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Folding domains within the ricin toxin A subunit as targets of protective antibodies

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ABSTRACT

Efforts to develop an effective vaccine against ricin are focused on the engineering of attenuated and stable recombinant forms of the toxin's enzymatic A subunit (RTA). While several candidate antigens are in development, vaccine design and efficacy studies are being undertaken in the absence of a fundamental understanding of those regions of RTA that are critical in eliciting protective immunity. In this present study, we produced and characterized a collection of monoclonal antibodies (MAbs) directed against five distinct immunodominant regions on RTA, and used these MAbs to identify several key neutralizing epitopes on the toxin. Protective MAbs were directed against α-helices located in RTA folding domains 1 and 2, whereas non-neutralizing antibodies recognized random coils and loops that were primarily confined to folding domain 3. These data offer insights into the immunodominant and structural determinants on RTA that give rise to protective immunity, and for the first time provide an immunological rationale for ricin vaccine design.

1. Introduction

Ricin toxin is a natural product of the castor bean plant, *Ricinis communis*, which is cultivated on an industrial scale around the world for the production of castor oil. The toxin constitutes up to 5% of the total protein of the castor bean and can be extracted from the mash produced as a by-product of castor oil production, through several simple enrichment steps. Ricin, in semi-purified or purified form, is extremely toxic [1–3]. Although few cases of ricin intoxication in humans have been reported [4], animal studies confirm that the toxin can be fatal by injection, inhalation or ingestion. Ricin has a history of being used both as a biological weapon and biothreat agent [5], a fact that is disconcerting considering no available vaccines or antidotes are currently available to prevent or counteract the effects of the toxin. For these reasons, the Centers for Disease Control and Prevention (CDC) and the Department of Health and Human Services (HHS) have classified ricin as a Category B select agent, and the National Institutes of Health consider the development of countermeasures against ricin toxin an integral part of their biodefense research program.

Ricin is a member of the family of type II ribosome-inactivating proteins, a family that includes abrin and shiga toxins [6,7]. Ricin toxin consists of a 32 kDA enzymatic A subunit (RTA) joined by a disulfide bond to a 34-kDA lectin B subunit (RTB). RTA is an RNA *N*-glycosidase whose target is a conserved adenine residue in the so-called sarcin/ricin loop (SRL) of eukaryotic 28S ribosomal RNA [8]. RTB recognizes, with low affinity, α(1-3)-linked galactose and *N*-acetylgalactosamine residues on the surface of almost every cell type, and it mediates toxin internalization via both clathrin-dependent and clathrin-independent mechanisms [9–11]. Once internalized, the toxin exploits multiple endocytic pathways, and traffics in a retrograde fashion from early endosomes to the *trans*-Golgi network, eventually reaching the endoplasmic reticulum (ER) [12,13]. In the ER, RTA and RTB dissociate, and the A-subunit is retro-translocated across the ER membrane to the cytoplasm [14,15].

Although a number of candidate ricin vaccines have been explored over the past several decades, current efforts are focused on the development of recombinant, attenuated derivatives of RTA. The most advanced vaccine in terms of clinical development is RiVax, a recombinant RTA subunit carrying two site-directed point mutations: one mutation in a catalytic tyrosine residue (Y80A) and the other in a valine (V76M) residue postulated to promote vascular leak syndrome [16–19]. This recombinant derivative of RTA is attenuated several thousand fold relative to the native protein, but

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retains immunogenicity. RiVax is currently in Phase I clinical trials (E. Vitetta and J. Smallshaw, UT Southwestern, personal communication). The second RTA derivative being pursued as a vaccine was developed by the U.S. Army and is called RTA 1-33/44-198 [20–22]. This variant carries a deletion of an N-terminal proximal exposed loop region (T34-P43) as well as a truncation of the C-terminus (A199-F267). Carra et al. have demonstrated that these deletions result in a thermostable protein that is both non-toxic and immunogenic in a mouse model [20].

While considerable effort has been invested in the engineering of attenuated derivatives of RTA, these studies are being conducted with only a limited understanding of the regions of the toxin that are important in eliciting protective antibodies. This is potentially problematic because immunization with RTA is known to elicit a mixture of neutralizing, non-neutralizing, and toxin-enhancing antibodies [23,24]. Indeed, we have speculated that there may be discrete regions or domains on RTA that are primarily responsible for stimulating antibodies capable of neutralizing the toxin [25]. It is therefore surprising that, to date, that only a handful of B cell epitopes on RTA have been identified. Lebeda and Olson described a 26-amino acid loop-helix-loop motif (Y91-T116) on RTA that was postulated to serve as an important target of neutralizing monoclonal antibodies (MAbs) [26]. In support of their hypothesis, we recently demonstrated that residues N97-F108 are the target of the protective MAb R70 [25,27]. In the same study, we also established that antibodies against amino acids T161-M175 are capable of conferring protection against both systemic and mucosal ricin challenge, at least in mice.

