Measurement of Brevetoxin Levels by Radioimmunoassay of Blood Collection Cards after Acute, Long-Term, and Low-Dose Exposure in Mice

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We developed a radioimmunoassay (RIA) using a sheep anti-brevetoxin antiserum to evaluate detection of brevetoxin on blood collection cards from mice treated with the brevetoxin congener PbTx-3. The RIA has high affinity for PbTx-3 [half-maximal effective concentration (EC_{50}) ± SE = 1.2 ± 0.2 nM; n = 10] and recognizes both type 1 and type 2 brevetoxins, but not ciguatoxin. Direct comparison of the RIA with a radiolabeled [^{3}H]-PbTx-3 receptor-binding assay (RBA) revealed excellent sensitivity, congener selectivity, and minimal interference from blood matrix. We first analyzed blood samples from an acute time course exposure, using a maximal nonlethal dose [180 µg/kg body weight (bw)] for 0.5, 1, 2, 4, and 24 hr. Mean blood brevetoxin levels were 36 nM at 30 min and stayed above 20 nM during the 1–4 hr time points. We next analyzed blood brevetoxin levels after longer exposure (0.5, 1, 2, 3, 4, or 7 days). Mean blood brevetoxin levels were 26.0 nM at 0.5 days, decreased to 8.2 nM at 1.0 day, and maintained a significant level ($p < 0.05$) of 1.3 nM at day 2. We next determined the lowest measurable dose using increasing concentrations of PbTx-3 (10–300 µg/kg bw). Analysis of the blood samples at 60 min revealed a linear relationship between administered and internal doses (r^2 = 0.993). All doses of brevetoxin administered were detectable at 1 hr, with significant levels found for the lowest administered dose of 10 µg/kg bw—a dose that was 10-fold lower than the lowest observable effect level. This RIA provides an optimal first-tier detection of brevetoxin from blood collection cards and, used in combination with the RBA and liquid chromatography–mass spectrometry, should provide a complete panel of methods to biomonitor brevetoxin exposure. *Key words:* blood, brevetoxin, radioimmunoassay. *Environ Health Perspect* 111:1595–1600 (2003). doi:10.1289/ehp.6166 available via *http://dx.doi.org/* [Online 2 July 2003]

Red tides have been documented on the Gulf Coast of Florida as early as 1530 (Taylor 1917). They occur nearly annually and often persist for many months (Woodcock 1948). The causative organism for these events, *Karenia brevis* (formerly *Gymnodinium breve* and *Ptychodiscus brevis*) produces a family of neurotoxins, collectively called brevetoxins (Martin and Chatterjee 1969; Steidinger and Joyce 1973). These events are responsible for fish, waterfowl, and marine mammal mortalities (Davis 1948) as well as human intoxication (Lin et al. 1981). The accumulation of brevetoxins in shellfish can lead to the neurotoxic shellfish poisoning syndrome in humans (McFarren et al. 1965), which, before 1993, was believed to be restricted to the southeastern United States. However, in 1993 neurotoxic shellfish poisonings and brevetoxin contamination of shellfish were reported in New Zealand (Mackenzie et al. 1996). The causative organism in the New Zealand waters was reported to be *K. brevis* or a closely related species (Satake et al. 1996). Subsequently, three different genera of the family Raphidophyceae (*Chattonella marina*, *Fibrocapsa japonica*, *Heterosigma akashiwo*) isolated from Japanese waters have been reported to produce a neurotoxin similar to brevetoxins (Kahn et al. 1995, 1996, 1997). In 2002, brevetoxin was confirmed to be produced by the raphidophytes *C. marina* and *Chattonella antiqua* (Haque and Onoue 2002), and samples from mid-Atlantic waters containing *Chattonella* species have been reported to contain brevetoxin (Bourdelais et al. 2002). The broad distribution of raphidophytes opens the concern of a widespread occurrence of this family of toxins.

Brevetoxins are analyzed largely to assure shellfish safety and on an as-needed basis for marine mammal and human intoxications. In the Gulf of Mexico, substantial monitoring is conducted for the causative organism, *K. brevis,* to prevent the harvest of contaminated shellfish beds. At present, toxin detection is conducted by the mouse bioassay (Delaney 1985); however, several functional assays are employed as potential alternatives to the mouse bioassay (Dickey et al. 2002). In the case of marine mammal and human intoxications, brevetoxin has been detected in isolated cases by functional assays and liquid chromatography–mass spectrometry (LC-MS) in tissues from bottlenose dolphins (Mase et al. 2000) and West Indian manatees (Landsberg and Steidinger 1997) and in urine from intoxicated humans (Poli et al. 2000).

A critical need exists for a simple and reliable detection method that can be used to routinely monitor aquatic animals and humans for brevetoxins. Fairey et al. (2001) described the application of a blood collection card method (Adam et al. 2000) to facilitate sampling and processing of blood samples from brevetoxin-exposed animals. The method of Fairey et al. (2001) uses a microplate receptor-binding assay (RBA) to screen samples for brevetoxin-like activity and LC-MS to confirm the presence of brevetoxin. In this article, we describe a brevetoxin radioimmunoassay (RIA) that we developed using a format parallel to the RBA, and compare the methods for detection of brevetoxin in the blood of exposed animals. We have determined that the RIA provides greater sensitivity and allows detection of blood brevetoxin at nonsymptomatic doses and for longer times postexposure.

