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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 IgG₁ (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 IgG₁ framework. The chimeric mAb, expressed and purified using a *Nicotiana*-based system retained 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 5 LD₅₀ 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.

Keywords

antibody; toxin; biodefense; therapeutics

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

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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 β 1,3-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 (TL163-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 IgG₁ 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 LD₅₀ 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 sequences were then grafted on to human constant γ 1(γ 1CR) and κ (κ CR) regions. Complete HC (SP+ V_H + γ 1CR) and LC (SP+ V_L + κ CR) sequences were cloned into *Nicotiana* expression vectors and transformed into *Agrobacterium tumefaciens* [15].

2.2 Expression and purification of cGD12

Two unique cultures of *Agrobacterium 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 (100mM Tris, 40 mM ascorbic acid, 1mM 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 $16,000 \times g$ 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 μ m filter prior to concentration via Minim Tangential Flow Filtration System (Pall, Port Washington, NY) then 0.2 μ m 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].

2.3 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.

2.4 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 $\mu\text{g}/\text{kg}$ or 100 $\mu\text{g}/\text{kg}$). 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 California).

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 IgG₁ framework. The chimeric mAb was expressed and purified using a *Nicotiana*-based system, as described in the Materials and Methods. 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 $1.3 \times 10^7 \text{ M}^{-1}$ and a dissociation constant (K_D) of 7.8×10^{-8} (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.

To assess the capacity of the chimeric mAb to neutralize ricin *in vitro*, we combined ricin (10 ng/ml) with cGD12 at a range of concentrations, and then applied this mixture in triplicate to Vero cells grown in 96-well microtiter plates. Murine GD12 protected Vero cells from the cytotoxic effects of ricin in a dose-dependent manner, and had an estimated

IC₅₀ of ~0.25 µg/ml (Fig. 2A), in accordance with what has been reported previously [6]. The chimeric mAb was marginally less effective than the murine counterpart with an estimated IC₅₀ of ~0.3 µg/ml, possibly because of slight differences in relative affinities for ricin holotoxin. Nonetheless, cGD12's capacity to neutralize ricin is still considered among one of the best described to date.

3.2 In vivo characterization of cGD12

We used an established mouse model of systemic ricin intoxication to assess the capacity of cGD12 to neutralize ricin *in vivo* [6, 17]. For these challenge studies, hypoglycemia was used as a quantitative measure of ricin intoxication [17]. Monoclonal antibody cGD12 was passively administered to female BALB/c mice (10 or 20 µg/animal) by i.p. injection. Twenty-four hours later, the animals were challenged by i.p. injection with the equivalent of 5 LD₅₀ of ricin (50 µg/kg) and blood glucose levels were subsequently assessed at 24 h intervals. Ricin-challenged control mice experienced a dramatic decline in blood glucose levels within 24 h and subsequently died or were euthanized (Fig. 2B, C). cGD12-treated animals, on the other hand, experienced only a moderate reduction in blood glucose levels and all survived toxin challenge. In fact, in the case of the group of mice given 20 µg of cGD12, blood glucose levels were not significantly different before and 24 h post ricin challenge, as determined by a paired t-test.

3.3 Post exposure therapeutic assessment of murine and cGD12

To examine the degree to which GD12 can rescue mice from ricin intoxication, groups of animals were administered the murine version of GD12 (10 µg/animal) by the i.p. route at 0, 2, 4 or 6 h post systemic challenge with 5 LD₅₀ of ricin. Control mice succumbed to ricin intoxication within 48 h. In contrast, mice treated with mGD12 at all time points tested experienced only a minimal drop in blood glucose levels and were completely protected from toxin-induced death (Fig. 3A, B). These data reveal the potential of GD12 to function as a post exposure therapeutic.

