MALAYSIAN MOSQUITOCIDAL SOIL BACTERIUM (*BACILLUS THURINGIENSIS)* STRAINS WITH SELECTIVE HEMOLYTIC AND LECTIN ACTIVITY AGAINST HUMAN AND RAT ERYTHROCYTES

VD Nadarajah [1], SH Chai [2], SM Mohammed[1], KK Chan[2] and K Kanakeswary[2]

[1]Human Biology Section, International Medical University, Kuala Lumpur; [2] IMU Research Laboratory, School of Postgraduate Studies and Research, International Medical University, Kuala Lumpur, Malaysia

Abstract. The objective of this study is to determine the role of carbohydrates on the toxic effect of parasporal inclusion proteins isolated from Malaysian mosquitocidal *Bacillus thuringiensis* (Bt) strains on erythrocytes (human and rat). Dose response analyses on the effect of these parasporal inclusions on human and rat erythrocytes suggest that toxin action is selective depending on bacterial strains and source of erythrocytes. Results from this study suggest Bt toxin is a lectin which recognizes specific plasma membrane glycoconjugate receptor(s) with a terminal residue of either D-mannose (Man), N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc) or even a combination of these monosaccharides.

INTRODUCTION

Bacillus thuringiensis (Bt) is found worldwide in many habitats, including soil, insect cadavers, stored-products, and deciduous and coniferous leaves (Bernhard *et al,*1997). Bt has a two-phase growth cycle, namely a vegetative and a stationary phase. When nutrients are abundant it grows vegetatively, but when the food supply runs short, it makes a dormant spore with one or more large crystalline parasporal inclusion proteins. These parasporal inclusions contain proteins, called δ-endotoxins (Heimpel, 1967), which are lethal when eaten by susceptible insect(s). The presence of parasporal inclusions readily distinguishes Bt from other spore forming bacilli, including the closely related *Bacillus cereus.* The insecticidal activity of Bt has made it a useful alternative or supplement to synthetic chemical pesticide application in commercial agriculture, forest management, and mosquito control (Lambert and Peferoen, 1992).

Correspondence: Dr Vishna Devi Nadarajah, International Medical University, Plaza Komanwel, 57000, Kuala Lumpur, Malaysia.
Tel: 03-5882 1481 ext 1156; Fax: 03-8656 7229
E-mail: vishnadevi_nadarajah@imu.edu.my

A nationwide screening program for microbial control agents for mosquitoes was initiated in Malaysia in 1986 and 20 Bt strains which produced larvicidal isolates were obtained. Out of these 20 Bt strains, a new Bt strain, named as subspecies *malaysianiensis,* was discovered (Lee and Seleena, 1990). Another subspecies of Bt, called *jegathesan*, was discovered in 1995 (Michael *et al,* 1995). The toxins from this new subspecies displayed mosquitocidal toxicity comparable to that of toxins from Bt subsp *israelensis* (Bti) which was intensively used in controlling blackfies in West Africa. The diversity of Malaysian strains was expanded with the discovery of the *cry16A* gene (a member of Bt Cry family of genes), which encodes the insecticidal Cry16A toxins in *Clostridium bifermentans,* isolated from the Malaysian soil.

Recently, the cytotoxic effect of Bt parasporal inclusions against cancer cells has been demonstrated (Lee *et al*, 2001; Okumura *et al*, 2004; Katayama *et al*, 2005). Earlier studies (Yokoyama *et al*, 1988, 1991) have shown that a 25 kDa parasporal inclusion from Bti has cytotoxic effects to cultured mammalian tumor cells and enhanced the effect of some antitumor agents (Bleomycin) at even non-toxic doses.

Mizuki *et al* (1999) reported the existence of a family of parasporal inclusions from Bt with potent anti-cancer activity. Lee *et al* (2001) suggested that the cytotoxic mechanisms of the anti-cancer parasporal inclusions resemble that of the Cry proteins, which is dependent on binding to receptor(s) on the cell membrane. It has been shown that the binding of the insecticidal Cry proteins to its putative receptor(s) is mediated by sugars (Jenkins *et al*, 2000). Akao *et al*, (2001) has reported that parasporal inclusions from Bt strain IBC-1456 (non insecticidal to lepidopteran larvae but specifically toxic to human cancer cells), recognizes D-galactose on the rabbit erythrocytes and N-acetyl-D-galactosamine (GalNAc) on sheep erythrocytes. By studying the toxin effect on erythrocytes, the authors have been able to show the types of monosaccarides that are able to inhibit toxin activity on the erythrocytes.

The aim of this study was to elucidate whether carbohydrate recognition plays a role in the toxin activity of parasporal inclusions from Malaysian mosquitocidal Bt strains on erythrocytes (human and rat). These Malaysian Bt strains are of interest because preliminary studies have shown that several of them have cytotoxic effects on leukemic cells. The role of carbohydrates on the toxic effects of these parasporal inclusions will be investigated through the hemolytic inhibition test (HIT).

MATERIALS AND METHODS

Bacterial strains and growth conditions

The Bt strains used in this study were Bt 1, Bt 2, Bt 4, Bt 7, Bt 8, Bt 9, Bt 10, Bt 18, Bt 19, Bt 20, Btj and IPS-82. They were from the Institute for Medical Research (IMR) Bt collection (Ms P Seleena and Dr Lee Han Lim). The Bt subtypes were determined using H antigen serotyping (Pasteur Institute) and have been shown to have larvicidal activity against *Aedes aegypti* (P Seleena and Dr Lee Han Lim, IMR, personal communication).

The Bt strains were grown on nutrient agar plate, pH7 at 30ºC until sporulation was complete (approximately 48-72 hours). A loopful of a sporulated Bt strain was placed in 0.5 ml sterile dH_2O in a 1.5 ml microcentrifuge tube and vortexed vigorously to disperse any clumps before heating in a waterbath at 75ºC for 30 minutes to kill vegetative cells and activate spores for germination. The spore-crystal suspension was allowed to cool to room temperature before being transferred into a 250 ml preaerated and prewarmed nutrient broth medium, pH7 in a 500 ml flat-bottom flask. This culture was then incubated at 30ºC with constant shaking at 150 rpm until greater than 95% free-phase-bright spores were produced (approximately 48 hours). 1M NaCl (w/v) was added to the culture to osmotically lyse the bacterium to release the spores and parasporal inclusions. The nutrient broth culture was then centrifuge at 13,000*g* for 10 minutes at 4ºC. The resultant spore-inclusion pellet was washed once with 1M NaCl (v/v), twice with ice-cold dH_2O and resuspended in an appropriate volume of Tris/KCl buffer (50 mM Tris/HCl, 10 mM KCl) before being stored at -20ºC for further use.

