Vaccine 29 (2011) 681–689

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Vaccine-induced intestinal immunity to ricin toxin in the absence of secretory IgA

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ARTICLE INFO

Article history:
Received 13 September 2010
Received in revised form 29 October 2010
Accepted 10 November 2010
Available online 27 November 2010

Keywords:
Biodefense
Vaccine
Mucosal
Toxin
Antibody

ABSTRACT

The RNA N-glycosidase ribosome inactivating proteins (RIPs) constitute a ubiquitous family of plant- and bacterium-derived toxins that includes the category B select agents ricin, abrin and shiga toxin. While these toxins are potent inducers of intestinal epithelial cell death and inflammation, very little is known about the mechanisms underlying mucosal immunity to these toxins. In the present study, we report that secretory IgA (SIgA) antibodies are not required for intestinal immunity to ricin, as evidenced by the fact that mice devoid of SIgA, due to a mutation in the polymeric immunoglobulin receptor, were impervious to the effects of intragastric toxin challenge following ricin toxoid immunization. Furthermore, parenteral administration of ricin-specific monoclonal IgGs, directed against either ricin's enzymatic subunit (RTA) or ricin's binding subunit (RTB), to wild type mice was as effective as monoclonal IgAs with comparable specificities in imparting intestinal immunity to ricin. These data are consistent with reports from others demonstrating that immunization of mice by routes known not to induce mucosal antibody responses (e.g., intramuscular and intradermal) is sufficient to elicit protection against both systemic and mucosal ricin challenges.

1. Introduction

The intestinal epithelium is constantly exposed to bacterium- and plant-derived toxins, many of which are capable of inducing epithelial destruction, severe mucosal inflammation, and often systemic complications. One of the most notorious classes of epithelial-damaging toxins is the RNA N-glycosidases, or ribosome inactivating proteins (RIPs). Although diverse in structure, the RNA N-glycosidases share a common mode of action: selective depurination of a conserved adenine residue within the so-called sarcin/ricin loop (SRL) of eukaryotic 28S ribosomal RNA [1]. Hydrolysis of the SRL results in an immediate arrest in ribosome progression and complete loss in protein translation [1]. The RNA N-glycosidase family includes shiga toxins (Stx), produced by *Shigella dysenteriae* and certain strains of foodborne enterotoxigenic *Escherichia coli*, as well as the plant-derived toxins, ricin and abrin. Ricin, which is found at high concentrations in the seeds of the castor bean plant, *Ricinus communis*, consists of a 32 kDa enzymatic A subunit (RTA) joined by a disulfide bond to a 34-kDa lectin B subunit (RTB). RTB binds to 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 [2–4]. 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) [5,6]. In the ER, RTA and RTB dissociate, and the A-subunit is retro-translocated across the ER membrane to the cytoplasm where it gains access to ribosomal RNA [7,8].

The sensitivity of the intestinal epithelium to ricin, and related RNA N-glycosidases, has been recognized for more than 100 years [9]. Rats challenged with ricin by gavage, for example, develop dose-dependent lesions in the proximal small intestine, including widespread villus atrophy, crypt elongation, sloughing of the epithelium, and occasional infiltration of inflammatory cells, including eosinophils and neutrophils [10–13]. We recently reported that similar histopathologic changes occur in mice following intragastric ricin challenge, although we failed to detect any evidence of mucosal ulceration and only rarely observed neutrophils in the intestinal lumen [14]. Epithelial cells themselves are directly affected by the toxin, based on the fact that application of ricin to the apical surfaces of polarized intestinal epithelial cell monolayers in vitro results in an arrest of protein synthesis within 3–4 h [15]. Others have shown that ricin activates cellular stress-activated protein kinase pathways (SAPKs) in intestinal epithelial cells, and induces them to secrete an array of pro-inflammatory cytokines [16–19]. These data suggest that ricin-induced epithelial destruction may be the consequence of direct cytotoxicity in combination with a local, acute inflammatory response.

Although it is well established that immunity to ricin is strictly antibody-mediated, neither the antibody classes nor the antibody specificities involved in the protection of the intestinal epithelium

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doi:10.1016/j.vaccine.2010.11.030

have been determined. In a previous report, we demonstrated that mice immunized intragastrically (i.g.) with ricin toxoid (RT) were impervious to the effects of a ricin challenge administered by gavage, and that protection was associated with elevated levels of anti-toxin IgA antibodies in fecal pellets and anti-toxin IgG antibodies in serum. We subsequently produced a collection of RTA-specific and RTB-specific monoclonal IgA and IgG antibodies (MAbs) from Peyer's patches and spleens of RT-immunized mice and characterized these MAbs in vitro [15,20]. Those studies identified a number of RTA-specific and RTB-specific IgA and IgG MAbs that were capable of neutralizing ricin in vitro, as determined using a Vero cell cytotoxicity assay. The MAbs also protected polarized epithelial cell monolayers from toxin-induced cell death, but only when the MAbs were applied to the apical (not basolateral) aspects of the epithelial monolayers [15]. The RTB-specific monoclonal IgGs (e.g., 24B11) and IgAs (e.g., 33G2 and 35H6) blocked ricin attachment to polarized epithelial cell monolayers and inhibited binding to the luminal aspects of human duodenum, indicating that this class of antibodies neutralizes ricin by preventing engagement of the toxin with host cell receptors. The RTA-specific monoclonal IgGs (e.g., R70 and GD12) and IgAs (e.g., 23D7 and 25A4), on the other hand, did not interfere with toxin attachment to cell surfaces, indicating that these MAbs neutralize ricin by an alternative, as of yet unknown, mechanism.

