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RICIN AND STAPHYLOCOCCAL ENTEROTOXIN B FATE IN WATER MATRICES

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14. ABSTRACT
This report is the final report for the Defense Threat Reduction Agency-funded project "Biological Toxin Fate in Water Matrices" (BA05TAS080) and summarizes research directed at characterizing toxin stability in deionized water (pH 7, 10 mM PO_4) with and without chlorine treatment in terms of hydrolysis/mass, denaturation, and activity as ascertained by polyacrylamide gel electrophoresis, circular dichroism, cell toxicity (ricin), and cell activity (SEB). Details of the research will be published separately. The SEB work was supplemented by liquid chromatography-mass spectrometry. Each toxin was stable for at least 30 days in 10 mM PO_4 (pH 7) at room temperature. For each toxin, the majority of the toxin precipitated when heated slowly to the toxin's T_m. The toxin remaining in solution retained some activity. Ricin remaining in solution maintained its native fold; SEB remaining in solution did not. Each toxin retained some activity after treatment at low initial chlorine:toxin molar ratios, with no activity observed for SEB after 5 day treatment with an initial chlorine:toxin ratio of 50:1 and for ricin after 24 hr treatment with an initial chlorine:toxin ratio of 100:1. As the initial chlorine:toxin ratio increased, the amount of native protein decreased.

15. SUBJECT TERMS

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PREFACE

The work described in this report was authorized under Contract No. DAAD13-03-D-0017 and Project No. 622622.55200,Threat Agent Science. This work was started in October 2004 and completed in March 2008.

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RICIN AND STAPHYLOCOCCAL ENTEROTOXIN B FATE IN WATER MATRICES

1. INTRODUCTION

Biological agent fate data is essential to the development of improved methods for simulation of battle space environment, environmental surveillance, decontamination, and individual protection related to these potential biological warfare agents. The objective of this project was to obtain stability data for the biological toxins ricin and staphylococcal enterotoxin B (SEB) to better understand the fate of these toxins in a water-based environment. Although there has been much work reported on the detection of these biological toxins and some research related to the stability of individual toxins, little has been done coordinating the study of different toxins under identical environmental conditions and/or identical chlorine treatment protocols.[1-10] Here we characterize toxin stability in deionized water (pH 7, 10 mM PO_4) with and without chlorine treatment in terms of hydrolysis/mass, denaturation, and activity as ascertained by polyacrylamide gel electrophoresis (PAGE), circular dichroism, cell toxicity (ricin), and cell activity (SEB) analysis. The SEB work was also supplemented by limited liquid chromatography-mass spectrometry (LC-MS). Appendix A summarizes publications from this work to date.

2. METHODS

Note: Ricin and SEB are extremely toxic. Their use is controlled under the Biological Select Agents and Toxins program in the United States by the Centers for Disease Control and Prevention (Atlanta, GA). Handling of these toxins should follow strict safety procedures determined in collaboration with the safety office of the research laboratory's organization.

2.1 Toxin Work Solutions.

Ricin (*Ricinus communis agglutinin II*, RCA) was received from the vendor (Vector Laboratories, Burlingame, CA) in 10 mM sodium phosphate, pH 7.8, 0.15 M NaCl, and 0.08% NaN_3. Staphylococcal enterotoxin B (SEB) was received from the vendor (Sigma-Alrich, St. Louis, MO) in dry form lyophilized from a solution originally containing 1.3 mg/mL SEB and 25 mM sodium phosphate, pH 7.4 (per discussion with Sigma-Aldrich Technical Support on 10 January 2006 and 26 March 2008). The vendor-supplied SEB was dissolved in deionized water to the desired SEB concentration from which the SEB working solution would be prepared. Toxin working solutions (Work Solutions) were prepared by dialyzing ricin or SEB into 10 mM sodium phosphate, pH 7.0 (Standard Buffer) or another water matrix as noted over a period of 11-14 hr with gentle stirring using three ~600–800 mL volumes of Standard Buffer (1.8-2.4 L total). Dialysis was carried out on ice using regenerated cellulose or cellulose ester Dispodialyzers® (Spectrum Laboratories, Rancho Dominguez, CA) with 5000 or 8000 molecular weight cutoff. Ricin Work Solutions were stored at 0-4 °C. SEB Work Solutions were kept at 0-4 °C until use, but used on the day prepared unless otherwise noted. The concentration of each Work Solution was ascertained by ultraviolet (UV) spectroscopy as described below. Standard Buffer was prepared in deionized water and stored at 0-4 °C.

