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Protective immunity to ricin toxin conferred by antibodies against the toxin's binding subunit (RTB)

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ABSTRACT

The B subunit (RTB) of ricin toxin is a galactose-/N-acetyl galactosamine-specific lectin that promotes attachment and entry of ricin into host cells. RTB is also the archetype of the so-called R-type lectin family, whose members include haemagglutinins of botulinum neurotoxin (BoNT) progenitor toxins, as well as the binding subunits of cytolethal distending toxins. Although RTB is an appealing subunit vaccine candidate, as well as a potential target for immunotherapeutics, the degree to which RTB immunization elicits protective antibodies against ricin toxin remains unresolved. To address this issue, groups of mice were immunized with RTB and then challenged with $5\times LD_{50}$s of ricin administered intraperitoneally. Despite high RTB-specific serum antibody titers, groups of RTB immunized mice were only partially immune to ricin challenge. Analysis of a collection of RTB-specific B cell hybridomas suggested that only a small fraction of antibodies against RTB have demonstrable neutralizing activity. Two RTB-specific neutralizing monoclonal IgG_1 antibodies, 24B11 and SylH3, when passively administered to mice, were sufficient to protect the animals against a $5\times LD_{50}$ dose of ricin. Both 24B11 and SylH3 blocked ricin attachment to terminal galactose residues and prevented toxin binding to the surfaces of bone marrow-derived macrophages (BMM), suggesting that they function by steric hindrance and recognize epitopes located on RTB's carbohydrate recognition sub-domains (1α or 2γ). These data raise the possibility of using specific RTB sub-domains, rather than RTB itself, as antigens to more efficiently elicit neutralizing antibodies and protective immunity against ricin.

1. Introduction

Ricin toxin, a natural by-product of the castor bean plant (*Ricinus communis*), is one of the most lethal toxins known [1,2]. The toxin's A subunit (RTA) is a 267-amino acid RNA *N*-glycosidase that functionally inactivates eukaryotic ribosomes by selective depurination of a highly conserved adenosine residue within ribosomal RNA [3,4]. The toxin's B subunit (RTB), a 262-amino acid galactose- and N-acetylgalactosamine-specific lectin, is linked to RTA via a single disulfide bond and mediates RTA attachment and entry into host cells. RTB consists of two globular domains with identical folding topologies (Fig. 1) [5]. Each of the two domains (1 and 2) are themselves comprised of three homologous sub-domains (α, β, γ) that probably arose by gene duplication from a "primordial" carbohydrate recognition domain (CRD) [5]. Only sub-domains 1α and 2γ retain functional carbohydrate recognition activity [6,7]. Sub-domain 1α binds only galactose and is considered a "low affinity" CRD, whereas sub-domain 2γ binds both galactose- and N-acetylgalactosamine and is considered a "high affinity" CRD [8–10]. The ricin-type (R-type) CRDs constitute a superfamily of lectins found in plants, animals, and toxins expressed by pathogenic bacteria, including *Campylobacter jejuni*, *Haemophilus ducreyi*, and *Clostridium botulinum* [11–16].

Ongoing efforts by public health and defense organizations in the United States and abroad to develop an effective vaccine [17,18] and immunotherapeutic [19,20] for ricin toxin, have focused almost exclusively on RTA, despite long-standing evidence for the existence RTB-specific antibodies that are capable of fully neutralizing ricin [21–25]. For example, in 1985, Foxwell et al. demonstrated that passive administration of polyclonal antibodies against RTB were as effective as antibodies against RTA in protecting mice against ricin intoxication [24]. In 1987, Colombatti et al. described a murine monoclonal IgG (mAb), 75/3B12 that blocked ricin binding to cell surfaces and neutralized ricin *in vitro* and *in vivo* [22,26]. More recently, we characterized a RTB-specific murine IgG mAb known as 24B11 that was also highly effective at inhibiting ricin attachment to host cells and at neutralizing ricin *in vitro* [25].

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While those studies highlight the potential of antibodies directed against RTB to interfere with the earliest events in ricin intoxication, our understanding of antibody-RTB interactions is far from complete. To date, only two RTB-specific mAbs, 75/3B12 and 24B11, have been characterized in detail, and only one, 75/3B12, has been tested *in vivo* [22,25,26]. Moreover, a recent study by Maddaloni et al. challenged the notion that RTB-immunization is sufficient to confer immunity to ricin [27]. Additionally, we and others have reported RTB-specific mAbs that bind ricin with high affinity but lack detectable neutralizing activity, although the epitopes on RTB recognized by these mAbs remain unknown [25,27].

Therefore, with the long-term objective of developing RTB-based vaccines and therapeutics as countermeasures against ricin toxin as a biothreat agent, the goal of this study was to better define the capacity of RTB to elicit immunity to ricin. In this study, we put forth evidence to suggest that only a very small proportion of antibodies elicited by RTB immunization are capable of neutralizing ricin and conferring protective immunity *in vivo*. We propose that neutralizing antibodies recognize epitopes near the CRDs within RTB sub-domains 1α and 2γ, whereas non-neutralizing antibodies bind sub-domains not involved in galactose recognition (*e.g.*, 1β, 2α). The fact that both neutralizing and non-neutralizing mAbs bound ricin with roughly equal affinities demonstrates that epitope specificity is likely the primary determinant of antibody-mediated protection. Finally, the results of this study suggest possible strategies to engineer RTB to more efficiently elicit neutralizing antibodies and protective immunity against ricin.

