Dot Blot Enzyme-Linked Immunosorbent Assay for Monitoring the Fate of Insecticidal Toxins from *Bacillus thuringiensis* in Soil

H. TAPP AND G. STOTZKY*

Laboratory of Microbial Ecology, Department of Biology, New York University,
New York, New York 10003

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The release of transgenic plants and microorganisms expressing truncated genes from *Bacillus thuringiensis* that code for active insecticidal toxins rather than for the inactive protoxins could result in the accumulation of these active proteins in soil, especially when bound on clay minerals and other soil particles. To monitor the fate of these toxins in soil, a dot blot enzyme-linked immunosorbent assay (ELISA) that detects free and particle-bound toxins from *B. thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* was developed. The lower limit of detection of the toxins, either free or adsorbed or bound on the clay minerals montmorillonite (M) or kaolinite (K) or on the clay-particle-size fraction separated from soil (by sedimentation according to Stokes' Law), was approximately 3 ng. Antibodies (Ab) to the toxins from *B. thuringiensis* subsp. *kurstaki* and from *B. thuringiensis* subsp. *thuringiensis* were raised in goats and rabbits, respectively, and each Ab was rendered specific by adsorption onto CNBr-activated Sepharose coupled with the other toxin. The preadsorbed Ab were specific for the toxins from both subspecies, both free and bound on M, K, or the clay-particle-size fraction of soil. The toxins that were added to sterile and nonsterile soil amended with M or K or not amended were detected on the clay-particle-size fraction of the soil after various periods of incubation by the dot blot ELISA. No toxins were detected on the silt- and sand-particle-size fractions. Each dot blot, containing various amounts of toxins and/or clays, was applied to a polyvinylidene difluoride membrane in a dot blot vacuum system. The toxins were still detectable on the clay-particle-size fraction of nonsterile soil after 40 days. This agreed with preliminary results of other studies in this laboratory that when these toxins bind on clay minerals, they become resistant to utilization by microorganisms.

To evaluate any potential adverse impacts on the environment from the release of organisms that have been genetically modified to contain the genes that code for the insecticidal toxins produced by subspecies of *Bacillus thuringiensis*, the adsorption and binding of these toxins on clay minerals and other soil particles and the effects of such surface interactions on the persistence and activity of the toxins must be established. *B. thuringiensis* forms crystalline protein parasporal inclusions that exhibit insecticidal activity (9). These inclusions (protoxins) are not toxic and require solubilization and enzymatic cleavage to yield the active toxins. Preparations of *B. thuringiensis*, usually as a mixture of cells, spores, and parasporal crystals, have been used as microbial insecticides for more than 30 years. Apparently, no unexpected toxicities have been noticed, probably because *B. thuringiensis* does not survive or grow well in natural habitats. Consequently, there is probably little or no production of the toxins in natural habitats, and the persistence of the introduced toxins is a function primarily of (i) the concentration added, (ii) the rate of consumption and inactivation by insect larvae, and (iii) the rate of degradation by the microbiota.

However, when the genes that code for the production of these toxins are genetically engineered into organisms, e.g., plants and bacteria, that are indigenous or adapted to a specific habitat and, therefore, can persist and proliferate, the toxins may continue to be synthesized in that habitat. In the case of transgenic plants, only the usable portions of the plants will be harvested, and the remainder of the plant biomass containing the toxins will be incorporated into soil. Hence, the toxins will

be present for longer periods and at higher concentrations in soil than toxins introduced with commercial preparations of *B. thuringiensis*. These concentrations could exceed the levels of toxins eliminated by consumption, inactivation, and degradation, resulting in levels of toxins that could constitute a hazard to nontarget organisms and enhance the selection of toxin-resistant target insects, especially if the toxins are bound on soil constituents.

The toxins from *B. thuringiensis* subsp. *kurstaki* (active against Lepidoptera) and *B. thuringiensis* subsp. *tenebrionis* (active against Coleoptera) are tightly bound on clay minerals (19, 21). The clay-bound toxins are more resistant to biodegradation than the free toxins (11), and they retain toxicity to insect larvae (unpublished data). Little information is available about the fate of the toxins from *B. thuringiensis* in soil. One reason for this may be that extraction and purification of organic materials, especially proteins, from soil are difficult. Significantly smaller amounts of the toxins from *B. thuringiensis* subsp. *kurstaki*, either purified or in transgenic cotton, were extracted from soil with a high clay content than from sandy soils (15). We describe the use of a method to detect and track the fate of these insecticidal toxins in soil by a dot blot enzyme-linked immunosorbent assay (ELISA) that does not require their extraction and purification.

MATERIALS AND METHODS

Purification of the toxins from *B. thuringiensis* subsp. *kurstaki*, *tenebrionis*, and *israelensis*. Cultures of *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* (active against members of the order Diptera) were grown in 1-liter flasks containing 300 ml of medium with shaking (250 rpm) at 28°C until sporulation (usually 5 days). The medium contained (per liter) 3 g of beef extract (Difco), 10 g of tryptose (Difco), 5 g of NaCl, and 0.2 ml of filter-sterilized 0.1 M $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ and 5.0 ml of filter-sterilized 1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, which were

* Corresponding author. Phone: (212) 998-8266. Fax: (212) 995-4015.

FIG. 1. Photograph of a dot blot ELISA using Ab raised against the toxins from *B. thuringiensis* subsp. *kurstaki* (Btk) of the toxins bound on the clay-particle-size fraction separated from 1 g of sterile (S) or nonsterile (NS) unamended K soil or K6K or K6M soil 24 h after the addition of 262 μ g of the toxins from *B. thuringiensis* subsp. *kurstaki* (Btk) to the soil. Reactions of the Ab with the free toxins, BSA, the clay minerals K and M, and the clay-particle-size fraction separated from soil not treated with toxins are also shown as controls. The amounts of the free toxins from *B. thuringiensis* subsp. *kurstaki* added to the dot blots in 4 μ l were 4 μ g (1/1 dilution), 0.4 μ g (1/10 dilution), and 0.04 μ g (1/100 dilution). A schematic of the dot blot is also shown to identify the locations of the variables. The apparently positive reactions with the soil-clay controls are caused by the grey shading of the black and white photograph. These dots are yellow, whereas the experimental variables are blue-purple, the color of the positive ELISA reaction. The dot blots for the soil-toxin treatments were done in duplicate. ●, positive reaction; ○, no reaction; ⊙, weak reaction.

added aseptically filter sterilized after autoclaving of the other components. The bacteria were maintained on 2% agar (Difco) slants of this medium.