2.2 Stability Studies.

Unless otherwise noted, studies on toxin stability (Stability Studies) were carried out by incubating toxin solutions in siliconized screw-cap microfuge tubes using a Boekel Tropicooler incubator, a Fisher Scientific Isotemp® heat block (Thermo Fisher, Waltham, MA), or a Fisher Scientific Dry Bath Incubator, with time = 0 taken to be the time at which the sample was placed in the incubator pre-regulated to 25 °C. Such samples will be referred to as Stability Samples. Prior to incubation (baseline) and at various time intervals, aliquots (Stability Aliquots) were withdrawn from the Stability Samples and solutions prepared for analysis by one or more of the following methods: pH, Coomassie® Plus total protein assay (CPA); bicinchoninic acid total protein assay (BCA); UV absorbance; circular dichroism (CD); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); liquid chromatography-mass spectroscopy (LC-MS, SEB only); neutral red uptake assay (NRU, for ricin); or T-cell proliferation assay (T-CA, for SEB). These analysis methods are described in detail below. The SEB Stability Aliquots for T-CA were sterile-filtered through a Whatman Puradisc 4 mm syringe filter (0.2 mm PVDF), quick-frozen on liquid nitrogen, and stored at -80 °C.

2.2.1 SEB Stability with Time at 25 °C.

2.2.1.1 SEB 30 Day Study.

One sample of SEB (1 mg/mL; 1.5 mL) in Standard Buffer was incubated at 25.5 °C (± 1.0 °C). Difficulty in maintaining more precise temperature regulation occurred as a result of heating, ventilation, and air conditioning system failure over several days during the experiment. In addition to the baseline aliquot, aliquots were drawn at the following time points after placing the sample in the incubator: 1 and 3 hr; 1, 2, 5, 9, 19, 23, and 30 days. Dilutions were made if appropriate, and the resulting Stability Aliquots were quick-frozen until analysis. Stability Aliquots were prepared for the following analyses: CPA, UV, CD, SDS-PAGE, and LC-MS.

2.2.1.2 SEB 33 Day Study.

Parallel samples of SEB (one per time point) with an initial concentration of 5 mg/mL in Standard Buffer were incubated at 25.0 °C (± 1.0 °C). In addition to the baseline aliquot, aliquots were drawn at the following time points after placing the samples in the incubator: 1, 5, 12, 19, 26, and 33 days. Stability Aliquots were prepared for the following analyses: pH, UV, CD, SDS-PAGE, and T-CA. The PAGE and T-CA aliquots were quick-frozen and stored at -80 °C for later analysis.

2.2.1.3 SEB 33 Day Study with Staggered Start.

This study was carried out in a similar manner to that described above for "SEB 33 Day Study." The initial SEB concentration was 4 mg/mL. For this study, baseline, 5, 19, and 33 day time point Stability Samples were prepared. However, rather than starting the incubation of all time points on the same day, the incubation for each time point Stability Sample was begun on a different day, but with all incubations scheduled to end within two days of each other so that the storage time did not vary significantly. The 33 and 5 day Stability Samples were initiated so that they would end on the same date; the 19 day Stability Sample was timed to end the next day; and the baseline sample was prepared on the same day as the 19 day Stability Aliquots.

2.2.2 SEB Stability with Temperature.

Melting experiments were carried out for SEB in 55 mM PO_4 ([SEB] = 0.4 mg/mL) and in 10 mM PO_4 ([SEB] = 0.2 mg/mL) as described in "Melt Studies" under "Circular Dichroism" below. In addition, a slow heating experiment was carried out. For each slow heating Stability Sample, the sample ([SEB] = 2 mg/mL) in a screw-cap siliconized microfuge tube was placed in a Fisher Scientific Isotemp® heat block (Thermo Fisher) pre-regulated to 25 °C and allowed to equilibrate for 5 min. The heat block temperature was then set to the desired temperature (55, 60, 68, or 95 °C), and the sample was brought slowly to that temperature at the heating rate of the heat block. Once the desired temperature was reached, the temperature was held constant for 5 min. At the 5 min mark, the sample was transferred to a Fisher Scientific Dry Bath Incubator (Thermo Fisher), pre-regulated to 25 °C, and allowed to equilibrate. A control SEB sample was placed at 25 °C in a Fisher Scientific Dry Bath Incubator, pre-regulated to 25 °C, and incubated at 25 °C for a length of time equal to that of the entire heating and cooling period required for the Stability Sample that had been brought to the highest temperature. For each Stability Sample and the control sample, after the final equilibration at 25 °C, the sample was centrifuged at 25 °C for 5 min at 10,000 rpm. Stability Aliquots were prepared for the following analyses: CD, UV, pH, T-CA, and PAGE. The T-CA and PAGE aliquots were quick-frozen and stored at -80 °C for later analysis.

2.2.3 SEB Stability Upon Chlorine Treatment.

2.2.3.1 Calcium Hypochlorite Treatment.

The SEB was dialyzed into distilled H_2O rather than Standard Buffer and calcium hypochlorite was added from a freshly prepared stock solution (Appendix B) at time = 0. The SEB sample (0.6 mg/mL; 1.5 mL) in distilled H_2O having an initial concentration of ~1.5 ppm free chlorine was incubated at 25.0 °C (± 0.5 °C). In addition to the baseline Stability Aliquot, aliquots were drawn at the following time points after placing the sample in the incubator: 1 and 3 hr; 1, 2, and 5 days. Dilutions were made if appropriate, and the resulting Stability Aliquots were quick-frozen and stored at -80 °C until analysis. Stability Aliquots were prepared for the following analyses: CPA, UV, CD, SDS-PAGE, and LC-MS. Chlorine concentration was determined by the Test Strip method described under "Chlorine Concentration Determinations" using Micro Check™ test strips.

