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CHO-MTT cytotoxicity screening test for bacillus toxins

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BROTH SUPERNATANTS FROM:
BACILLUS LICHENIFORMIS DSM13
BACILLUS CEREUS DSM4312
BACILLUS SUBTILIS XAS-1
BHIG BROTH

CHO-MTT CYTOTOXICITY SCREENING TEST FOR BACILLUS TOXINS

Sponsor

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The work performed in this study generally followed Good Laboratory Practice principles, however, no specific study-related Quality Assurance procedures were performed and the report may not contain all of the elements required by GLP. This is not considered to have compromised the scientific integrity of the study.

I consider the data generated by Huntingdon Life Sciences during the course of this study to be valid and that the final report fully and accurately reflects the raw data.

.....Susan Wilkins.....
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15 April 2003
Date

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SUMMARY

The Sponsor prepared bacterial broth (BHIG) supernatants from three strains of bacteria. These included Bacterial strains referenced as positive and negative for toxin production which were included as culture condition controls.

The culture supernatants were tested for the presence of toxins (enterotoxins or emetic toxins) using the CHO-MTT cytotoxicity test. The method detects toxin induced cell damage to CHO-K1 cells (Chinese Hamster Ovary, epithelial cell line) using the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay which is sensitive to both gross cytotoxicity (cell death) and reduction in mitochondrial activity. Doubling dilutions (50% to 0.781% broth in culture medium) of blank broth or test broth were applied to duplicate cultures of CHO cells. After 24 hours, viability/mitochondrial activity of the CHO cells was measured using the MTT assay. Absorbance values (proportional to mitochondrial activity) were expressed as % activity of control cultures (cells + culture medium).

From the dose response curves, IC_{50} values were calculated (the concentration of broth required to cause 50% reduction in activity). Since broth alone is expected to have some effect on the CHO cells, and this effect may vary from batch to batch, the toxicity of broths from bacterial cultures were compared to the toxicity of the relevant blank broth. The ratio of IC_{50} blank broth/IC_{50} test broth was calculated for each bacterial strain tested. Values of 2 or less indicate no toxicity above background, greater than 2 indicate the presence of toxin.

The results for toxin production from the Sponsor's culture controls were negative for the non-toxin producing strain *Bacillus licheniformis* DSM13 and positive for the toxin producing strain *Bacillus cereus* DSM4312, thus showing that culture conditions were suitable for the detection of toxin.

The result for the test strain *Bacillus subtilis* XAS-1 was negative for toxin production under the conditions described in this report.

INTRODUCTION

The objective of this cytotoxicity assay was to test for the presence of toxins (enterotoxins or emetic toxins) in supernatants from bacterial broth cultures (*Bacillus* species). The method detects toxin induced cell damage to CHO-K1 cells (Chinese Hamster Ovary, epithelial cell line) using the MTT assay which is sensitive to both gross cytotoxicity (cell death) and reduction in mitochondrial activity. The MTT assay technique (Mossman 1983) depends on the ability of viable cells to metabolise a water-soluble tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), into an insoluble formazan salt by the action of mitochondrial succinate dehydrogenase. The formazan produced is extracted and quantified by spectrophotometric measurement. The quantity of formazan produced is considered to be proportional to the number and/or metabolic activity of viable cells present in each culture.

The toxicity of filter-sterilised bacterial culture supernatant from the test strain was compared to the background toxicity of the broth alone. Broths from reference Bacillus strains (negative and positive for toxin production) were included as controls for both the bacterial culture method and the cytotoxicity assay.

Cell cytotoxicity assays were among the recommendations from the document: Opinion of the Scientific Committee on Animal Nutrition on the safety of use of *Bacillus* species in animal nutrition. (Expressed on 17 February 2000), published by the European Commission Health and Consumer Protection Directorate General.

The experimental phase of the study was undertaken between 7 and 9 April 2003.

TEST ARTICLES

Identity: Filtered BHIG broth supernatants from bacterial cultures, prepared and supplied by the Sponsor

Genus and Species	Sample Code	Additional information
Bacillus licheniformis	DSM13	ATCC 14580 (Negative strain) DS-number 03417 30C, RG 1 /17-03-2003
Bacillus cereus	DSM4312	(Positive strain) DS-number 38608 37C, RG 2 /17-03-2003
Bacillus subtilis	XAS-1	(Test strain) DS-number 47435 37C, VMT-rDNA /17-03-2003
Blank broth	BHIG	(Reference/blank broth) Brain Heart Infusion + 1% glucose

Appearance: Clear light brown liquids

Storage conditions: Frozen (ca -20°C)

Batch number: See additional information above

Expiry: Taken as 6 months from receipt/production

Purity: Not applicable

Date received: 27 March 2003

Supplier: DSM Food Specialties/Strain Conservation Unit

EXPERIMENTAL PROCEDURE

CHO-K1 STOCK CELL CULTURES

The cells used in this assay were the ECACC No 85051005 CHO-K1 cell line (Chinese Hamster Ovary, epithelial cell line. These cells were cultured in Ham's F12 medium with 2 mM L-glutamine, Penicillin (100 IU/ml)/Streptomycin (100 IU/ml) and supplemented with 10% foetal calf serum (FCS). Cultures were incubated at 37±1°C with a gaseous phase of 5% CO_2 in air.