Solubilization and activation of Bt toxin

The spore-inclusion mixture of Bt was allowed to thaw at room temperature (approximately 2-3 minutes). These spore-inclusion mixtures were solubilized with freshly prepared 50 mM Na_2CO_3/HCl (pH10.5) buffer containing 10 mM DTT at 37ºC in a water-bath for 1 hour. Insoluble materials were sedimented by centrifugation at 13,000*g* for 5 minutes at 4ºC. The soluble parasporal inclusion was activated with either trypsin or proteinase-K [1:10 proteases to parasporal inclusion ratio (v/v)] at 37ºC for 1 hour. This was followed by further centrifugation at 13,000*g* for 5 minutes at 4ºC and the resultant supernatant was designated as activated parasporal inclusion. This activated product was filter sterilized in a 0.2 μm syringe filter and stored in -20ºC until further use.

Determination of protein concentration and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Bradford (1976) using a Bradford reagent and BSA as the standard was used to determine the protein concentration of solubilized, activated and sterilized parasporal inclusions. Solubilized and activated Bt parasporal inclusions were analysed using SDS-

PAGE in 10% polyacrylamide gel according to the modified method of Laemmli and Favre (1973) as described by Thomas and Ellar (1983). All gels consisted of a 4% (w/v) acrylamide stacking gel and a 10% (w/v) resolving gel.

Preparation of erythrocytes and erythrocyte degradation level

Human and white rat blood were used in this study. Human blood was obtained from a volunteer regardless of gender, blood group, age or medical condition. White rats were obtained from the Institute for Medical Research regardless of gender or age. Five milliliters of erythrocytes were mixed with EDTA and kept on ice before spinning at 1,400*g* for 5 minutes at 27ºC. The resultant supernatant (serum) was removed and the pellet was washed three times with TBS (20 mM Tris-buffered saline, pH7.8) by centrifugation under the same conditions as above. The erythrocytes were suspended in TBS at a concentration of 2% (v/v) and stored at 4ºC until further use. To determine erythrocyte degradation, 20 ml of 2% erythrocytes was prepared (and spun at 1,400*g* for 5 minutes at 27ºC) and harvested over time (0, 2, 8, 24, 48, and 72 hours). The resultant supernatant was measured at 540 nm (spectrophotometer uv-vis Auto, USA) to check the erythrocyte hemolysis level. All experiments were performed in duplicate.

Hemolytic assay (HA)

The hemolytic assay was performed using a modified method of Yu *et al* (1991). Parasporal inclusions from respective Bt strains (Bt 1, 2, 4, 7, 8, 9, 10, 18, 19, 20, j and IPS-82) were freshly solubilized and activated before each hemolytic assay. The activated parasporal inclusion was incubated with 2% erythrocytes for 1 hour at 27ºC. The reaction was stopped by centrifugation at 3,000*g* for 3 minutes at 4ºC. The hemolytic activity of the Bt parasporal inclusion was determined by measuring the absorbance of supernatant at 540 nm. All experiments were performed in duplicate.

Hemolytic inhibition test (HIT)

Bt 1, 2, 4, 7, and j hemolytic to either human, rat or both types of erythrocytes were selected for this study. Parasporal inclusions from Bt 1, 2, 4 and 7 were solubilized and activated with trypsin whilst Btj was solubilized and activated with proteinase-K as described earlier. Parasporal inclusions from Bt 1, 4, 7 and j were tested for a monosaccharide effect against human and rat erythrocytes whilst parasporal inclusions from Bt2 were tested against human erythrocytes only.

The seven monosaccharides (sugars) used in this study were: D-glucose (Glc), D-mannose (Man), D-galactose (Gal), L-fucose (Fuc), N-acetylgalactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc) and N-acetylneuraminic acid (NeuNAc). These sugars were solubilized in TBS (20 mM TBS, pH7.8). Human and rat erythrocytes were washed three times with TBS (20 mM TBS, pH7.8) by centrifugation at 1,400*g* for 5 minutes at 27ºC. The erythrocytes were suspended in TBS at a concentration of 2% (v/v) and stored at 4ºC until further use.

The HIT utilized solubilized and activated (trypsin or proteinase-K) Bt parasporal inclusions, which was pre-incubated for 30 minutes at 27ºC with the same volume of either 50 mM (final concentration) Glc, Gal, Man, Fuc, GalNAc, GlcNAc or NeuNAc. 2% of human or rat erythrocytes (v/v) were incubated with the inclusion-monosaccaride mixture and this mixture was harvested (over 3 hours). The reaction was stopped via centrifugation (at 3,000*g* for 3 minutes at 4ºC). The inhibition of the hemolytic activity from selected Bt strains was determined by measuring the absorbance of supernatant at 540 nm. All experiments were performed in duplicate.

RESULTS

Bt culture and protein analysis

Different Bt strains replicate at different speeds, with some Bt strains growing faster than others. However, all the Bt strains (Bt 1, Bt 2, Bt 4, Bt 7, Bt 8, Bt 9, Bt 10, Bt 18, Bt 19, Bt 20, Btj and IPS-82) achieved greater than 95% free-phase-bright spores after 48 hours of incubation in nutrient broth, therefore, the Bt culture used in this study was harvested 48 to 72 hours after growing in nutrient broth.

