An activity-dependent assay for ricin and related RNA N-glycosidases based on electrochemiluminescence

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Abstract

Synthetic biotinylated RNA substrates were cleaved by the combined actions of ricin holotoxin and a chemical agent, N,N′-dimethylethylenediamine. The annealing of the product with a ruthenylated oligodeoxynucleotide resulted in the capture of ruthenium chelate onto magnetic beads, enabling the electrochemiluminescence (ECL)-based detection of RNA N-glycosidase activities of toxins. ECL immunoassays and the activity assay exhibited similar limits of detection just below signals with 0.1 ng/ml of ricin; the ECL response was linear as the ricin concentration increased by two orders of magnitude. Activities were detected with other adenine-specific RNA N-glycosidases, including *Ricinus communis* agglutinin (RCA), saporin, and abrin II. The substrate that provided the greatest sensitivity was composed of a four-residue loop, GdAGA, in a hairpin structure. When the 2′-deoxyadenosine (dA) was substituted with adenosine (A), 2′-deoxyinosine, or 2′-deoxyuridine, toxin-dependent signals were abolished. Placing the GdAGA motif in a six-residue loop or replacing it with GdAdGA or GdAAA resulted in measurable activities and signal patterns that were reproducible for a given toxin. Data indicated that saporin and abrin II shared one pattern, while ricin and RCA shared a distinct pattern. A monoclonal antibody that enhanced the activities of ricin, RCA, and abrin II to different extents, thus improving the diagnostic potential of the assay, was identified.
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Keywords: Ricin; Abrin; Saporin; Electrochemiluminescence; Activity; N-Glycosidase; Ribosome inactivating protein; Immunoassay; Bioterrorism

Ricin and abrin are enzymatic toxins that include RNA N-glycosidase components, or A chains. These A chains catalyze the removal of a specific adenine base from mammalian 28S ribosomal RNA, irreversibly altering its interactions with elongation factors [1,2]. Each A chain is linked by a disulfide bond to a B chain, which binds to galactose moieties on cell surfaces and aids in delivering the N-glycosidase to the cytoplasm where it inactivates ribosomes. These heterodimeric, two-chain toxins are referred to as type II ribosome-inactivating proteins (RIPs).[1]

Ricin is derived from castor beans of *Ricinus communis*, whereas abrin is derived from precatory beans of *Abrus precatorius*. Variants of the two-chain toxins exist for both ricin and abrin [3]. Both plants also produce four-chain forms that more effectively agglutinate red blood cells but are less toxic than the two-chain forms [2,4].

The worldwide availability of ricin as a by-product of castor oil production renders its use as a biological weapon more feasible [5]. A lethal dose of ricin for a human adult (70 kg) is 350–700 μg by inhalation and about three orders of magnitude greater by ingestion [6]. Abrin is also a potential threat agent [7,8]. Abrin II is the most toxic form of abrin [4]. Type I RIPs, such as saporin, lack B chains and cannot effectively intoxicate cells without an alternative cell-targeting moiety. Indeed, such moieties have been linked to saporin—and ricin A

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14. ABSTRACT
Synthetic biotinylated RNA substrates were cleaved by the combined actions of ricin holotoxin and a chemical agent, N,N'-dimethylethylenediamine. The annealing of the product with a ruthenylated oligodeoxynucleotide resulted in the capture of ruthenium chelate onto magnetic beads, enabling the electrochemiluminescence (ECL)-based detection of RNA N-glycosidase activities of toxins. ECL immunoassays and the activity assay exhibited similar limits of detection just below signals with 0.1ng/ml of ricin; the ECL response was linear as the ricin concentration increased by two orders of magnitude. Activities were detected with other adenine-specific RNA N-glycosidases, including Ricinus communis agglutinin (RCA), saporin, and abrin II. The substrate that provided the greatest sensitivity was composed of a four-residue loop, GdAGA, in a hairpin structure. When the 2'-deoxyadenosine (dA) was substituted with adenosine (A), 2'-deoxyinosine, or 2'-deoxyuridine, toxin-dependent signals were abolished. Placing the GdAGA motif in a six-residue loop or replacing it with GdAdGA or GdAAA resulted in measurable activities and signal patterns that were reproducible for a given toxin. Data indicated that saporin and abrin II shared one pattern, while ricin and RCA shared a distinct pattern. A monoclonal antibody that enhanced the activities of ricin, RCA, and abrin II to different extents, thus improving the diagnostic potential of the assay, was identified.

15. SUBJECT TERMS
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chain—to create therapeutic constructs designed to eliminate specific cell types [9,10].

