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Antibody/doxycycline combined therapy for pulmonary ricinosis: Attenuation of inflammation improves survival of ricin-intoxicated mice

Yoav Gal, Ohad Mazor, Ron Alcalay, Nehama Seliger, Moshe Aftalion, Anita Sapoznikov, Reut Falach, Chanoch Kronman*, Tamar Sabo

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

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ABSTRACT

Ricin, a highly toxic plant-derived toxin, is considered a potential weapon in biological warfare due to its high availability and ease of preparation. Pulmonary exposure to ricin results in the generation of an acute edematous inflammation followed by respiratory insufficiency and death. Passive immunization with polyclonal anti-ricin antibodies conferred protection against pulmonary ricinosis, however, at clinically-relevant time points for treatment, survival rates were limited. In this study, intranasal instillation of a lethal dose of ricin to mice, served as a lung challenge model for the evaluation and comparison of different therapeutic modalities against pulmonary ricinosis. We show that treatment with doxycycline resulted in a significant reduction of pro-inflammatory cytokines, markers of oxidative stress and capillary permeability in the lungs of the mice. Moreover, survival rates of mice intoxicated with ricin and treated 24 h later with anti-ricin antibody were significantly improved by co-administration of doxycycline. In contrast, co-administration of the steroid drug dexamethasone with anti-ricin antibodies did not increase survival rates when administered at late hours after intoxication, however dexamethasone did exert a positive effect on survival when applied in conjunction with the doxycycline treatment. These studies strongly suggest that combined therapy, comprised of neutralizing anti-ricin antibodies and an appropriate anti-inflammatory agent, can promote high-level protection against pulmonary ricinosis at clinically-relevant time points post-exposure.

1. Introduction

Ricin, a toxin derived from the plant *Ricinus communis*, is an N-glycosidase that irreversibly inactivates the 28S rRNA of the mammalian 60S ribosome subunit, subsequently arresting cell protein synthesis [1]. The toxicity of ricin depends on the route of exposure, inhalatory exposure being considered most dangerous, the estimated dose causing death to 50% of the population (LD_{50}) being within the range of several microgram/kilogram [2]. Pathological studies of pulmonary ricin intoxication have demonstrated

Abbreviations: BALF, bronchoalveolar lavage fluid; ChE, cholinesterase; MMP-9, matrix-metalloproteinase-9; PBS, phosphate-buffered saline; $sPLA_2$, secretory phospholipase A_2; RCA, *ricinus communis* agglutinin; VEGF, vascular endothelial growth factor; XO, xanthine oxidase.

* Corresponding author. +972 89381522.
E-mail address: chanochk@iibr.gov.il (C. Kronman).

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that injury is mostly confined to the lungs [3]. The damage inflicted to the lungs is manifested by perivascular, interstitial and alveolar edema, influx of neutrophils to the lungs and the mounting of an acute inflammatory response. Flooding of the lungs leads to respiratory insufficiency and death [3].

Prophylactic anti-ricin vaccines have been developed and are now in human phase I studies [4], however, post-exposure medical countermeasures are needed for treatment of victims after pulmonary exposure to lethal doses of the toxin. Previous studies have examined the possibility to protect animal models against pulmonary ricinosis by passive immunization with polyclonal anti-ricin antibodies. Protection levels declined with the passage of time after intoxication, and when the anti-ricin antibodies were administered 24 h after exposure, survival rates were within the 30–50% range [5,6]. At this late time point, the pathophysiological state of some of the intoxicated mice may have deteriorated so that the loss of function of the lungs is irreversible. Conversely, it may be that higher survival rates can be attained even at this late time point if the raging pulmonary inflammation is assuaged through additional medical intervention.

A growing body of studies supports the notion that antibiotic tetracyclines, restrain inflammatory responses of various etiologies. Doxycycline, a tetracycline derivative, has been shown to inhibit staphylococcal exotoxin-induced production of cytokines and chemokines by peripheral blood mononuclear cells [7] and to attenuate polymorphonuclear cell recruitment in models of lung injury secondary to LPS, bleomycin or *Streptococcus pneumoniae* pneumonia [8–10]. Recently, it was reported that doxycycline exhibits anti-inflammatory activity in CF bronchial epithelial cells by inhibiting ERK 1/2, P38 and JNK dependent cell signaling (Bensman *et al.*, 2012). Interestingly, signaling pathways involving ERK, P38 and JNK were shown in the past to be stimulated by ricin [11].