Fig 1 shows the SDS-PAGE profiles of solubilized and trypsin activated parasporal inclu-

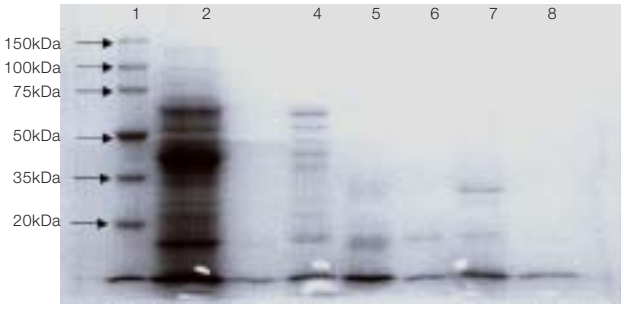
sions harvested from Bt 1, Bt 2, Bt 4, Bt 7, Bt 8 and Bt 9. The polypeptide profile of Bt 1 appears to be no different before or after solubilization and activation. There are ample polypeptide bands between 70 and 20 kDa. Bt 2 however showed a clearer polypeptide profile with bands between 60 and 20 kDa after solubilization and activation. Bt 4 and Bt 7 showed polypeptides with molecular weights of lesser than 20 kDa, whilst Bt 8 showed two major bands at 28 and 18 kDa. Bt 9 showed no clear bands under the given conditions.

Fig 2 shows the SDS-PAGE profiles for Bt 10, Bt 18, Bt 19, Bt 20, IPS82 and Btj after solubilization and activation with trypsin. As observed, Bt 10 shows a clear band with a molecular weight of lesser than 20 kDa, whilst Bt 18 shows polypeptides of molecular weights of 65 and 28 kDa, respectively. Bt 19 and 20 do not show clear bands, whilst IPS82 shows polypeptide profile of bands between 68 and 23 kDa, approximately. Btj showed a band of approximately 28 kDa. With the exception of Btj, all the other Bt strains showed a protein profile of lower than 25 kDa after treatment with proteinase-k (data not shown).

The results also show both human and rat erythrocytes will gradually degrade although kept at 4ºC (data not shown). The degradation process accelerates after 48 hours. Rat erythrocytes will degrade earlier human erythrocytes. Therefore, human and rat erythrocytes should only be used for a maximum of 24 hours after preparation.

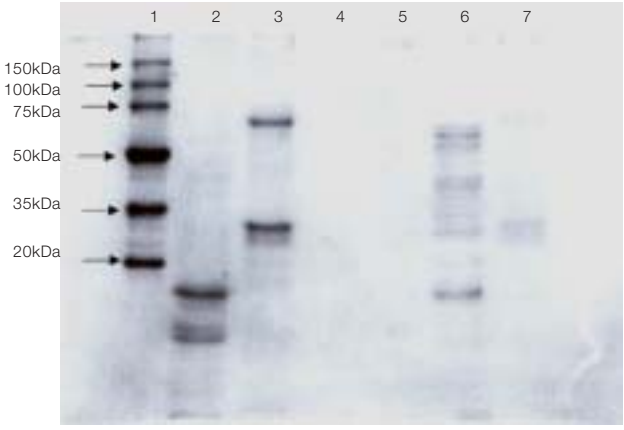
Hemolytic assay (HA)

Parasporal inclusions from 12 Bt strains (Bt 1, Bt 2, Bt 4, Bt 7, Bt 8, Bt 9, Bt 10, Bt 18, Bt 19, Bt 20, Btj and IPS-82) were selected to determine the hemolytic activity against erythrocytes. The selectivity of the Bt parasporal inclusions was assessed against human and rat erythrocytes. The hemolytic assay was performed using solubilized and activated (either trypsin or proteinase-K) Bt parasporal inclusions. The resultant supernatant was measured at 540 nm. The absorbance measured after the reaction was stopped for the respective Bt strains was divided by the standardized absorbance value, which is 0.4 nm (under the conditions tested, 100% erythrocyte lysis will



Parasporal inclusions from Bt 1, Bt 2, Bt 4, Bt 7, Bt 8 and Bt 9 were solubilized at a concentration of 0.2 mg/ml in 50 mM Na_2CO_3/HCl (pH10.5) buffer containing 10 mM DTT and later activated with trypsin at 1:10, proteases to parasporal inclusion ratio (v/v) at 37ºC for 1 hour. Twenty microliters of each sample was loaded onto 10% SDS PAGE gel, which was performed as described in the methods section.
Lane 1-perfect marker; lane 2-Bt 1; lane 4-Bt 2; lane 5 Bt 4; lane 6; Bt 7; lane 7-Bt 8; lane 8-Bt 9.

Fig 1–SDS-PAGE analysis of solubilized and activated Bt parasporal inclusions.



Parasporal inclusions from Bt 10, Bt 18, Bt 19, Bt 20, IPS 82 and Btj were solubilized at a concentration of 0.2 mg/ml in 50 mM Na_2CO_3/HCl (pH10.5) buffer containing 10 mM DTT and activated with trypsin at 1:10, proteases to parasporal inclusion ratio (v/v) at 37ºC for 1 hour. Twenty microliters of each sample was loaded onto 10% SDS PAGE gel, which was performed as described in the methods section.
Lane 1-perfect marker; lane 2-Bt 10; lane 4-Bt 18; lane 4 Bt 19; lane 5; Bt 20; lane 6-Bt 82; lane 7-Btj 9.

Fig 2–SDS-PAGE analysis of solubilized and activated Bt parasporal inclusions.

Table 1
Hemolytic activity of Bt parasporal inclusions with trypsin activation.

Trypsin activation		
Parasporal inclusions	Hemolytic activity	
	Human	Rat
Bt 1	+++	+++
Bt 2	+++	-
Bt 4	+++	+++
Bt 7	+++	+++
Bt 8	-	-
Bt 9	-	+
Bt 10	++	-
Bt 18	-	-
Bt 19	-	+++
Bt 20	-	++
Bt j	-	+++
IPS-82	-	+

0.2 mg/ml parasporal inclusions from various Bt strains was solubilized in 50 mM Na_2CO_3, 10 mM DTT, pH 10.5 and activated with 0.02 mg/ml trypsin. Two hundred mircoliters of the solubilized and activated inclusions from each Bt strain was incubated separately with 2% human or rat erythrocytes for 1 hour at 27ºC. The reaction was stopped by centrifugation at 3,000*g* for 3 minutes at 4ºC. The absorbance of the supernatant was measured at 540 nm.
+ indicated mild hemolytic activity (<50% hemolysis); ++ indicated moderate hemolytic activity (50% to 75% hemolysis); +++ indicated high hemolytic activity (>75% hemolysis); - Indicated non-hemolytic (<20% hemolysis).