Biothreat detection can lead to the misallocation of costly resources if false positive signals cannot be quickly and independently identified. Our activity assay offers confirmatory capability for an electrochemiluminescence (ECL)-based immunological system for ricin analysis which provides high sensitivity in a simple and robust format (BioVeris Corp., Gaithersburg, MD; http://www.bioveris.com; formerly known as Igen Corp.) [11]. Immunoassay reagents (antibodies) recognize surface features of proteins (epitopes) that may be unrelated to any enzymatic activity or other mechanism of toxicity. Thus, it may be possible for inactive protein toxins to cause positive signals with immunoassays, resulting in an overestimation of the threat. Our work describes a toxin assay that is dependent on RNA *N*-glycosidase activity, which mediates intracellular toxicity. Previously reported assays for such deadenylating toxins involve detection chemistries that are not suited to the ECL platform [12–14 and references therein]. Also, many of these methods involve multiple steps, time-consuming separations of products, and/or hazardous reagents. Our assay involves nonradioactive reagents, simple procedures without product separations, a one-tube-per-test ECL format, and easily interpreted results because the substrates have a defined site of toxin action. Furthermore, the convenience of using the commercially available ECL immunoassays in combination with our activity-based assay should considerably promote confidence in threat assessments involving ricin.

Materials and methods

Materials

Ricin holotoxin (a.k.a. RCA II or RCA_{60}) and RCA (a.k.a. RCA I or RCA_{120}) were obtained from Vector Laboratories (Burlingame, CA). Saporin (Advanced Targeting Systems, San Diego, CA) and uracil DNA glycosylase (UDG; a.k.a. uracil *N*-glycosylase; Epicentre, Madison, WI) were also obtained commercially. Abrin II, which was purified as described previously [4], was provided by Dr. Eric Garber (Food and Drug Administration, College Park, MD).

Mouse monoclonal antibody 9C3 (Mab 9C3) was provided by Dr. Mark Dertzbaugh (U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD) [15]. The antibody was affinity purified using an UltraLink Immobilized Protein A/G column according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL). The eluate was dialyzed against distilled water and then frozen and lyophilized. The purified protein residue was weighed and then dissolved in nuclease-free water (Ambion, Inc., Austin, TX) to a concentration of 35 µg/ml. Aliquots (1 ml) in 1.5-ml microcentrifuge tubes were vortexed 5 s after additions of diethylpyrocarbonate (0.3 µl of DEPC per tube; Sigma–Aldrich, St. Louis, MO); afterward, tubes were rotated at 20 rpm at room temperature for 1 h and then stored at 4 °C.

Activity assay

Unless otherwise indicated, reagents were obtained from Sigma–Aldrich (St. Louis, MO). Substrate oligonucleotides were obtained from Integrated DNA Technologies with purification by RNase-free high-performance liquid chromatography (Coralville, IA). Reactions were performed in quadruplicate samples in 0.2-ml PCR tubes in eight-tube strips (USA Scientific Inc., Ocala, FL). Total reaction volumes were 10 µl, with 5 µl of reagent solution plus 5 µl of sample in diluent (nuclease-free water with 0.01% v/v Triton X-100, or "Triton" hereafter). Reagent solution for 100 reactions, freshly combined before use, included 200 µl of 200 mM sodium citrate with 10 mM EDTA (pH 4.1 after dilution to the final reaction concentrations of 40 mM Na citrate, 2 mM EDTA), 293 µl of 35 µg/ml Mab 9C3, and 7 µl of 50 pmol/µl RNA substrate (in 10 mM Tris, 1 mM EDTA, pH 8, or "TE" buffer, Ambion). Tubes were incubated in a thermocycler at a constant 37 °C with a lid temperature of 105 °C.

Reactions were typically stopped by adding 240 µl of combined "stop/detection" reagents to each tube after a 3-h incubation at 37 °C. For Fig. 2 data, reactions were stopped by adding 220 µl of stop solution to each tube; stopped tubes were recapped, mixed by repeated inversions, and kept at room temperature. After all reactions were stopped, detection reagents were added (20 µl per tube), followed by mixing. Stop solution for 100 tubes consisted of reagents added in the following order: 20.2 ml TE buffer containing 1.09 M NaCl, 1.54 ml 2 N HCl, 260 µl *N*,*N*′-dimethylethylenediamine, and 48 µl of Triton. Stop solution (pH 8) was filtered through a 0.2-µm-pore-size filter. Detection reagents for 100 tubes consisted of 1.94 ml TE buffer with 1.09 M NaCl, 55 µl of 10 mg/ml Dynabeads M-270 Streptavidin (Invitrogen Corp., Carlsbad, CA), and 10.5 µl of 50 pmol/µl ruthenylated oligodeoxynucleotide in TE buffer (Ru-ODN, Table 1; Biosource Int., Camarillo, CA; ruthenium in BV-TAG label from BioVeris Corp.). Tubes were then incubated 15 min at 37 °C (lid temp., 45 °C).