2.2.3.2 Sodium Hypochlorite Treatment, 2 Day Study.

Sodium hypochlorite (NaOCl, reagent grade, 10-13% available chlorine) stock solution was obtained from Sigma-Aldrich (St. Loius, MO). The free chlorine concentration of the NaOCl stock solution was determined on the first day of the experiment, and NaOCl spiking solutions were then prepared by dilution from the NaOCl stock solution. The free chlorine concentration of each NaOCl spiking solution was measured three times and averaged immediately prior to spiking a Stability Sample. The volume required to spike a given Stability Sample was determined based on this averaged free chlorine measurement. For each Stability Sample ([SEB] = 2 mg/mL), the sample was placed in a heat block pre-regulated to 25 °C as noted above under "Stability Studies" and equilibrated for 5 min. The sample was then spiked with the appropriate volume of NaOCl spiking solution to give the desired chlorine:SEB molar ratio and the sample mixed by inversion three times. Stability Aliquots of the Stability Sample were removed at 2.5 min and 3 min after the spike for chlorine concentration and pH determinations, respectively. At the ~47 hr mark after addition of the chlorine spike, the Stability Sample was centrifuged (5 min, 25 °C, 10,000 rpm), and Stability Aliquots were prepared for

chlorine concentration determination, CD, UV, pH, and PAGE. The PAGE aliquots were quick-frozen and stored at -80 °C for later analysis. A control SEB Stability Sample was also prepared that was spiked with 10 mM PO_4 rather than NaOCl. In addition 1-3 blank samples were prepared that did not contain SEB. Chlorine concentration was determined by the Spectrophotometric Assay method described under "Chlorine Concentration Determinations."

2.2.3.3 Sodium Hypochlorite Treatment, 5 Day Study.

Experiments were carried out as described under "Sodium Hypochlorite Treatment, 2 Day Study" above, except that Stability Aliquots were prepared at the ~118 hr mark rather than the ~47 hr mark. Additionally, a Stability Aliquot was prepared for T-CA. The PAGE and T-CA aliquots were quick-frozen and stored at -80 °C for later analysis. Chlorine concentration was determined by the Spectrophotometric Assay method described under "Chlorine Concentration Determinations."

2.2.4 Ricin Stability with Time at 25 °C, 32 Day Study.

Parallel samples of ricin (one per time point) with an initial concentration of 2 mg/mL in Standard Buffer were incubated at 25.0 °C (± 1.0 °C). In addition to the baseline aliquot, aliquots were drawn at the following time points after placing the samples in the incubator: 1, 4, 11, 18, 26, and 32 days. Stability Aliquots were prepared for the following analyses: pH, UV, CD, SDS-PAGE, and NRU. The PAGE and NRU aliquots were quick-frozen and stored at -80 °C for later analysis.

2.2.5 Ricin Stability with Temperature.

A melting experiment was carried out for ricin (0.3 mg/mL) in 10 mM PO_4 as described in "Melt Studies" under "Circular Dichroism" below. In addition, a slow heating experiment was carried out in a similar manner to that described for SEB above under "SEB Stability with Temperature". A starting ricin concentration of 4 mg/mL was used and slow heating was carried out for Stability Samples to temperatures of 37, 55, 73, and 95 °C. Stability Aliquots were prepared for the following analyses: CD, UV, pH, NRU, and PAGE. The PAGE aliquots were quick-frozen and stored at -80 °C for later analysis. The NRU aliquots were stored at 4 °C (as is the vendor-supplied ricin) for later analysis.

2.2.6 Ricin Stability Upon Chlorine Treatment.

Experiments were carried out in a similar manner to that described for SEB above in "Sodium Hypochlorite Treatment, 2 Day Study" under "SEB Stability Upon Chlorine Treatment". Starting Stability Sample ricin concentrations of 3-4 mg/mL were used. Stability Aliquots were prepared at the ~24 hr mark rather than the ~47 hr mark. Additionally, a Stability Aliquot was prepared for NRU. The PAGE aliquots were quick-frozen and stored at -80 °C for later analysis. The NRU aliquots were stored at 4 °C for later analysis. Chlorine concentration was determined by the Spectrophotometric Assay method described under "Chlorine Concentration Determinations."

2.3 pH.

The pH was determined with an Accumet AP61 Series Portable pH meter (Thermo Fisher, Waltham, MA) after standardization with pH 4, 7, and 10 buffers (Buffer-Pac Color-Coded Solutions, Thermo Fisher) using either an MI-412 Micro-combination pH microelectrode (Microelectrodes, Inc., Bedford, NH) or a Thermo Scientific 3P Cole Parmer 1-1/2 microprobe (Thermo Fisher) electrode.

