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Extended therapeutic window for post-exposure treatment of ricin intoxication conferred by the use of high-affinity antibodies

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ABSTRACT

The plant toxin ricin is considered a potential bioterror agent against which there is no available antidote. To date, neutralizing antibodies are the most promising post-exposure treatment for ricin intoxication, yet so far they were shown to be effective only when given within several hours post exposure. As part of an ongoing effort to develop efficient ricin-countermeasures, we tested whether high-affinity antibodies that were previously isolated from immunized non-human primates, may confer effective post-exposure therapy for ricin-intoxicated mice treated at late time-points after exposure. While each antibody is capable of providing high protection rate by itself, a formulation consisting of three neutralizing antibodies that target different epitopes was tested to provide therapeutic coverage against different variants of the malicious pathogen. Indeed, the tri-antibody based cocktail was highly effective, its administration resulting in very high survival rates (>70%) when animals were treated as late as 48 h post exposure and significant protection (>30%) even at 72 h. This study establishes for the first time that anti-ricin antibodies can serve as a highly effective antidote at such late time-points after exposure. From the clinical point of view, the extended therapeutic window documented here is of high importance allowing adequate time to accurately identify the causative agent and may permit initiation of life-saving treatment with these antibodies even after the onset of clinical signs.

1. Introduction

Ricin toxin, derived from the castor bean plant *Ricinus communis*, is a type 2 ribosome-inactivating protein that consists of two subunits: the catalytically-active ricin A subunit (RTA) that depurinates a conserved adenine residue in the 28S ribosomal RNA of the 60S subunit, thereby leading to irreversible inhibition of protein synthesis and cell death; and the ricin B subunit (RTB), a lectin that binds to galactose residues at the cell surface, allowing the toxin to be internalized and transported to the endoplasmic reticulum (Olsnes and Kozlov, 2001). The high toxicity, availability and ease of preparation, rendered ricin classification as a Category B agent by the U.S. Center for Disease Control and Prevention (CDC) and it is considered a potential bioterror agent of concern.

Thus far, there is no effective treatment against ricin poisoning, and in animal models, passive immunization was shown to be the most effective post-exposure therapy. We, as well as others have demonstrated that administration of anti-ricin antibodies preparations, consisting of either polyclonal or monoclonal antibodies, can provide high survival rates for ricin-intoxicated mice (Gal et al., 2014; Pratt et al., 2007; Beyer et al., 2009; Respaud et al., 2016). However, since ricin induces a fast-progressing disease, the therapeutic window for post exposure intervention was found to be rather narrow. Accordingly, when antibody-based treatment was initiated 24 h post pulmonary ricin-exposure, only 30–60% of the intoxicated animals survived (Gal et al., 2014; Pratt et al., 2007; Respaud et al., 2016). It was also reported that the addition of anti-inflammatory agents can synergize the therapeutic activity of anti-ricin antibodies (Gal et al., 2014, 2016). Yet, this narrow therapeutic window necessitates rapid identification of the toxin in event of intoxication and limits the capability to initiate effective treatment upon the appearance of clinically relevant markers.

In a recent study, we isolated a panel of anti-ricin antibodies from immune scFv phage-displayed libraries that were derived from the antibody-encoding genes of two ricin-immunized non-human primates (Noy-Porat et al., 2016). Out of the 10 isolated antibodies (five directed against the A subunit of ricin and five

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against the B subunit), four antibodies (anti-RTA MH1 and the anti-RTB MH73, MH75 and MH77) were found to target different epitopes and were able to confer high protection rates to ricin-intoxicated mice when given six hours after exposure. Moreover, these antibodies were found to possess exceptionally high affinity toward the toxin, with K_D values below pM ($k_{off} < 1 \times 10^{-7}$ s^{-1}) that were well correlated with their ability to neutralize ricin.

Here, as part of an ongoing effort to develop ricin-countermeasures, we tested whether a cocktail comprising the four different high-affinity antibodies will convey effective post-exposure therapy for ricin-intoxicated mice and provide an extended therapeutic window for intervention.

2. Material and methods

2.1. In vitro ricin neutralization assay

Pure ricin was prepared as described previously (Gal et al., 2014). HeLa Ub-FL cells (Luker et al., 2003) were a kind gift from Professor Piwnica-Worms (University of Texas, MD Anderson Cancer Center, Austin, TX, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal calf serum (FCS). For cytotoxicity studies (Gal et al., 2015), cells were seeded in 96-well plates (1.5×10^4 cells/well) in medium containing ricin (30 ng/ml) and incubated at 37 °C in the presence or absence of the anti-ricin antibody. Six hours later, the medium was removed; the cells were lysed; and the residual intracellular ubiquitin-luciferase fusion protein activity was determined using *D*-luciferin as a substrate by measuring luminescence levels and expressed as percent activity determined for untreated cells.