To maintain a one-tube-per-test format, quadruplicate tubes were cut from the eight-tube strips, uncapped, and transferred to a modified Costar round-bottomed polypropylene 96-well microtiter plate (Corning Inc., Corning, NY; product No. 3365). The modification involved drilling a 3.5-mm-diameter hole at the bottom (center) of each well such that the PCR tubes could be inserted as four-tube strips held upright across four wells. The plate was then analyzed on the BioVeris M-SERIES M1R instrument using the following parameters: plate type, standard round 1; plate layout, standard BioVeris; volume in well, 250 µl; volume sampled, 200 µl; bead type, 2.8;

Table 1
Oligonucleotides used in this study

Name	Sequence[a,b]
RNA GdAGA	5′/Biotin/<u>AGC</u>*GGGAGAG***dAGA***UCUCCC*
RNA GAGA	5′/Biotin/<u>AGC</u>*GGGAGAG***GAGA***UCUCCC*
RNA GdAAA	5′/Biotin/<u>AGC</u>*GGGAGAG***dAAA***UCUCCC*
RNA GdAdGA	5′/Biotin/<u>AGC</u>*GGGAGAG***dAdGA***UCUCCC*
RNA AGdAGAC	5′/Biotin/<u>AGC</u>*GGGAGA***GdAGAC***CUCCCG*
RNA GdUGA	5′/Biotin/<u>AGC</u>*GGGAGAG***dUGA***UCUCCC*
RNA GdIGA	5′/Biotin/<u>AGC</u>*GGGAGAG***dIGA***UCUCCC*
DNA GAGA(CU)	5′/Biotin/<u>dGdAdGdCdC</u>*dGdGdGdAdGdAdG***dGdAdGdA***dCdUdCTdCdCdC*
RNA product	5′/Biotin/<u>AGCGGGAGAG</u>
Ru-ODN	5′/Ruthenium/TTTTTdAdCdCTdCTdC(T)dCdGdCTdC

[a] d, 2-deoxyribosyl moiety (*T* = dT).
[b] Oligonucleotide segments: bold face, hairpin loops; italics, stem segments; underline, segments that hybridize to Ru-ODN. The T in parentheses in the Ru-ODN sequence participates in a G-T mismatch (upon annealing to a product oligo) that disfavors the annealing of Ru-ODN to unreacted substrate molecules, wherein the hairpin configuration is maintained without mismatches.

clean type, level 2; bead wash, level 5; detection sequence, standard.

Immunoassays

Ricin detection kits were obtained from BioVeris (BioVerify Ricin Test) in which reagents were lyophilized in tubes. Similar kits, albeit using different antibodies, were obtained through the Department of Defense's Critical Reagents Program (CRP; Ricin Toxin ECL Minitube Immunoassay). Kits were used according to instructions as provided, and ECL measurements were made on the M1R instrument using the recommended parameters. M-SERIES BV-DILUENT solution (BioVeris) was used in place of "ECL Buffer" in the CRP protocol.

Results and discussion

The goal of our work was to provide an ECL-based assay for ricin that could be used to independently confirm positive results obtained from an ECL immunoassay. The assay format depicted in Fig. 1 involves the RNA *N*-glycosidase-dependent immobilization of ruthenium onto paramagnetic particles (e.g., Dynabeads). In contrast, the ECL immunoassays for ricin involve ruthenium immobili-

zation via a classical format in which the ricin molecule is sandwiched between a capture (polyclonal) antibody bound to paramagnetic particles and a free ruthenium-labeled antibody. The premise of the activity assay is that *N*-glycosidase-dependent cleavage of a biotinylated hairpin substrate leads to the unblocking of the 5′ half of its stem once the 3′ half diffuses away, which allows Ru-ODN to anneal to the RNA product. Upon addition of the streptavidin-labeled Dynabeads, the biotinylated product noncovalently cross-links Ru-ODN to the beads. Indeed, an artificial RNA product (Table 1), when mixed with RNA substrate to simulate enzyme-dependent conversions, yielded an ECL response that was directly proportional to the product concentration (data not shown).