Table 2
Hemolytic activity of Bt parasporal inclusions with proteinase-K activation.

Proteinase-K Activation		
Parasporal inclusions	Hemolytic activity	
	Human	Rat
Bt 1	+++	+++
Bt 2	++	-
Bt 4	-	+++
Bt 7	+++	+++
Bt 8	-	-
Bt 9	-	+++
Bt 10	-	+++
Bt 18	-	+
Bt 19	-	+++
Bt 20	-	-
Bt j	++	+++
IPS82	-	+++

0.2 mg/ml parasporal inclusions from various Bt strains was solubilized in 50 mM Na_2CO_3, 10 mM DTT, pH 10.5 and activated with 0.02 mg/ml proteinase K. Two hundred microliters of the solubilized and activated inclusions from each Bt strain was incubated separately with 2% human or rat erythrocytes for 1 hour at 27ºC. The reaction was stopped by centrifugation at 3,000*g* for 3 minutes at 4ºC. The absorbance of the supernatant was measured at 540 nm.
Solubilized and activated various Bt parasporal inclusions was used to determine the hemolytic activity against human and rat erythrocytes.
+ indicated mild hemolytic activity (<50% hemolysis); ++ indicated moderate hemolytic activity (50% to 75% hemolysis); +++ indicated high hemolytic activity (>75% hemolysis); - indicated non-hemolytic (<20% hemolysis).

show this absorbance value) and was calculated as a percentage:

$$\frac{\text{Absorbance}}{0.4\ \text{nm}} \times 100\% = \text{Hemolytic activity (\%)}$$

It is proposed that the higher the hemolytic activity induced by Bt parasporal inclusion, the higher the absorbance measured due to release of components in the erythrocyte's cytoplasm (mainly consisting of hemoglobin) into the supernatant. The treated erythrocyte cell population was compared with the control population, which was erythrocytes and TBS only, without the parasporal inclusions.

There were Bt strains (Bt 1, Bt 4, Bt 7 and Btj) that demonstrated hemolytic activity of both human and rat erythrocytes whilst other Bt strains (Bt 2, Bt 9, Bt 10, Bt 19, Bt 20, IPS-82) demonstrated hemolytic activity either of only human erythrocytes or rat erythrocytes (Table 1 and Table 2). Bt 4 only showed hemolytic activity against human erythrocytes after activation by trypsin, whilst Btj showed hemolytic activity only with proteinase-K treatment. Bt 8 was the

Table 3
Comparison of the effect of monosaccarides on hemolytic activity against (A) human and (B) rat erythrocytes.

	Effect of monosaccarides on the hemolytic activity of Bt parasporal inclusions (%) (90 minutes) against								
	(A) human					(B) erythrocytes of rat			
Sugar	Bt 1	Bt 2	Bt 4	Bt 7	Bt j	Bt 1	Bt 4	Bt 7	Bt j
D-glucose	-4.6	-0.8	0.0[a]	-4.6	-9.0	0.0[a]	0.0[a]	-26.1	-1.1
D-mannose	-67.5	-29.5	0.0[a]	-6.0	-5.8	0.0[a]	0.0[a]	-30.1	+3.4
D-galactose	+7.6	-15.4	0.0[a]	-6.2	-5.6	0.0[a]	0.0[a]	-26.4	+3.4
L-fucose	-1.0	+1.5	0.0[a]	+8.8	-8.2	+0.3	-1.0	-21.4	+1.0
N-acetyl-D-glucosamine	+0.9	+9.7	-1.3	-36.3	-0.3	-2.1	0.0	-12.3	-15.1
N-acetyl-D-galactosamine	-2.7	+1.2	0.0[a]	-6.9	-26.2	-9.5	-4.3	-29.6	-19.4
N-acetylneuraminic acid	-64.1	-35.6	-91.7	-35.8	-4.6	-39.5	-92.6	-39.3	-56.4

Bt parasporal inclusions was pre-incubated for 30 minutes in 27ºC with either 50 mM D-glucose, D-mannose, D-galactose, L-fucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine or N-acetylneuraminic acid. Two percent of human erythrocytes was added to the monosaccaride-parasporal inclusion mixture and further incubated in the same condition. After 90 minutes of incubation, the reaction was stopped via centrifugation at 3,000*g* for 3 minutes at 4ºC. The absorbance of the supernatant was taken at 540 nm.
- Indicates protection from hemolysis; + Indicates hemolytic activity was more than the control; [a] Indicates no protection.

Bt strain which was non-hemolytic against human or rat erythrocytes. Bt 18 showed mild hemolytic against rat erythrocytes only after treatment with proteinase-K.

The hemolytic inhibition test (HIT)

Bt 1, Bt 2, Bt 4, Bt 7 and Btj which are hemolytic against human or rat erythrocytes, were selected to conduct the HIT as described in the methods section. The seven monosaccharides used were D-glucose (Glc), D-mannose (Man), D-galactose (Gal), L-fucose (Fuc), N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-galactosamine (GalNAc) and N-acetylneuraminic acid (NeuNAc). In this study, selected Bt parasporal inclusions was preincubated with the respective monosaccharide prior to addition of the erythrocytes for further incubation. The inclusion-monosaccaride-erythrocyte mixture was then harvested at intervals. Figs 3 and 4 show the time course for Bt 1 and Btj. Similar experiments were repeated for Bt 2, Bt 4 and Bt 7 (data not shown).

Table 3 summarizes the results of the HIT study for Bt 1, Bt 2, Bt 4, Bt 7 and Btj after 90 minutes. The values given in Table 3 indicate the blocking effect (protection) of the monosaccharide on the hemolytic activity of the Bt inclusions compared to the control [control was assumed as 0% (control population consisted of Bt parasporal inclusions and erythrocytes only), no protection against hemolysis]. The lower the percentage of erythrocyte lysis compared to the control (indicated with a minus sign in Table 3) the higher the blocking effect of the monosaccharide towards Bt toxin, indicating more erythrocytes have been protected from hemolysis. There were instances, as shown in Table 3, when the monosaccarides had either no blocking effect (shown as 0%) or had higher hemolytic activity than the control (indicated with a plus sign).