2.4 Protein Concentration Determination.

2.4.1 UV Absorbance.

The absorbance was measured using a JASCO Model J-810 spectropolarimeter (JASCO Analytical Instruments, Easton, MD) equipped with a PTC-423S Peltier thermoelectric temperature control system. In each case, 15 measurements on the solvent solution without any protein (blank) at the wavelength of interest were recorded at intervals of 1 s and averaged. Fifteen measurements were then recorded on the sample and averaged. The blank average was subtracted from the sample average. For SEB, the blank-subtracted A_{277} was used with $E^{1\%} = 14$ to calculate the concentration.[11] For ricin, the blank-subtracted A_{280} was used with $E^{1\%} = 14$.[12]

2.4.2 Coomassie® Plus Assay (CPA) and Bicinchoninic Acid Assay (BCA).

These total protein assays used a VersaMax™ microplate reader (Molecular Devices) and commercially available reagents, Pierce Coomassie® Plus Protein Assay Reagent for CPA (Pierce, Rockford, IL) and QuantiPro™ bicinchoninic acid (BCA) Assay Kit (Sigma-Aldrich, St. Louis, MO). During method development, studies showed that bovine serum albumin (BSA) could be used as a calibration standard for SEB and ricin for the BCA. For the CPA, BSA was a suitable standard for SEB, but ricin results required a scaling factor of 1.8 when using BSA as a standard. For each day of analysis, calibration standards were prepared by serial dilution of a stock BSA solution (2.0 mg/mL, Sigma-Aldrich, St. Louis, MO) that was opened immediately prior to use. The calibration curve was linear in the 0.5-25 μg/L range. Protein solutions (BSA, ricin, or SEB) were prepared in 10 mM phosphate buffer, pH 7.0, to fall within the linear range of this curve. To carry out the assay, aliquots of each sample solution and calibration standard solution were transferred to a minimum of 8 individual wells (140 μL/ well) of a flat bottom microplate. In addition, 16 blank wells of each plate were prepared using 140 μL of 10 mM phosphate buffer per well. For the Coomassie® Plus plates, 140 μL of the reagent was then added to each well. The plate was shaken for 30 s, incubated for 10 min at 37 °C, and the endpoint absorbance recorded at λ = 595 nm. For the QuantiPro™ BCA assay, 140 μL of the mixed reagent was added to each well. The plate was incubated for 60 min at 60 °C, cooled, and the endpoint absorbance recorded at λ = 562 nm at 25 °C. The average plate blank absorbance was subtracted from the average absorbance for each sample/standard. Protein concentration was determined based upon comparison to the calibration curve in the linear range. The concentrations of the undiluted samples were calculated based on the dilution factor used to prepare the samples for the assay.

2.5 Circular Dichroism (CD).

Circular dichroism experiments used a JASCO Model J-810 spectropolarimeter (JASCO Analytical Instruments, Easton, MD) equipped with a PTC-423S Peltier thermoelectric temperature control system.

2.5.1 General Scan.

The CD spectra were obtained for staphylococcal enterotoxin B and ricin at 25 °C from 260 nm to 190 nm or from 285 nm to 190 nm with a data pitch of 0.5 nm, a band width of 1 nm, a response time of 1 s, and a scanning speed of 10 nm/min. Typically, a given sample was diluted with Standard Buffer to an approximate concentration of 0.1-0.2 mg/mL, which provided an appropriate signal for each toxin. The CD scans were carried out in 0.1 cm cuvettes. Prior to each sample scan, a blank scan was recorded for the same volume of Standard Buffer. The blank scan was subtracted from the sample scan and molar ellipticities were determined using the JASCO *Spectral Analysis* Program, which is part of the *Spectra Manager* software (JASCO Analytical Instruments, Easton, MD).

2.5.2 Time Course Experiments.

Samples were prepared and up to 100 consecutive scans were completed using the Macro Command software, which is part of JASCO's *Spectra Manager* program (JASCO Analytical Instruments). Each CD scan was completed at 25 °C from 285 nm to 190 nm with a data pitch of 0.5 nm, a band width of 1 nm, a response time of 1 s, and a scanning speed of 10 nm/min.

2.5.3 Melt Studies.

Thermal denaturation experiments from 25.0 to 95.0 °C at 1 °C/min were carried out on ricin and SEB to determine the degree of protein unfolding that occurs during the temperature change. The millidegrees at 222 nm and 205 nm for SEB and ricin, respectively, were automatically measured every 0.1 °C over the temperature range. For ricin, 205 nm was used because the millidegrees at 222 nm were constant over the temperature range studied. At 95.0 °C, a General Scan (see "General Scan" above) was collected with the standard parameters as described previously. The sample was cooled back to 25.0 °C with monitoring at the same wavelength. A final spectrum was collected with the general parameters at 25.0 °C. The procedure was completed within 1.5 hr.

2.6 SDS-PAGE.

SDS-PAGE was carried out by one of the following three methods.

