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Quantitative profiling of the *in vivo* enzymatic activity of ricin reveals disparate depurination of different pulmonary cell types

Reut Falach, Anita Sapoznikov, Yoav Gal, Ofir Israeli, Moshe Leitner, Nehama Seliger, Sharon Ehrlich, Chanoch Kronman*, Tamar Sabo

Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, Ness-Ziona, Israel

HIGHLIGHTS

- Maximum depurination in lungs of mice exposed to ricin, is reached at 48 h post exposure.
- The extent of ricin-induced depurination in different cells populating the lungs, varies greatly.
- Anti-ricin antibody treatment, rapidly halts further depurination in the cells of the lung.
- The magnitude of depurination induced by abrin is significantly lower than that of ricin.

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ABSTRACT

The plant-derived toxins ricin and abrin, operate by site-specific depurination of ribosomes, which in turn leads to protein synthesis arrest. The clinical manifestation following pulmonary exposure to these toxins is that of a severe lung inflammation and respiratory insufficiency. Deciphering the pathways mediating between the catalytic activity and the developing lung inflammation, requires a quantitative appreciation of the catalytic activity of the toxins, *in-vivo*.

In the present study, we monitored truncated cDNA molecules which are formed by reverse transcription when a depurinated 28S rRNA serves as template. We found that maximal depurination after intranasal exposure of mice to $2LD_{50}$ ricin was reached 48 h, where nearly 40% of the ribosomes have been depurinated and that depurination can be halted by post-exposure administration of anti-ricin antibodies. We next demonstrated that the effect of ricin intoxication on different cell types populating the lungs differs greatly, and that outstandingly high levels of damage (80% depurination), were observed in particular for pulmonary epithelial cells. Finally, we found that the magnitude of depurination induced by the related plant-derived toxin abrin, was significantly lower in comparison to ricin, and can be attributed mostly to reduced depurination of pulmonary epithelial cells by abrin. This study provides for the first time vital information regarding the scope and timing of the catalytic performance of ricin and abrin in the lungs of intact animals.

1. Introduction

Ricin, a potent protein toxin derived from the seeds of the castor bean plant, comprises a catalytic A chain (RTA), disulfide-bonded to a cell-binding B chain (RTB). The A chain possesses RNA N-glycosidase activity which depurinates a single adenine residue located near the 3′ terminal end of the 28S rRNA within the α-sarcin/ricin loop (Olsnes et al., 1975; Endo and Tsurugi 1987; Olmo et al., 2001; Hartley and Lord, 2004). This site-specific depurination event prevents binding of elongation factor-2 to the ribosome, thereby causing translational arrest (Oliviery et al., 1996). Pulmonary ricin intoxication is considered most hazardous, the estimated LD_{50} being within the μg/kg range (Audi et al., 2005). The clinical expression following inhalatory exposure in animals is that of severe lung inflammation, which in turn leads to

Abbreviations: RTA, ricin toxin A chain; RTB, ricin toxin B chain; RT, reverse transcriptase; BALF, brobchoalveolar lavage fluid; PBS, phosphate buffered saline; AUC, area under the curve.

* Corresponding author at: Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, Reuven Lerer 1, Ness-Ziona, Israel.
E-mail address: chanochk@iibr.gov.il (C. Kronman).

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respiratory insufficiency and death (Lindauer et al., 2009). Although some theories have been promulgated as to how ribosomal damage may induce inflammation (Iordanov et al., 1997; Shifrin and Anderson 1999; Zhou et al., 2003; Wang et al., 2005; Cherla et al., 2006; Li and Pestka 2008; Bae and Pestka 2008; Jandhyala et al., 2008), the actual cellular events that bridge between the molecular activity of ricin and its clinical manifestations are yet to be resolved. In-depth appreciation of the relationship between ricin catalytic activity and the developing lung inflammation following pulmonary exposure to the toxin, requires that the scope and timing of the *in-vivo* catalytic performance of the toxin, be well-defined. We therefore set out to delineate the progress of ribosome depurination following pulmonary exposure of mice to a lethal dose of ricin or abrin.

The catalytic activity of ricin was assessed in the past, directly or indirectly, by different approaches. Tracking ricin activity by measuring its ability to prevent protein synthesis was examined *in-vitro* in radioactive amino acid(s) incorporation assays (Endo and Tsurugi 1987; Iordanov et al., 1997) or in luciferase-based assays for measuring protein synthesis in cell-culture (Saenz et al., 2007). Visualization of the depurinated 28S rRNA product was achieved by incubation with aniline acetate which induces cleavage at depurinated sites, followed by SDS-PAGE analysis of the cleaved RNA (Stripe et al., 1988; Taylor and Irvin 1990; Li and Pestka, 2008). Another method is based on the identification of adenine residues released during depurination of ribosomes (Brigotti et al., 1988). All of these assays are semi-quantitative and/or adapted for *in-vitro* samples and are not compatible with the quantification of depurination in intoxicated animals.

In the present study, we developed a quantitative method which allows evaluation of ricin activity within the lungs of intranasally intoxicated mice. This method was also employed to assess the ribosomal damage in different cell types populating the lung and to follow the *in-vivo* catalytic activity of the closely-related toxin, abrin. These studies reveal noticeable differences between the intoxications induced by these two toxins.

