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 antiqua, Ptychodiscus brevis, and Chattonella marina) have been reported in isolated cases by functional assays and liquid chromatography–mass spectrometry. 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). The broad distribution of raphidophytes opens the concern of a widespread occurrence of this family of toxins.

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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 (Bourdélais et al. 2002). The broad distribution of raphidophytes opens the concern of a widespread occurrence of this family of toxins.

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Brevetoxin levels were measured in blood samples 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.
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 (Avec, 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 desiccatant 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 Turboblow 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 to the semitendinous 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. RIA were run in 12 × 75 borosilicate glass tubes in PBS containing 137 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 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 [3H]-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 [3H]-PbTx-3 tracer was added. The tubes were placed on a shaker (Titramax 100; Heidolph Instruments, Cinnaminson, NJ, USA) and then 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 separation of bound and unbound brevetoxin. The Sac-Cel was filtered onto 25-mm GF/C glass fiber filters (Whatman, Newton, MA, USA) and incubated for another hour after the addition of the radioactive toxin. The RIA antisera was directed against a PbTx-3 antibody for 1 hr before the [3H]-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. The RIA antisera was directed against a PbTx-3 antibody for 1 hr before the [3H]-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. The RIA antisera was directed against a PbTx-3 antibody for 1 hr before the [3H]-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. The RIA antisera was directed against a PbTx-3 antibody for 1 hr before the [3H]-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.

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 EC50 (p < 0.001). The RIA produces a standard curve for 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 (EC50) 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).

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; EC50 = 1.2 nM) displays a higher sensitivity than does the RBA (B; EC50 = 4.0 nM). Data shown are individual standard curves with EC50 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 similar 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. Five brevetoxin congeners of approximate microgram levels (PbTx-1: 26.4, PbTx-2: 1.2, PbTx-3: 1.1, PbTx-9: 0.8, PbTx-6: 0.4) were administered to ICR mice and applied to blood collection cards. The RIA was used to detect brevetoxin levels using blood collection cards. The results are shown in Table 1 and illustrated in Figure 5.

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 nonradioisotopic 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 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).

**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.
Dose Difficulty Hindlimb Rolling Forelimb Hindlimb

Results shown are mean ± SE. Lower than symptomatic levels (100 µg/kg bw).

We found that the difference between type 2 and type 1 brevetoxins is approximately 4-fold for our assay system. 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 EC50 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 (EC50 of 20 vs. 12 nM). The differences may be due to the format in which the assays were conducted. When comparing the selectivity of conjugating PbTx-2 to fetoins, the EC50 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 assay system. 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.

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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 brevetoxins in mice. This delayed response was persistent hyperthermia, which was distinct from the rapid onset hyperthermia 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 [3H]-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 observ- able 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 under non-symptomatic 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 tauro conjugate, an N-sulfocysteine 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 Plasak 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.

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