The monosaccaride D-mannose, GlcNAc, GalNAc and NeuNAc, can block the hemolytic action of selected Bt strains on human erythrocytes (Table 3). Interestingly, the blocking effect of mannose was selective towards the parasporal inclusions for Bt 1 (Fig 1) and Bt 2, but not for Bt 4 or Bt 7 whilst GlcNAc had a blocking effect towards parasporal inclusions for Bt 7 only (Table 3). The blocking effect shown

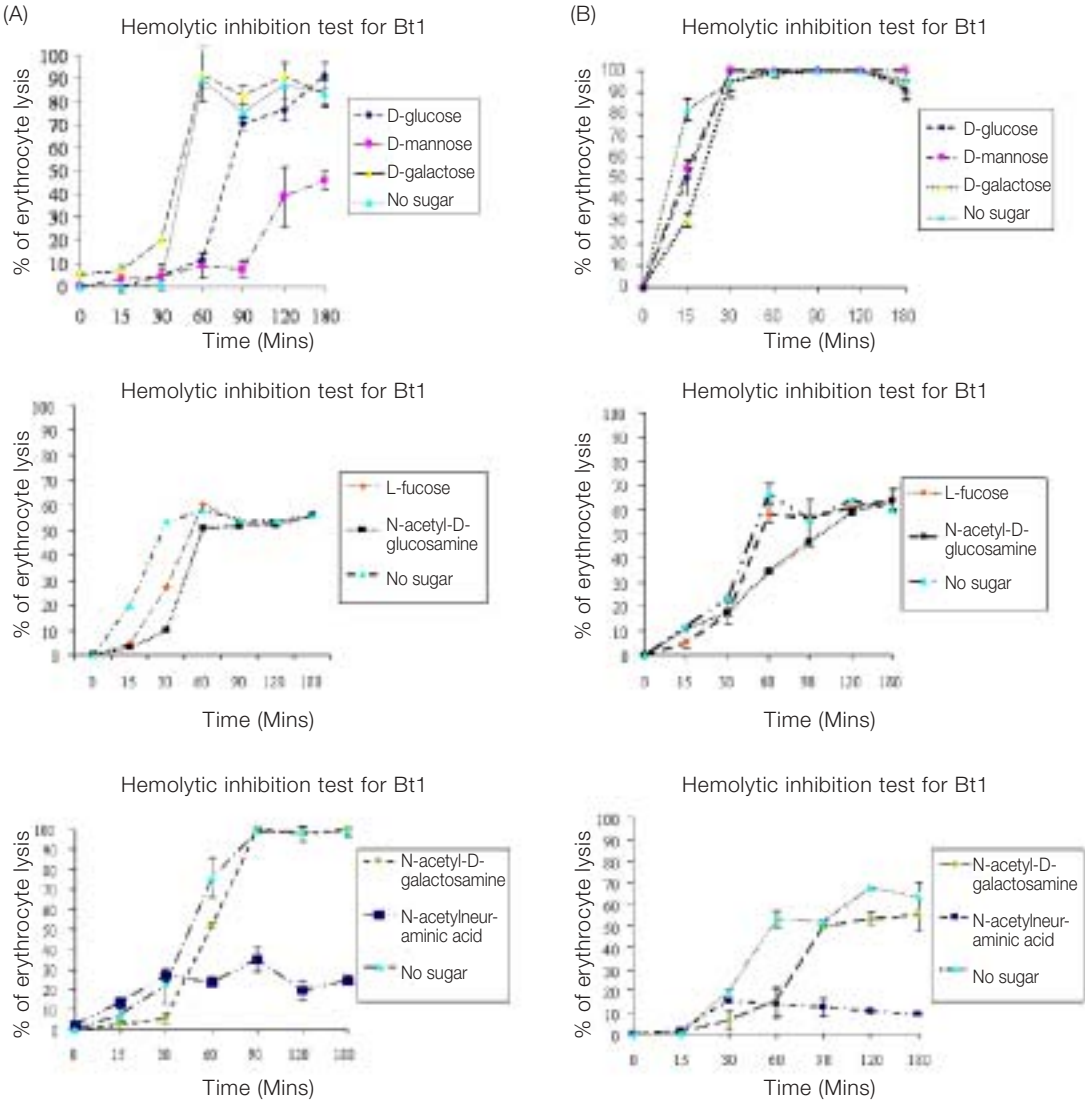
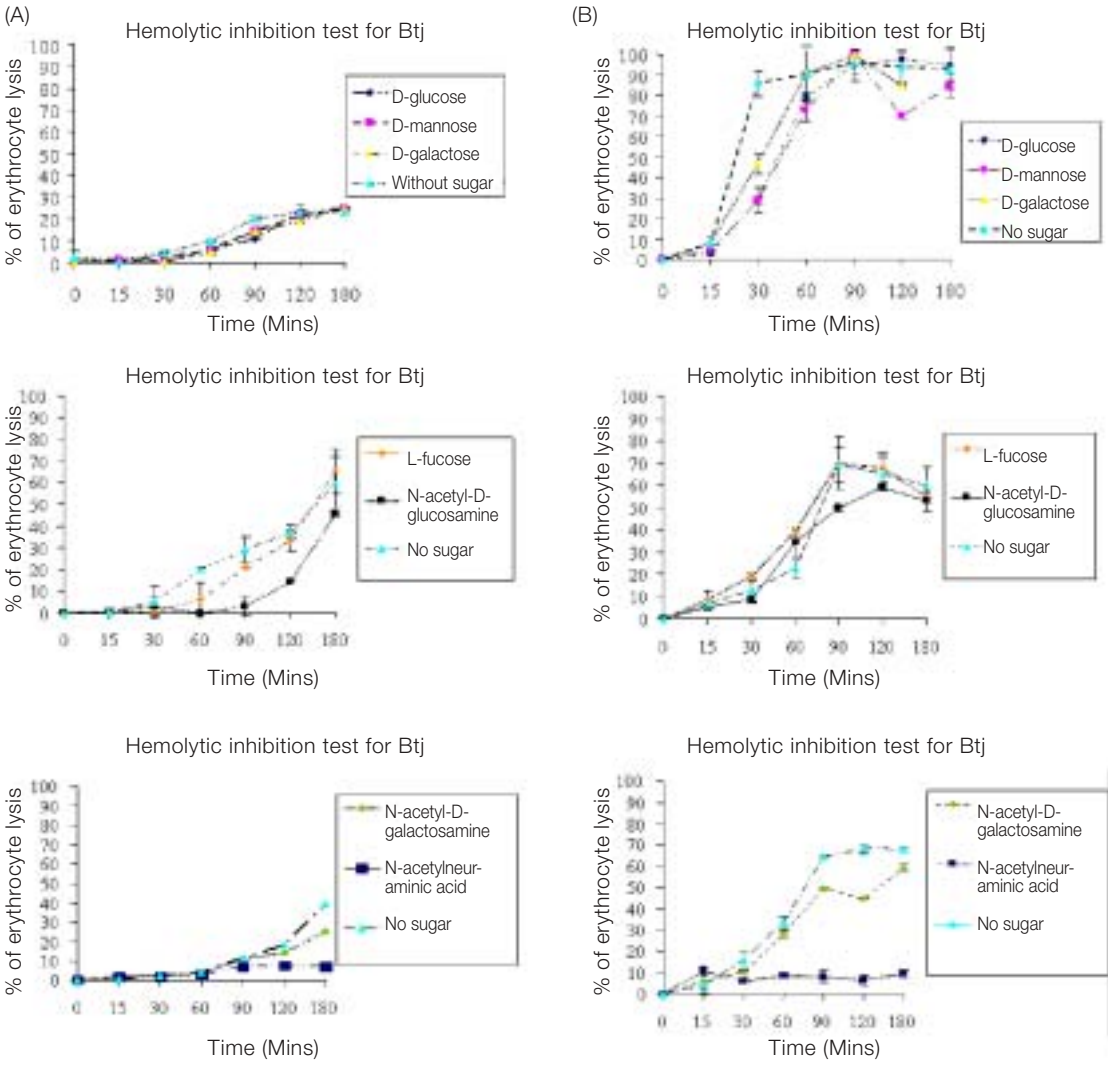