In the present study, we examined the possibility to improve survival of mice exposed intranasally to a lethal dose of ricin, by co-administration of doxycycline together with polyclonal anti-ricin antibodies. Survival rates of mice subjected to this combination treatment were compared to those attained by co-administration of the highly potent anti-inflammatory steroid, dexamethasone, with the anti-ricin antibodies. We demonstrate that co-administration of doxycycline together with anti-ricin antibodies significantly improved the ability to protect mice even when treatment with the drug commenced at late hours (24 h post-intoxication), while dexamethasone confers slightly improved survival only when administered early after exposure. Nevertheless, dexamethasone did exert some positive effect on survival when administered late after intoxication in conjunction with both doxycycline and anti-ricin antibodies.

2. Methods

2.1. Ricin preparation

Crude ricin was prepared from seeds of endemic *R. communis*, essentially as described before [12]. Briefly, seeds were homogenized in a Waring blender in 5% acetic acid/phosphate buffer (Na_2HPO_4, pH 7.4) the homogenate was centrifuged and the clarified supernatant containing the toxin was subjected to ammonium sulphate precipitation (60% saturation). The precipitate was dissolved in phosphate-buffered saline (PBS) and dialyzed extensively against the same buffer. The toxin preparation appeared on a Coomassie blue stained non-reducing 10% polyacrylamide gel as 2 major bands of molecular weight approximately 65 kDa (= ricin toxin, ~80%) and 120 kDa (=*R. communis* agglutinin (RCA), ~20%). Protein concentration was determined as 2.86 mg/ml by 280 nm absorption (Nanodrop). Pure toxin was prepared as described previously [12,13]. Briefly, the crude ricin preparation was loaded onto a gel-filtration column (Superdex 200HR 16/60 Hiload 16/600 superdex 200 pg on an AKTA explorer, GE Healthcare Bio-Science AB; Uppsala; Sweden) and washed out with PBS to yield two well-separated protein peaks corresponding to RCA and ricin. The purity of the ricin fraction was estimated by SDS-PAGE analysis to be >98%.

2.2. Anti-ricin antibodies

Rabbits were immunized with pure ricin toxin with Freund's adjuvant in a stepwise manner, injections 1, 2 and 3 containing 4, 16 and 16 μg toxin/rabbit respectively and subsequent injections containing 100 μg toxin/rabbit, with 4-week intervals between injections. Blood samples were collected (1 week after injection) to ascertain anti-ricin antibody titer build-up. Immunization was continued until steady high anti-ricin titers were observed.

Anti-ricin antibody titers were determined by ELISA. Microtiter plates (Nunc) were coated with pure ricin (2.5 ng/ml in carbonate buffer pH 9.6, overnight incubation at room temperature), washed 3 times in wash buffer (0.8%NaCl + 0.05% Tween-20) and then incubated with blocking buffer (PBS + 0.05% Tween 20 + 2% BSA) for 1 h at 37 °C. Rabbit antisera samples were added in 2-fold serial dilutions and incubated at 37 °C for 1 h. Plates were then washed 3 times with wash buffer and incubated at 37 °C for 1 h with AP-conjugated goat anti-rabbit immunoglobulin (Sigma, 1:500 in blocking buffer). After washing as above, the microtiter plates were developed with substrate (p-NPP, Sigma) and optical densities were measured at 405 nm using an ELISA reader (Molecular Devices).

Concentrated anti-ricin IgG preparations were generated from pooled hyperimmune antisera by precipitation of the proteins with ammonium sulfate (40% saturation, overnight with constant stirring). Following centrifugation (5000 rpm, 50 min, 4 °C), the pellet was dissolved in purified water, and subjected to dialysis (overnight, 30 mM phosphate buffer pH = 7.4). The dialyzed proteins were applied on an anion-exchange column (Express-Ion, exchanger D; Whatman) and eluted with 60 mM phosphate buffer containing 1 M NaCl. The sample was precipitated by the addition of ammonium sulfate (40%, 3 h), and following centrifugation (5000 rpm, 60 min, 4 °C) the pellet was dissolved in 300 mM glycine buffer (pH = 7.4) and dialyzed (300 mM glycine buffer pH = 7.4 overnight). The