We have postulated, based primarily on our in vitro studies, that only SIgA is present in the intestinal lumen at sufficient concentrations to effectively protect the epithelium from ricin. However, Vitetta and colleagues recently reported that mice immunized with a candidate RTA subunit vaccine by the intramuscular (i.m.) or intradermal (i.d.) routes were protected against a lethal dose of ricin by the oral route [21,22]. Those mice had high-titer serum antitoxin antibody levels. While those investigators did not measure SIgA in intestinal secretions of vaccinated mice, i.m. or i.d. immunizations generally do not elicit mucosal antibody responses. Therefore, those findings suggest that serum antibodies play a role in protecting the intestinal epithelium from ricin intoxication in vivo. We wished to examine the relative contributions of SIgA and IgG in intestinal immunity to ricin in detail, particularly in light of the fact that relatively little is known in general about toxin–antibody interactions at mucosal surfaces. Taking advantage of a recently developed mouse model of intestinal ricin intoxication, in conjunction with available polymeric immunoglobulin knock-out mice lacking SIgA, as well as our collection of ricin-specific IgA and IgG MAbs, we report here that IgG antibodies directed against either ricin's enzymatic subunit or ricin's binding subunit are sufficient to protect the intestinal epithelium from the effects of ricin. This study contributes to an emerging body of literature demonstrating the importance of serum antibodies in mucosal immunity to a variety of pathogenic and toxic agents, and has implications for the development of vaccines and immunotherapeutics against the family of RNA N-glycosidases, three of which are considered potential biothreat toxins.

2. Materials and methods

2.1. Chemicals and reagents

Ricin (*Ricinus communis* agglutinin II) was purchased from Vector Laboratories (Burlingame, CA). RiVax^{TM} was kindly provided by Dr. Robert Brey (Soligenix, Inc., Princeton, NJ). Phenylmethylsulphonylfluoride (PMSF) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO). Tween-20 was obtained from BioRad (Torrance, CA), and protease inhibitor cocktails were purchased from Calbiochem-EMD Biosciences (La Jolla, CA). Paraformaldehyde (16%) was purchased from Electron Microscopy Sciences (Fort Washington, PA), and Bouin's fixative was obtained from Krackeler Scientific (Albany, NY). Dialysis was performed using Slide-a-lyzer from Pierce Chemical (Rockford, IL).

2.2. Production of ricin toxoid (RT)

To produce RT, ricin (1 mg/ml) was dialyzed in a Slide-a-lyzer dialysis unit (MWCO 10,000; Pierce Chemical) against 4% paraformaldehyde for 18 h at 47 °C, followed by 30 h at 42 °C. Dialysis was then continued against 0.1 M glycine for 4 days in the cold room, to quench residual paraformaldehyde in the RT preparations. RT preparations (1 mg/ml) were stored at −80 °C and were thawed immediately prior to use.

2.3. Hybridomas and MAbs

The hybridomas UNIVAX 70/138 (hereafter referred to as R70), originally described by Lemley et al. [23], and TFTB-1, originally described by Fulton et al. [24], were purchased from the ATCC (Manassas, VA) and were maintained in CD Hybridoma serum-free, protein-free, antibiotic-free medium (Gibco-Invitrogen, Carsbad, CA). All other ricin-specific IgG and IgA MAbs were produced in our laboratory and have been described previously [15,20]. IgG MAbs were purified from serum-free, protein-free hybridoma supernatants by means of a HiTrap Protein G sepharose column (GE Healthcare Life Sciences, Piscataway, NJ). Purity of the MAb preparations was determined by SDS-PAGE, and concentrations were determined by absorbance spectroscopy [25]. Antibody preparations were endotoxin-free, as determined by the Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD).

2.4. Intragastric ricin challenge and tissue collection

All animals used in this study were housed under conventional, specific pathogen-free conditions and were treated in strict compliance with guidelines established by the Institutional Animal Care and Use Committee (IACUC) at the Wadsworth Center. Wild-type BALB/c and pIgR knock-out (BALB/c-*pIgR^{tm1}* or pIgR$^{-/-}$) mice (females, 5–6 weeks of age) were purchased from Taconic Laboratories (Hudson, NY). β2 microglobulin deficient (β2$^{-/-}$) mice (females, 5–6 weeks of age) [26] and C57Bl/6 age and sex-matched control animals were purchased from Jackson Laboratories (Bar Harbor, ME).

For RT immunization studies, groups of mice (6–10 mice per group) received RT (50 μg per animal per immunization) without adjuvant by the i.g. route three times at 10–14 day intervals. For RiVax studies, groups of mice (6–10 mice per group) were immunized with RiVax adsorbed to alum (10 μg/dose) by the subcutaneous route (s.c.) three times at 10–14 day intervals. Ricin-specific serum and fecal antibody titers were determined by ELISA, as described previously [14,15]. Toxin challenge studies were performed 10–14 days following the last immunization, and involved administration of ricin (5 mg/kg diluted in PBS) to mice by the i.g. route using a 22 G × 1.5-in. blunt-end feeding needle (Popper Scientific, New Hyde Park, NY) [14]. Twenty-four hours later, the animals were euthanized by CO_2 asphyxiation. Freshly excised segments of the proximal small intestines of the mice were immersed in Bouin's fixative and embedded in paraffin, or homogenized in ice-cold cell lysis buffer (Cell Signaling, Beverly, MA) supplemented with protease inhibitors, and then frozen at −20 °C. MCP-1 levels in intestinal homogenates were determined by the BD cytometric bead array (CBA) flex set (BD Biosciences, San Jose, CA), as described previously [14]. Flow cytometric analysis was done using a FACSCalibur in the Wadsworth Center Immunology Core. Hematoxylin and eosin (H&E) stained sections of the small intestine were scored for ricin intoxication according to a 12-point histologic