Fig 3–The effect of monosaccarides on Bt 1 hemolytic activity. The hemolytic inhibition test for Bt 1 was performed on (A) human erythrocytes and (B) rat erythrocytes. 76.7ug/ml of parasporal inclusion were incubated with L-fucose and N-acetyl-D-galactosamine, respectively, prior to incubation with erythrocytes. The effect of the monosaccarides on the hemolytic activity of the various strains were compared with the control (toxin and erythrocytes only).

by NeuNAc was found against all the Bt subtypes studied, except for the Btj parasporal inclusions (Fig 4). For Btj, GalNAc showed a blocking effect against hemolytic activity (Fig 4).

Interestingly, the carbohydrate blocking effects shown by D-mannose, GalNAc and GlcNAc on human erythrocytes treated with Bt 1, Bt 2 and Bt 4 does not happen in rat erythrocytes, except with Bt7 (Table 3). However, the blocking effect shown by NeuNAc acid was same in human and rat erythrocytes, except for Btj. For Btj, while there was no blocking in human erythrocytes by NeuNAc, there was an effect on rat erythrocytes (Fig 4).



DISCUSSION

Bt constitutes a large family of bacterial strains found in different habitats, some are highly specialized as insect pathogens (Wasano *et al,* 2000). Michael *et al* (1995) demonstrated that in the SDS-PAGE profile of the parasporal inclusion crystals from Malaysian soil isolates, Bt subspecies *jegathesan* (Btj) ranged from 23 kDa to 77 kDa after activation by chymotrypsin. All the Malaysian soil Bt isolates in this study demonstrated low molecular weight polypeptides ranging from 20 kDa to 75 kDa after activation by trypsin or proteinase-K. Trypsin activation of parasporal inclusions from Btj showed a band of approximately 28 kDa.

Alkaline-solubilized and protease activated parasporal inclusions of 12 Malaysian soil Bt isolates were examined for their hemolytic activity against human and rat erythrocytes. The hemolytic activity exhibited by the Bt strains in this study showed that it is dependent of the type of erythrocytes used. There were Bt strains (Bt 1, Bt 4, Bt 7 and Btj) that demonstrated hemolytic activity against both human and rat erythrocytes, whilst other Bt strains (Bt 2, Bt 9, Bt 10, Bt 19, Bt 20, IPS-82) demonstrated hemolytic activity either for human erythrocytes

or rat erythrocytes only. The choice of proteolytic enzyme used to activate the parasporal inclusions seem to play a role in determining the hemolytic activity, for example for Bt 4 and Btj.

Takaaki and Ohba (1994) demonstrated that three Bt isolates designated as 89-T-18-2, 89-T-18-8, and 89-T-18-20, Bt subspecies *israelensis* (Bti) and Bt subspecies *kyushuensis* demonstrated hemolytic activity against horse, sheep and human erythrocytes. However, the degree of activity varied depending on the bacterial strains and the source of the erythrocytes. The *israelensis* strain showed high hemolytic activity against all erythrocytes. In contrast, *kyushuensis* and the other three soil isolates had only moderate to low-level hemolytic activity. This is consistent with our findings, which show that different bacterial strains exhibited different hemolytic activities, being toxic against human erythrocytes, rat erythrocytes or both. The parasporal inclusion selectivity of erythrocyte-agglutination has been previously reported by Akao *et al* (2001) in which Bt strains showed selectivity for sheep erythrocytes but no toxic effect was seen to horse, cow, guinea pig or chicken erythrocytes. However, it was shown that the Bt isolate IBC-1456 has agglutination-selectivity towards both sheep and rabbit erythrocytes.

It has been proposed that the mosquitocidal strains, Bti and Bt subspecies *kyushuensis,* showed cytolytic activity, which is very likely attributable to the broad-spectrum cytolysins, Cyt1 and Cyt 2 proteins (Koni and Ellar, 1993). It also has been demonstrated that parasporal inclusions from Bt isolates contain hemolysins, which are immunogically related to the CytB toxin (Takaaki and Ohba, 1994). Therefore, it is suggested that parasporal inclusions isolated from this study contain Cyt toxin. There is a possibility that the isolated inclusions in this study were novel proteins which do not belong to any Cyt group. Amino acid sequencing is required to confirm the sequence of the Bt parasporal inclusions used in this study.