Having established that mGD12 was capable of rescuing mice up to 6 h post ricin exposure, we next sought to establish the degree to which cGD12 can protect mice when administered at clinically-relevant concentrations [18] and against a more stringent toxin challenge [10]. Groups of mice were administered cGD12 at doses of 100 or 200 µg/animal by i.p. injection at 0, 4, 6, 8 or 10 h after ricin (10 LD₅₀) challenge (Fig. 3 C,D). Control mice that received ricin only had a mean survival time of 48 h. It should be noted that survival curves for toxin control mice given 5 LD₅₀ of ricin were not significantly different from those given 10 LD₅₀ of ricin, as determined by a Gehan-Breslow-Wilcoxon Test.

Administration of 100 µg of cGD12 to mice at times 0 or 4 hr post ricin challenge was sufficient to completely protect the animals from toxin-induced death. Administration of cGD12 at 6 hr post ricin challenge conferred partial protection (2/5 survived) and extended the mean time to death to 96 h. Delivery of 100 µg of cGD12 at 8 or 10 h post-ricin challenge offered no measureable protection or increase in mean survival time (Fig. 3C). Doubling the amount of cGD12 that was administered to the mice afforded greater protection and increased survival time. In particular, 4/5 mice that received 200 µg of cGD12 at 6 hr post-ricin challenge survived toxin-induced death, and the mean time to death in the group of mice administered cGD12 8 hr after toxin challenge was extended to 96 h (Fig. 3D). These data demonstrate a clear dose-dependent benefit of cGD12 as a therapeutic in response to a high-dose ricin challenge. Based on these results, we estimate that cGD12 protected mice at a molar toxin:antibody ratio of ~1:43, which is almost 7 times more effective than other murine mAbs tested in a post-exposure challenge mouse model [12].

4. Discussion

There is an urgent need for the development of countermeasures against potential biothreat agents, particularly highly lethal and fast-acting toxins like ricin [19]. The murine mAb GD12 was first identified as recognizing a human immunodominant linear B-cell epitope on RTA, defined as residues 163-TLARSFIICIQM-174. GD12 proved to be highly effective at neutralizing ricin *in vitro* and in conferring passive protection to mice against systemic and mucosal ricin challenge. To explore GD12 as a post exposure therapeutic, we produced and characterized a chimeric derivative of GD12 in which the murine V_H and V_L regions were fused to a human IgG₁ framework. The chimeric mAb was successfully expressed in a *Nicotiana*-based system and was nearly as capable as its murine counterpart in neutralizing ricin *in vitro* and *in vivo*. More importantly, when administered as much as 4 h following challenge with 10 LD₅₀ of ricin, cGD12 proved completely effective at protecting mice against toxin-induced death. GD12 is therefore the first partially humanized anti-ricin mAb with the potential capacity to serve as an immunotherapeutic in response to a high dose ricin challenge.

Several specific attributes of cGD12 make it an appealing candidate for further development as a therapeutic for ricin intoxication. First, both the murine and chimeric versions of GD12 were extremely effective at neutralizing ricin *in vitro* and *in vivo*. In particular, the murine version of GD12 was capable of protecting mice at a toxin:mAb molar ratio of ~1:4, as evidenced by the fact that 10 µg of mGD12 was sufficient to protect mice against a challenge of 1 µg of ricin, which was equivalent to 5 LD₅₀ in our model. Thus, mGD12 is roughly 2 times more effective than RAC18, 15 times more effective than R70, and possibly 70 times more effective than a cocktail of RTA- and RTB-specific mAbs at inactivating ricin in a mouse model [10, 12, 13]. Although cGD12 was slightly less potent at neutralizing ricin than was its murine counterpart, it was still highly effective at rescuing mice from ricin intoxication. We are currently producing different glycoforms and subclasses of cGD12 in an effort to enhance the mAb's ability to neutralize ricin.