Stock cells were stored frozen in cryotubes at -196°C under liquid nitrogen, in medium containing 5% dimethyl sulphoxide. Frozen vials of cells were thawed rapidly at 37°C and grown in tissue culture flasks in medium as described above. Actively growing cell stocks were maintained and expanded by subculturing (passage) using 0.05% trypsin/0.02% EDTA to harvest the cells. The passage procedure was repeated to provide a sufficient number of cells for a test.

BACTERIAL CULTURES/SUPERNATANT PREPARATION

Supernatants for use as internal assay controls were prepared at Huntingdon Life Sciences as described below.

Supernatants from the test strains of bacteria were prepared by the sponsor in BHIG broth (supplied frozen).

Aliquots of blank broth from each production were provided for background toxicity measurement.

Bacterial culture/supernatant production (HLS)

The broth used for bacillus cultures at HLS was brain heart infusion broth (Oxoid CM225) with 1% glucose (BHIG).

Bacterial seed stocks were stored frozen and were transferred to tryptone soya agar plates or slopes prior to use. For each bacterial strain, a single colony was transferred to 10 ml BHIG broth and cultured overnight at 32°C. Growth was confirmed by visual inspection of turbidity and an aliquot of overnight culture transferred to BHIG in the ratio of 1 ml culture to 50 ml broth in a 500 ml flask. Each culture was incubated with shaking at 32°C for 6 hours. The culture was then centrifuged at approximately 5000 x g for 10 minutes at 4°C, the supernatant harvested and filter sterilized using 0.2 µm filters. Aliquots of supernatant (and blank broth) were stored at -20°C prior to testing.

INTERNAL ASSAY CONTROLS

Negative for toxin production (reference Finlay et al 1999):
Bacillus licheniformis NCTC 6346, equivalent to ATCC 9800

Positive for diarrhoeagenic/enterotoxigenic toxin production (reference Beattie and Williams 1999):
Bacillus cereus NCTC 11145

Positive control for emetic toxin production (reference Beattie and Williams 1999):
Bacillus cereus NCTC 11143

CHO-MTT CYTOTOXICITY ASSAY

The supernatants supplied by the Sponsor were tested in a single assay in parallel with the internal assay controls.

The cells from flasks of actively growing CHO-K1 cultures were harvested and the number of viable cells determined by counting trypan blue-stained cell preparations using a haemocytometer. The cell suspension was then diluted with medium to provide 2 x 10^5 cells/ml and 100 µl aliquots plated out into flat-bottomed, 96-well microtitre plates (except for 2 wells which received medium only as reagent blanks). Plates were incubated at 37±1°C in a humidified atmosphere of 5% CO_2 in air, overnight.
The following day the test supernatants were thawed, mixed by vortexing and duplicate dilutions prepared in tissue culture medium in 96-well round-bottomed microtitre plates. After inspecting the CHO-K1 cells, 100 µl aliquots of test broth/broth dilutions were transferred from the dilution plates to the CHO-K1 culture plates. Since the culture plates already contained 100 µl medium over the cells, the test samples were diluted 1 in 2 on addition to the assay plates. The concentrations of test sample in the dilution plates and final concentration in the assay plates were as follows:

	Broth concentrations in duplicate wells	
Row	Dilution plate	Assay plate
A	Neat	50%
B	1 in 2	25%
C	1 in 4	12.5%
D	1 in 8	6.25%
E	1 in 16	3.125%
F	1 in 32	1.563%
G	1 in 64	0.781%
H	Medium control wells	

Each assay plate included duplicate dilutions of the relevant blank broth and the relevant negative culture control supernatant.

The treated plates were incubated for 24 ± 0.5 hours at 37 ± 1°C in a humidified atmosphere of 5% CO_2 in air.

On completion of the incubation period each plate was given a preliminary visual examination, then 50 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) 2 mg/ml was added to each well and the plates returned to the incubator for 3 hours ± 10 minutes. The medium was then replaced with 100 µl dimethyl sulphoxide (DMSO) and shaken for a minimum of 15 minutes to elute any formazan from the cells. The absorbance was read using a 96-well plate reader at 540 nm with a 620 nm reference filter.

ASSESSMENT OF RESULTS

The absorbance value from each test well was expressed as a percentage of the mean medium control absorbance to give % viability. The concentration of broth which inhibited viability by 50% (IC_{50}) was calculated from dose response curves using SAS statistics software. The toxicity of the test broth was compared to the toxicity of the relevant blank broth (the same broth batch as used for the bacterial cultures) by calculating the ratio IC_{50} blank/IC_{50} test broth. A greater than two-fold increase in toxicity over the background of the blank broth was taken as a positive result (ratios less than or equal to 2 indicating no toxicity above background and ratios greater than 2 indicating toxicity).

