. *B. sphaericus* spores that are eaten by mosquito larvae release toxins into the mosquito gut, causing the larvae to stop feeding and die. *B. sphaericus* is only effective against feeding larvae, and does not affect mosquito pupa or adults. No measurable health effects were seen in laboratory animals that were exposed to larvicidal concentrations of *B. sphaericus* by multiple routes of exposure. The larvicide property of *B. sphaericus* consists of two proteins of 51 and 42 kDa. Within the midgut the 51 and 42 kDa proteins are converted to 42 and 39 kDa proteins, respectively, resulting in a major toxicity (Baumann et al. 1991).

Oliveira-Filho (2007) presents an interesting review about the ecotoxicity of bioinsecticides indicating absence of adverse effects on aquatic and terrestrial species. But the release of microbial toxins in the environment constantly generates the need for more data on biosafety, and consequently new toxicological evaluations should be undertaken to support the risk assessment of each new strain and toxin discovered.

Bone marrow is a highly vascularized tissue, and it contains a population of rapidly cycling cells that can be readily isolated and processed. The kinetics of bone marrow cells' progression can be measured through the ratio between polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE), which is about 1:1 in the young adult mouse. PCE is a transient cell precursor of NCE (mature erythrocyte). Declines in PCE frequency compared with NCE after 24 h of exposure to a toxic chemical compound means inhibition of the kinetics of cell progression, which can be recognized as cytotoxicity (Schmid 1975, Salamone and Heddle 1983; OECD 1997). The cell viability assay is recommended to study cell death induced by toxin exposure.

Cell viability assessment based on the double-stain with fluorescent DNA-binding acridine orange/ethidium bromide (AO/EtBr-1:1) allows viable cells to be distinguished from necrotic and apoptotic cells. Cell death caused by an exogenous damage will result in necrosis. Otherwise, cells with DNA severely damaged are arrested at the G_1 phase. If the DNA repair process fails, the p53 gene is activated and initiates apoptosis (Cotran et al. 1999; Müllauer et al. 2001; Golstein and Kroemer 2007; Puttonen et al. 2008).

The aim of this study was to carry out an investigation on the acute toxicity and cytotoxicity of strains of *B. thuringiensis* serotypes *kurstaki* and *israelensis* and *B. sphaericus* serotype H5 on the survival of *Danio rerio,* and on cell viability of peripheral blood erythrocytes of *Oreochromis niloticus* and cell proliferation of mouse bone marrow.

Material and methods

Bacillus strains tested

Three Brazilian entomopathogenic strains were used in this study. Each one represented a different serotype: *B. thuringiensis* serotype *kurstaki*, encoding Cry1Aa, Cry1Ab, Cry1Ac, and Cry1B proteins, toxic to lepidopteran larvae (Monnerat et al. 2007), *B. thuringiensis* serotype *israelensis,* encoding Cry4A, Cry4B, Cry11, and cyt1 proteins, toxic to dipteran larvae (Monnerat et al. 2005) and *B. sphaericus* serotype H5 encoding 51 and 42 kDa proteins, toxic to dipteran larvae (Monnerat et al. 2004). These strains were isolated from Brazilian soils and are stored at a collection of entomopathogenic *Bacillus* spp. of Embrapa Genetic Resources and Biotechnology, Brazil.

The strains were tested in a maximum hazard concentration (10^6 spores/ml) as proposed by USEPA (1996) for fish acute toxicity and 10^8 spores/ml for cytotoxicity studies. Concentrations were determined by serial dilutions after counting the number of spores in a concentrate of around 10^8–10^{10} spores/ml in NYSM medium (Silva et al. 2002).

Acute toxicity assay in *Danio rerio*

Thirty day static-renewal acute assays with zebrafish (*D. rerio*) were conducted as reported in a standardized protocol (USEPA 1996). Zebrafish used in this study was a wild type purchased from a local commercial supplier in Brasília. After a week of acclimatization on laboratory conditions, tests were performed in 3,000 ml beakers containing synthetic softwater (pH 7.5 ± 0.1, water hardness 40–48 mg l^{-1} as $CaCO_3$), maintained at 25 ± 1°C under a 16 h light/8 h dark cycle. Twenty fish were exposed in duplicate (10 per beaker) to each concentration (1×10^6 and 5×10^6 spores/ml^{-1}) of the tested strains. Testing solutions were replaced once a week and fish mortality was recorded daily until the 30th day.