2.6.1 Method 1.

Gel eletrophoresis was carried out using the Mini-PROTEAN® 2 Cell and 4-20% Ready Gel® precast Tris-HCl gels from BioRad (Hercules, CA). Samples for PAGE were prepared by diluting the sample two fold into Laemmli sample buffer, which contained 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, and 0.01% bromophenol blue (BioRad). The samples were heated at 95 °C for 5 min and cooled to room temperature prior to loading. Running buffer was prepared by diluting the concentrated mixture of 10x Tris/glycine/SDS solution containing

250 mM Tris, pH 8.3, 1.9 M glycine, and 0.1% SDS (BioRad). Kaleidoscope Standards (BioRad), which are prestained, broad range molecular weight standards, were diluted two-fold into the loading buffer and heated at 95 °C with the other samples. The Kaleidoscope Standard solution was loaded into at least one lane of each gel. Electrophoresis was carried out at 160 V for 48 min. The gels were rinsed with three 50 mL washes of distilled water followed by 50 mL of Coomassie R250 stain to visualize the proteins in the gel. Coomassie R250 stain was composed of 684 mg Coomassie Blue R250 stain, 85.5 mL formaldehyde, 154.2 mL absolute ethanol, and 360 mL deionized water. The gel was allowed to develop for at least 1 hr and was then rinsed with deionized water. The gel was destained for at least 2 hr with a solution composed of 10 mL formaldehyde, 250 mL absolute ethanol, and 750 mL deionized water. Finally, the gel was washed twice with deionized water. Using the molecular weight standards, the molecular weights of the major bands were determined.

2.6.2 Method 2.

Gel eletrophoresis was carried out using a Mini-PROTEAN® 3 Cell and 4-20% Ready Gel® precast Tris-HCl gels from BioRad. Samples for PAGE were prepared by diluting the sample two-fold into Laemmli sample buffer, which contained 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, and 0.01% bromophenol blue (BioRad). The samples were heated at 95 °C for 10 min and cooled prior to loading. Running buffer was prepared by diluting the concentrated mixture of 10x Tris/glycine/SDS solution contained 25 mM Tris, pH 8.3, 192 mM glycine, and 0.1% SDS (BioRad). Mark12 (Invitrogen), which are unstained, wide range molecular weight standards, were loaded into at least one lane after heating at 95 °C for 10 min. Electrophoresis was carried out at 100 V for 90 min. The gels were rinsed with three 50 mL washes of distilled water followed by 50 mL of BioSafe™ Coomassie stain (BioRad) to visualize the proteins in the gel. The gel was allowed to develop for at least 1 hr and then rinsed with distilled water. Using the molecular weight standards, the molecular weights of the samples were calculated.

2.6.3 Method 3.

This method was the same as Method 2 with the following exceptions: samples were heated at 95 °C for 5 min and cooled prior to loading; Kaleidoscope Standards (BioRad) were loaded into at least one lane of each gel after being heated at 40 °C for 1 min; electrophoresis was carried out at 200 V for 35 to 45 min.

2.7 SEB Liquid Chromatography-Mass Spectroscopy (LC-MS).

A Finnigan-MAT Quantum Triple Quadrupole Mass Spectrometer (MS) was used. To develop a protocol for SEB analysis, an SEB Work Solution was prepared by dissolving 1 mg SEB in 1 mL deionized H_2O. SEB Work Solutions were analyzed by one of four procedures: 1) injection of SEB Work Solution onto a C_4 Biobasic column linked to the MS; 2) desalting of SEB Work Solution by dialysis, followed by infusion onto the MS; 3) desalting of SEB Work Solution via a Speedisk column, followed by infusion onto the MS; and 4) dilution of SEB Work Solution and injection onto a Phenomenex Jupiter C_4 HPLC column linked to the MS. Of these procedures, the final method proved to be the most straight forward and was used for the remainder of the study as described below.

For the analysis of SEB stability samples, Stability Aliquots were diluted so that the concentration of SEB was expected to fall between 10 and 100 μg/mL. Cytochrome C (100 μg/mL) was used as an internal standard. Fifty microliter volumes were injected onto a Phenomenex Jupiter C4 HPLC column (5 μm particle size, 300Å pore size, 50 x 1.0 mm) and separated using a linear gradient of aqueous acetonitrile acidified with 1% glacial acetic acid at a flow rate of 150 mL/min. The initial conditions were 100% H_2O, 35% acetonitrile at 10 min, and 65% acetonitrile at 18 min. The eluent from the column was directly connected (no split) to the electrospray (ESI) ionization source of the MS. Positive ion ESI mass spectra were acquired over the 600-1400 or 700-1400 m/z range. Linearity evaluations were carried out periodically throughout the course of the stability experiments. Linearity solutions were prepared from post-dialysis SEB solutions with each group of linearity solutions having three or five concentrations of SEB and 100 μg/mL Cytochrome C.