Materials and Methods

Brevetoxin mouse exposure. For the short-term study and the dose response, female ICR mice, 18–20 g, were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). The mice were kept for 24 hr with food and water given *ad libitum.* The mice were injected intraperitoneally (IP) with the brevetoxin congener PbTx-3 in phosphate-buffered saline (PBS) at 180 µg/kg body weight (bw) for the 24-hr study and at 10–300 µg/kg bw for the dose response. The control mice were injected with 1.66% methanol in PBS. The behavior and symptomatology of the mice were recorded for both studies. The blood was collected after 0.5, 1, 2, 4, and 24 hr for the short-term study, and

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after 1 hr for the dose response. At each time point or PbTx-3 concentration, four mice were anesthetized with 2.0 mg ketamine (Parke-Davis, Morris Plains, NJ, USA) and 0.2 mg Prom ACE (Aveco, Fort Dodge, IA, USA) in a volume of 100 µL PBS. The mice were then exsanguinated via cardiac puncture to the left ventricle with a lithium heparinized 1-cc syringe. We applied 100 µL of blood to each spot on the blood spot collection card. For the long-term study we used female CD-1 mice (Charles River Laboratories, Raleigh, NC, USA); the procedure used for treatment of these animals was previously described by Gordon et al. (2001).

Blood collection. Blood (100 µL) was applied to each circle on the grade-903 filter-paper blood collection card (Schleicher & Schuell, Keene, NH, USA). The cards were then allowed to dry overnight in a cool, dark environment. Once the cards were dry, they were separated by 6-in. × 6-in. weighing paper (VWR Scientific Products, Suwanee, GA, USA) and transferred to airtight plastic bags (VWR Scientific Products) containing desiccant packages (Multisorb Technologies Inc., Buffalo, NY, USA) and humidity cards (Multisorb Technologies Inc.). The cards were stored at –20°C until use.

Brevetoxin extraction from blood collection cards. The dried blood spots were prepared and processed the same way for both the RIA and the RBA. The entire 100 µL dried blood spot was cut from the cellulose blood collection card and extracted overnight in 2 mL methanol in a 12 × 75 mm test tube. The spots were removed from the tubes, and the methanol extracts were brought to dryness with nitrogen using a Turbovap LV evaporator (Zymark, Hopkinton, MA, USA) and then stored at –20°C until use. The blood spot extracts were resuspended in the original spot volume of 100 µL containing 90 µL RIA or RBA assay buffer and 10 µL methanol. Whole mouse blood (Harlan Bioproducts, Indianapolis, IN, USA) was used for blood spots spiked with 20 nM PbTx-3 (Calbiochem, San Diego, CA, USA), which were used as a quality check.

Immunizations. Groups of four sheep were immunized with PbTx-2 conjugated to ovalbumin and fetuin. For primary immunizations, immunogens were prepared as water-in-oil suspensions by injecting the conjugate, dissolved in PBS (1 mg protein in 1 mL), into 2 mL Freund's complete adjuvant, followed by vortex mixing. Immunogens for secondary and subsequent immunizations (up to eight boosts) were administered as emulsions in Freund's incomplete adjuvant.

The immunogen emulsion (0.5 mL) was administered intramuscularly into the semitendinosus muscle, 0.25 mL per hind leg, via a 20-gauge needle. Test bleeds (10 mL Vacutainer, no. 366430; Becton Dickinson, Franklin Lakes, NJ, USA) were taken from the jugular vein 14 days after the third and subsequent immunizations, and antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). Larger volumes of blood for antiserum production were obtained by venipuncture and collected into blood bags (Blood bag no. 4R0001; Baxter, Deerfield, IL, USA) under negative pressure, from sheep identified as having high titers. A series of three injections at 4-week intervals was followed by a rest period, with subsequent boosts at no less than 4-week intervals.

Antiserum from animal 7098, immunized with PbTx-2–fetuin conjugate, was chosen for use in the development of both the ELISA and the RIA because of its high antibody titer and competitive binding of both free PbTx-2 and the PbTx–protein conjugates. All animal manipulations were performed under the authority of the AgResearch Ruakura Animal Ethics Committee.

Radioimmunoassay. RIAs were run in 12 × 75 borosilicate glass tubes in PBS containing 137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl (all from Sigma Chemical Company, St. Louis, MO, USA), and 0.01% Emulphor-EL 620 (GAF, New York, NY, USA. The assay tubes consisted of PbTx-3 standard or blood spot extract (50 µL), anti-PbTx antiserum (1:4,000), and [^{3}H]-PbTx-3 (0.4 nM) in PBS (final assay volume of 500 µL). The six PbTx-3 standards ranged from 0.01 nM to 100 nM. The PbTx-3 standards and blood spot extracts were allowed to preincubate in buffer at room temperature with the anti-PbTx-3 antibody for 1 hr before the [^{3}H]-PbTx-3 tracer was added. The tubes were placed on a shaker (Titramax 100; Heidolph Instruments, Cinnaminson, NJ, USA) and incubated for another hour after the addition of the radioactive toxin. Sac-Cel (Alpco Diagnostics, Windham, NH, USA) was then added to the assay tubes to allow for the separation of bound and unbound brevetoxin. The Sac-Cel was filtered onto 25-mm GF/C glass fiber filters (Whatman, Newton, MA, USA). Each assay tube was rinsed three times with 2 mL PBS. The filters were placed in 3.5 mL Scinti-verse (Fisher, Suwanee, GA, USA), and the radioactivity was counted on a 1211 RackBeta liquid scintillation counter (Wallace-Perkin Elmer, Wellesley, MA, USA).