Since *N*-glycosidase activity produces an abasic site with an aldehyde group and does not cleave the sugar-phosphate backbone of the oligo, a chemical agent was added to cleave the backbone at the labile abasic site. McHugh and Knowland [16] showed that *N,N′*-dimethylethylenediamine (Me$_2$ED; 100 mM) efficiently cleaves abasic sites at pH 7.4 during 15-min incubations at 37 °C. Our data demonstrated that Me$_2$ED enhanced ECL signals at pH 7.5 or 8.0 and that signal-to-background ratios were optimal at about 100 mM compared to 0.5- and 1.5-fold variations of the Me$_2$ED concentration (not shown). All other
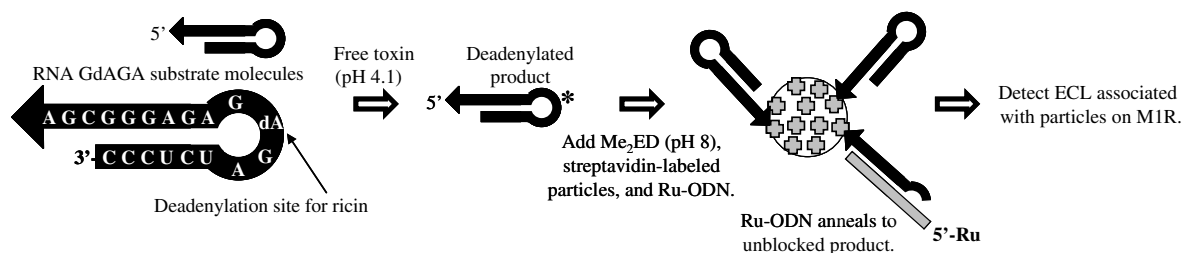

Fig. 1. Electrochemiluminescence (ECL)-based RNA *N*-glycosidase activity assay format. Activity-dependent unblocking of a RNA hairpin substrate yielded a product that cross-linked a ruthenylated oligodeoxynucleotide to the paramagnetic particles. All steps occurred in one tube and included toxin reaction at 37 °C (3 h), addition of stop/detection reagents and further incubation at 37 °C (15 min), and quantification of ECL (ca. 1.5 h per 96-well plate). Table 1 lists the oligonucleotides depicted in this figure. Triangle (arrowhead on substrate), biotin; cross-shaped dodecagon, streptavidin; Ru, ruthenium (BV-TAG) label; Ru-ODN, ruthenylated oligodeoxynucleotide; circle, paramagnetic particle (coated with streptavidin); Me$_2$ED, *N,N′*-dimethylethylenediamine; *, abasic site (cleaved by Me$_2$ED).

reagents used in the assay were similarly optimized (not shown).

Previous research showed that RNA substrates for ricin A chain containing a GdAGA loop exhibited large rate enhancements relative to substrates with a GAGA loop [17,18]. Therefore, we designed a synthetic biotinylated RNA substrate containing a 2′-deoxyadenosine (dA) residue within the four-residue loop, GdAGA, in a hairpin structure (RNA GdAGA; Table 1). In step 1 of our assay (Fig. 1), ricin holotoxin cleaved an adenine base from the RNA substrate at 37 °C to produce an intermediate containing an abasic site. In step 2, the addition of stop/detection reagents brought the pH up to 8 to stop the reaction, cleaved the sugar-phosphate backbone of the intermediate, and immobilized substrate molecules and product molecules (cross-linked to Ru-ODN) onto the particles. Tubes were then incubated at 37 °C for 15 min. Step 3 involved the automated analysis of tubes on the M-SERIES M1R

instrument: its robotic arm transferred liquid from sample tubes to a flow cell where paramagnetic particles were magnetically separated from solution. The instrument injected a solution containing tripropylamine, which served as an electron source in cyclic light-generating redox reactions of the ruthenium label. Because the reactions were electrochemically initiated at an electrode surface, only ruthenium immobilized on the particles that were magnetically appressed to the electrode contributed to luminescence. Repeated light emission by each ruthenium label conferred high sensitivity.

To define the site of action of ricin, the dA residue was replaced with adenosine (GAGA loop), 2′-deoxyinosine (GdIGA loop), or 2′-deoxyuridine (GdUGA loop; Table 1); no activity was detected when these variations were exposed to ricin (5 ng/ml in 5-μl sample), saporin (1 ng/ml), abrin II (26 ng/ml), or RCA (25 ng/ml; data not shown). To further verify that enzymatic hydrolysis at the second loop residue would cause signal generation, three RNA substrates (RNA GdAGA, RNA GAGA, and RNA GdUGA) and the DNA substrate (DNA GAGA(CU)) were exposed to UDG in TE buffer, which cleaves uracil from DNA [19]; only RNA GdUGA and the DNA substrate yielded signals above background (data not shown). Thus, the uracil at the dU residue in RNA GdUGA was cleaved by UDG. Observations of ECL signals with the dU-containing DNA substrate suggest that the U residues in the stems of the RNA substrates (GAGA, GdAGA) were also available to UDG but not cleaved by it, so the 5′ halves of their stems remained blocked to Ru-ODN. These predictable results show that base cleavage at sites other than the second loop residue can lead to signal generation. On the other hand, the data also demonstrate that alternative substrates can be used to define the substrate specificity and site(s) of action of an enzyme or toxin.