2. Materials and methods

2.1. Chemicals, biological reagents and cell lines

Ricin, RTA, and RTB were purchased from Vector Laboratories (Burlingame, CA). Ricin toxoid (RT) was produced by treatment of holotoxin with paraformaldehyde (4%, v/v), as described previously [25]. Ricin and RT were dialyzed against PBS at 4 °C in 10,000 MW cutoff Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL), prior to use in cytotoxicity studies. Paraformaldehyde (16%) was purchased from Electron Microscopy Sciences (Fort Washington, PA). GlutaMax™, fetal calf serum and goat serum were purchased from Gibco-Invitrogen (Carlsbad, CA). A ClonaCell HY™ kit for hybridoma production was purchased from STEMCELL Technologies (Vancouver, BC, Canada). Unless noted otherwise, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Vero cells, THP-1, and the murine myeloma cell line P3X63.Ag8.653 were purchased from the American Type Culture Collection (Manassas, VA). Cell culture media were prepared by the Wadsworth Center Media Services facility. Monoclonal antibody SylH3 was affinity-purified on a protein G column by the Wadsworth Center protein expression core. Unless otherwise noted, all cell lines and hybridomas were maintained in a humidified incubator at 37 °C with 5% CO_2.

2.2. Mouse strains, animal care and immunizations

Female BALB/c mice approximately 6–8 weeks of age were purchased from Taconic Labs (Hudson, NY). Animals were housed under conventional, specific pathogen-free conditions and were treated in compliance with the Wadsworth Center's Institutional Animal Care and Use Committee (IACUC) guidelines. For serum profiling by RTB peptide array and antibody competition analysis by Biacore, female BALB/c mice were immunized by the intraperitoneal (i.p.) route with RTB or RT (50 μg per animal) without adjuvant three times at 10-day intervals. Ten days after the third immunization, blood was collected from the tail vein. For

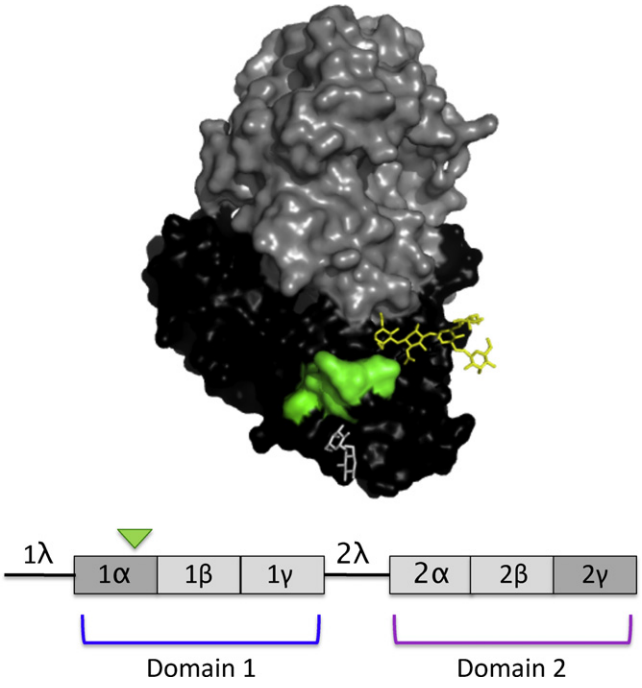

Fig. 1. Structure of ricin and RTB. (Upper panel) A 3D depiction of ricin toxin constructed using PyMOL. The subunits are highlighted: RTA (grey), RTB (black), epitope recognized by 24B11 (green), lactose within CRD (white), and mannose side chain (yellow). (Lower panel) Linear depiction of RTB showing domains (1 and 2), as well as individual sub-domains (1α, 1β, 1γ, 2α, 2β, 2γ). 1λ is a peptide linker connecting RTA to RTB in the ricin pre-protein, while 2λ connects the two RTB domains. Only sub-domains 1α and 2γ retain carbohydrate recognition activity. The green arrowhead indicates the 24B11 epitope. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

hybridoma production, female BALB/c mice were primed i.p. with RT (50 μg) on day 0, and then boosted by the same route with RT (50 μg) on days 10 and 20.

2.3. B-cell hybridoma production

Four days after the second boost with RT (50 μg), mice were euthanized, and total splenocytes were fused with the myeloma cell line P3X63.Ag8.653, using polyethylene glycol (PEG) as described previously [28]. The resulting hybridomas were seeded in methylcellulose and cloned as per the instructions in the ClonaCell -HY™ hybridoma cloning manual (STEMCELL Technologies, Vancouver, BC, Canada). Hybridomas secreting antibodies of interest were expanded and cultured in either RPMI medium containing 10% fetal calf serum, oxaloacetate, pyruvate, and insulin (OPI), 8 mM GlutaMax™, and penicillin-streptomycin, or in medium A (STEMCELL Technologies) before being transitioned to CD Hybridoma, a serum-free, protein-free, antibiotic-free medium (Gibco-Invitrogen, Carlsbad, CA).

2.4. ELISAs and RTA peptide arrays

ELISAs and peptide arrays were performed as previously described [25]. Briefly, Nunc Maxisorb F96 microtiter plates (ThermoFisher Scientific, Pittsburgh, PA) were coated overnight with ricin, RTA, RTB, BSA (0.1 μg/well) or individual peptides (1 μg/well) in PBS (pH 7.4) before being treated with primary mouse sera, hybridoma supernatants, or purified mAbs. Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG-specific polyclonal antibodies (SouthernBiotech, Birmingham, AL) were used as the secondary reagent. The ELISA plates were developed using the colorimetric detection substrate 3,3′,5,5′-tetramethylbenzidine (TMB;