Receptor-binding assay. RBAs were run using the same per-tube assay volume and the same six RIA PbTx-3 standards. The RBAs were carried out in 12 × 75 mm tubes in binding buffer consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/mL bovine serum albumin, and 0.01% Emulphor EL 620. Each assay tube contained PbTx-3 standards or blood spot extract (50 µL), [^{3}H]-PbTx-3 (1 nM), and rat brain membrane preparation (80–100 µg protein/mL) in binding buffer (500 µL total volume). Tubes were incubated for 1 hr at 4°C. The membranes were filtered and counted as described above for RIA.

Toxin congener recognition. The RIA and the RBA were used to evaluate toxin congener recognition using PbTx-1, PbTx-2, PbTx-6, and PbTx-9 (Molecular Probes, Inc., Eugene, OR, USA). Caribbean ciguatoxin (CCTX-1) was provided by R. Dickey (Food and Drug Administration, Gulf Coast Seafood Laboratory, Dauphin Island, AL).

Data analysis. All concentrations and half-maximal effective concentration (EC_{50}) values were determined using Prism Graph Pad 3.0 (GraphPad Software, Inc., San Diego, CA, USA). Where appropriate, significance values were determined by analysis of variance using JMP statistical software (SAS Institute, Cary, NC, USA).

Results

Using a sheep antibody directed against a PbTx-2–fetuin conjugate, we have developed an immunoassay to analyze blood cards for brevetoxin. In order to facilitate comparison with the RBA, we developed the RIA to be a competitive binding assay, run in solution with separation by filtration. Comparison of standard curves for PbTx-3 revealed high sensitivity for both assays (Figure 1). However, there was a significant difference between the RIA and RBA in the calculated EC_{50} ($p < 0.001$). The RIA produces a standard curve for PbTx-3

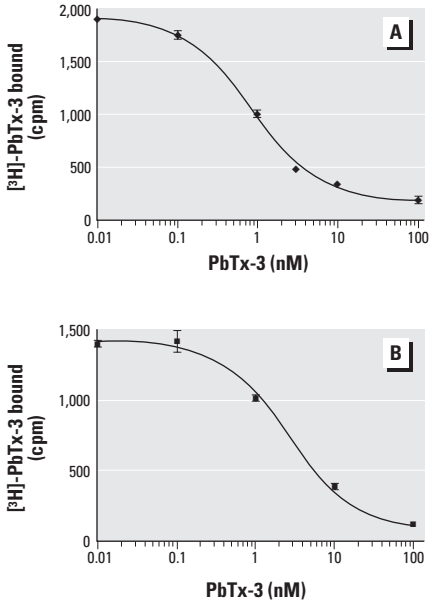

Figure 1. Comparison of standard curves for the RIA (*A*) and the RBA (*B*). The two standard curves were derived as described in "Materials and Methods." The RIA (*A*; EC_{50} = 1.2 nM) displays a higher sensitivity than does the RBA (*B*; EC_{50} = 4.0 nM). Data shown are individual standard curves with EC_{50} that have been repeated multiple times. Error bars indicate SE.

with a limit of detection (± SE) of 0.31 ± 0.06 nM and EC_{50} = 1.2 ± 0.2 nM (n = 10). Using the RBA, the calculated limit of detection for PbTx-3 was 1.01 ± 0.38 nM and EC_{50} = 4.0 ± 1.5 nM (n = 7), which is 3-fold higher than that for the RIA.

We next examined the matrix effect of the blood cards on the RIA and RBA. Dried blood spots collected from control animals were extracted and added to assay tubes containing the PbTx-3 standard curve. The presence of matrix caused a minor reduction in total binding in the RIA (11%) and RBA (15%), with no apparent effect on relative affinity (Figure 2).

We next examined toxin congener recognition between the two assays. The affinity of the antibody for each toxin was determined by reporting the ratio of PbTx-3 equivalents detected by RBA to those detected by RIA. Three brevetoxin congeners (PbTx-2, PbTx-3, and PbTx-9) showed very comparable affinities for each assay method. However, the RIA had a higher affinity for PbTx-6 and a lower affinity for PbTx-1 (Table 1). Overall, this comparison of the RBA with the RIA yields a rank order of affinity of PbTx-6 > 3 = 2 = 9 > 1. Unlike the RBA, the RIA did not recognize the CCTX-1 (data not shown).

We next analyzed a sample set from an acute time-course exposure that was identical to the sample set previously analyzed by Fairey et al. (2001) using the microplate receptor-binding assay and LC-MS. Mice were treated IP with a high, sublethal dose of PbTx-3 (180 µg/kg bw), and blood was collected at 0.5, 1, 2, 4, and 24 hr. Blood brevetoxin levels were at mean levels of 36 M at 30 min and maintained levels near 25 nM during the 1-, 2-, and 4-hr time points (Figure 3). At 24 hr, brevetoxin was still detectable (3.3 nM) at levels significantly different from those in nontreated animals (p = 0.002).

Given that we could still detect brevetoxin in blood a full day after exposure, we analyzed blood brevetoxin exposure after longer durations. Using a sample set collected 2.5 years earlier for experiments described by Gordon et al. (2001), we extracted the blood and conducted brevetoxin analysis by RIA. Mice (CD-1 rather than ICR strain) were treated with 180 µg/kg bw PbTx-3, and groups of four mice were bled at 0.5, 1, 2, 3, 4, or 7 days. The mean blood brevetoxin value at 0.5 days was 26.0 nM and then decreased to 8.2 nM at 1 day and maintained levels around 1.3 nM until day 3 (Figure 4). The brevetoxin was detected in animals 3, 4, and 7 days after treatment; however, these levels did not significantly (p > 0.05) differ from the vehicle-treated animals.