2.8 T-Cell Proliferation Assay (T-Cell) for SEB Activity.

The SEB concentration in each of the SEB Stability Aliquots to be assayed was verified by UV absorbance using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) at 277 nm. The proliferative response of murine splenocytes to SEB was determined as described by Garcia, *et al.*[13] Briefly, splenocytes were prepared from BALB/c mice. Dilutions of the SEB Stability Aliquots were made, and cultures of the toxin and splenocytes were prepared in a volume of 200 μL in a 96-well plate. The splenocytes were cultured at a density of 8×10^5 cells/well. These cultures were incubated for 2 days, and tritiated thymidine was added to pulse the cells, at 0.2 μCi of tritiated thymidine (2 Ci/mmol, 8.3 mCi/mg per well), by addition of the tritiated thymidine diluted in tissue culture fluid. On the third day, the cells were harvested in an automatic cell harvestor in which the cells were precipitated on filter paper, and the unincorporated thymidine was removed to liquid waste. The filter papers containing the cells were deposited in plastic scintillation vials, and the radioactivity of the tritiated thymidine associated with the cells was determined in a liquid scintillation spectrometer. Triplicate experiments were carried out for each sample. The assays were carried out using either 4 or 6 replicates and variable dose ranges so that responses at the low or high concentration range might be detected.

2.9 Neutral Red Uptake Assay (NRU) for Ricin Activity.

The protocol used was the "Test Method Protocol for the BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test" prepared by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The complete protocol and list of materials are available at:

http://iccvam.niehs.nih.gov/methods/invidocs/phIIIprot/3T3Protocol.doc

Briefly, BALB/c 3T3 Murine Fibroblasts (CCL-163 American Type Culture Collection [ATCC], Manassas, VA) were passaged a minimum of three times after thawing prior to use in the assay. Ninety-six well microplates were seeded at 2.5×10^3 cells per well. The plated cells were maintained in culture in DMEM supplemented with 10% newborn calf serum and 4 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 hr prior to treatment. Exposures were performed 24 ± 2 hr after seeding the 96 well plates. All ricin Stability Aliquots to be analyzed were sterile-filtered through a Whatman Puradisc 4 mm syringe filter (0.2 mm PVDF). The concentration of ricin was determined on the day of exposure to the

3T3 cells by UV spectroscopy as described above. Eight dilution concentrations per ricin Stability Aliquot were tested in individual 96 well plates with 6 wells per concentration. The NRU was determined by using a microtiter plate reader to measure the optical density (at 540 nm) of eluted NR dye in the 96-well plates. Calculation of cell viability, expressed as NRU, was made for each ricin concentration by using the mean NRU of six replicate values per test concentration. Cell viability was compared with the mean NRU of all vehicle control (VC) values and then relative cell viability was expressed as percent of untreated VC. The concentration of the toxin reflecting a 20, 50, and 80% inhibition of cell viability (IC_{20}, IC_{50}, and IC_{80}) was determined by applying a Hill function to the concentration response data using GraphPad PRISM® version 4. The Hill function is a four parameter logistic mathematical model relating the concentration of test substance to the response being measured in a sigmoidal shape:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC50 - \log X)\text{HillSlope}}}$$

where Y = response (i.e., % viability), X is the substance concentration producing the response, Bottom is the minimum response (0% viability, maximum toxicity), Top is the maximum response (maximum viability), EC_{50} is the substance concentration at the response midway between Top and Bottom, and HillSlope describes the slope of the curve. When Top = 100% viability and Bottom = 0% viability, the EC_{50} is then equal to the IC_{50}.

2.10 Chlorine Concentration Determinations.

The free chlorine concentration was determined by one of the two methods described below. Although Cl_2 was not present in the reaction mixtures, molar ratios of chlorine:toxin are reported here in terms of Cl_2:toxin because the standard methods of concentration determination are calibrated relative to ppm Cl_2.

2.10.1 Spectrophotometric Assay.

This method used a Hach® DR 2800 Spectrophotometer using stored method "80 Chlorine, F&T PP". For each sample, an aliquot of the sample was transferred into a round sample cell containing 10 mM phosphate buffer to total 10 mL. The sample cell exterior was wiped with laboratory tissue, placed into the instrument cell holder, and the instrument zeroed. The sample cell was removed from the instrument. The contents of a DPD Free Chlorine Powder Pillow (Hach Cat. No. 21055-69) were added to the sample cell and the solution mixed gently for 20 s, wiped with laboratory tissue, and placed into the cell holder. Within 1 min of adding the reagent, the sample was read and the results were displayed in mg Cl_2/L. Corrections for the dilution were applied to obtain the original sample concentration.

2.10.2 Test Strips.

Free chlorine concentration was determined using either Free Chlorine Micro Check™ test strips (HF Scientific, Inc., Ft. Myers, FL) or AquaChek™ test strips (Hach, Loveland, CO). The Micro Check™ test strips (0.0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.5, 2.0, 2.6, 4.0, 6.0, and 10.0 ppm color pads) were used according to the method supplied by the vendor by dipping in the test solution and swishing back and forth. The resulting strip color was

compared to the chart provided to determine the free chlorine concentration. For the AquaChek™ test strips (0, 0.5, 1.0, 2.0, 4.0, and 10.0 ppm color pads), 15 μL of sample was placed onto the appropriate test pad on the strip. After 10 s, the color was compared to the color chart provided to determine the free chlorine concentration.