Sedimented 5-day-old cultures of *B. thuringiensis* subsp. *kurstaki* or 4 g of Dipel 2 $\times$ (Abbott Laboratories) were washed twice with 1 M NaCl and twice with double-distilled water (ddH₂O). The toxins produced by *B. thuringiensis* subsp. *kurstaki* (M_r , 66,000) were isolated by extracting the washed sediment overnight (18 h) with 50 ml of MOPS buffer (0.1 M 3-*N*-morpholinopropanesulfonic acid [Sigma; pH 7.8] containing 0.5 M dithiothreitol [Boehringer Mannheim Corp.] and 1 M KSCN [Sigma]), by dialyzing the extract against ddH₂O for 6 to 8 h with hourly changes of ddH₂O, and by adding 17.5 g of (NH₄)₂SO₄ per 100 ml of dialyze to precipitate the proteins. After 2 to 3 h, the precipitate was centrifuged at 27,000 $\times$ g, resuspended in a minimum amount of ddH₂O, dialyzed for 8 h against ddH₂O with several changes of ddH₂O, and lyophilized (Thermovac Industries Corporation [model FD-6A]) (19–21).

The toxins from *B. thuringiensis* subsp. *tenebrionis* (M_r , 68,000) were purified from commercial M-One (Mycogen Corporation) by a precipitation method (14) by adding 100 ml of 1 M Na₂CO₃ to 500 ml of M-One, diluting to 1 liter with ddH₂O (the pH was maintained at 10), stirring for 2 to 3 h, and centrifuging at 16,300 $\times$ g for 15 min. The supernatant was dialyzed against 3 liters of 10 mM sodium phosphate buffer (pH 6.0) for 3 days, with a daily change of buffer. The precipitated proteins were recovered by centrifugation at 26,300 $\times$ g for 30 min, redissolved in a minimum amount of 100 mM Na₂CO₃ (pH 10), dialyzed for 8 h against ddH₂O with several changes of ddH₂O, and lyophilized (14, 19).

Toxins from *B. thuringiensis* subsp. *israelensis* (M_r , 25,000 to 130,000) were purified by gradient separation on Ludox (Dupont) (22). Sporulation followed by lysis of 3- to 5-day-old cultures, which were harvested by centrifugation at 3,200 $\times$ g for 10 min, was induced by resuspension in ddH₂O and shaking at 30°C for 4 days. The lysed cultures were centrifuged at 1,600 $\times$ g for 10 min, and the pellets were suspended in 1 M NaCl and vigorously shaken in a separatory funnel to produce foam, which was enriched in spores. The foam was removed, the remaining suspension was centrifuged, the pellet was suspended in ddH₂O, and the crystals and spores were disaggregated by sonication at 100 W for 30 s (Branson microprobe sonicator). Ludox HS-40 (40% sodium silica; pH 9.7), which precipitates below pH 7, was adjusted to pH 8.0 with 1 M Tris-HCl (pH

2.5). Gradients of pH-adjusted Ludox (defined as a 100% solution which was then diluted with ddH₂O) were prepared by layering 5 ml of 40% Ludox onto 5 ml of 50% Ludox in 40-ml tubes. The suspension of crystals and spores (2 to 4 ml) was layered onto the gradient and centrifuged in a swinging bucket rotor at 12,000 $\times$ g for 30 min. The spores sedimented to the bottom of the tube, and the crystals, which were concentrated at the interface between the Ludox layers, were collected and washed several times with ddH₂O, with centrifugation at 26,300 $\times$ g, to remove the Ludox and to pellet the crystals. Samples of the pellets were stained with safranin (crystals stain red) and malachite green (spores stain green) to estimate the purity of the crystals (2).

Preparation of clay minerals. The <2- μ m fractions of montmorillonite (M) and kaolinite (K) were prepared from bentonite and kaolin, respectively (Fisher Scientific Co.) by differential centrifugation (3, 8, 10, 17). The clays contained a mixed cation complement consisting primarily of sodium and calcium as the charge-compensating cations.

Soils. Studies were conducted in Kitchawan soil (a sandy loam soil collected at the Kitchawan Research Laboratory of the Brooklyn Botanic Garden, Ossining, N.Y.) that was not amended (K soil) or that was amended to 6% (vol/vol) with M (K6M soil) or with K (K6K soil). These soil-clay mixtures have been used extensively in this laboratory in numerous studies of the effects of various physicochemical factors on the ecology and other aspects of microbes and viruses, and, therefore, there is a large database available on these mixtures (17), the physicochemical characteristics of which have been described elsewhere (1, 18). The soil-clay mixtures (stored in plastic-lined garbage cans at 24 $\pm$ 2°C) were passed through a 2-mm sieve, brought to their –33-kPa water tension (determined with a pressure membrane) by the addition of a dilute glucose-mineral salts solution and an inoculum (ca. 1%) of a microbiologically active soil, and incubated at 24 $\pm$ 2°C, with periodic mixing, for 1 to 2 weeks. The rejuvenated mixtures were passed through a 2-mm sieve, placed in plastic bags, adjusted to their –33-kPa tension with tap water, and, after 24 h at 4°C with periodic kneading (to enhance the uniform distribution of water), stored in open plastic bags in a high-humidity chamber (5, 18).