As a final experiment, we examined the dose response for brevetoxin to determine the lowest dose at which we could detect blood brevetoxin levels using blood collection cards and RIA detection. ICR mice were treated with increasing concentrations of PbTx-3 (10–300 µg/kg bw), and their symptoms were recorded by observation over the course of 60 min (Table 2). Blood was collected at 60 min and applied to blood collection cards. The lowest observable effect level was 100 µg/kg bw PbTx-3, with the symptoms of hind limb paralysis, chewing, and seizures observed. Three mice in the 200 µg/kg bw group and all four mice in the 300 µg/kg group died within the 60-min trial. All doses of brevetoxin administered were detectable at 1 hr, with significant levels found for the lowest administered dose of 10 µg/kg bw, a dose that was 10-fold lower than the lowest observable effect dose of 100 µg/kg bw (Figure 5). Analysis of the blood samples by RIA revealed a linear relationship (r^2 = 0.993) between administered and internal dose (Figure 6).

Table 1. Relative affinities of brevetoxin congeners for the RBA and RIA.

Congener	RBA:RIA
PbTx-1	26.4
PbTx-2	1.2
PbTx-3	1.1
PbTx-9	0.8
PbTx-6	0.4

Five brevetoxin congeners of approximate microgram weight suspended in methanol were tested in the RBA and RIA and calibrated to PbTx-3 equivalents. The data are reported as ratio of the PbTx-3 equivalents as determined by the RBA versus RIA. The data are provided as ratios rather than traditional ED_{50} values because two of the congeners were of too small a mass to be dried and weighed.

Discussion

In this article, we have compared an antibody-based assay with an RBA for the detection of brevetoxins from blood collection cards of laboratory mice exposed to brevetoxin. The RIA provides more sensitive detection with minimal interference from residual matrix after extraction of blood from the cards. The RIA allowed detection of brevetoxin at doses 10 times lower than the lowest observable effect level and at times up to 2 days after exposure.

Toxin detection methods are classified as analyses or assays, based on whether a sample value is measured with or without separation of active components. Functional, nonanimal assays for brevetoxins include RBAs, immunoassays (including both solid-phase nonradiometric ELISA and liquid-phase radiometric RIA), and cell-based assays (cytotoxicity and reporter gene; Van Dolah and Ramsdell 2001). A primary difference in the assays is the initial level of recognition at the receptor target

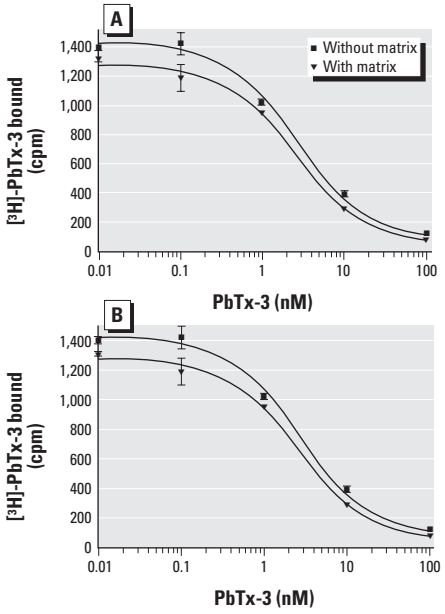

Figure 2. Comparison of brevetoxin standard curves in the absence and presence of dried blood matrix. The RIA (*A*) and the RBA (*B*) yield a small reduction in total [^{3}H]-PbTx-3 binding, 11% and 15%, respectively. Nonetheless, there was no significant difference in EC_{50} between the curves with matrix as opposed to those without matrix. Error bars indicate SE.

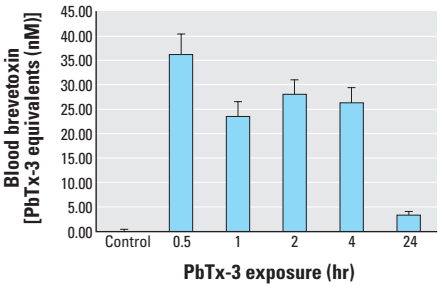

Figure 3. Blood brevetoxin levels after acute brevetoxin exposure. Mice were treated with 180 µg/kg bw PbTx-3 IP and blood was collected from four mice at each time point (0.5, 1, 2, 4, and 24 hr). The blood PbTx-3 level remained near 25 nM for the first 4 hr and declined to 3.3 nM by 24 hr. Results shown are mean ± SE for four animals at each time point from a single experiment.

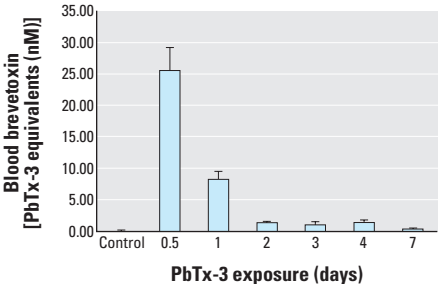

Figure 4. Blood brevetoxin levels after long-term brevetoxin exposure. Mice were treated with 180 µg/kg bw PbTx-3 IP, and blood was collected from four mice at each time point (0.5, 1, 2, 3, 4, and 7 days). A significant level of brevetoxin, 1.3 nM, could be detected up to 2 days after exposure. Days 3, 4, and 7 all showed detectable brevetoxin, but not in concentrations significantly different than that of the control. Results shown are for four animals at each time point from a single experiment. Results shown are mean ± SE.