3. RESULTS AND DISCUSSION

3.1 SEB Stability with Time.

No significant difference in mass or fold, as judged by LC-MS and CD, respectively, was found for SEB incubated at 25 °C in 10 mM PO_4 for 30 days. Activity was not ascertained for the 30 day study. Activity was ascertained for the 33 day study and appeared to decrease slightly and then increase to 1.8 times the initial value over 33 days. It is possible that the long -80 °C storage length for the earlier time points relative to the 33 day time point resulted in decreased activity for the earlier time point Stability Aliquots. Therefore, the 33 day experiment was repeated, this time staggering the start times for the time point Stability Samples so that all time points would end within 2 days of each other, resulting in very similar storage periods for all samples. No significant change in mass (as judged by SDS-PAGE), fold (as judged by CD), or activity (as judged by T-CA) was found for SEB incubated at 25 °C for this 33 day experiment.

3.2 SEB Stability with Temperature.

SEB was irreversibly denatured upon heating to 95 °C in either 10 mM or 55 mM PO_4. The T_m was 68 °C for SEB in 10 mM PO_4 and was 60 °C for SEB in 55 mM PO_4. Upon slow heating to 37, 55, and 60 °C in 10 mM PO_4, no precipitation occurred and no significant difference in fold was observed compared to control SEB (as judged by CD). Upon heating to 68 °C, 88% of the SEB precipitated and the SEB remaining in solution had a significant difference in fold compared to control SEB, with the heated SEB having more random coil character (based on CD). No significant change in SEB mass occurred (as judged by PAGE) for samples heated to 37, 55, and 60 °C. The majority of the SEB in solution for the Stability Sample heated to 68 °C also had no significant change in mass compared to control SEB; however, a faint new band appeared at ~34,000 MW. Compared to the control sample, the activity of SEB heated to 55 °C was not significantly different and the activity of SEB heated to 60 °C was 80%. The activity of the SEB that remained in solution after heating to 68 °C was 35% relative to that of the control.

3.3 SEB Stability Upon Chlorine Treatment.

3.3.1 Calcium Hypochlorite Treatment.

As the chlorine concentration increased, the mass and number of SEB species increased. For SEB treated with an initial chlorine:SEB ratio of 56:1, the mass of the centroid of the envelope of the species peaks in the mass spectrum was ~500 mass units greater than the mass of untreated SEB. The activity for SEB treated at a chlorine:SEB molar ratio of 2:1 was determined and no difference in activity was found relative to control SEB (MS centroid mass increase was 25 mass units relative to control SEB).

3.3.2 Sodium Hypochlorite Treatment.

For the 2 day and 5 day studies, as the chlorine:SEB ratio was increased from 1:1 to 1000:1, the PAGE results showed less unmodified SEB and a smeared appearance indicating multiple species at higher molecular weights than un-modified SEB. At 500:1 and 1000:1, no unmodified SEB band appeared. At 100:1, a very faint smeared band indicated the presence of some remaining SEB having the same molecular weight as the control. The CD results indicated no significant change in fold at 1:1, 5:1, and 10:1, but significant change in fold at 25:1 and increasing random coil character as the chlorine:SEB ratio increased from 25:1 to 1000:1. At 5 days, the free chlorine concentration for the 10:1, 25:1, 50:1, and 100:1 Stability Samples was undetectable. Sodium thiosulfate from a freshly prepared stock solution (1.6×10^{-02} M in deionized water) was added to quench any residual chlorine (final thiosulfate concentration in Stability Sample = 32 mM). This quenching step was included because the affect of residual chlorine on the T-CA had not been determined, whereas previous preliminary experiments had shown that this level of thiosulfate relative to SEB did not affect the activity of SEB. The activity of SEB Stability Samples treated for 5 days with initial chlorine:SEB ratios of 10:1, 25:1, 50:1 and 100:1 was determined. For SEB treated at 10:1 and 25:1, the activity was 55%, and 16% relative to the control sample, respectively. There was no observed activity for the SEB treated at 50:1 and 100:1.

3.4 Ricin Stability with Time.

No significant difference in mass (as judged by PAGE), fold (as judged by CD) or toxicity (as judged by NRU) was found for ricin incubated at 25 °C in 10 mM PO_4 for 30 days.

3.5 Ricin Stability with Temperature.

Upon heating from 25.0 °C to 95.0 °C, essentially all of the ricin precipitated. The T_m was 72.5 °C. Upon heating to 37 °C and 55 °C, no precipitation occurred. Upon heating to 73 °C, ~77% of the ricin precipitated. Upon heating to 95 °C, 95% of the ricin precipitated and the CD spectrum could not be obtained. The PAGE results showed no difference in control ricin compared to ricin heated to 37 and 55 °C, as well as to the ricin that remained in solution upon heating to 73 °C. No bands were visible in the solution from the Stability Sample that was heated to 95 °C. The CD spectra for Stability Samples heated to 37 and 55 °C, and for the ricin in solution from the Stability Sample that was heated to 73 °C, showed no difference in fold relative to control ricin. No significant difference in toxicity occurred for ricin heated to 37 or 55 °C. The ricin remaining in solution after heating to 73 °C had slightly decreased activity relative to the control ricin. Ricin heated to 95 °C was not tested for toxicity.