2.2. In vivo protection assay

Female outbred ICR mice (Charles River Laboratories, Canterbury, UK) were maintained at 20–22 °C and a relative humidity of 50± 10% on a 12-h light/dark cycle, fed with commercial rodent chow (Koffolk Inc., Rancho Santa Fe, CA, USA) and tap water *ad libitum*. Treatment of animals was in accordance with regulations outlined in the U.S. Department of Agriculture (USDA) Animal Welfare Act and the conditions specified in the Guide for Care and Use of Laboratory Animals (National Institute of Health, 2011). Animal studies were approved by the local ethical committee on animal experiments.

Anesthetized mice, 27–30 g, were intoxicated by intranasal instillation (50 μl/mice) of the indicated ricin dose (LD_{50} = 2.55 μg/kg) that was slowly applied (25 μl/nostril) using a gel-loading tip (Cohen et al., 2014; Sapoznikov et al., 2015) and treated at the indicated time points after intoxication. For the mono-antibody treatment, each antibody was diluted in PBS to a concentration of 0.5 mg/ml and for the antibody cocktail therapy, the antibodies were diluted to a final concentration of 1, 1.5 or 2 mg/ml (for the cocktail of 2, 3 or 4 antibodies, respectively). At the indicated time points after intoxication, mice were treated with 100 μg of each antibody (alone or in a cocktail) by intravenous injection in a final volume of 200 μl. The mice were monitored for 14 days, and the protection conferred by each antibody was calculated as the percent of surviving mice. Mice intoxicated with ricin without antibody treatment were used as control. Survival plots were calculated using Prism software (Version 5.01, GraphPad Software Inc., La Jolla, CA, USA, 2007).

2.3. Epitope binning

Binding studies were carried out using the Octet Red system (ForteBio, Version 8.1, Menlo Park, CA, USA, 2015) that measures biolayer interferometry (BLI), essentially as described before (Noy-Porat et al., 2016). All steps were performed at 30 °C with shaking at 1500 rpm in a black 96-well plate containing 200 μl solution in each well. Streptavidin-coated biosensors were loaded with biotinylated antibody (5 μg/ml) for 300 s followed by a wash. The sensors were then reacted for 300 s with ricin (10 μg/ml), moved to buffer-containing wells for another wash step and reacted with non-labeled antibody pair (300 s followed by another short wash). Binding and dissociation were measured as changes over time in light interference after subtraction of parallel measurements from unloaded biosensors.

3. Results

In a previous study it was demonstrated that treatment of mice intoxicated by $2LD_{50}$ ricin, at six hours post exposure, with each of the antibodies MH1, MH73, MH75 or MH77 at a dose of 100μg/mouse, resulted in high protection efficacy (85–100%) (Noy-Porat et al., 2016). Therefore, the treatment efficacy of a cocktail comprising these four antibodies (total 400μg/mouse) was first tested under the same conditions. As expected, 100% of the animals that were treated with the antibody cocktail had survived, while untreated mice succumbed within 7 days (Fig. 1). This treatment conferred full protection to animals that were intoxicated with ricin at a higher dose of $3LD_{50}$ and high protection rates of 86% and 73% were achieved even when the challenge doses of ricin were increased to $4LD_{50}$ and $5LD_{50}$, respectively.

These results encouraged us to determine whether the treatment with this antibody cocktail may enable us to widen the therapeutic window, allowing effective therapy at significantly later time points after ricin intoxication. To this end, mice were intoxicated with $2LD_{50}$ of ricin and treated with the antibody cocktail at 24 h post intoxication. Indeed, it was found that 56% of the treated animals had survived the challenge (Fig. 2A). These results are considerably higher when compared to the treatment efficacy of polyclonal anti-ricin antibodies at this time point, about 30% survival (Gal et al., 2014; Pratt et al., 2007). Yet, given that at the six-hour time point treatment with the antibody cocktail provided full protection against $3LD_{50}$ of ricin, it was anticipated that a higher protection rate would be conferred when treatment was initiated 24 h post exposure. It was therefore decided to compare

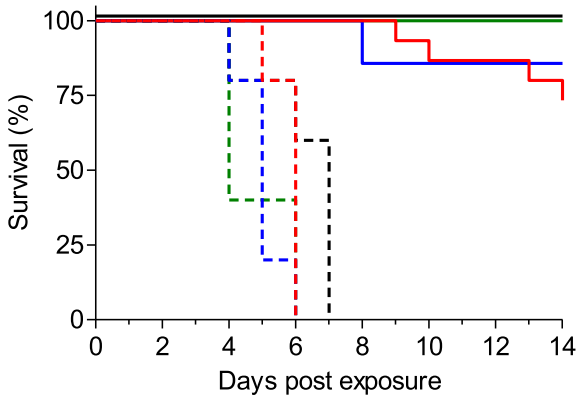

Fig. 1. Post-exposure treatment of ricin-intoxicated mice. Mice were intranasally instilled with the indicated doses of ricin (Black lines = 2 LD_{50}; Green lines = 3 LD_{50}; Blue lines = 4 LD_{50}; Red lines = 5 LD_{50}). Six hours later, the ricin-intoxicated mice were either treated by intravenous administration of the four antibody cocktail (solid lines, n = 10, 5, 7, or 15 for 2, 3, 4 and 5 LD_{50}, respectively) or left untreated (dashed lines, n = 5 for each ricin dose). Animal survival was monitored for 14 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)