To effectively complement the ECL immunoassays, the activity assay must have a comparable sensitivity toward ricin. Fig. 2 shows that, for various toxin incubation times of 1 to 4 h, the activity-dependent ECL response was proportional to the toxin concentration; also, 3-h incubations were sufficient to detect ricin concentrations as low as those detectable with the ECL immunoassays (Table 2).
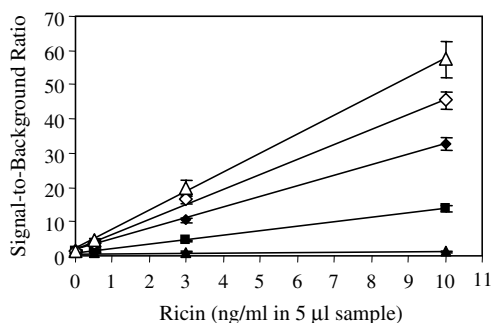

Fig. 2. Effect of varying incubation time at 37 °C and ricin concentration on signal-to-background ratios from the electrochemiluminescence-based activity assay. As described under Materials and methods, reactions included 3.5 pmol RNA GdAGA substrate per tube (0.35 μM). The signal average for a given treatment ($n = 4$) was divided by the signal average for background samples lacking toxin ($n = 4$), with propagation of the standard deviations (SD). Error bar length equals $2 \times$ SD. Equations obtained by linear regression analysis are shown in parentheses. Closed triangle, 0-h incubations ($y = 0.022x + 1.003$, $R^2 = 0.754$); square, 1-h incubations ($y = 1.275x + 1.021$, $R^2 = 0.998$); closed diamond, 2-h incubations ($y = 3.139x + 1.176$, $R^2 = 1.000$); open diamond, 3-h incubations ($y = 4.366x + 2.068$, $R^2 = 0.998$); open triangle, 4-h incubations ($y = 5.549x + 2.205$, $R^2 = 0.999$). Ricin concentration is given for the sample volume (5 μl).

Table 2
Limits of detection and coefficients of variation for the activity assay and both immunoassays (from data used to calculate signal-to-background ratios in Fig. 3)

Assay	Limits of detection[a]		Ricin (0.1 ng/ml)	Coefficients of variation	
	Bkg avg + ($3 \times$ SD)	Bkg avg $\times$ 1.2	Signal avg − SD	0.1 ng/ml ricin (%)	10 ng/ml ricin (%)
Activity (inc. Mab 9C3)[b]	3653	3664	3915	8	8
Activity (no Mab 9C3)	2952	3082	3043	6	8
CRP Immunoassay	138	140	173	7	6
BioVerify Immunoassay	274	275	403	9	17

[a] Average ECL signal values for background samples ("Bkg avg"; $n = 6$) were used to calculate limits of detection by two simple formulas given in the subheadings (SD, standard deviation).

[b] Activity assay reactions were performed with and without Mab 9C3 (100 ng per reaction) as described under Materials and methods. Fig. 3 does not include activity data without the Mab.

Moreover, data in Fig. 2 can be replotted to show that, for a given ricin concentration, the rate of increase in signal-to-background ratio was constant over time up to 4 h (not shown). Background signals typically ranged from 2000 to 4000 ECL units in different experiments. In one experiment, factors that contributed to an average background signal of 2600 ± 100 included baseline signal ($4 \pm 0\%$ of signal; no beads or Ru-ODN, $n = 4$), interaction of Ru-ODN with beads in the absence of RNA GdAGA substrate ($3 \pm 0\%$ of signal), said interaction in the presence of unreacted substrate or perhaps a trace contaminant similar to RNA product ($70 \pm 4\%$; see Table 1 footnotes), and abiotic hydrolysis that occurred at pH 4.1 at 37 °C over 3 h ($23 \pm 5\%$; TE buffer, pH 8, provided the control for no hydrolysis). Thus, commercially prepared substrate is sufficiently pure to achieve the required sensitivity. Amukele and Schramm [17] showed that dA undergoes abiotic hydrolysis (deadenylation) much faster than A residues at pH 4. Longer incubations (6 h; not shown) did not improve the sensitivity due to a higher background signal, which we attributed primarily to abiotic hydrolysis of RNA GdAGA.