(RBAs and cell-based assays) or by an antibody (RIA and ELISA). In our previous study (Fairey et al. 2001), we examined brevetoxin in mouse blood extract by RBA and by analysis by LC-MS. In an effort to maintain similar formats for comparison, we developed an RIA using the same radioligand and separation method as we used in the RBA. The antibody used for this work had previously been used in an ELISA format (Garthwaite et al. 2001)

RIAs have previously been described for brevetoxins to include two types, based on the brevetoxin conjugate linkage used for antibody production. Baden et al. (1984) prepared a PbTx-3–succinate derivative coupled by amine bond to bovine albumin by carbodiimide condensation and produced antibodies in goats, whereas Levine and Shimizu (1992) prepared a PbTx-2 conjugate coupled via an amide bond to bovine albumin by sodium borohydride reduction and produced antibodies in rabbits. Both conjugation methods link albumin to the C-42 on the K-ring of brevetoxin; however, the chemical linkage differs both in composition and length in the two preparations. Poli et al. (1995) compared the two antibody preparations and found EC_{50} values about 10 times lower for the rabbit antibody directed against the PbTx-2 conjugate (0.2 vs. 3.0 ng/mL). To produce the antiserum used in our study, we followed Levine and Shimizu's (1992) method of conjugating PbTx-2 to fetuin. The EC_{50} of 1.5 nM for this antibody falls within the range of the previously reported RIAs.

Antisera produced using both conjugation procedures detect the type 2 brevetoxins (PbTx-1, PbTx-7, and PbTx-10) poorly relative to the type 1 brevetoxins (PbTx-2, PbTx-3, PbTx-5, PbTx-6, and PbTx-8). We found that the difference between type 2 and type 1 brevetoxins is approximately 4-fold for our antiserum. The differences range from > 500-fold reported by Levine and Shimizu (1992), to 100-fold reported by Poli and Hewetson (1992), to 4.5-fold reported by Baden et al. (1984). This indicates that the sheep antibody described here shows a lower degree of selectivity for the type 2 backbone of brevetoxins and may be more applicable for detection of toxins containing both backbone structures.

In the first phase of our investigation, we compared this RIA with the RBA using an identical format. We found that the RIA had an EC_{50} that was three times lower than the RBA (1.2 nM vs. 4.0 nM). In an earlier comparison of the RIA (goat antibody) and the RBA, Baden et al. (1988) found that the RIA was less sensitive than the RBA (EC_{50} of 20 vs. 12 nM). The differences may be due to the format in which the assays were conducted. When comparing the selectivity for brevetoxin congeners between the two assay methods, we found that the differences between the methods for brevetoxin type 1 and type 2 backbones differ by approximately 26-fold, a value consistent with that reported by Baden et al. (1988). Thus, although the RIA detects both brevetoxin backbone structures, in terms of pharmacologic activity the RIA substantially underestimates the type 2 brevetoxins. This critical difference underlies a major difference in methods based on receptors versus antibodies: receptor-based assays are more closely correlated to animal-based assays and toxicity than are assays based on antibodies (Van Dolah and Ramsdell 2001).

Biologic monitoring for brevetoxins requires measurement of toxin levels in accessible tissues (e.g., blood, hair, urine, and circulating or accessible cells) in order to assess exposure. Toxicokinetic studies have been conducted using radiolabeled brevetoxin by several routes of administration [oral (Cattet and Geraci 1993), intravenous (Poli et al. 1990), inhalation (Benson et al. 1999)]; however, only Poli et al. (2000) have quantified brevetoxins in clinical samples after exposure. Poli et al. (2000) examined urine and serum samples from two individuals with neurotoxic shellfish poisoning and found that the urine analysis was positive, but brevetoxins were not detectable in serum because of more rapid clearance from serum and matrix interference of serum. We have applied the blood collection card method for marine toxin analysis to allow monitoring of brevetoxins in whole blood with minimal matrix interference. The extraction of dried blood from cellulose cards with methanol provides an excellent separation because the methanol fixes much of the blood matrix to cellulose while providing high recovery of the toxin. The extracts from control blood have caused a small inhibitory effect in the RBA (Fairey et al. 2001). We have examined this matrix effect by constructing brevetoxin standard curves in the absence and presence of extracted blood matrix for both the RBA and the RIA. In the RIA we observed an 11% decrease in total binding with the extracted blood matrix and no significant change in EC_{50} for brevetoxin. These data indicate that blood may indeed provide the optimal collection fluid for biomonitoring of brevetoxin exposure. Furthermore, blood collection cards provide a simple and effective method of sample collection and storage for both the clinic and the field.

Our first exposure experiment for the brevetoxin RIA of blood cards was a time-course study designed to reproduce the data obtained by the RBA and LC-MS by Fairey et al. (2001). Using the RIA, we were able to detect brevetoxin in blood at all time points (0.5, 1, 2, 4, and 24 hr) and for each of the four animals per treatment group. By contrast,

Table 2. Observable symptoms in mice after brevetoxin exposure.