There were some limitations in this study. The erythrocytes were obtained from volunteers regardless of blood group, gender, age or medical condition. Secondly, the parasporal inclusion expressions varied among the Bt strains during sporulation. Some Bt strains may produce higher concentrations of Bt parasporal inclusions whilst others may not. Hence, there was difficulty in standardizing the protein concentration for all the Bt strains, however this study ensured that the same concentration of protein from a single strain (Bt 1, Bt 2, etc) was used to compare between the human and rat erythrocyte activity. The present study is limited to comparing the selectivity of respective Bt parasporal inclusions at certain concentrations against human and rat erythrocytes. This study was also able to determine the role of proteases in parasporal inclusion activation.

The binding mechanism of the Malaysian Bt strains was investigated through the hemolytic inhibition test (HIT). We found parasporal inclusion crystals from Malaysian Bt strains have novel lectin activity. Carbohydrate-specific lectin molecules are able to react with glycoproteins or glycolipids and the binding is inhibited by specific monosaccharides. Knowles *et al* (1984) has reported that lectin-like binding of Bt δ-endotoxin is an initial step in its insecticidal action, and GalNAc is a part of the receptor for the Bt toxin. The molecular mechanism of insecticidal activity in Bt Cry1A toxin has been well understood by examining the three domain structures of this protein (Jenkins *et al*, 2000). It is now widely accepted that the cell receptor for Bt Cry1Ac, a Bt δ-endotoxin specific to lepidopteran insects, is aminopeptidase N, a glycoprotein that resides on susceptible cells (Schnepf *et al*, 1998). Burton *et al* (1999) reported that a site on the domain III lectin-like fold of the Cry1Ac protein recognizes GalNAc on aminopeptidase N. Mutations on the Cry1Ac GalNAc binding site can result in loss of receptor binding, resulting in non insecticidal effects for Cry1Ac. This indicates that carbohydrate recognition is essential for the toxin-receptor interaction. It has been suggested that the binding mechanism of Bt lectins is analogous to that of the Cry protein in the initial step of the insecticidal action. Results from the present study strongly suggest that the receptors on human erythrocytes for Bt 1 and Bt 2 are glycoconjugated terminating in a residue of mannose, whilst for Bt 7 it might be GlcNAc and

for Btj it might be GalNAc. Interestingly, an anionic carbohydrate, NeuNAc, also shows inhibitory effects against Bt 1, Bt 2, Bt 4 and Bt 7 but not for Btj.

Among these monosaccharides, NeuNAc was the most efficient inhibitor. Drobniewski *et al* (1987) reported that a non-specific inhibition of the cytotoxicity of a δ-endotoxin by a galacturonic acid and D-glucuronic acid is due to the presence of negative charges on this anionic carbohydrate. Although Akao *et al* (1999) had reported the blocking effect of N-acetylneuraminic acid on a Japanese isolate of Bt subspecies *kurstaki*, the authors suggest that as an anionic carbohydrate, the blocking effect shown by N-acetylneuraminic acid could be through non-specific binding via the strong negative charge of the carbohydrate.

D-Mannose has been previously suggested to be a residue on the receptor for the lepidopteran specific Bt parasporal inclusions (Wasano *et al,* 2003). Results from the present study show Malaysian mosquitocidal Bt strains (Bt 1 and Bt 2) recognize D-mannose on the receptor of human erythrocytes. Since D-mannose has partially inhibited the Bt toxin, it seems likely to be part of the receptor.

Akao *et al* (2001) has demonstrated that the hemagglutination assay activities against sheep erythrocytes and rabbit erythrocytes were inhibited by GalNAc and D-galatose, respectively. The authors strongly suggested that the receptor on the sheep and rabbit erythrocytes for the lectin of tested Bt toxin (IBC-1456) is a glycoconjugate terminating in a residue of GalNAc and D-galactose, respectively. We found receptor recognition by mosquitocidal Btj analogous to the observations by Akao *et al* (2001) which recognized GalNAc. This suggests the lectin of Btj is a glycoconjugate terminating in a GalNAc residue. The monosaccharide GlcNAc showed a blocking effect on Bt 7 parasporal inclusions, which suggests the terminal residue for the Bt 7 strain is GlcNAc. Akao *et al* (2001) has suggested that the toxin from the tested isolate (IBC-1456) contains two different lectins, one active to D-galactose and another to GalNAc. This is not observed in the study with human erythrocytes suggesting that the lectin activity from the Bt strains tested are limited to single carbohydrate recognition. However, proof of this must await isolation of the lectins.

The carbohydrate-recognizing mechanism is different between human erythrocytes and rat erythrocytes. The monosaccharide recognized by all the Bt parasporal inclusions on rat erythrocytes is NeuNAc and hence it is very likely that the binding is due to non-specific binding through the strong negative charge of the monosaccharide. Interestingly, parasporal inclusions from Bt 7 recognized D-mannose and GalNAc on rat erythrocytes but not a human erythrocytes. Since parasporal inclusions from these Bt strains have shown cytotoxic activity against leukemic cell lines, it is possible, that carbohydrates play a role in the initial reaction between the parasporal inclusions and the leukemic cells.

There are some limitations to the hemolytic inhibition test (HIT). For example, it cannot determine the role of carbohydrate for parasporal inclusions that are non-hemolytic but yet cytotoxic to leukemic cell lines. Another factor is, the study was limited to the number and types of carbohydrates used, which means conclusions are made based on the seven monosaccarides.

Observation of these findings reveal that parasporal inclusions from Malaysian mosquitocidal Bt strains consist of lectins which bind to carbohydrates to initiate hemolytic activity. The results suggest binding of the Malaysian mosquitocidal Bt parasporal inclusions to a specific glycoconjugate on the plasma membrane of susceptible cells is an essential feature for hemolytic activity.

The possible relationship between carbohydrate-recognizing and cancer cell killing activities for the non-hemolytic strains has been identified as a subject for future work. This preliminary study on hemolytic activity identified the monosaccarides involved in lectin recognition. It can later be applied to toxin-cancer cell lines binding mechanism research.

In conclusion, several of the Malaysian mosquitocidal Bt parasporal inclusions examined in this study showed hemolytic selectivity. The findings also suggest these Bt strains produce

parasporal inclusions that are lectins, and can inhibit the hemolytic activity of parasporal inclusions.

