Anti-Ricin Protective Monoclonal Antibodies

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1. Abstract

Development of anti-ricin protective monoclonal antibodies (mAbs) started in the early 1980s. Much progress has been made since then. Antibodies represent a great potential to be developed as antidotes against toxins. They can be used either prophylactically to prevent or therapeutically to treat toxin-mediated intoxications in an emergency situation. Unlike many other therapeutic products, antibodies offer unique and high target specificity, and long half-life in serum. There are a dozen of mAbs, which are currently in the discovery stage for medical countermeasures against ricin intoxication. This review summarizes these mAbs, including their anti-ricin mechanism, generation, and efficacies *in vivo.*

2. Introduction

Antibodies can provide immediate neutralization against toxins. The history of using antibodies as effective antidotes against toxins can be dated back to 1890 (2), when German physiologist, Emil Adolf von Behring discovered a therapeutic effect against diphtheria toxin using the serum. He was then awarded the first-ever Nobel Prize in Physiology or Medicine in 1901 for his contributions (1). At that time, human hyperimmune sera containing a small portion of specific antibodies obtained from the convalescent donors was used as antidotes to combat toxins produced by microorganisms, such as diphtheria and tetanus with a remarkable record of safety, efficacy, and versatility. At the present time, antibody-based products continue to be used as antidotes against toxins (3-5), including tetanus, diphtheria, botulism, and venomous bites. Antibodies can be used either prophylactically to prevent or therapeutically to treat toxin-mediated intoxications in an emergency situation. The major advantages of antibody-based products as antidotes against toxins are exquisite specificity to the target and prolong half-life in serum.

The development of monoclonal antibodies (mAbs) by mouse hybridoma technology in the late 1970s opened a new era in antibody therapy. MAbs are monospecific,

homogeneous, and reproducible (6). The major benefit afforded by this technology is that it is possible, in principle, to develop a mAb against any target of choice and to produce it in unlimited amounts. MAbs have been developed as therapeutic agents for various clinical applications, initially from murine origin, later chimeric between murine and human, and now humanized or fully human antibodies (7-9). The purpose of this review is to summarize the development of protective mAbs against ricin intoxication, including their action mechanism, generation, and efficacy *in vivo*. This manuscript reviews the literatures on mAbs that provide protection to mice against ricin intoxication. MAbs that did not have *in vivo* protection data were not included in the review.

3. Mechanism of antibody neutralizing ricin

Antibodies, which are glycosylated proteins, naturally produced in the body, have a high specificity and affinity to foreign substances, playing an important role in the immune defence. Practically for any pathogen or toxin, there could be an antibody which functions against it. Antibody biological functions include two principal actions. The first is direct effects (10), which appear to be a function of the antibody antigen-binding alone, such as toxin neutralization, viral neutralization, and interference with microbial attachment or replication. The second is indirect effects (11), which are called effector functions, resulting from the consequence of crosslinking crystallisable fragment (Fc) receptors, such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP). In fact, most antibodies work by indirect effects via binding to antigens and sending signals to other parts of the immune system to attack and eliminate the pathogenic antigens by CDC, ADCC, or/and ADCP. Only a very few antibodies possess the direct effects, the neutralizing functions. Therefore, antibodies can be divided into two groups based on their functions, neutralizing antibodies and non-neutralizing antibodies. The former not only neutralize the biological effect of antigens, but also flag antigens to destroy them through their indirect effects, while the latter only have the indirect effects to destroy pathogenic antigens.

For development of antibody-based antidotes against toxins like ricin, neutralizing antibodies are desirable. As a matter of fact, the non-neutralizing antibodies are not helpful, but some of them even harmful due to the assistance of ricin to enter cells, which was confirmed by Colombatti, *et al* in 1986 (12). Therefore, not every single anti-ricin mAb can be developed as an antidote against ricin intoxication.