Dose (μg/kg bw)	Difficulty breathing	Hindlimb paralysis	Chewing	Rolling on back	Forelimb extension	Hindlimb extension	Seizing
0 (control)	NO	NO	NO	NO	NO	NO	NO
10	NO	NO	NO	NO	NO	NO	NO
25	NO	NO	NO	NO	NO	NO	NO
50	NO	NO	NO	NO	NO	NO	NO
100	NO	30 min	25 min	NO	NO	15 min	15 min
200	NO	5 min	NO	15 min	NO	NO	10 min
300	10 min	5 min	15 min	10 min	15 min	NO	5 min

NO, not observed more than 60 min after toxin administration. Mice were treated IP with increasing doses of brevetoxin, and observable symptoms were recorded at the earliest onset of occurrence. The observation period was time of injection to 60 min postinjection, at which time the animals were sacrificed for blood collection.

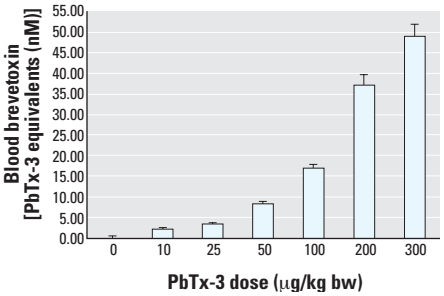

Figure 5. Blood brevetoxin levels after low-dose brevetoxin exposure. Mice were treated with PbTx-3 IP, and blood was collected from all four mice at each dose [0 (control) and 10, 25, 50, 100, 200, 300 μg/kg bw] after 1 hr exposure. The plot of average blood PbTx-3 concentration demonstrates that PbTx-3 can be detected at doses 10 times lower than symptomatic levels (100 μg/kg bw). Results shown are mean ± SE.

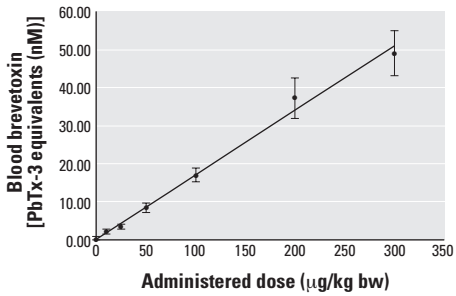

Figure 6. Blood brevetoxin levels after low-dose brevetoxin exposure. The plot of the internal dose versus external dose yields an r^2 value of 0.993. The PbTx-3 concentrations were calculated by subtracting the control value (0 dose) from the mean for duplicate blood spots from one animal. The results indicate mean ± SE for four animals at each concentration from a single experiment.

in our earlier study using a microplate receptor assay (Fairey et al. 2001), although brevetoxin activity was detectable in all experimental groups, only in the 4.0-hr treatment was brevetoxin detected in all four animals. In this earlier study, the mean brevetoxin level (± SE) measured by the RBA at 4 hr treatment was 25 ± 7 nM, which compares well with that found in the present study measured by RIA, a mean value of 27 ± 7 nM at 4 hr treatment. The similar measurements, yet less consistent brevetoxin detection of the RBA in our earlier report, may be due, at least in part, to the lower reaction volume of the microplate format.

We next decided to examine RIA detection of blood cards at longer periods of brevetoxin exposure. In an earlier study (Gordon et al. 2001), we reported a delayed thermoregulatory response to brevetoxin in mice. This delayed response was persistent hyperthermia, which was distinct from the rapid onset hypothermia commonly reported in response to polyether toxins. At that time, we collected blood from four animals each at the 0.5-, 1-, 2-, 3-, 4-, and 7-day time points. The samples were applied to blood collection cards that were stored 32 months before measurement by RIA. The level of brevetoxins detected by RIA were elevated for 12 hr, were reduced by one-third by 24 hr, maintained a significant level above control by day 2, and were detectable but not at significant levels for days 3, 4, and 7. These findings are consistent with the toxicokinetic study of Cattet and Geraci (1993), who found that radiolabeled [^{3}H]-PbTx-3 was detectable up to 8 days after oral exposure. Taken together, these findings indicate that nonlethal exposures to brevetoxin may be measurable up to 2 days after exposure using RIA measurement by blood collection cards.

The final exposure we conducted was a dose dependency. The doses chosen ranged from 10 to 300 µg/kg bw PbTx-3, which is 5.5–167% of the maximally tolerable dose (180 µg/kg bw) used for our previous studies (Fairey et al. 2001; Gordon et al. 2001). We chose blood measurements for 60 min based on our findings of a plateau value of 25–30 nM for time points between 1 and 12 hr after exposure. Based on observable symptoms, we were able to establish 100 µg/kg bw as the lowest observable effect level. An acute decrease in core body temperature was observable only at 200 µg/kg bw and 300 µg/kg bw. Measurement of blood brevetoxin by RIA revealed a linear relationship between administered and internal dose for the 1-hr exposure. Brevetoxin was measurable by RIA at all tested doses with significant amounts at the lowest dose of 10 µg/kg bw. These results indicate that brevetoxin can be measured from blood collection cards at doses 10 times lower than the dose causing the lowest observable symptoms in mice. Taken together with the finding that blood brevetoxin levels are comparable between 1 and 12 hr, this indicates that biomonitoring of brevetoxin by RIA of blood cards may be feasible at nonsymptomatic exposure levels.

