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Short communication

Plant-based expression of a partially humanized neutralizing monoclonal IgG directed against an immunodominant epitope on the ricin toxin A subunit

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ABSTRACT

GD12 is a murine monoclonal IgG_1 (mAb) that recognizes an immunodominant linear neutralizing epitope (163-TLARSFIICIQM-174) on the A subunit (RTA) of ricin toxin. With the long-term goal of using GD12 as a potential countermeasure against ricin intoxication, we have produced a chimeric derivative of GD12 (cGD12) in which the murine heavy and light chain variable regions were fused to a human IgG_1 framework. The chimeric mAb, expressed and purified using a *Nicotiana*-based system demonstrated epitope specificity and ricin neutralizing activity similar to the parental murine mAb. Passive administration of cGD12 (10 μg) to mice by intraperitoneal injection protected the animals against a systemic ricin challenge. In a post-exposure setting, the murine and chimeric mAbs administered as much as 6 h after toxin challenge were each capable of rescuing mice from toxin-induced death, revealing the potential of GD12 to serve as both a prophylactic and therapeutic for ricin intoxication.

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1. Introduction

Ricin is a member of the type II ribosome-inactivating protein (RIP) family of toxins that includes abrin, Shiga toxin from *Shigella dysenteriae*, and Shiga-like toxins from *Escherichia coli* [1,2]. Because the toxin can be lethal to humans following injection, inhalation and possibly ingestion, ricin has been classified by the Centers for Disease Control and Prevention as a Category B biothreat agent. The mature form of ricin consists of two subunits. The A-subunit (RTA, 267 amino acids) is a RNA N-glycosidase, which mediates the selective depurination of a conserved adenosine residue in the so-called sarcin/ricin loop (SRL) of 28S ribosomal RNA [3]. The B-subunit (RTB, 262 amino acids) is a lectin specific for β1,3-linked galactose and *N*-acetylgalactosamine (Gal/GalNac) residues on both glycolipids and glycoproteins on the surface of cells [4].

We are interested in the development of an antibody-based therapeutic for treatment of individuals following exposure to ricin via the systemic or mucosal routes. Towards achieving this long term goal, we recently produced and characterized a collection of ricin-neutralizing monoclonal antibodies (mAbs) against both RTA and RTB [5–8]. One of these mAbs, GD12, is of particular interest because it recognizes a linear (continuous) epitope (T163-I174) on RTA that is known to be immunodominant in humans [6,9]. GD12 is also sufficient to passively protect mice against lethal doses of ricin administered by systemic (intraperitoneal) or mucosal (intragastric) routes [6]. Furthermore, GD12 is one of the most potent anti-RTA mAbs identified to date [10–13].

In this communication, we describe the production and characterization of a chimeric derivative of GD12 (cGD12) in which the murine heavy (V_H) and light chain (V_L) variable regions were fused to a human IgG_1 framework. The chimeric mAb was expressed in a *Nicotiana*-based system, which results in the rapid (days) production of extremely high amounts of mAbs. We report that cGD12 is capable of rescuing mice from a 10 LD_{50} challenge with ricin, when the mAb was administered within 6 h following toxin challenge. To our knowledge, GD12 is the first partially humanized anti-ricin mAb with demonstrated capacity to serve as an immunotherapeutic in response to a high dose ricin challenge.

2. Materials and methods

2.1. Construction of chimeric GD12 expression vectors

The V_H and V_L coding sequences were amplified by PCR using murine specific primers [14]. The V_H and V_L, GD12 deduced amino acid sequences were fused to a unique N-terminal murine signal peptide (SP) sequence (MGWSWIFLFLLSGAAGVHC) known to function in the *Nicotiana* expression system. The SP + V_H and SP + V_L

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sequences were then grafted on to human constant γ1 (γ1CR) and κ (κCR) regions. Complete HC (SP + V_H + γ1CR) and LC (SP + V_L + κCR) sequences were cloned into *Nicotiana* expression vectors and transformed into *Agrobacterium tumefaciens* [15].

2.2. Expression and purification of cGD12

Two unique cultures of *A. tumefaciens*, each transfected with either the HC or LC expression vectors, were grown as described [15] and co-infiltrated into 4–6 week old transgenic *Nicotiana benthamiana* plants lacking plant-specific N-glycan residues [16]. Eight days post-infiltration, the leaf tissue was extracted in a juicer (Model GS-1000, Green Star, Tribest Corp., Anaheim, CA), using 25 ml of chilled extraction buffer (100 mM Tris, 40 mM ascorbic acid, 1 mM EDTA) per 100 g of green leaf tissue. The plant-derived extract was clarified by lowering the pH of the extract to pH 4.8 with 1 M phosphoric acid then re-adjusting it to pH 7.5 with 2 M Tris base to insolubilize plant debris. The mixture was then subjected to centrifugation at 16,000 × g for 30 min. The resulting supernatant was then subjected to a second round of centrifugation under the same conditions. The clarified extract was filtered through a 0.2 μm filter prior to concentration via a Minim Tangential Flow Filtration System (Pall, Port Washington, NY), then 0.2 μm filtered again before loading onto a 5 ml HiTrap MabSelect SuRe Protein A column (GE Healthcare, Piscataway, NJ) at 2 ml/min. The column then was washed with running buffer (50 mM HEPES/100 mM NaCl, pH 7.5) and eluted with 0.1 M acetic acid, pH 3.0. The resulting eluate was neutralized to pH 7 using 2 M Tris, pH 8.0 and supplemented with Tween 80 to 0.01%. The mAb solution was then polished via Q filtration (Mustang Acrodisc Q membrane; Pall), aliquoted and stored at −80 °C.