To provide the equivalent of 0.5 or 1.0 g of oven-dry soil, the mixtures at their –33-kPa water tension were dispensed into 20- or 50-ml screw-cap test tubes to

TABLE 1. Schematic representation of dot blot ELISA showing the lower limits of detection of the toxins from *B. thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* that were free, adsorbed at equilibrium, and bound on the clay minerals K or M or bound on the clay-particle-size fraction separated from K soil or K6K or K6M soil^a

Source of toxin	Pure clay or clay-size fraction from soil with which toxin was adsorbed or bound	Amt (reaction) of toxin in samples no.:					
		1 (μg)	2 (μg)	3 (ng)	4 (ng)	5 (ng)	6 (ng)
<i>B. thuringiensis</i> subsp. <i>tenebrionis</i>	Free	4.22 (●)	0.422 (●)	42.2 (●)	4.22 (●)	0.422 (○)	0.042 (○)
	Adsorbed on K	1.84 (●)	0.184 (●)	18.4 (●)	1.84 (○)	0.184 (○)	0.018 (○)
	Bound on K	1.76 (●)	0.176 (●)	17.6 (●)	1.76 (○)	0.176 (○)	0.018 (○)
	Adsorbed on M	4.77 (●)	0.477 (●)	47.7 (●)	4.77 (●)	0.477 (○)	0.048 (○)
	Bound on M	4.17 (●)	0.417 (●)	41.7 (●)	4.17 (●)	0.417 (○)	0.042 (○)
	Bound on K soil	0.28 (●)	0.028 (●)	2.88 (●)	0.288 (○)	0.028 (○)	0.003 (○)
	Bound on K6K soil	0.56 (●)	0.056 (●)	5.63 (●)	0.563 (○)	0.056 (○)	0.006 (○)
	Bound on K6M soil	1.00 (●)	0.100 (●)	10.0 (●)	1.000 (○)	0.100 (○)	0.010 (○)
<i>B. thuringiensis</i> subsp. <i>kurstaki</i>	Free	4.95 (●)	0.495 (●)	49.50 (●)	4.950 (●)	0.495 (○)	0.050 (○)
	Adsorbed on K	1.02 (●)	0.102 (●)	10.20 (○)	1.020 (○)	0.102 (○)	0.010 (○)
	Bound on K	0.70 (●)	0.070 (●)	7.07 (●)	0.700 (○)	0.070 (○)	0.007 (○)
	Adsorbed on M	5.14 (●)	0.514 (●)	51.40 (●)	5.140 (●)	0.514 (○)	0.051 (○)
	Bound on M	3.69 (●)	0.369 (●)	36.90 (●)	3.690 (●)	0.369 (○)	0.037 (○)
	Bound on K soil	5.68 (●)	0.568 (●)	56.80 (●)	5.680 (●)	0.568 (○)	0.057 (○)
	Bound on K6K soil	6.13 (●)	0.613 (●)	61.30 (●)	6.130 (●)	0.613 (○)	0.061 (○)
	Bound on K6M soil	15.40 (●)	1.540 (●)	15.40 (●)	1.540 (●)	0.154 (○)	0.015 (○)

^a Known amounts of free, adsorbed, and bound toxins were used to prepare the dilutions, 10 μl of which was used for each dot blot. ●, positive reaction; ○, no reaction; ○, weak reaction.

which 0.5 or 1.0 ml, respectively, of known amounts of toxins from *B. thuringiensis* subsp. *kurstaki* or *tenebrionis* which were dissolved in phosphate buffer (pH 7) were added, and the tubes were vortexed to distribute the toxins uniformly. In some studies, the soil tubes were sterilized by autoclaving before the addition of the toxins. The tubes were incubated at 24 ± 2°C, and duplicate tubes were randomly analyzed after various times.

Fractionation of soil into clay-, silt-, and sand-size particles. The clay-size fraction was separated from the soil-clay mixtures by sedimentation according to Stokes' Law (4). Small spherical particles of density (ρ_s) and diameter (X) settle through a liquid of density (ρ_L) and viscosity (η) at a velocity (v), where $v = X^2 g(\rho_s - \rho_L)/18\eta$ and g is the acceleration of gravity. The clay-particle-size fraction (<2-μm effective diameter) was separated by sedimentation after homogenization of a soil suspension and removal of the suspension above a certain level (h) after a period of time (t) as follows: $t = 18\eta h/g(\rho_s - \rho_L)X^2$. Thus, a clay particle with an assumed density of 2.60 g/cm³ and an effective diameter of <2 μm will settle 10 cm in 8 h at 20°C.

After various periods of incubation of the mixtures, 10 or 20 ml of ddH₂O was added to the 20- or 50-ml soil tubes, respectively, and the suspensions were vortexed and allowed to settle. After 8 h, 5 ml of the suspension was removed with a pipette from the 0- to 10-cm depth and centrifuged at 26,300 × g for 15 min, the supernatant was discarded, and 5 ml of ddH₂O was added to the remaining soil suspension, which was again vortexed and allowed to settle. After the second 8-h settling period, 5 ml of the suspension was again removed from the 0- to 10-cm depth, combined with the first pelleted sample, and centrifuged as described above. The combined pellet was assayed by the dot blot ELISA.

The K6M soil was also separated by sedimentation into clay-, silt- (2- to 20-μm effective diameter), and sand-(>20-μm effective diameter) size particles. After removal of the clay-size fraction, the silt-size fraction was removed from the top 10-cm depth after vortexing and settling for 4 min and 48 s. The remainder of the particles was the sand-size fraction. All fractions were centrifuged, and the pellets were assayed by the dot blot ELISA.

Adsorption studies. The lyophilized toxins were dissolved in 0.1 M phosphate buffer (pH 6), and any insoluble material was discarded after centrifugation at 26,300 × g for 20 min. The protein content of the toxin preparations was determined by the Lowry method (12) with bovine serum albumin (BSA), which has an M_r of 68,000, which is similar to that of the toxins, as the standard and adjusted to the desired concentrations with the buffer. The toxins were added to suspensions of M and K in a total volume of 1 ml. For most adsorption studies, 0.1 mg of M and 1 mg of K per ml were used to obtain maximum adsorption at the concentrations of toxins added. The clay-toxin mixtures were rotated in test tubes (8-ml volume) at 40 rpm on a motorized wheel at 24 ± 2°C for 3 h. After adsorption, the mixtures were centrifuged at 26,300 × g , and the concentrations of the toxins in the supernatants were determined. The differences between the amounts of toxins added and the amounts of toxins detected in the supernatants were used to calculate the amounts of toxins adsorbed at equilibrium on the clays (19).