A relevant question exists as to how well the IP dosing of PbTx-3 in mice models the exposure of humans and aquatic species to toxin in algae, toxin released from algae into the water or aerosol, and toxin accumulated in the food web? Preliminary studies in our laboratory with mice given PbTx-3 orally and finfish exposed to *K. brevis* in aquaria (unpublished data) gave results similar to those reported here. Another potential concern lies in the detection of brevetoxin metabolites. *K. brevis* is reported to produce brevetoxin congeners 2, 1, and 3 in the ratio of 20:4:1 (Roszell et al. 1989). The predominantly occurring brevetoxin in algae, PbTx-2, is rapidly reduced to the more stable PbTx-3. Following the neurotoxic shellfish poisoning in New Zealand (Mackenzie et al. 1996; Satake et al. 1996), four brevetoxin metabolites were described, including a taurine conjugate, an *S*-cysteine conjugate, and fatty acid esters (Ishida et al. 1996; Morohashi et al. 1995, 1999; Murata et al. 1998). Subsequently, Poli et al. (2000) reported brevetoxin activity consistent with metabolites from shellfish from Florida coastal waters, and Plakas et al. (2002) identified these as cysteine conjugates. In addition, Poli et al. (2000) purified the two metabolites and compared their activity by RIA and RBA. The metabolites had activity comparable with PbTx-3 by RIA; however, they had a third less activity by RBA. Thus, RIA is likely to detect the major brevetoxin conjugates found both in algae and shellfish; however, receptor-based assays are necessary for precise toxicity measurement.

In summary, we have found that the RIA is more sensitive and has less matrix effect than does the RBA when detecting PbTx-3 in exposed mice using blood collection cards. Nevertheless, the two assays complement one another well when analyzing different PbTx congeners. The RIA provides detection in blood at brevetoxin exposures well below symptomatic levels and allows detection up to 2 days after exposure. Taken together, these results support the merits of tier-based testing for brevetoxins: antibody methods provide a good screening method, whereas receptor-based methods provide a good toxicity measurement, and LC-MS provides absolute confirmation of toxin congeners.

References

Adam BW, Alexander RJ, Smith SJ, Chance DH, Loeder JG, Elvers LH, et al. 2000. Recoveries of phenylalanine from two sets of dried-blood spot reference materials: prediction from hematocrit, spot volume, and paper matrix. Clin Chem 46:126–128.

Baden DG, Mende TJ, Szmant AM, Trainer VL, Edwards RA, Roszell LE. 1988. Brevetoxin binding: molecular pharmacology versus immunoassay. Toxicon 26:97–103.

Baden DG, Mende TJ, Walling J, Schultz DR. 1984. Specific antibodies directed against toxins of *Ptychodiscus brevis* (Florida's red tide dinoflagellate). Toxicon 22:783–789.

Benson JM, Thischler DL, Baden DG. 1999. Uptake, tissue distribution, and excretion of brevetoxin-3 administered to rats by intratracheal instillation. J Toxicol Environ Health 56:345–355.

Bourdelais AJ, Tomas CR, Naar J, Kubanek J, Baden DG. 2002. New fish-killing alga in coastal Delaware produces neurotoxins. Environ Health Perspect 110:465–470.

Cattet M, Geraci JR. 1993. Distribution and elimination of ingested brevetoxin (PbTx-3) in rats. Toxicon 31(11):1483–1486.

Davis CC. 1948. *Gymnodinium breve*: a cause of discolored water and animal mortality in the Gulf of Mexico. Bot Gaz 109:358–360.

Delaney JE. 1985. Bioassay procedures for shellfish toxins. In: Laboratory Procedures for the Examination of Seawater and Shellfish (Greenberg AE, Hung DA, eds). Washington, DC:American Public Health Association, 64–80.

Dickey RW, Plakas SM, Jester ELE, El Said KR, Johannessen JN, Flewelling LJ, et al. 2002. Multilaboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts [Abstract]. In: 10th International Conference on Harmful Algae, 21–25 October 2002, St. Petersburg, FL. Copenhagen:ISSHA, 72.

Fairey ER, Shuart NG, Busman M, Moeller PD, Ramsdell JS. 2001. Biomonitoring brevetoxin exposure in mammals using blood collection cards. Environ Health Perspect 109:717–720.

Garthwaite I, Ross KM, Miles CO, Briggs LR, Towers NR, Borrell T, et al. 2001. Integrated enzyme-linked immunosorbent assay screening system for amnesic, neurotoxic, diarrhetic, and paralytic shellfish poisoning toxins found in New Zealand. J AOAC Int 84:1643–1648.

Gordon CJ, Kimm-Brinson KL, Padnos B, Ramsdell JS. 2001. Acute and delayed thermoregulatory response of mice exposed to brevetoxin. Toxicon 39:1367–1374.

Haque SM, Onoue Y. 2002. Variation in toxin compositions of two harmful raphidophytes, *Chattonella antiqua* and *Chattonella marina*, at different salinities. Environ Toxicol 17:113–118.

Ishida H, Muramatsu N, Nukaya H, Kosuge T, Tsuji K. 1996. Study on neurotoxic shellfish poisoning involving the oyster, *Crassostrea gigas*, in New Zealand. Toxicon 34:1050–1053.

Kahn S, Ahmed MS, Arakawa O, Onoue Y. 1995. Properties of neurotoxins separated from a harmful red tide organism *Chattonella marina*. Isr J Aquacult 47:137–141.

Kahn S, Arakawa O, Onoue Y. 1997. Neurotoxins in a toxic red tide of *Heterosigma akashiwo* (Raphidophyceae) in Kagoshima Bay, Japan. Aquacult Res 28:9–14.

———. 1996. Neurotoxin production by a chloromonad *Fibrocapsa japonica* (Raphidophyceae). J World Aquacult Soc 27:254–263.