2.3. ELISA, pepscan analysis and SPR

Ricin, RTA, and RTB were purchased from Vector Laboratories (Burlingame, CA). ELISA and peptide array analysis were performed exactly as described previously [6,7], except that detection of cGD12 was achieved using horseradish peroxidase (HRP)-labeled goat polyclonal anti-human IgG-specific secondary antibodies (Southern Biotech, Birmingham, AL). The affinity of cGD12 for ricin toxin was determined by surface Plasmon resonance (SPR) using a Biacore 3000 (GE Healthcare) with ricin attached to a CM5 chip surface, as described [7].

2.4. Ricin cytotoxicity assays

Vero cell cytotoxicity assays were performed as previously described [6,7]. All treatments were performed in triplicate, and 100% viability was defined as the average value obtained from wells in which cells were treated with medium only.

2.5. Passive protection studies

Murine GD12 (mGD12) or cGD12 (0.2 or 0.4 ml final volume) was administered to 8–12 week old female BALB/c mice (Taconic Labs, Hudson, NY) by intraperitoneal (i.p.) injection, at specific time points before or after the animals had received a single i.p. injection of ricin toxin (50 μg/kg or 100 μg/kg). Blood glucose levels, used as a surrogate makers of intoxication, and survival were measured daily over a 3-day period [7,17]. Mice were euthanized when they became overtly moribund and/or blood glucose levels fell below 25 mg/dl. 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. Statistical analysis and graphic representation of the data was performed using GraphPad Prism version 5.00 for Macintosh (GraphPad Software, San Diego, CA).

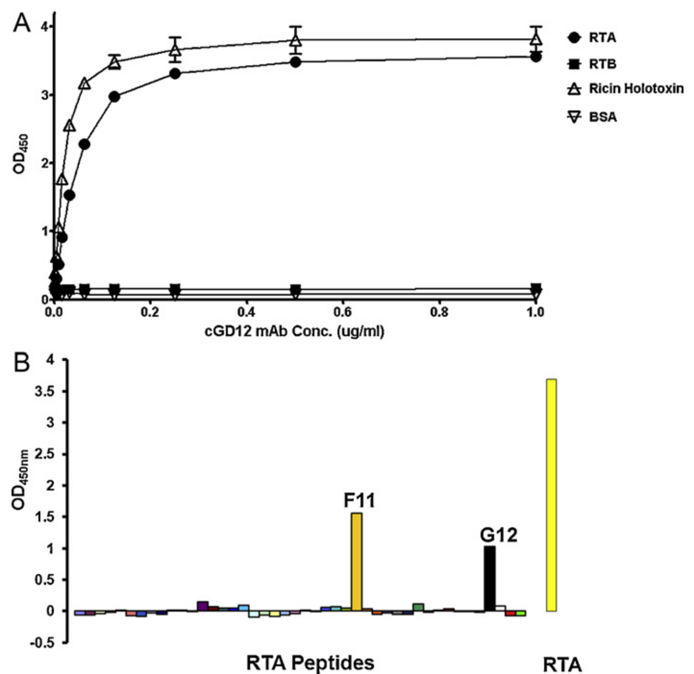

Fig. 1. Epitope specificity of cGD12. (A) Reactivity of cGD12 with ricin holotoxin, RTA, RTB, or BSA, as determined by ELISA. Microtiter plates were coated with 0.1 μg/well of each of the target antigens. Each datum point represents the average value of two replicate wells, with error bars representing the standard deviation from the mean. (B) Reactivity of cGD12 with an RTA peptide array. ELISA plates were coated with 44 overlapping 12-mer peptides spanning the length of RTA. cGD12 bound preferentially to peptide "F11" (gold), encompassing residues T163-M174 (TLARSFIICIQM), but showed additional reactivity with "G12" (black), a particularly isoleucine rich peptide (SVYDVSILIPII). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results

3.1. In vitro characterization of cGD12

We produced a chimeric derivative of GD12 in which the murine heavy and light chain variable regions were fused to a human IgG_1 framework. The chimeric mAb was expressed and purified using a *Nicotiana*-based system, as described in Section 2. To validate the specificity of cGD12, we examined the reactivity of the chimeric mAb with ricin holotoxin, RTA, and RTB by ELISA. As expected, cGD12 bound to ricin holotoxin and RTA, but not to RTB (Fig. 1A). The actual affinity of cGD12 for ricin was determined by Biacore analysis, which revealed that cGD12 had an association constant (K_A) of 1.3×10^7 M^{-1} and a dissociation constant (K_D) of 7.8×10^{-8} M (Table 1). Thus, the affinity of cGD12 for ricin was slightly less than that of its murine counterpart. As confirmation of the mAb's epitope specificity, cGD12 was used to probe a peptide array consisting of 44 overlapping 12-mers that collectively span the length of the RTA sequence. cGD12 preferentially bound peptide "F11", corresponding to residues T163-M174 (Fig. 1B) [6]. Like its murine counterpart, cGD12 also has demonstrable reactivity with an isoleucine-rich peptide towards the C-terminus of RTA.

Table 1
Affinities of murine and chimeric GD12 for ricin holotoxin.

mAb	K_D (M)	K_A (M^{-1})
mGD12	2.9×10^{-9}	3.5×10^8
cGD12	7.8×10^{-8}	1.3×10^7

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