Necrosis/apoptosis assay in *Oreochromis niloticus*

Oreochromis niloticus used in this study were obtained from a fish farm of the local municipality, where breeding conditions were controlled and monitored constantly. The criterion for fish selection was body length of 7–10 cm. Fish were acclimatized in the Genetics Laboratory of the University of Brasilia for a week in tanks of 250 l volume, with filtered and dechlorinated tapwater continuously aerated. Fish were maintained at a constant temperature of 25 ± 2°C and fed with fish chow. The ammonium level in the water was constantly monitored and the water was periodically renewed. Fish were randomly placed in other aquaria in groups of 7 and treatments were carried out

through intra-abdominal injection of 0.2 ml of the Bt *israelensis*, Bt *kurstaki*, and Bs H5, at a concentration of 1×10^8 spores/ml and observed for 72 h. For positive chemical control, fish were treated intra-abdominally with cyclophosphamide (CP) at 30 mg kg^{-1} body weight. CP (Genuxal–Asta Medica) is a well-known DNA alkylating agent commonly used as positive control in genotoxicity studies. For the apoptosis test, 0.1 ml of peripheral blood was obtained from cardiac puncture and diluted in 2.0 ml of fetal bovine serum at room temperature of 23°C. A smear of 15 µl of cell suspension was made immediately and 1 µl of AO/EtBr stain was added and the slides were covered with a coverslip. Slides were analyzed with a fluorescence Axioskop 2 Zeiss microscope with 1000× magnification using a wavelength of 510–560 nm. Around 500 peripheral erythrocytes were analyzed and classified as viable, necrotic or apoptotic.

Mouse bone marrow assay

Swiss mice from the Central Animal Facility of the University of Brasília were acclimatized to laboratory conditions for 1 week prior to the study. Males and females (10–12 weeks old), weighing 30 ± 2 g, were fed Purina mice chow and filtered water ad libitum. The negative control received filtered water (100 µl via gavage). They were housed at random, in groups of 6, dosed with test-solution of Bt *israelensis*, Bt *kurstaki*, and Bs H5 at a volume of 100 µl, via gavage for 24 h. Animals were sacrificed by cervical dislocation and the bone marrow preparations for polychromatic and normochromatic erythrocyte identification followed the protocol proposed by Schmid (1975). After smear, slides were fixed with methanol and stained with Giemsa. One thousand cells per animal were scored and classified as polychromatic (PCE) or normochromatic (NCE). The relationship between PCEs/NCEs was determined by the first 1,000 PCEs or NCEs counted.

Statistical analysis

Results of necrosis/apoptosis assay in *O. niloticus* were statistically analyzed by *t*-test for paired samples. Differences among treated and control groups of mice exposed to Bt and Bs-toxins were analyzed by *t*-test for paired samples. Both analysis were performed through computer software package SigmaStat 3.5.

Results

Neither mortality nor visible adverse effects were observed in *D. rerio* or *O. niloticus* exposed to tested concentrations of all bacterial strains. This observation supports that the lethal concentrations (LC_{50}) of these strains to these fish species are higher than 5×10^6 spores/ml.

In the necrosis–apoptosis study on peripheral erythrocytes of *O. niloticus*, an increased frequency of necrotic cells caused by exposure to strains Bt *israelensis* and Bt *kurstaki* was found (Table 1, *t*-test $P < 0.05$). On the other hand, exposure to Bs H5 did not cause cytotoxic effect in either tested system, compared with the controls (*t*-test $P > 0.05$). Thus, none of studied strains induced apoptosis, which means no genotoxicity. In contrast, fish exposed to cyclophosphamide, a positive chemical control, presented a statistically significant increased number of both necrotic and apoptotic cells (Table 1, *t*-test $P < 0.05$).

The data on cell cycle kinetics in mouse bone marrow cells shown in Table 2 indicate no effect on cell proliferation by the three strains tested Bt *israelensis*, Bt *kurstaki*, and Bs H5 compared to control, which means absence of

Table 2 Means and percentage of NCEs in mouse bone marrow cells, showing that for all treatments no cytotoxicity was found

Strains/treatments	Mean of NCE ± SD	% of NCE	*t*-Test (*P*)
Control	622 ± 95	38.3	–
Bt *kurstaki* 48 h	564 ± 72	36.0	0.5416
Bt *kurstaki* 96 h	617 ± 110	38.1	0.9564
Bt *israelensis* 48 h	722 ± 180	41.9	0.3909
Bt *israelensis* 96 h	578 ± 120	36.6	0.7163
Bs H5 48 h	441 ± 98	30.6	0.0676
Bs H5 96 h	475 ± 87	31.3	0.0734

* $P > 0.05$, no significant

Table 1 Percentage of viability, necrosis, and apoptosis in 500 analyzed cells from peripheral erythrocytes of *O. niloticus*

Treatments	Viable	Necrosis	Apoptosis	% Viability	% Necrosis	% Apoptosis
Bt *israelensis*	465.2 ± 68	36.0 ± 7	2.8 ± 1.1	93.4	7.2*	0.5
Bt *kurstaki*	473.8 ± 75	25.6 ± 6	2.1 ± 0.9	94.5	5.1*	0.4
Bs H5	477.2 ± 26	22.5 ± 9	0.5 ± 0.2	95.4	4.5	0.1
Water	483.0 ± 18	12.3 ± 5	4.6 ± 1.8	96.8	2.7	0.5
Cycloph.	443.3 ± 32	29.4 ± 9	27.0 ± 10	88.8	5.8*	5.4*

* $P < 0.05$, *t*-test significant

cytotoxicity. There was also no increased toxicity as a function of time, comparing results of 48 with 96 h of exposure. Our analysis was based in the higher variations of NCEs population in comparison with PCE population.