The results for an assay were considered valid if the ratio IC_{50} blank broth/IC_{50} test broth of the in-house negative control (*Bacillus licheniformis* NCTC 6346) was less than or equal to 2, and the results for the in-house positive controls (*Bacillus cereus* NCTC 11145 or *Bacillus cereus* NCTC 11143) were greater than 2.

ARCHIVES

All raw data, samples and specimens arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen that are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences, Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain a copy of the final report in its archive indefinitely.

RESULTS

Absorbance values (proportional to mitochondrial activity/viability) were expressed as a percentage of medium control values and are shown in the following tables. The concentration of broth required to cause 50% reduction in viability (IC_{50}) was calculated from each dose response curve (Appendix 1). Each assay plate contained the relevant blank broth and culture negative control broth. The ratio of IC_{50} blank broth/ IC_{50} test broth was calculated for each control or test broth. Values of 2 or less indicate no toxicity above background, greater than 2 indicate the presence of toxin. Each Broth sample were graded as positive or negative for toxin production.

HLS assay acceptance controls

	% Viability (abs values as % medium control values)							
	HLS Broth		HLS 6346		HLS 11143		HLS 11145	
% Broth	blank broth		Neg con		Pos con (emetic)		Pos con (entero)	
50.0	24.5	22.6	20.8	20.2	0.0	0.0	0.0	0.0
25.0	69.3	65.5	54.0	55.3	1.6	0.9	0.2	0.4
12.5	83.7	83.1	88.7	79.8	6.1	5.1	4.3	4.3
6.25	82.1	81.7	86.8	82.9	12.8	11.8	7.4	7.8
3.125	84.8	108.1	90.9	88.8	25.1	23.2	12.5	13.4
1.563	96.3	84.8	94.5	93.4	47.9	48.8	32.3	32.2
0.781	98.1	93.4	98.6	97.8	84.7	84.1	72.7	70.6
IC50 (%)	32.60		27.40		1.50		1.07	
Broth/test	N/A		1.19		21.60		30.40	
POS/NEG			NEG		POS		POS	

Samples from Sponsor

	% Viability (abs values as % medium control values)							
	Ref. broth		DSM 13		DSM 4312		XAS-1	
% Broth	blank BHIG (Sponsor)		Neg con (Sponsor)		Pos con (Sponsor)		Test (Sponsor)	
50.0	41.2	38.9	41.6	43.0	4.2	4.6	29.2	12.3
25.0	79.1	79.3	68.4	68.6	4.4	4.1	69.6	65.3
12.5	94.8	88.5	86.4	84.7	5.7	5.3	88.5	85.4
6.25	107.0	91.7	100.1	97.0	8.5	7.0	98.8	95.2
3.125	101.7	92.0	97.0	96.1	15.2	12.0	98.8	95.9
1.563	105.6	93.9	98.4	91.9	26.5	25.6	94.0	96.9
0.781	105.7	95.8	104.2	97.7	37.7	35.7	93.0	97.4
IC50 (%)	42.70		41.10		< 0.78 (~0.414)		31.90	
Broth/test	N/A		1.04		~103		1.34	
POS/NEG			NEG		POS		NEG	

CONCLUSION

The results for the internal assay controls showed no toxin production for the reported negative strain *Bacillus licheniformis* NCTC 6346 and indicated toxicity for the reported toxin producing strains *Bacillus cereus* NCTC 11145 (enterotoxigenic) and *Bacillus cereus* NCTC 11143 (emetic). Therefore the results for the assay were considered valid.

The results for toxin production from the culture controls supplied by the sponsor were negative for the non-toxin producing strain *Bacillus licheniformis* DSM13 and positive for the toxin producing strain *Bacillus cereus* DSM4312, thus showing that culture conditions by the Sponsor were suitable for toxin detection.

The result for the test strain *Bacillus subtilis* XAS-1 was negative for toxin production under the conditions described in this report.

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APPENDIX 1

IC_{50} values – Statistics Table

Plate number	Broth Source	Treatment name	IC_{50}		Ratio of Broth alone	
			Estimate	95% C.I.	Estimate	95% C.I.
1	HLS	Broth alone	32.6	28.2,37.6		
1	HLS	11143	1.5	1.34,1.7	21.6	17.7,26.5
1	HLS	11145	1.07	0.998,1.15	30.4	26.2,35.3
1	HLS	6346	27.4	24.6,30.6	1.19	0.995,1.42
2	Sponsor	Broth alone	42.7	38.2,47.7		
2	Sponsor	DSM 13	41.1	36.7,46.1	1.04	0.884,1.22
2	Sponsor	DSM 4312	0.414	0.301,0.57	103	68.1,156
2	Sponsor	XAS-1	31.9	29.1,35.1	1.34	1.16,1.54

C.I. = Confidence Interval