Landsberg JH, Steidinger KA. 1997. A historical review of red tide events caused by *Gymnodinium breve* as related to mass mortalities of the endangered manatee (*Trichechus manatus latirostris*) in Florida, USA. In: Harmful Algae, the Proceedings of the Eighth International Conference on Harmful Algae, 25–29 June 1997, Vigo, Spain (Reguera B, Blanco J, Fernandez M, Wyatt T, eds). Santiago de Compostela, Spain:Xunta de Galicia and IOC of UNESCO Publishers, 97–100.

Levine L, Shimizu Y. 1992. Antibodies to brevetoxin B: serologic differentiation of brevetoxin B and brevetoxin A. Toxicon 30:411–418.

Lin Y, Risk M, Ray SM, Van Engen D, Clardy J, Golik J, et al. 1981. Isolation and structure of brevetoxin B from the "red tide" dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*). J Am Chem Soc 103:6773.

Mackenzie L, Haywood AJ, Adamson J, Truman P, Till D, Seki T, et al. 1996. Gymnodimine contamination of shellfish in New Zealand. In: Harmful and Toxic Algal Blooms, The Proceedings of the Seventh International Conference on Toxic Phytoplankton, 12–16 July 1995, Sendai, Japan (Yasumoto T, Oshima Y, Fukuyo Y, eds). Paris:International Oceanographic Commission of UNESCO, 97–100.

Martin DF, Chatterjee AB. 1969. Isolation and characterization of a toxin from the Florida red tide organism. Nature 221:59.

Mase B, Jones W, Ewing R, Bossart G, Van Dolah F, Leighfield T, et al. 2000. Epizootic in bottlenose dolphins in the Florida panhandle: 1999–2000. In: Proceedings of the American Association of Zoo Veterinarians and International Association for Aquatic Animal Medicine, 17–21 September 2000, New Orleans, LA (Baer CK, ed). New Orleans,

LA:American Association of Zoo Veterinarians and International Association for Aquatic Animal Medicine, 522–525.

McFarren EF, Tanabe H, Silva FJ, Wilson WB, Campbell JE, Lewis KH. 1965. The occurrence of a ciguatera-like poison in oysters, clams, and *Gymnodinium breve* cultures. Toxicon 3:111–123.

Morohashi A, Satake M, Murata K, Naoki H, Kaspar HF, Yasumoto T. 1995. Brevetoxin B3, a new brevetoxin analog isolated from the greenshell mussel *Perna canaliculus* involved in neurotoxic shellfish poisoning in New Zealand. Tetrahedron Lett 36(49):8995–8998.

Morohashi A, Satake M, Naoki H, Kaspar HF, Oshima Y, Yasumoto T. 1999. Brevetoxin B4 isolated from greenshell mussels *Perna canaliculus*, the major toxin involved in neurotoxic shellfish poisoning in New Zealand. Nat Toxins 7:45–48.

Murata K, Satake M, Naoki H, Kaspar HF, Yasumoto T. 1998. Isolation and structure of a new brevetoxin analog, brevetoxin B2 from greenshell mussels from New Zealand. Tetrahedron 54:735–742.

Plakas SM, El Said KR, Jester ELE, Grande HR, Musser SM, Dickey RW. 2002. Confirmation of brevetoxin metabolism in the eastern oyster (*Crassostrea virginica*) by controlled exposures to pure toxins and *Karenia brevis* cultures. Toxicon 40:721–729.

Poli MA, Hewetson JF. 1992. Antibody production and development of a radioimmunoassay for Pbtx-2 type brevetoxins. In: Proceedings of the Third International Conference on Ciguatera Fish Poisoning (Tosteson TR, ed). Quebec, Canada:Polyscience Publishers, 115–127.

Poli MA, Musser SM, Dickey RW, Eilers PP, Hall S. 2000. Neurotoxic shellfish poisoning and brevetoxin metabolites: a case study from Florida. Toxicon 38:981–993.

Poli MA, Rein KS, Baden DG. 1995. Radioimmunoassay for PbTx-2-type brevetoxins: epitope specificity of two anti-PbTx sera. J AOAC Int 78:538–542.

Poli MA, Templeton CB, Thompson WL, Hewetson JF. 1990. Distribution and elimination of brevetoxin PbTx-3 in rats. Toxicon 28:903–910.

Roszell LE, Schulman LS, Baden DG. 1989. Toxin profiles are dependent on growth stages in cultured *Ptychodiscus brevis*. In: Toxic Marine Phytoplankton (Granelli E, Sunstrom B, Edler L, Anderson D, eds). New York:Elsevier Science, 403–406.

Satake M, Morohashi A, Murata K, Kaspar HF, Yasumoto T. 1996. Chemical studies on the NSP toxins in the green shell mussels from New Zealand. In: Harmful and Toxic Algal Blooms, The Proceedings of the Seventh International Conference on Toxic Phytoplankton, 12–16 July 1995, Sendai, Japan (Yasumoto T, Oshima Y, Fukuyo Y, eds). Paris:International Oceanographic Commission of UNESCO, 487–490.

Steidinger KA, Joyce EA Jr. 1973. Florida Red Tides. Educational Series no. 17. St. Petersburg, FL:Florida Department of Natural Resources, 1–26.

Taylor HF. 1917. Mortality of Fishes on the West Coast of Florida. Report of the U.S. Commissioner of Fisheries. Appendix III. Bureau of Fisheries Document 848. Washington, DC:Government Printing Office.

Van Dolah FM, Ramsdell JS. 2001. Review and assessment of in vitro detection methods for algal toxins. J AOAC Int 84:1617–1625.

Woodcock AH. 1948. Note concerning human respiratory irritation associated with high concentrations of plankton and mass mortality of marine organisms. J Mar Res 7(1):56–62.