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Technical Note

Rapid assessment of antibody-induced ricin neutralization by employing a novel functional cell-based assay

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ABSTRACT

Ricin is one of the most potent and lethal toxins known against which there is no available antidote. Currently, the most promising countermeasures against the toxin are based on neutralizing antibodies elicited by active vaccination or administered passively. A cell-based assay is widely applied for the primary screening and evaluation of anti-ricin antibodies, yet such assays are usually time-consuming (18–72 h). Here, we report of a novel assay to monitor ricin activity, based on HeLa cells that stably express the rapidly-degraded ubiquitin-luciferase (Ub-FL, half-life of 2 min). Ricin-induced arrest of protein synthesis could be quantified within 3 to 6 h post intoxication (IC_{90} of 300 and 100 ng/ml, respectively). Furthermore, by stabilizing the intracellular levels of Ub-FL in the last hour of the assay, a 3-fold increase in the assay sensitivity was attained. We applied this assay to monitor the efficacy of a ricin holotoxin-based vaccine by measuring the formation of neutralizing antibodies throughout the immunization course. The potency of anti-ricin monoclonal antibodies (directed to either subunit of the toxin) could also be easily and accurately measured in this assay format. Owing to its simplicity, this assay may be implemented for high-throughput screening of ricin-neutralizing antibodies and for identification of small-molecule inhibitors of the toxin, as well as other ribosome-inactivating toxins.

1. Introduction

Ricin, derived from the plant *Ricinus communis*, is one of the most lethal toxins known. It consists of two covalently-linked subunits: the A-subunit (RTA) is an N-glycosidase that irreversibly inactivates the 28S rRNA of the mammalian 60S ribosome subunit, and the B-subunit (RTB) is a galactose-specific lectin that mediates the binding of the toxin to cell membranes (Olsnes and Kozlov, 2001). Due to its high toxicity, availability and ease of production and dissemination, ricin is considered a potential bio-terror agent and is classified as a category B select agent by the Center for Disease Control and Prevention (CDC). Currently, there is no available antidote against ricin exposure, underlining the need to develop effective countermeasures. Toward this end, efforts were made to identify small molecules, aptamers or sugar-analogs that inhibit ricin toxicity (Wahome et al., 2010), none of which were shown to be effective *in vivo*. To date, the most promising anti-ricin therapy is based on neutralizing antibodies elicited by active vaccination or administered passively (Smallshaw and Vitetta, 2011; Gal et al., 2014). An *in vitro* assay to measure ricin-neutralization efficacy is a necessary and invaluable step in the development of ricin countermeasures in general and for antibody-based therapy in particular.

Over the years, several assays were developed in order to measure the biological activity of ricin, either for forensic purposes or as tools to evaluate inhibitors of ricin (Bozza et al., 2015). The vast majority of these assays measure the RTA enzymatic activity using cell-free methods, either by monitoring the inhibition of protein synthesis in an *in vitro* transcription/translation system or by detecting the release of adenine or the formation of depurination reaction products from ribosomal RNA (Brigotti et al., 1998; Hale, 2001). These assays were applied with success to evaluate the potency of ricin-neutralizing antibodies (Pelat et al., 2009). Yet, ricin intoxication is a multi-step process that involves cell binding, cytoplasmatic transport and ribosomal inactivation. Therefore, to accurately assess the full potential of ricin-neutralizing antibodies to serve as therapeutic agents, they should be tested in a cell-based assay. The most common cell based assays for ricin toxicity measure cell survival as the intoxication end point, within 48–72 h post exposure (Prigent et al., 2011; Vance et al., 2013). However, ricin-mediated protein arrest is an early cellular event that precedes cell death by many hours, enabling accurate measurements of marker protein levels within 18–24 h (Wahome et al., 2010; Cohen et al., 2014). Similarly, it was shown that imaging cytometry enables monitoring the intracellular levels of a short half-life protein, such as a modified-GFP. This assay allowed detection of ricin activity 6 h post-exposure, even though full protein synthesis inhibition was reached only 18 h

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post-exposure (Halter et al., 2009). Yet, a ricin-neutralization assay that is sensitive, robust, enabling accurate and quantitative measurements within several hours is a strong necessity. Here, we present the development and implementation of such a simple and short-term cell-based assay to characterize the neutralizing activity of anti-ricin antibodies.

2. Materials and methods

2.1. Anti-ricin antibodies

All animal experiments were performed in accordance with the Israeli law and were approved by the ethics committee for animal experiments at the Israel Institute for Biological Research. Animals were maintained at 20–22 °C and a relative humidity of 50 ± 10% on a 12 hour light/dark cycle. Pure ricin was prepared as described previously (Gal et al., 2014). Cynomolgus macaque (*Macaca fascicularis*) was subcutaneously injected with 2 μg of ricin mixed with complete Freud's adjuvant followed by two monthly booster injections of 5 and 80 μg purified ricin mixed with incomplete Freud adjuvant. Blood samples were drawn 21 days following each boost and the serum was collected.

For the production of chimeric (macaque-human) monoclonal antibodies, FreeStyle Max 293 cells (Life Technologies, USA) were transiently transfected with a plasmid encoding for a full length IgG. After a week, the supernatant was collected and the antibodies were purified on HiTrap Protein-A column (GE healthcare, Sweden).

2.2. In vitro ricin-toxicity assay

Ub-FL cells (HeLa cells stably expressing ubiquitin-luciferase) (Luker et al., 2003) were a kind gift from Professor Piwnica-Worms (University of Texas, MD Anderson Cancer Center, USA). Cells were cultured in DMEM (Biological Industries, Beit Haemek, Israel) supplemented with 10% FCS.