Fig. 3 shows that a 3.2-h incubation provided activity data complementary to both ECL immunoassays. In the range of 0.1 to 10 ng/ml ricin, the activity assay and the immunoassays exhibited signal-to-background ratios that were comparable and directly (linearly) proportional to the ricin concentration. Table 2 shows that two methods of determining the limit of detection (LOD) gave similar values for each assay; thus, signal-to-background ratios (Fig. 3) greater than 1.2 represented positive signals. For the activity assay including Mab 9C3 and the two immunoassays, 0.1 ng/ml ricin gave signals above the LODs (Table 2). Only the BioVerify assay gave signals significantly above the LOD when samples with 0.05 ng/ml ricin were tested (not shown). This result is attributed to the fact that

the BioVerify assay uses 100 μl of sample per test, whereas the CRP assay uses 50 μl; however, the smaller volume is more conducive to preparing replicate tests with the limited amounts of material that typically reach the diagnostic laboratory (C. Rossi, personal communication). Coefficients of variation in Table 2 indicate that individual tests performed with all of the assays were highly reproducible.

Datapoints for the three assays beyond 10 ng/ml of ricin (up to 1000 ng/ml) are not shown in Fig. 3 but asymptotically approached maximum signals. Because nonspecific cleavage of adenine residues near the 5′ (biotinylated) end of the substrate RNA GdAGA would yield a product incapable of cross-linking biotin to Ru-ODN, we infer from the asymptotic response that nontarget adenine residues were not significantly cleaved by ricin. If the opposite was true, false negative signals would be possible with high concentrations of ricin in test samples. The high apparent specificity of ricin toward the dA target residue is consistent with previous findings [17,18]. Indeed, these rate differences may account for the undetectable activity with ricin and RNA GAGA as tested in our study (Fig. 4).

The ECL immunoassays rely in part upon an immuno-capture strategy based on paramagnetic particles to confer sensitivity, whereas the activity assay relies upon the catalytic generation of product molecules by the toxin. Enzyme catalysis is generally more sensitive to the reaction environment than antigen–antibody binding interactions. We sought to define the effects of representative substances that might interfere with activity and to identify means of mitigating their effects. Potential mechanisms of interference include solvent effects (e.g., pH, salinity), reagent degradation (e.g., by nucleases), or direct interactions with the toxin (e.g., dithiothreitol). As an example, the ricin-catalyzed deadenylation of RNA GdAGA was optimal at pH 4.1 in sodium citrate buffer (not shown), which is consistent with previous work [20]. This observation is reflected in Table 3, which shows that about 75% of ricin activity remained when 10 mM acid (HCl) or base (NaOH) was added to reagent solution with the unbuffered 5-μl sample. Less inhibition was observed when representative salts were added (10 mM KCl or NaCl). Still, clinical samples containing 0.9% saline (154 mM NaCl; e.g., nasal swabs) may require desalting or dilution. Dithiothreitol, which can reductively cleave the disulfide bond linking the A and B chains of ricin, moderately inhibited ricin activity (Table 3). In contrast to the need to reductively separate ricin's chains for optimal activity on ribosomes, Barbieri et al. [21] showed that reduction is not needed when artificial substrates are transformed by ricin and related type II RIPs. Thus, advantages of synthetic substrates include long-term storability and a simpler format that does not require reductive activation of the toxin.

DNases reportedly contaminate many preparations of RIPs, but DNase I had no effect on the assay (Table 4) [22]. The chelating agent EDTA was included in the toxin reaction (2 mM) and in the stop/detection reagents (0.7 mM) to avoid degradation of oligonucleotides due to
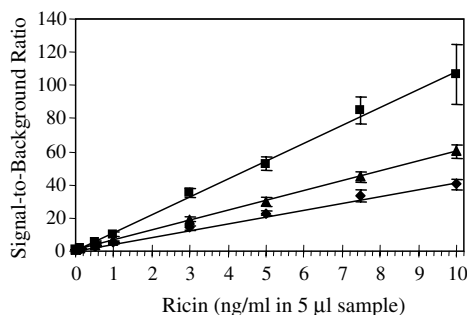