Discussion

The mode of action of Cry-toxins on susceptible insects is dependent on enzymatic activation after ingestion. The crystalline inclusions are dissolved and then converted to active toxins by insect proteases. The active Cry-toxins bind to specific receptor sites and produce pores in the insect gut which results in loss of homeostasis and septicemia, which are lethal to the insect (Broderick et al. 2006). There are no known equivalent receptor sites for binding of the δ-endotoxins in mammals (Noteborn et al. 1995; Gill and Ellar 2002; Broderick et al. 2006). The mode of action also appears to be insect specific due to the reliance of the lepidopteran midgut on unique ATPases for potassium influx regulation and the insect midgut's unique susceptibility to ionic stress (Knowles and Ellar 1986), plus the observations that even when Cry toxin binding site proteins are expressed in mammalian cells, the mammalian cells are unable to express them in a form that allows the toxins to bind to them (Keeton and Bulla 1997; Gill and Ellar 2002; Broderick et al. 2006). On the other hand, Mizuki et al. (1999) and Kim et al. (2000), reported in vitro studies on cytotoxicity of Bt-protein on human cells. After inclusion into the cytoplasm, these Bt-proteins were activated through an alkali-proteolytic process becoming highly cytocidal. This study was carried out with leukaemic T cells, Hela cells, and normal T cells, and the major cytocidal activity was found only on leukemic T cells.

In the mouse bone marrow assay, evidence of toxicity is given by reduction of %PCE (Salamone and Hiddle 1983). Population of PCE was not affected because the relationship between PCEs/NCEs determined by the first 1,000 PCEs or NCEs counted always showed more PCEs than NCEs. Thus, no bone marrow suppression was noted.

Apoptosis is a distinct form of cell death that proceeds along a genetically determined execution program. It is a form of cell death designated to eliminate unwanted cells in a tissue through activation of a coordinated, internally programmed series of events genetically controlled (Cotran et al. 1999). Exposure of cells to genotoxic agents, such as radiation and chemotherapeutic compounds induces apoptosis by a mechanism that is initiated by DNA damage. Apoptosis–necrosis analysis to study cell viability and mode of cell death induced by toxins using fluorescent double stain is rapid, repeatable and easy to perform. It allows the determination of the viability/death ratio and also it is possible to distinguish apoptosis from necrosis. Acridine orange reaches the nucleus of intact cells and binds both RNA and DNA, whereas ethidium bromide is generally excluded from those cells with an intact plasma membrane. If the membrane is broken due to a necrotic effect, the dye readily reaches the nucleus to intercalate with DNA. Dead cells, necrotic and late apoptotic cells have disrupted cellular membranes, which allow EtBr reach the nucleic acid in the nucleus. The incoming DNA-dye complex intensifies the level of fluorescence over that of the dye alone. In the early apoptosis only AO reaches the nucleus showing the fragmentation of chromatin and the apoptotic bodies, through green fluorescence given exclusively by AO. This method provides more realistic information about the mode of action of cell death (Puttonen et al. 2008). Necrosis is a cell death due to progressive degradative action of enzymes, characterized by denaturation of cytoplasmic proteins caused by exogenous injury. Necrosis can be started by non-specific external stimuli, such as ischemia, trauma, infection, cellular membrane receptor blockade or surface receptor damage (Cotran et al. 1999).

Fish treated with CP at 30 mg kg^{-1} presented necrotic as well as apoptotic effects, probably due to the high dose administered. Lower doses probably could cause more apoptosis than necrosis, because CP works as a clastogenic agent causing chromatin fragmentation. Probably, 30 mg kg^{-1} is above the apoptotic threshold dose. Brockmann et al. (2006) showed that apoptosis initiates at much lower doses than cytotoxic doses when cells are exposed to alkylating chemicals. Positive control is used to demonstrate the sensitivity of the test-system. No induction of apoptosis could mean that toxins from *B. thuringiensis* and *B. sphaericus* to not reach the cell nucleus causing DNA or chromatin damage. It should be pointed that cell death by necrosis involved a large population of cells due to the presence of the Bt and Bs-toxins in high concentrations, contrasting to apoptosis, which is an individual event. Thus, necrosis was observed due to an extremely invasive route for fish exposure.

Conclusions

This study showed that acute toxicity of *Bacillus* strains against fish were not observed in the concentrations tested, suggesting that LC_{50} s of these strains are higher than 5×10^6 spores/ml. On the contrast, by non-conventional routes of exposure (e.g., injection) Bt *israelensis* and Bt *kurstaki* toxins showed toxicity causing peripheral erythrocyte cells death by necrosis. Thus, our results are coherent with the toxicological mechanism of action of such toxins on target cells, which means binding with the cell receptor causing cell membrane disruption. In fish exposed through whole body, no toxicity was found confirming literature data that Bt and Bs have low toxicity to non-target aquatic species.

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