Preparation of bound clay-toxin complexes. After equilibrium adsorption, the clay-toxin complexes were sequentially washed with 1 ml of ddH₂O (pH 5.8),

with centrifugation at 26,300 × g , until no more toxins were desorbed and were then washed several more times. The supernatants were analyzed after each wash for the presence of protein. The amounts of the toxins bound on the clays were calculated by subtracting the total amount of the toxins recovered in the equilibrium supernatant and in all washes from the amount of toxins added (19).

Preparation of Ab to the toxins. Antibodies (Ab) against the toxins from *B. thuringiensis* subsp. *kurstaki* were supplied by Abbott Laboratories (raised in goats with crystal protoxin protein). Ab against the toxins from *B. thuringiensis* subsp. *tenebrionis* (purified in this laboratory) were raised in rabbits by HRP Inc. Laboratories (Denver, Pa.) as follows: 1 mg of the toxins in 1 ml of Freund's complete adjuvant was injected into a female New Zealand White rabbit; a second injection of 1 mg of toxins in 1 ml of Freund's incomplete adjuvant was administered 1 month later; and test bleeds 2 weeks later showed that the serum contained the appropriate polyclonal Ab. All Ab preparations gave positive results against the toxins from *B. thuringiensis* subsp. *tenebrionis* or *kurstaki* in both Western blot (immunoblot) and dot blot ELISA (19, 20).

Removal of cross-reacting Ab. One gram of CNBr-activated Sepharose 4B (Pharmacia) was washed with four 50-ml aliquots of 1 mM HCl on a sintered-glass filter, and 30 mg of the purified toxins from *B. thuringiensis* subsp. *kurstaki* or *tenebrionis*, which was dissolved in 5 ml of coupling buffer (0.1 M NaHCO₃ [pH 8.3] containing 0.5 M NaCl), was added to the Sepharose gel, which was mixed end over end at 24 ± 2°C for 1 h. Excess toxins were removed by washing twice with 20 ml of coupling buffer. The gel was then immersed in 20 ml of 0.1 M Tris-HCl buffer (pH 8) for 2 h to bind Tris-HCl to any remaining active sites on the gel. The coupled gel was washed alternatively, three times each, with 0.1 M sodium acetate-acetic acid buffer (pH 4) containing 0.5 M NaCl and with 0.1 M Tris-HCl buffer (pH 8) containing 0.5 M NaCl.

A 5-ml slurry of toxin-Sepharose gel, consisting of 75% settled gel and 25% buffer, was poured into 10-ml columns fitted with a 0.45-μm-pore-size filter membrane (Gelman) and was washed with 10 ml of phosphate-buffered saline (PBS; pH 7.5). The Ab (80 μl) to the toxins from either *B. thuringiensis* subsp. *kurstaki* or *tenebrionis* were loaded onto the columns, which were then eluted with 15 ml of PBS. The eluate was collected at a flow rate of approximately 0.1 ml/min (faster flow rates were less efficient for complete binding of the cross-reacting Ab).

Dot blot ELISA. Dot blot ELISA was used to confirm the presence of the toxins in the clay-toxin complexes. Rectangles (12 by 6 cm) of a protein-binding polyvinylidene difluoride (PVDF) membrane were cut, rinsed in 10 ml of methanol for 1 to 3 s, equilibrated in PBS for 1 to 2 min, and placed in a dot blot apparatus (Hybri-Dot Manifold; GIBCO-BRL Life Technologies, Inc.). Samples (usually 4 μl) of free toxins, free clays, or clay-toxin complexes were spotted with an Eppendorf pipette onto the PVDF membrane through the sample wells of the dot blot apparatus to obtain reproducible 3-mm-diameter dots in the pattern of a 96-well microtiter plate. The following series of controls was used to ensure that nonspecific binding of the Ab did not occur: M and K; clay-, silt-, and sand-particle-size fractions separated from soil that had received no toxins; and BSA. The control samples were prepared and diluted identically to the experi-

TABLE 2. Schematic representation of dot blot ELISA showing cross-reaction before and after preadsorption of the Ab raised against the toxins from *B. thuringiensis* subsp. *tenebrionis* on CNBr-activated Sepharose coupled with the toxins from *B. thuringiensis* subsp. *kurstaki*^a

Source of toxin[s] or control	Pure clay or clay-size fraction from soil with which toxin was adsorbed or bound	Reaction with Ab before preadsorption with the following sample dilutions:			Reaction with Ab after preadsorption with the following sample dilutions:		
		1/1	1/10	1/100	1/1	1/10	1/100
<i>B. thuringiensis</i> subsp. <i>tenebrionis</i>	Free	●	●	●	●	●	●
	Adsorbed on K	●	●	●	●	●	●
	Bound on K	●	●	●	●	●	●
	Adsorbed on M	●	●	●	●	●	●
	Bound on M	●	●	●	●	●	●
	Bound on K6M soil	●	●	○	●	●	○
<i>B. thuringiensis</i> subsp. <i>kurstaki</i>	Free	●	●	○	○	○	○
	Adsorbed on K	●	○	○	○	○	○
	Bound on K	●	○	○	○	○	○
	Adsorbed on M	●	○	○	○	○	○
	Bound on M	●	○	○	○	○	○
	Bound on K6M soil	●	○	○	○	○	○
<i>B. thuringiensis</i> subsp. <i>tenebrionis</i> and <i>kurstaki</i>		●	●	○	●	●	○
Controls							
<i>B. thuringiensis</i> subsp. <i>israelensis</i> (free)		●	●	●	●	●	●
BSA		○	○	○	○	○	○
K		○	○	○	○	○	○
M		○	○	○	○	○	○
K6M soil		○	○	○	○	○	○

^a Dilutions of the toxins from these two subspecies and *B. thuringiensis* subsp. *israelensis* that were free, adsorbed at equilibrium and bound on the clay minerals K or M, or bound on the clay-particle-size fraction separated from 0.5 g of K6M soil 48 h after the addition of 316 µg of the toxins from *B. thuringiensis* subsp. *kurstaki* and 234 µg of the toxins from *B. thuringiensis* subsp. *tenebrionis*, either separately or together, are compared. Reactions of the Ab with the free toxins from these three *B. thuringiensis* subspecies, BSA, K, M, and the clay-particle-size fraction separated from soil not treated with toxins are also shown as controls. The amounts of the free toxins from the *B. thuringiensis* subspecies added to the dot blots in 4 µl were 4 µg (1/1 dilution), 0.4 µg (1/10 dilution), and 0.04 µg (1/100 dilution). ●, positive reaction; ○, no reaction; ○, weak reaction.

mental samples. The apparatus was connected to a water pump, and a vacuum was applied for 3 to 5 min until all samples were impacted on the membrane.