With the long-term goal of both improving and evaluating the humoral response to candidate ricin vaccines, the immediate objective of this present study was to better define the neutralizing and non-neutralizing B-cell epitopes on RTA. Since the two best characterized protective B-cell epitopes on RTA are linear determinants, we sought to identify the immunodominant linear regions on RTA so as to gain insight into the regions that are critical for eliciting both neutralizing and non-neutralizing antibodies. In this study we have produced and characterized a collection of MAbs directed against five of the six (I–VI) distinct immunodominant linear regions on RTA. We used both cell-based cytotoxicity assays, as well as animal challenge studies, to determine which of these MAbs was capable of neutralizing ricin *in vitro* and *in vivo*. We found that neutralizing MAbs were primarily directed at α-helices located in RTA folding domains 1 and 2, whereas non-neutralizing antibodies recognized random coils and loops that were primarily confined to folding domain 3. These data provide insight into immunodominant and structural determinants on RTA that give rise to protective immunity, and for the first time provide an immunological rationale for ricin vaccine design.

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 [28]. 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). RiVax™ vaccine, from Soligenix, Inc. (Princeton, NJ), was prepared by adsorption of recombinant RTA (Y80A; V76M) to AlOH (Alhydrogel® in 10 mM histidine, pH 6.0, 144 mM NaCl, so that the total vaccine contained 0.85 mg aluminum equivalents/ml and 200 μg RTA protein/ml. 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 specifically, all other chemicals were obtained from the Sigma–Aldrich Company (St. Louis, MO). Vero cells, irradiated MRC-5 human lung fibroblast cells, 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 SyH7 was affinity-purified on a protein G column by the Wadsworth Center protein expression core. Cell lines and hybridomas were maintained in a humidified incubator at 37 °C with 5% CO_2. Pooled affinity-purified rabbit anti-RiVax polyclonal antibody (PAb) was provided by Drs. Ellen S. Vitetta and Joan Smallshaw (University of Texas Southwestern Medical Center, Dallas, TX).

2.2. Mouse strains, animal care and immunizations

Female BALB/c mice approximately 10–12 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 RTA peptide array, and antibody competition analysis by BIAcore, female BALB/c mice were immunized by either the subcutaneous (sc) or intraperitoneal (i.p.) route with RiVax (10 μg per animal) or with RT (50 μg per animal) three times at 10 day intervals. Ten days after the third immunization, blood was collected from the tail vein. For hybridoma production, female BALB/c mice were primed parenterally (i.p.) with either RT (50 μg) or RiVax (10 μg) on day 0, and then boosted by the same route with RT (50 μg) or RiVax (10 μg) on days 10 and 20.

2.3. B-cell hybridoma production

Four days after the second boost with either RT (50 μg) or RiVax (10 μg), mice were euthanized, and total splenocytes were fused with the myeloma cell line P3X63.Ag8.653, using polyethylene glycol (PEG). The resulting hybridomas were either seeded into wells of 96-well cell culture-treated microtiter plates containing a layer of irradiated MRC-5 feeder cells and cloned by limiting dilution [28,29], or else seeded in methylcellulose and cloned as per the instructions in the ClonaCell-HY™ hybridoma cloning manual (STEMCELL Technologies, Vancover, BC, Canada). Hybridomas secreting antibodies of interest were expanded and cultured in either a 1:1 mixture of NCTC (Sigma Co.) and RPMI medium containing 10% fetal calf serum, oxaloacetate, pyruvate, and insulin (OPI), 8 mM GlutaMax™, and penicillin–streptomycin, or else in medium A (STEMCELL Technologies) before being transitioned to CD Hybridoma, a serum-free, protein-free, antibiotic-free medium (Gibco-Invitrogen).

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) were coated overnight with ricin, RTA, RTB, BSA (0.1 μg/well) or individual peptides in PBS (pH 7.4) before being treated with primary mouse or rabbit sera, hybridoma supernatants, or purified MAbs. Horseradish peroxidase (HRP)-labeled goat anti-mouse or rabbit IgG-specific polyclonal antibodies (SouthernBiotech) were used as secondary reagents. The ELISA plates were developed using the colorimetric detection substrate 3,3′,5,5′-tetramethylbenzidine (TMB; Kirkegaard & Perry Labs, Gaithersburg, MD) and were analyzed with a SpectroMax 250 spectrophotometer, with Softmax Pro 5.2 software (Molecular Devices, Sunnyvale, CA). The RTA peptide array used in this study consisted