Fig. 3. Comparison of the responses to varying ricin concentrations for two electrochemiluminescence-based immunoassays and the activity assay using the RNA GdAGA substrate (in 3.2-h incubations). Assays are described under Materials and methods. Mab 9C3 was included in reactions for activity data shown. Calculations are described in Fig. 2 legend, except $n = 6$ for background samples. Equations in parentheses were obtained by linear regression analysis with y intercepts fixed at 1.0. Diamond, activity assay ($y = 4.124x + 1.0$, $R^2 = 0.995$); triangle, CRP ECL Minitube Immunoassay ($y = 5.882x + 1.0$, $R^2 = 1.000$); square, BioVerify Ricin Test ($y = 10.736x + 1.0$, $R^2 = 0.998$).
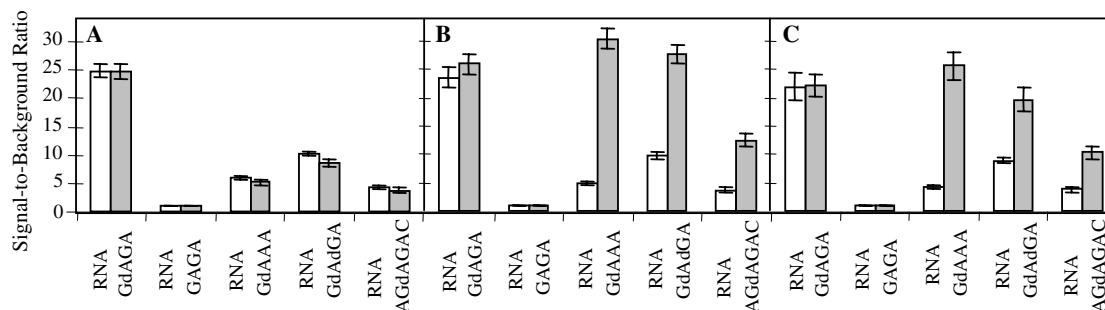
Fig. 4. Toxin differentiation by activity profiling with RNA substrates in separate experiments. Substrates are named according to the residue sequence in the hairpin loop (d, 2-deoxyribosyl moiety; Table 1). (A) Ricin (RT, 5 ng/ml; white bars) vs *Ricinus communis* agglutinin (RCA, 25 ng/ml; gray bars); (B) Ricin (5 ng/ml; white bars) vs saporin (1 ng/ml; gray bars); (C) Ricin (5 ng/ml; white bars) vs abrin II (26 ng/ml; gray bars). Error bar length = 2 × SD.

Table 3
Residual activity of ricin with RNA GdAGA substrate in the presence of various chemical inhibitors

Agent	Residual ricin activity at selected agent concentrations[a] (mM)				
	200[b]	100	20	10	4
NaCl	7% ± 1[c]	30% ± 2	90% ± 6	94% ± 10	n.d.[d]
KCl	5% ± 1	25% ± 2	79% ± 8	90% ± 7	n.d.
NaOH	n.d.	n.d.	41% ± 2	77% ± 7	99% ± 4
HCl	n.d.	n.d.	38% ± 5	74% ± 8	93% ± 10
DTT[e]	n.d.	n.d.	74% ± 7	81% ± 6	92% ± 7
MgCl$_2$	n.d.	n.d.	n.d.	65% ± 9	91% ± 7

[a] Ricin incubations at 37 °C (3 h) and assay procedures are described under Materials and methods.
[b] Concentrations of agents are given for a 5-μl sample volume.
[c] Values (±propagated SD) were determined by subtracting the background signal average from the signal averages for ricin (5 ng/ml) with the agent ($n = 4$) and then dividing these differences by the difference for ricin only (no agent or DEPC); values are expressed as a percentage (residual activity).
[d] Not determined.
[e] Dithiothreitol.

Table 4
Residual activity of ricin with RNA GdAGA substrate in the presence of nucleases

Agent	Residual activity at selected nuclease concentrations[a] (pg/μl)				
	5000[b]	50	10	2	0.4
DNase I	101% ± 8[c]	n.d.[d]	n.d.	n.d.	n.d.
DNase I + 4 mM MgCl$_2$	98% ± 5	n.d.	n.d.	n.d.	n.d.
RNase A	n.d.	−4% ± 0	−4% ± 0	3% ± 1	87% ± 7
RNase A + DEPC[e]	n.d.	−4% ± 0	−4% ± 0	12% ± 1	60% ± 5

[a] Ricin incubations at 37 °C (3 h) and assay procedures are described under Materials and methods. Ricin was present at 5 ng/ml.
[b] Concentrations of agents are given for a 5-μl sample volume.
[c] Values were determined as described in the Table 3 footnotes. Negative percentages reflect substrate degradation.
[d] Not determined.
[e] Mixtures of ricin and RNase A in 1-ml aliquots were treated with diethylpyrocarbonate (DEPC) as described for Mab 9C3 under Materials and methods.

divalent cations (e.g., Mg^{2+}; Table 3) or to magnesium-dependent DNase activity [23]. A reaction buffer pH of 4.1 should be suboptimal for most nucleases. Nonetheless, RNase A caused degradation of the substrate, RNA GdAGA. Although ricin A chain has been reported to be unaffected by diethylpyrocarbonate [22], we observed inhibition of ricin (not shown) that limited the usable DEPC concentration such that treatment with this chemical rendered only a slight mitigation of the interference by RNase

A (Table 4). Since RNases are known to be present in castor bean extracts [24] and perhaps in illicit weapons made therefrom, we investigated whether a polyclonal antiricin antibody linked to Dynabeads might be useful in removing ricin from potential interferences in solution. Briefly, ricin (1 ng in 1 ml) was concentrated from solutions onto the beads, eluted with a low-strength glycine buffer (pH 3.1), and then added directly to a reagent solution containing RNA GdAGA; minimum signals (avg − SD) were 1.4-fold

above the LOD (background avg + [3 × SD]). Future experiments will expand on these preliminary findings and examine various sample matrices that are relevant to ricin intoxication (e.g., blood).