Chimeric GD12 also has the added benefit over other ricin-specific mAbs of being expressed in a *Nicotiana*-based expression system [15, 18]. As compared to conventional mammalian-based expression systems, expression in *Nicotiana* results in the production of extremely high amounts of mAbs in a matter of days, especially with the recent advent of viral-based transient expression vectors. Second, the availability of transgenic plants with altered glycosylation pathways enables the production of mAbs with mammalian glycoforms. Finally, large scale manufacturing of antibody under current Good Manufacturing Practice is now routine [20]. Thus, development of partially humanized antibody based therapeutics against biothreat agents such as ricin via this plant based method offers a rapid, versatile, low cost and large capacity system to rapidly address a bioterror event.

While we demonstrated in this report that cGD12 mAb can rescue mice from ricin intoxication when administered up to 4 h post-toxin exposure, we expect that this therapeutic window could be extended by increasing the amount of antibody and/or co-administering cGD12 with one or more other ricin-neutralizing antibodies. Of particular interest would be combining cGD12 with mAbs that target other immunodominant regions of RTA, as there is evidence that they may neutralize ricin by different mechanisms from GD12 [7]. Alternatively, cGD12 could be mixed with one or more RTB-specific mAbs [8]. There is evidence that RTB-specific mAbs can act synergistically with RTA-specific mAbs to neutralize ricin [5]. Thus, we are currently investigating the use of a partially humanized neutralizing anti-RTB mAb in combination with cGD12.

Finally, the partially humanized GD12 may prove valuable as a RTA-specific human IgG standard as serum samples from clinical trials of two leading ricin vaccines are underway [21]. Assessing ricin-specific antibody titers and toxin neutralizing activities in the sera of immunized individuals will serve as the sole indicator of vaccine efficacy in humans. At the present time there is currently no accepted standardized ricin ELISA protocols or cytotoxicity assays within the ricin scientific-clinical community. In order to enable comparative analysis of vaccine efficacies, it is imperative that a single standard be adopted across institutions.

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Highlights

- >GD12 is a murine monoclonal antibody that protects mice from ricin toxin.
- >A partially humanized version of GD12 was produced in plants.
- >Chimeric GD12 neutralizes ricin toxin in vitro and in vivo.
- > Chimeric GD12 protected mice when administered 6 hr post ricin challenge.

Figure 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 μ g/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 (TLARSFIIICIQM), but showed additional reactivity with “G12” (black), a particularly isoleucine rich peptide (SVYDVSILIPII).

Figure 2. Neutralizing and protective capacity of cGD12

(A) Mouse or chimeric GD12, at the indicated concentrations, were mixed with ricin (10 ng/ml) for 1 h, and then applied in triplicate to Vero cells grown in 96-well microtiter plates. Cell viability was assessed 48 h later. Each data point represents the average of three replicate wells. The error bars represent the standard deviation. (B, C) Female BALB/c mice (n=4 per group) were passively immunized with cGD12 (20 or 10 μg/ animal) and then challenged 24 h later with 5 LD₅₀s of ricin (50 μg/kg). (B) Blood glucose levels in the groups of mice were determined at time zero and 24 h intervals following ricin challenge. (C) The percent survival of mice described in the experiment in panel B was assessed at 24 hr intervals.

Figure 3. Capacity of murine and chimeric GD12 to rescue mice from ricin toxin-induced death Female BALB/c mice (n=5 per group) were administered mGD12 (**A,B**) or cGD12 (**C,D**) before, concurrently, or at intervals after ricin challenge. (**A**) Blood glucose levels in the groups of mice treated with mGD12 (10 μ g/animal) were determined at time 0 and then at 24 h intervals following challenge with 5 LD₅₀ of ricin. Each symbol represents an individual mouse. (**B**) Percent survival of mice described in Panel A. (**C-D**) Protection afforded by cGD12. BALB/c mice were administered cGD12 at (**C**) 100 μ g/animal or (**D**) 200 μ g/animal concurrently or at indicated intervals after challenge with 10 LD₅₀ of ricin.

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