The dot blots were developed by ELISA by incubating the membranes for 1 h in PBS containing 3% gelatin (wt/vol), by washing in PBS, and by incubating for 1 h in PBS containing 1% gelatin and the appropriate Ab. The membranes were then washed several times in PBS and incubated for 1 to 2 h in PBS containing goat anti-rabbit (*B. thuringiensis* subsp. *tenebrionis* Ab) or rabbit anti-goat (*B. thuringiensis* subsp. *kurstaki* Ab) immunoglobulin G conjugated to horseradish peroxidase (Sigma). After three more rinses in PBS, the color was developed by incubating the membranes for 1 to 2 min in 60 ml of PBS containing 45 µl of H₂O₂ and 50 mg of 4-chloro-1-naphthol (Bio-Rad) in 10 ml of methanol.

To determine the lower limits of detection of the toxins (free or adsorbed or bound on the clays), 10 µl of serial 10-fold dilutions of each sample was spotted onto PVDF membranes. The limit of detection was estimated by visual comparison of the intensity of the color after ELISA.

For ease of comparing the dot blots, they are presented as tables. A representative photograph of a dot blot with its schematic is shown to illustrate the relation between actual dot blots and their schematics (Fig. 1).

Use of dot blot ELISA to detect toxins in soil. Various concentrations of the toxins from *B. thuringiensis* subsp. *kurstaki* or *tenebrionis*, either separately or together, were added to different amounts of sterile or nonsterile K, K6M, or K6K soil, and the soil samples were incubated for different periods of time at 24 ± 2°C. The clay-particle-size fraction was then separated, and 4 to 10 µl of the 1/1, 1/10, and 1/100 dilutions of the fraction, containing ca. 0.2 to 20 ng of clay, was assayed by dot blot ELISA using Ab that were preadsorbed or not preadsorbed against the toxins from *B. thuringiensis* subsp. *kurstaki* and *tenebrionis*.

Insect bioassays. Eggs of the tobacco hornworm (*Manduca sexta*) and food medium were obtained from Carolina Biological Supply Company. The eggs, dispensed uniformly on solidified medium in petri dishes, were incubated at 29 ± 1°C under a 40-W lamp for 3 to 5 days, until the eggs hatched. The medium (in 10-ml amounts) was dispensed, after microwaving, into vials (3-cm diameter and 6-cm height) and was allowed to solidify. Toxins (free, adsorbed, or bound) were serially diluted with ddH₂O to yield 2.5 to 1,000 ng/100 µl. Aliquots (100 µl) of each dilution were distributed over the surface of the medium, and after air drying, four to five second-instar larvae were added to each of triplicate vials. Mortality was determined after 3 and 7 days. The 50% lethal concentrations were calculated from plots of the log₁₀ of the dose against the probit of the percent

mortality (6, 16). Controls consisted of the particles and toxins alone at the same concentrations as those in the particle-toxin complexes. The toxicities of various concentrations of purified toxins bound on M, K, and the clay-particle-size fraction from soil have been successfully evaluated, and the 50% lethal concentrations of toxins bound on M and K were in agreement with those obtained by another laboratory (unpublished data).

RESULTS

Lower limit of detection of toxins. The lower limit of detection of the toxins from *B. thuringiensis* subsp. *kurstaki* or *tenebrionis*, which were either free or adsorbed or bound on M, K, and the clay-particle-size fraction separated from soil, was approximately 3 ng (Table 1). The sensitivity of the assay appears, therefore, to be unaffected by the presence of clay.

Purification of cross-reacting Ab. The Ab raised against the protoxins from *B. thuringiensis* subsp. *kurstaki* against *tenebrionis* cross-reacted to different extents with the toxins from *B. thuringiensis* subsp. *tenebrionis*, *kurstaki*, and *israelensis*, which were either free or adsorbed or bound on clay (Tables 2 and 3). After the Ab raised against the protoxins from *B. thuringiensis* subsp. *kurstaki* or *tenebrionis* reacted with CNBr-activated Sepharose to which the toxins from the other subspecies were coupled, the cross-reaction between the Ab and toxins was eliminated (Tables 3 and 4). The Ab preadsorbed by the toxins from *B. thuringiensis* subsp. *kurstaki* (Table 2), but not from *B. thuringiensis* subsp. *tenebrionis* (Table 3), still reacted with the toxins from *B. thuringiensis* subsp. *israelensis*, indicating that to render an Ab specific to the toxins from one subspecies of *B. thuringiensis* in the presence of toxins from other subspecies (e.g., in soil), preadsorption of the Ab with the toxins from all

TABLE 3. Schematic representation of dot blot ELISA showing cross-reaction before and after preadsorption of the Ab raised against the toxins from *B. thuringiensis* subsp. *kurstaki* on CNBr-activated Sepharose coupled with the toxins from *B. thuringiensis* subsp. *tenebrionis*^a