ACKNOWLEDGEMENTS

The authors would like to express their thanks and gratitude to Dr Lee Han Lim and Ms P Seleena from the Institute for Medical Research for providing us with the Bt strains. We would also like to thank the IMU Research and Consultative Committee for their support and funding of this research.

REFERENCES

Akao T, Mizuki E, Yamashita S, Saitoh H, Ohba M. Lectin activity of *Bacillus thuringiensis* parasporal inclusion proteins. *FEMS Microbiol Lett* 1999; 179: 415-21.

Akao T, Mizuki E, Yamashita S, Ho-San Kim, Lee DW, Ohba M. Specificity of lectin activity of *Bacillus thuringiensis* parasporal inclusion proteins. *J Basic Microbiol* 2001; 41: 3-6.

Bernhard K, Jarett P, Meadows M, *et al*. Natural isolates of *Bacillus thuringiensis* worldwide distribution, characterization, and activity against insect pests. *J Invertebr Pathol* 1997; 70: 59-68.

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.

Burton SL, Ellar DJ, Li J, Derbyshire DJ. N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognized by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. *J Mol Biol* 1999; 287: 1011-22.

Drobniewski FA, Knowles BH, Ellar, DJ. Nonspecific ionic effects on the cytolytic and hemolytic properties of *Bacillus thuringiensis* subsp *darmstadiensis* 73-E10-2. *J Bacteriol* 1987; 171: 3060-7.

Heimpel, AM. A critical review of *Bacillus thuringiensis* var *thuringiensis* Berliner and other crystalliferous bacteria. *Ann Rev Entomol* 1967; 12: 287-322.

Jenkins JL, Lee MK, Valaitis AP, Curtiss A, Dean D. Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gpsy moth aminopeptidase N receptor. *J Biol Chem* 2000; 275: 14423-31.

Knowles BH, Thomas WE, Ellar, DJ. Lectin-like binding of *Bacillus thuringiensis* var *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action. *FEBS Lett* 1984; 168: 197-202.

Knowles BH, Nicholls CN, Amstrong G, Tester M, Ellar DJ. Broad spectrum cytolytic toxins made by *Bacillus thuringiensis.* In: Proceedings of the Vth International Colloquim on Invertebrate Pathology and Microbial Control, Adelaide, Australia: Society for Invertebrate Pathology, 1990: 283-7.

Koni PA, Ellar DJ. Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic delta-endotoxin. *J Mol Biol* 1993; 229: 319-27.

Lambert B, Peferoen M. Insecticidal promise of *Bacillus thuringiensis.* Facts and mysteries about a successful biopesticide. *Bio Sci* 1992; 42: 112-21.

Laemmli UK, Favre M. Maturation of the head of Bacteriophage T4. *J Mol Biol* 1973; 80: 575-99.

Lee HL, Seleena P. Isolation of indigenous larvicidal microbial control agents of mosquitos: the Malaysian experience. *Southeast Asian J Trop Med Pub Health* 1990; 21: 281-7.

Lee D, Katayama H, Akao T. A 28kDa protein of the *Bacillus thuringiensis* induces a human leukaemic cell-specific cytotoxicity. *Biochem Biophys Acta* 2001; 1547: 57-63.

Michael DK, Seleena P, Lee HL, Sarjeet SG. Isolation and identification of novel toxins from a new mosquitocidal isolate from Malaysia, *Bacillus thuringiensis* subsp *Jegathesan. Appl Env Microbiol* 1995: 2965-69.

Mizuki E, Ohba M, Akao T, Yamashita S, Saitoh H, Park YS. Unique activity of non-insecticidal Bt parasporal inclusions: in vitro cell-killing action on cancer cells. *J Appl Microbiol* 1999; 86: 477-86.

Ohba M, Aizawa K. Distribution of *Bacillus thuringiensis* in soils of Japan. *J Invertebr Pathol* 1986; 47: 277-82.

Okumura D, Akao T, Higuchi K, *et al*. *Bacillus thuringiensis* serovar *shandongiensis* strain 89-T-34-22 produces multiple cytotoxic proteins with similar molecular masses against human cancer cells. *Lett Appl Microbiology* 2004; 39: 89-92.

Schnepf E, Crickmore N, Van Rie J, *et al*. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* 1998; 62: 775-98.

Takaaki I, Ohba M. Hemolytic activity associated with parasporal inclusion proteins of mosquito-specific *Bacillus thuringiensis* soil isolates: A comparative neutralization study. *FEMS Microbiol*

Lett 1994; 116: 195-200.

Thomas WE, Ellar DJ. *Bacillus thuringiensis var. israelensis* crystal δ-endotoxin: Effects on insect and mammalian cells *in vitro* and *in vivo*. *J Cell Sci* 1983; 60: 181-97.

Wasano N, Chisa YA, Sato R, Ohba M, Takeshi K, Hidenori I. Spherical parasporal inclusions of the lepidoptera-specific and coleopteran-specific *Bacillus thuringiensis* strains: A comparative electron microscopic study. *Curr Microbiol* 2000; 40: 128-31.

Wasano N, Ohgushi A, Ohba M. Mannose-specific lectin activity of parasporal proteins from a lepidoptera-specific *Bacillus thuringiensis* strain. *Curr Microbiol* 2003; 46: 43-6.

Yokoyama Y, Ohmari I, Kohda K, Kawazoe Y. Potentiation of the cytotoxic activity of anticancer drugs against cultured L1210 cells by *Bacillus thuringiensis* subsp *israelensis* endotoxin. *Chem Pharm Bull* 1988; 36: 4499-504.

Yokoyama Y, Ohmari I, Kohda K, Kawazoe Y. Potentiation of antitumor activity of bleomycin toward solid tumors in mice by *Bacillus thuringiensis* subsp. *israelensis* endotoxin. *Anticancer Res* 1991; 11: 1625-8.

Yu YM, Ohba M, Gill SS. Characterization of mosquitocidal activity of *Bacillus thuringiensis* subsp *fukuokaensis* crystal proteins. *Appl Env Microbiol* 1991; 57: 1075-81.

Yudina TG, Burtseva LI. Activity of δ-endotoxin of four *Bacillus thuringiensis* subspecies against prokaryotes. *Mikrobiologiya* 1997; 66: 25-31.