Another strategy for avoiding potential RNase interference would be to use the DNA GAGA(CU) substrate instead of RNA GdAGA. However, the DNA substrate allows a (minimal) detectable ricin concentration that is about two orders of magnitude higher than the concentration detectable with RNA GdAGA (data not shown). Similarly, the DNA substrate "dA12" was reported to have a much lower turnover number (k_{cat}) for ricin A chain than an RNA substrate with a GdAGA loop [17].

Because previous studies showed that slight modifications of RNA substrates altered their interactions with ricin, we hypothesized that similarly modified substrates could be combined with the convenience of the ECL platform to enable the rapid differentiation of adenine-specific *N*-glycosidases. For instance, Orita et al. [18] showed that replacing the second G of the GAGA loop with riboinosine dramatically reduced the cleavage rate by ricin, suggesting that the amino group of that G is important. Similarly, we replaced the second G with an A residue—the amino groups on A and G are situated differently—to obtain a substrate with a GdAAA loop. In a second alternative, the second G residue was replaced with dG to give the substrate RNA GdAdGA. We also tested a substrate with a six-residue loop, RNA AGdAGAC, because previous work showed diminished recognition of the hexaloop structure by ricin A chain [25]. Our approach was to test ricin as a standard against RCA, saporin, and abrin II as unknowns: the concentration of the "unknown" toxin was adjusted so that the signal with RNA GdAGA substrate was comparable to that obtained with 5 ng/ml ricin. As shown in Fig. 4, these alternative substrates yielded a reproducible pattern with ricin. Fig. 4A reveals that the patterns for ricin and RCA are very similar, which might be expected given that the A chains of the two toxins share 93% homology [26]. Figs. 4B and C illustrate the patterns for saporin and abrin II, which are indistinguishable from one another yet clearly different from the ricin/RCA pattern. Thus, a small set of substrates was used to partially differentiate adenine-specific *N*-glycosidases with very similar specificities, given that all selectively cleaved at the dA residue within the RNA GdAGA substrate.

Antibody-based differentiation of the toxins is shown in Fig. 5. Mab 9C3 enhanced signals by 20–25% for ricin and saporin but caused more dramatic enhancements with RCA and abrin II. When combined with the data from the alternative substrate tests (Fig. 4), these four toxins could be fully differentiated under the reaction conditions used. We observed that Mab 9C3 enhanced *N*-glycosidase activities toward an artificial substrate, whereas antiricin Mabs have typically been shown to neutralize the toxicity of ricin toward whole cells or cell-free ribosomes [15,27]. Mab 9C3 may have acted as a specific noninhibitory solubilizing agent for the toxins. Though the epitope
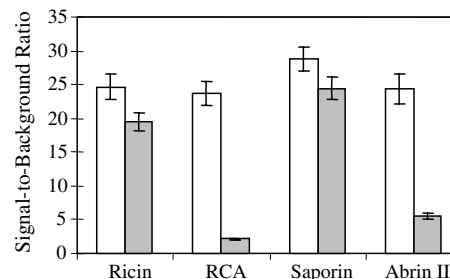

Fig. 5. Effects of Mab 9C3 on the activities of various toxins. Reactions included RNA GdAGA substrate and 100 ng Mab 9C3 (white bars) or no antibody (gray bars). Toxin concentrations were adjusted such that signals with Mab 9C3 were similar to those observed for ricin (5 ng/ml). Concentrations: RCA (25 ng/ml); saporin (1 ng/ml); abrin II (22 ng/ml).

recognized by Mab 9C3 and the mechanism(s) of activity enhancement are unknown, our observations suggest that the four toxins share an epitopic determinant.

In conclusion, our activity assay offers a facile means of corroborating ECL immunoassay data by a mechanism distinct from that of the immunoassay. This is necessary to overcome the cross-reactivity issues inherent to some degree in all immunoassays. The combination of these methods should provide greater confidence in sampling efforts. The assays are also complementary in practical terms in that the protocol for the activity assay can be completed in 5.3 h (96-tube experiment; Fig. 1 legend), while the easy-to-use immunoassays require about 2.3 h. Additional specificity, if needed, can be obtained with activity tests involving alternative substrates or antibody-based differentiations. Ongoing efforts to improve the usefulness of the activity assay include the design and testing of nuclease-resistant substrates. This approach or the aforementioned antibody-based purification strategy should aid in addressing the potential problems with interfering substances. In addition to improving diagnostic testing for ricin, the activity assay may be applied to toxin decontamination efforts and to evaluations of antiricin drugs or ricin-based vaccine candidates.

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