Source of toxin[s] or control	Pure clay or clay-size fraction from soil with which toxin was adsorbed or bound	Reaction with Ab before preadsorption with the following sample dilutions:			Reaction with Ab after preadsorption with the following sample dilutions:		
		1/1	1/10	1/100	1/1	1/10	1/100
<i>B. thuringiensis</i> subsp. <i>tenebrionis</i>	Free	●	⊙	○	○	○	○
	Adsorbed on K	●	⊙	○	○	○	○
	Bound on K	●	⊙	○	○	○	○
	Adsorbed on M	●	⊙	○	○	○	○
	Bound on M	●	⊙	○	○	○	○
	Bound on K6M soil	●	⊙	○	○	○	○
<i>B. thuringiensis</i> subsp. <i>kurstaki</i>	Free	●	●	●	●	●	●
	Adsorbed on K	●	●	●	●	●	●
	Bound on K	●	●	●	●	●	●
	Adsorbed on M	●	●	●	●	●	●
	Bound on M	●	●	●	●	●	●
	Bound on K6M soil	●	●	○	●	●	○
<i>B. thuringiensis</i> subsp. <i>tenebrionis</i> and <i>kurstaki</i>		●	●	○	●	●	○
Controls							
Free <i>B. thuringiensis</i> subsp. <i>israelensis</i>		●	⊙	○	○	○	○
BSA		○	○	○	○	○	○
M		○	○	○	○	○	○
K		○	○	○	○	○	○
K6M soil		○	○	○	○	○	○

^a Dilutions of the toxins from *B. thuringiensis* subsp. *tenebrionis*, *kurstaki*, and *israelensis* that were free, adsorbed at equilibrium and bound on the clay minerals K or M, or bound on the clay-particle-size fraction separated from 0.5 g of K6M soil 48 h after the addition of 316 µg of the toxins from *B. thuringiensis* subsp. *kurstaki* and 234 µg of the toxins from *B. thuringiensis* subsp. *tenebrionis*, either separately or together, are compared. Reactions of the Ab with the free toxins from these three *B. thuringiensis* subspecies, BSA, K, M, and the clay-size fraction separated from soil not treated with toxins are also shown as controls. The amounts of the free toxins added to the dot blots in 4 µl were 4 µg (1/1 dilution), 0.4 µg (1/10 dilution), and 0.04 µg (1/100 dilution). ●, positive reaction; ○, no reaction; ⊙, weak reaction.

appropriate subspecies of *B. thuringiensis* is necessary. Toxins bound on the clay-particle-size fraction separated from K6M soil to which the toxins from *B. thuringiensis* subsp. *kurstaki* and *tenebrionis* had been added separately also showed no cross-reaction with preadsorbed Ab (Tables 2 and 3). Therefore, the use of preadsorbed Ab in this dot blot ELISA enables the detection in soil of toxins from one subspecies of *B. thuringiensis* in the presence of toxins from another subspecies.

Soil studies. The results of studies to evaluate the applicability of this dot blot ELISA to soils showed that after 1 or 2 days of incubation of the free toxins from *B. thuringiensis* subsp. *kurstaki* and *tenebrionis* in sterile and nonsterile soil that was either amended with M or K or not amended, the toxins were detected on the clay-particle-size fraction separated from all soils (Tables 4 and 5). Moreover, the intensity of the color of the dot blots did not differ significantly between equivalent dilutions of sterile and nonsterile soil samples that were either amended with clay or not amended, indicating that the binding of the toxins onto the clay-particle-size fraction of soil provided protection against their biodegradation.

The specificity of the dot blot ELISA was shown by the absence of a positive reaction with the various controls (i.e., M, K, the various size fractions from soil, and BSA). Attempts were made to obtain quantitative data by comparing the intensities of the colors of dot blots from serial 10-fold dilutions of free and bound toxins. Although the concentration of toxins present in each dot blot prepared with free toxins was known, it was not possible to obtain quantitative data on the amounts of toxins present in the various particle-toxin complexes by comparison with the color intensities of dot blots of the free

toxins, since some particles of the complexes were often dislodged from the membrane during the ELISA. Even though positive results were still apparent under these conditions, this dislodgement prevented obtainment of quantitative data, both visually and with densitometric methods.

The silt- and sand-particle-size fractions separated from nonsterile K6M soil to which the toxins from *B. thuringiensis* subsp. *kurstaki* and *tenebrionis* had been added, either separately or together, were also assayed after 2 days of incubation to determine whether the toxins bound on these fractions. The toxins were detected only on the clay-particle-size fraction (Table 5).

To verify the results of the 1- and 2-day incubation studies that indicated that the toxins bound on the clay-particle-size fraction were protected against biodegradation in nonsterile soil, longer periods of incubation were evaluated. The toxins were still detectable on the clay-particle-size fraction from nonsterile soil after 40 days (Table 6). These results agreed with those of other studies in this laboratory with pure and mixed cultures of microbes, which indicate that the toxins are protected from microbial utilization when bound on clay minerals (11).

When 107 µg of the toxins from *B. thuringiensis* subsp. *kurstaki* was added to various amounts of nonsterile clay-amended soil (K6M) and unamended soil (K), the toxins were detected on the clay-particle-size fraction of essentially all dilutions prepared from 0.5 to 6 g of soil and on the 1/1 dilution from 10 g of unamended soil (Table 7). Insect bioassays confirmed the presence of the toxins. However, the toxins were not detected by dot blot ELISA on the clay-particle-size fraction

TABLE 4. Schematic representation of dot blot ELISA using Ab raised against the toxins from *B. thuringiensis* subsp. *kurstaki* or subsp. *tenebrionis* of the toxins bound on the clay-particle-size fraction separated from 1 g of sterile or nonsterile K soil or K6K or K6M soil^a

Source of toxin Ab or control	Treatment (soil/toxin)	Reaction with the following sample dilutions:		
		1/1	1/10	1/100
BTT	K/BTT (S)	●	●	●
	K/BTT (NS)	●	●	●
	K6K/BTT (S)	●	●	●
	K6K/BTT (NS)	●	●	●
	K6M/BTT (S)	●	●	●
	K6M/BTT (NS)	●	●	●
Controls				
Free BTT		●	●	●
BSA		○	○	○
K		○	○	○
M		○	○	○
K soil		○	○	○
K6K soil		○	○	○
K6M soil		○	○	○
BTK	K/BTK (S)	●	●	●
	K/BTK (NS)	●	●	●
	K6K/BTK (S)	●	●	●
	K6K/BTK (NS)	●	●	●
	K6M/BTK (S)	●	●	●
	K6M/BTK (NS)	●	●	●
Controls				
Free BTK		●	●	●
BSA		○	○	○
K		○	○	○
M		○	○	○
K soil		○	○	○
K6K soil		○	○	○
K6M soil		○	○	○

^a Results are from 24 h after the addition of 262 µg of the toxins from *B. thuringiensis* subsp. *kurstaki* (BTK) or 154 µg of the toxins from *B. thuringiensis* subsp. *tenebrionis* (BTT) to the soil. Reactions of the Ab with the free toxins, BSA, the clay minerals K and M, and the clay-particle-size fraction separated from soil not treated with toxins are also shown as controls. The amounts of the free toxins from the two *B. thuringiensis* subspecies added to the dot blots in 4 µl were 4 µg (1/1 dilution), 0.4 µg (1/10 dilution), and 0.04 µg (1/100 dilution). ●, positive reaction; ○, no reaction; S, sterile; NS, nonsterile.

separated from 10 g of the K6M soil, even though this fraction showed insecticidal activity (Table 7).