2. Materials and methods

2.1. Ricin and abrin preparation

Toxins were prepared as previously described (Gal et al., 2014; Sapoznikov et al., 2015; Sabo et al., 2015). Briefly, seeds of *Ricinus communis* or *Abrus precatorius* were homogenized in a Waring blender in 5% acetic acid/phosphate buffer (Na2 HPO4, pH 7.4) the homogenate was centrifuged and the clarified supernatant containing the toxin was subjected to ammonium sulphate precipitation (60% and 80% saturation, for ricin and abrin, respectively). The precipitate was dissolved in phosphate-buffered saline (PBS) and dialyzed extensively against the same buffer.

In-vitro activity of the two toxins was quantitated in a HEK293-acetylcholinesterase cell-based assay (Cohen et al., 2014). Half-maximal effective concentrations (EC_{50}) of ricin and abrin were determined to be 0.14 ± 0.05 and 0.1 ± 0.03 ng/ml, respectively (Gal et al., 2014; Sabo et al., 2015).

2.2. Animal studies

Animal experiments were performed in accordance with the Israeli law and approved by the Ethics Committee for animal experiments at IIBR. Treatment of animals was in accordance with regulations outlined in the USDA Animal Welfare Act and the conditions specified in the National Institute of Health Guide for Care and Use of Laboratory Animals. All animals in this study were female CD-1 mice (Charles River Laboratories Ltd., UK) weighing 27–32 g. Prior to exposure, animals were habituated to the experimental animal unit for 5 days. All mice were housed in filter-top cages in an environmentally controlled room and maintained at 21 °C and $55 \pm 10\%$ humidity. Lighting was set to mimic a 12/12 h dawn to dusk cycle. Animals had access to food and water *ad libitum*. Mice were anesthetized by an intraperitoneal injection of ketamine (1.9 mg/mouse) and xylazine (0.19 mg/mouse), and were intranasally intoxicated with $2LD_{50}$ of crude ricin or abrin. LD_{50} values of ricin and abrin were previously determined to be 3.5 (95% confidence intervals of 2.3–4.4) and 4.0 (95% confidence intervals of 3.43–4.7) μg/kg body weight, respectively (Gal et al., 2014; Sabo et al., 2015).

2.3. Anti-ricin antibodies

Anti-ricin Abs were prepared from serum of rabbits that were immunized with purified ricin, as previously described (Gal et al., 2014). The anti-ricin antibodies displayed ELISA titers of 5×10^6 and a dissociation constant (K_D) of ~1 nM (Sabo et al., 2015).The neutralizing titer of these antibodies was determined to be 5×10^5 (Sabo et al., 2015) in an *in-vitro* HEK293-acetylcholinesterase cell-based assay described previously (Cohen et al., 2014).Treatment of ricin-intoxicated mice with anti-ricin Abs was performed by intravenous application of 100 μl of antibody preparation at 3 or 24 h following intoxication. The molar access of anti-ricin antibodies (determined to be ~25% of the IgG in the antibody preparation) to exposure dose of ricin is within the range of 10^3–10^4.

2.4. RNA purification

RNA was isolated from the left lungs of mice using Qiagen RNeasy mini kits (Qiagen, CA, USA), with an on-column DNase step (Qiagen, CA, USA). Samples were recovered in 100 μl H_2O and stored at −70 °C.

2.5. Cell sorting

Mice were exposed to ricin and 24 h later lungs were harvested, entire left lungs were cut into small pieces and digested for 2 h at 37 °C with 4 mg/ml collagenase D (Roche, Mannheim, Germany) in PBS containing Ca^{2+} and Mg^{2+} (Biological Industries, Beit-Haemek, Israel). The tissue was then meshed through a 40 μm cell strainer and red blood cells were lysed with ACK lysis buffer (150 mM NH4Cl, and 10 mM KHCO3). Cells were stained using antibodies coupled to: PE [anti-Gr-1 (RB6-8C5), CD31 (PECAM-1, 390)]; APC [anti-CD11c (N418), CD326 (EpCAM, G8.8)]; PerCP-Cy5.5 [streptavidin]; biotinylated [anti-CD45 (30-F11)]. All reagents were obtained from BioLegend (San-Diego, CA, USA) and eBioscience (San-Diego, CA, USA). Cells were isolated according to the following markers: neutrophils, $CD11c^-$ and Gr-1^{high}; alveolar macrophages, autofluorescent, $CD11c^{high}$, Gr-1^{int}, DCs, $CD11c^{int}$, Gr-1^-; endothelial cells, $CD45^-$ and $CD31^+$ and epithelial cells, $CD45^-$, $CD31^-$, $CD326^+$ by high-speed sorting with a FACSAria (BD Biosciences, San Jose, CA, USA) using FlowJo software (version 7.1.2, Tree Star, Ashland, OR, USA).

2.6. Depurination assay

Reverse transcriptase (RT) reaction was conducted with two oligonucleotide primers: the first, R-1 - CGATGGTAGACACCCTAATACT marked with FAM, and the second, R-HEX – CTTTGATTGGTCCTAAGGGAGTCATT marked with HEX. Lung derived RNA was incubated in the presence of both primers in an RT mix containing M-MLV reverse transcriptase (RT), DTT, dNTPs and RNAsin (Promega, Madison,WI, USA), for 20 min at 37 °C and then for 20 min at 48 °C. cDNA was resolved by a capillary electrophoresis fragment analysis