To determine ricin cytotoxicity, cells were seeded in 96-wells plates (2×10^4 cells/well), and 16 h later the medium was replaced by culture medium containing increasing concentrations of purified ricin. At the indicated time points the cell-culture medium was removed and the cells were lysed by the addition of 50 μl lysis buffer (Promega, USA). The residual luciferase activity in each well was then determined by mixing equal volumes of the cell lysate and luciferine (Promega, USA) followed by immediate measurement of the luminescence levels. In the assays where a proteasome-inhibition step was included, at the end of the incubation period the culture medium was aspirated and replaced by a fresh culture medium containing 10 μM of MG132 (Sigma-Aldrich, Israel) and the cells were incubated for another hour.

2.3. In vitro neutralization assay

In order to determine the neutralization activity of the anti-ricin antibodies, ricin (30 ng/ml) was mixed with increasing concentrations of the monoclonal antibodies or with a serial dilutions of the serum samples. The mixtures were then added to the Ub-FL cultured cells and the assay was continued as described above (6 hour incubation with the addition of MG132).

The residual luciferase activity in the cell-culture wells was plotted as percent of luminescence levels obtained from untreated cells and the curves were fitted using Prism software (GraphPad Software Inc.). Each experiment was repeated several times and the graphs are of representative experiment.

3. Results and discussion

3.1. Kinetics of protein synthesis inhibition

The key feature needed in order to develop a rapid yet sensitive assay to measure inhibition of protein synthesis, is an intracellular probe with the shortest possible half-life. We therefore chose to base our assay on cells that constitutively express ubiquitin-luciferase (Ub-FL), a firefly luciferase fused to four copies of the mutant G76V hydrolase-resistant ubiquitin (Luker et al., 2003). It has a rapid turnover rate within the cytoplasm, with a half-life of about 2 min. Yet, it possesses full enzymatic activity, thus provides marked signal amplification that enables to accurately measure any change in cellular enzyme levels. In order to determine the sensitivity of these cells to ricin and to find the optimal time frame for the neutralization assay, cells were incubated with a wide range of ricin concentrations. At different time points (1, 3 or 6 h) the cells were lysed and the residual intracellular luciferase levels were determined. At 1 h post ricin exposure, no reduction in luciferase levels was seen even at the highest ricin concentrations (Fig. 1). However, at 3 and 6 h post exposure, there is a clear dose–response reduction in luciferase levels as a function of ricin concentrations. The toxin concentration needed to reduce luciferase levels to 50% (IC_{50}) following incubation in the presence of toxin for 3 and 6 h was 30 ng/ml and 7 ng/ml, respectively.

Since this assay was intended to be used for evaluation of anti-ricin antibodies exhibiting various neutralizing potencies, the lowest ricin concentrations that provide the needed sensitivity in a short-time frame should be used. In order to maximize sensitivity, a toxin concentration that results in 90% reduction of protein synthesis (IC_{90}) is beneficial. Therefore, even though the 3 hour incubation assay is sufficient to record a significant decrease in luciferase levels, we decided to continue with the 6 hour incubation period in which the IC_{90} value is 100 ng/ml (as compared to 300 ng/ml for the 3 hour assay).

3.2. Optimization of assay sensitivity

The bioluminescence values obtained in the assay range from 200 cps in the untreated control cells to 5 cps in the cells exposed to the highest ricin concentrations (coinciding with the level of the noise signal in this assay). These values allow to measure ricin activity in a very accurate manner with a dynamic range factor of over 40 between control and the ricin-treated samples. Yet, to further increase sensitivity, an additional step which inhibits Ub-FL degradation for a short time period just before terminating the assay was introduced. This step results in an increase of the bioluminescence signal in the control cells while the signal will remain low in the ricin-treated cells. To this end, we used MG132, a reversible proteasome inhibitor which was previously shown to increase the bioluminescence signal in Ub-FL cells by several orders of magnitude (Luker et al., 2003). Ub-FL cells were exposed to increasing concentrations of ricin for 6 h, and then the ricin-containing

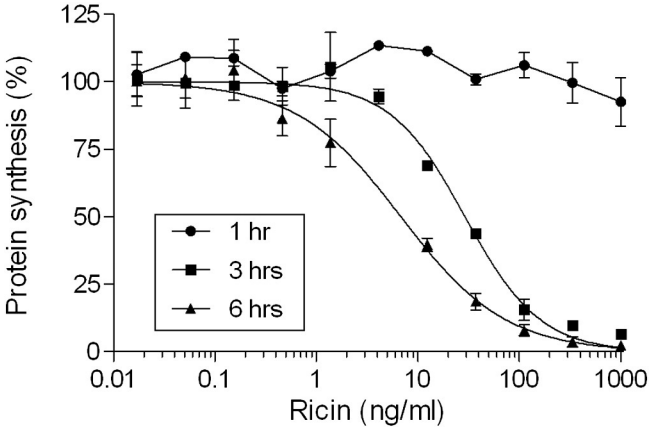

Fig. 1. Time-dependent ricin-induced inhibition of protein synthesis. Cultured Ub-FL cells were exposed to increasing concentrations of ricin for the indicated durations. Following incubation, residual activity of intracellular luciferase was determined and expressed as percent of the activity determined for untreated cells. Points are mean ± SEM of triplicates.

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