Although greater amounts of the toxins from *B. thuringiensis* subsp. *kurstaki* are adsorbed and bound on clays than the amounts of the toxins from *B. thuringiensis* subsp. *tenebrionis* (19), similar amounts of both toxins added to soil were detected on the separated clay-particle-size fraction, and there were no apparent differences in detection between the two toxins in any of the studies. In general, there were also no apparent differences in the detection of the toxins added at the same concentrations to the K, K6M, and K6K soil samples, even though more clay-size particles were separated from the K6M and K6K soils than from the K soil.

DISCUSSION

The dot blot ELISA used in this study enables rapid (3 to 4 h, after separation of the various size fractions from soil) and highly specific determination of the presence and, therefore,

TABLE 5. Schematic representation of dot blot ELISA using Ab raised against the toxins from *B. thuringiensis* subsp. *kurstaki* or subsp. *tenebrionis* of the toxins bound on the clay-, silt-, and sand-particle-size fractions separated from 0.5 g of K6M soil^a

Source of toxin Ab or control	Soil fraction ^b	Treatment (soil/toxin[s])	Reaction with the following sample dilutions:		
			1/1	1/10	1/100
BTT	Clay	K6M/BTT	●	●	●
	Clay	K6M/BTT and BTK	●	●	●
	Silt	K6M/BTT	○	○	○
	Silt	K6M/BTT and BTK	○	○	○
	Sand	K6M/BTT	○	○	○
	Sand	K6M/BTT and BTK	○	○	○
Controls					
Free BTT			●	●	○
BSA			○	○	○
K6M soil (clay)			○	○	○
K6M soil (silt)			○	○	○
K6M soil (sand)			○	○	○
	Clay	K6M/BTK	●	●	●
	Clay	K6M/BTK and BTT	●	●	●
	Silt	K6M/BTK	○	○	○
	Silt	K6M/BTK and BTT	○	○	○
	Sand	K6M/BTK	○	○	○
	Sand	K6M/BTK and BTT	○	○	○
Controls					
Free BTK			●	○	○
BSA			○	○	○
K6M soil (clay)			○	○	○
K6M soil (silt)			○	○	○
K6M soil (sand)			○	○	○

^a Results are from 48 h after the addition of 316 µg of the toxins from *B. thuringiensis* subsp. *kurstaki* (BTK) and 234 µg of the toxins from *B. thuringiensis* subsp. *tenebrionis* (BTT), separately or together, to the soil. Reactions of the Ab with the free toxins, BSA, and the various size fractions separated from soil not treated with toxins are also shown as controls. Amounts of the free toxins added to the dot blots in 10 µl were 49.5 and 42.2 ng (1/1 dilution), 4.95 and 4.22 ng (1/10 dilution), and 0.495 and 0.422 ng (1/100 dilution) for *B. thuringiensis* subsp. *kurstaki* and *tenebrionis*, respectively. ●, positive reaction; ○, no reaction; ○, weak reaction.

^b Effective diameters were as follows: clay, <2 µm; silt, 2 to 20 µm; and sand, >20 µm.

the persistence of the toxins from subspecies of *B. thuringiensis* in soil. The clay-particle-size fraction of soil appears to be more effective in binding the toxins than the silt- or sand-particle-size fractions. This assay was effective in both clay-amended and unamended soils, indicating its applicability for use with soils that vary in the amounts or types of clays. The toxins from *B. thuringiensis* subsp. *kurstaki* and *tenebrionis* are tightly bound on clay minerals, and greater amounts of the toxins from *B. thuringiensis* subsp. *kurstaki* than from subsp. *tenebrionis* are bound (19). Greater amounts of the toxins are bound on M than on K, since M has a significantly higher specific surface area and cation exchange capacity than K. Consequently, less M than K is required to bind similar amounts of the toxins, and, therefore, dot blots of the M- or K-toxin complexes with similar protein contents will contain

TABLE 6. Schematic representation of dot blot ELISA using Ab raised against the toxins from *B. thuringiensis* subsp. *kurstaki* or subsp. *tenebrionis* of the toxins bound on the clay-particle-size fraction separated from 0.5 g of nonsterile K soil or K6K or K6M soil^a

Source of toxin Ab or control	Incubation time (days)	Treatment (soil/toxin)	Reaction with the following sample dilutions:		
			1/1	1/10	1/100
BTT	3	K/BTT	●	●	○
	3	K6K/BTT	●	●	●
	3	K6M/BTT	●	●	○
	7	K/BTT	●	●	●
	7	K6K/BTT	●	●	○
	7	K6M/BTT	●	●	○
	10	K/BTT	●	●	●
	10	K6K/BTT	●	●	●
	10	K6M/BTT	●	●	○
	14	K/BTT	●	●	●
	14	K6K/BTT	●	●	○
	14	K6M/BTT	●	●	○
	40	K/BTT	●	●	●
	40	K6K/BTT	●	●	●
	40	K6M/BTT	●	●	●
Controls					
Free BTT			●	●	●
BSA			○	○	○
K			○	○	○
M			○	○	○
K soil			○	○	○
K6K soil			○	○	○
K6M soil			○	○	○
BTK	3	K/BTK	●	●	●
	3	K6K/BTK	●	●	●
	3	K6M/BTK	●	●	●
	7	K/BTK	●	●	○
	7	K6K/BTK	●	●	○
	7	K6M/BTK	●	●	○
	10	K/BTK	●	●	●
	10	K6K/BTK	●	●	○
	10	K6M/BTK	●	●	○
	14	K/BTK	●	●	●
	14	K6K/BTK	●	●	●
	14	K6M/BTK	●	●	●
	40	K/BTK	●	●	●
	40	K6K/BTK	●	●	●
	40	K6M/BTK	●	●	●
Controls					
Free BTK			●	●	●
BSA			○	○	○
K			○	○	○
M			○	○	○
K soil			○	○	○
K6K soil			○	○	○
K6M soil			○	○	○