3.6 Ricin Stability Upon Chlorine Treatment.

At 24 hr, the free chlorine concentration for all samples was undetectable. No quenching step was included for the ricin samples as was done for the SEB experiments above because it had previously been determined that chlorine at any possible residual levels remaining in the NRU Stability Aliquots would not affect the toxicity assay. As the initial chlorine:ricin ratio was increased, the PAGE results showed less un-modified ricin and a smeared appearance indicating multiple species at higher molecular weights than un-modified ricin. At 100:1 and 1000:1, no unmodified ricin remained. At 1:1, there was no difference in molecular weight from the control ricin. At 50:1, a very faint peak indicated the presence of some ricin having the same molecular weight as the control. The CD indicated no significant change in fold at 1:1, but significant change in fold at 50:1 and higher. There was no significant

difference in toxicity for ricin treated at 1:1 relative to control ricin. There was significant toxicity for ricin treated at 50:1 and no observed toxicity for ricin treated at 100:1. Ricin treated at 1000:1 was not tested for toxicity.

4. CONCLUSIONS

We have found that staphylococcal enterotoxin B (SEB) and ricin are each stable for at least 30 days in 10 mM PO_4 (pH 7) at room temperature. Not surprisingly, based on the polyacrylamide gel electrophoresis (PAGE) results, heating did not affect the molecular weight of either toxin. The melting temperatures (T_m) for SEB and ricin in 10 mM PO4 (pH 7) were 68 and 73 °C, respectively. For each toxin, the majority of the toxin precipitated when heated slowly to the toxin's T_m. However, the toxin remaining in solution retained some activity. In addition, the ricin remaining in solution maintained its native fold, whereas the SEB remaining in solution did not. Each toxin was stable upon slow heating to 55 °C. Finally, each toxin retained some activity after treating at low initial chlorine:toxin molar ratios. For SEB, some activity was observed after treatment for 5 days with an initial chlorine:toxin of 25:1, but not 50:1. For ricin, some activity was observed after treatment for 1 day with an initial chlorine:toxin of 50:1, but not 100:1. As the initial chlorine:toxin increased, less of the native protein remained. Taken together, the protein assay and PAGE results for each toxin, and mass spectrometry (MS) results for SEB, indicate that multiple higher molecular weight species are formed upon reaction with chlorine and the number of species increases as the initial chlorine:toxin ratio is increased.

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APPENDIX A
PUBLICATIONS

The following Technical Reports have been published:

Morrissey, K.M.; Schenning, A.M.; Bevilacqua, V.L.H.; Rice, J.S. *Evaluation, Validation and Demonstration of a Total Protein Assay for Application to Biotoxin Fate Studies*; ECBC-TR-576; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2007; UNCLASSIFIED Report (AD-A475 212).

Schenning, A.M.; Bevilacqua, V.L.H.; Morrissey, K.M.; Rice, J.S. *Evaluation of ToxTrak™ for Analysis of Protein Toxin Toxicity*; ECBC-TR-578; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2007; UNCLASSIFIED Report (AD-A475 390).

The above papers are related to method development that occurred as part of this project that is not included in the final report. At least one additional paper is planned upon completion of data analysis.

Two platform presentations and five poster presentations have been presented at international conferences.

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APPENDIX B
PREPARATION OF WATER WITH RESIDUAL CHLORINE

The stock calcium hypochlorite water used for this study was prepared according to the procedure below but using distilled deionized water rather than reverse osmosis purified water.

Provided by EAI Corporation, Project 100008.002, Sub-Task 002.

Preparation of Reverse Osmosis Water with Residual Chlorine for the JSAWM Program

NOTE: The preparation outlined below was developed for a specific water source using a specific lot of HTH (calcium hypochlorite). The exact amount of stock solution to add will need to be experimentally determined.

Preparation of Stock Solution

Weigh 1.00 g HTH (Arch Chemical, Granular HTH, 65% available chlorine, item number 30929, lot HTH-2088-0799) into a 22 mL glass vial. Add 20.0 mL of ASTM Type I deionized water, cap, and shake vigorously for 1-2 min. Let the solids settle for 5-10 min before using. NOTE: This stock solution is not stable, and is made up just prior to preparing the water matrix.

Preparation of Water Matrix

Using a 100 mL graduated cylinder, add 100 mL of reverse osmosis water to an 125 mL amber, glass bottle (see note 1 below). Add 15 µL of HTH stock solution (see note 2 below), cap, and swirl to mix. Remove the cap, and check the free residual chlorine level with a test strip (see note 3 below). The free residual chlorine should be 1-3 ppm. NOTE: This water matrix is not stable, and is only to be used for 1 work day (8-10 hr).

(1) The amber bottle should be conditioned with the chlorinated water prior to the first use (let it soak overnight). In addition, the old chlorinated water is left in the bottle until a new batch is made. This will reduce the chlorine demand of the bottle surfaces.

(2) Pipet the supernatant. Do not resuspend the solids prior to removing the 15 µL.

(3) HF Scientific, Free Chlorine Micro-Check test strip, or equivalent.