^a Results are from different times after the addition of 212 µg of the toxins from *B. thuringiensis* subsp. *kurstaki* (BTK) or 160 µg of the toxins from *B. thuringiensis* subsp. *tenebrionis* (BTT) to the soil. Reactions of the Ab with the free toxins, BSA, the clay minerals K and M, and the clay-particle-size fraction

less M than K. Although binding of the toxins on organic matter has not yet been determined, the organic matter contents of these soil-clay mixtures were similar.

This method is relatively simple and does not require prior extraction or purification of the toxins from soil. Extraction of the toxins from *B. thuringiensis* subsp. *kurstaki* from soil amended with transgenic cotton expressing the toxins from *B. thuringiensis* subsp. *kurstaki* or with purified toxins had an apparent limit of detection of 0.5 ng/g of soil (15). The dot blot ELISA is being used in this laboratory to track the fate of these toxins in soil, especially their binding on soil particles, in conjunction with insect bioassays. Although insect bioassays can provide sensitive and specific toxicity data (e.g., a lower limit of detection of 1 ng/mg of plant protein of the toxins from *B. thuringiensis* subsp. *kurstaki* in transgenic plants [7]), bioassays are expensive, laborious, and time-consuming, with at least 72 h of larval exposure (depending on the target insect) required before 50% lethal concentrations can be determined.

The lower limit of detection of the toxins by the dot blot ELISA was approximately 3 ng, which should enable the detection of the toxins in soil. The amounts of the toxins in transgenic plants containing toxin genes from *B. thuringiensis* subsp. *kurstaki* or *tenebrionis* that could accumulate in soil have been calculated to range from 5 to 100 ppb in the top 3-in. (ca. 8-cm) plow depth (6a). The weight of an acre-furrow slice to a depth of 3 in. (ca. 8 cm) is assumed to be approximately 10⁶ lb (1 lb = 453.59237 g) (13). Therefore, even at the lowest concentration of 5 ppb of the toxins that could accumulate in soil, the toxins would be present at 5 ng/g of soil, on the basis of a uniform distribution of the toxins throughout an acre-furrow slice. Inasmuch as the results of these studies indicate that the toxins concentrate on the clay-particle-size fraction of soil, it should be possible to detect concentrations below the average of 5 ppb when this fraction is separated from soil and analyzed by the dot blot ELISA. Although insect bioassays appear to be more sensitive than the dot blot ELISA (Table 7), the relative rapidity and lower cost of the method indicate that it can be used as an initial screen for the presence of the toxins before further verification by bioassays.

The dot blot ELISA enabled the detection of clay-bound toxins from the subspecies of *B. thuringiensis* evaluated; however, only semiquantitative data were obtained. Although the amounts of toxin proteins added to soil can be controlled, the amounts present on the clay-particle-size and other fractions separated from soil cannot be accurately determined, since the amounts of the fractions separated and which will stick on the PVDF membrane are not always consistent. Consequently, the amounts of toxins present can be estimated only by comparison of the color intensities of dot blots with those containing known amounts of free toxins.

The Ab raised against the toxins from one subspecies of *B. thuringiensis* was rendered specific to these toxins by preadsorption with toxins from another subspecies. Consequently, toxins from one subspecies of *B. thuringiensis* should be detectable in soils that have been exposed to multiple toxins from several subspecies if the Ab has been preadsorbed by the toxins from all other subspecies involved.

In addition to enabling the detection of the toxins from subspecies of *B. thuringiensis* in soil, the results of this study

separated from soil not treated with toxins are also shown as controls. The controls were evaluated on each day that the soils were evaluated. The amounts of the free toxins from the two subspecies that were added to the dot blots in 4 µl were 4 µg (1/1 dilution), 0.4 µg (1/10 dilution), and 0.04 µg (1/100 dilution). ●, positive reaction; ○, no reaction; ○, weak reaction.

TABLE 7. Schematic representation of dot blot ELISA using Ab raised against the toxins from *B. thuringiensis* subsp. *kurstaki* of the toxins from this subspecies bound on the clay-particle-size fraction separated from different amounts of K soil or K6M soil^a

Treatment (soil/toxin) and soil amt (g) or control	Reaction with Ab to BTK with the following sample dilutions:			Bioassay result
	1/1	1/10	1/100	
K/BTK (0.5)	●	●	●	+
K6M/BTK (0.5)	●	●	●	+
K/BTK (3.0)	●	●	●	+
K6M/BTK (3.0)	●	●	●	+
K/BTK (6.0)	●	●	●	+
K6M/BTK (6.0)	●	●	○	+
K/BTK (10.0)	●	○	○	+
K6M/BTK (10.0)	○	○	○	+
Controls				
Free BTK	●	●	●	+
K soil	○	○	○	—
K6M soil	○	○	○	—

^a Results are from 24 h after the addition of 107 µg of the toxins to the soil. Reactions of the Ab with the free toxins and the clay-particle-size fraction separated from soil not treated with toxins are also shown as controls. The amounts of the free toxins from *B. thuringiensis* subsp. *kurstaki* (BTK) added to the dot blots in 4 µl were 4 µg (1/1 dilution), 0.4 µg (1/10 dilution), and 0.04 µg (1/100 dilution). Bioassay data show killing (+) or no killing (—) of second-instar larvae of *M. sexta* exposed to the clay-toxins, toxins, and clay. ●, positive reaction; ○, no reaction.

agreed with those of other studies in this laboratory, which indicate that when these toxins bind on clay minerals, they are protected from utilization by microorganisms (11).

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