Ricin is a 60-65 kDa glycoprotein derived from beans of the castor plant (13). It consists of a ricin toxin A (RTA) protein and a ricin toxin B (RTB) protein linked by a disulfide bond. RTB binds to galactose residues on the mammalian cell surfaces not only triggering cellular uptake of ricin (14), but also facilitating transport of the RTA from the endoplasmic reticulum (ER) to the cytosol (15, 16), where RTA then enzymatically cleaves ribosomal RNA to stop protein synthesis (17). Mounting evidence has shown that antibodies against either subunit can neutralize ricin (18-22). Regarding neutralizing efficacy, some reports demonstrated anti-RTA neutralizing antibodies were more efficacious than RTB neutralizing antibodies (19), but others showed the opposite way, that is, RTB neutralizing antibodies being more effective in protection than RTA neutralizing antibodies (21). Theoretically, RTB is the logical target for neutralizing antibodies, as these would block the entry of ricin into cells and the transportation of RTA to the cytosol. However, it seems to be more difficult to develop anti-RTB neutralizing mAbs than anti-RTA neutralizing mAbs. One of the reasons is that the immunodominant epitopes on RTB might not provide any neutralizing protection. In other words, RTB is poor in eliciting anti-ricin neutralizing antibodies although it is highly immunogenic in eliciting non-neutralizing anti-ricin antibodies (22). To date, only a few anti-RTB neutralizing antibodies have been reported (21, 23-26).

4. Immunogens

In order to make anti-ricin mAbs, mice need to be immunized with ricin to elicit anti-ricin immune response. However, ricin is too toxic to be used directly as a primary immunogen to immunize mice. In other words, primary immunization with either holotoxin or a mixture of purified RTA and RTB is lethal in an immunogenic dose.

Instead, purified or recombinant RTA or RTB can be used as an immunogen to immunize mice (18, 19, 27) or a primary immunogen and then boosted with holotoxin (19). Ricin can also be inactivated with formalin and then ricin toxiod, which is not lethal, can be used as an immunogen to immunize mice (24). Furthermore, there is a developing vaccine, RiVax, which is a recombinant RTA subunit with two residue mutations, resulting in attenuated toxicity with retaining immunogenicity. Rivax was used to immunize mice (28). In our laboratory, mice were found to survive a large dose of ricin poisoning if the mice were poisoned by a stepwise increase dosage of ricin. In this way, mice were immunized by an intraperitoneal (i.p.) injection of ricin from 0.2×LD50 to 25×LD50 and a high anti-ricin antibody titer was obtained (29).

5. Animal models

Mice are good models for evaluation of mAb efficacy against ricin intoxication. The severity of ricin intoxication in mice depends on species, genders, and ages. In general, inbred, female, and younger mice are more sensitive to ricin challenge than outbred, male and elder mice. Besides, ricin challenge routes are also contributed to the severity of ricin intoxication. The most lethal challenge route is inhalation, followed by injection and then ingestion. For example, in mouse models, the LD50 for inhalation, injection, and ingestion is around 3-5 μg/kg, 5-10 μg/kg, and 30 g /kg respectively (30, 31).

6. Evaluation of efficacy

There are a couple of ways to evaluate antibody efficacy against ricin intoxication. In the earlier publications (24, 27), the antibody titration against a fixed amount of ricin was applied, in which, a series of different antibody concentrations were mixed with a fixed amount of ricin, or vice versa and then the antibody ricin mixtures were injected into mice to observe mouse survival rate. The lowest antibody administration dose or highest ricin challenge does, which could provide the full protection to the mice, is the antibody efficacy titer.

The therapeutic efficacy of anti-ricin antibody-based treatment is largely dependent on timing of administration of rescuing antibodies relative to the ricin exposure. A relatively wide therapeutic window will provide necessary time for the exposed victims to obtain anti-ricin antibody treatment in the event of a ricin attack. Therefore, later on, the therapeutic window of antibodies was used to describe mAb efficacy. The longest time of administration of anti-ricin antibodies after ricin exposure, which can rescue 100% of the ricin-intoxicated mice or keep blood glucose level unchanged, is the antibody efficacy indicator. The blood glucose concentration within 36 hr after ricin challenge might be used as a surrogate for lethal challenge as a measure of ricin toxicosis (32). It is quite understandable that the wider therapeutic window is, the better the antibody efficacy.

7. Protective mAbs evaluated against ricin intoxication *in vivo*

The first protective anti-ricin mAb was reported by Colombatti, *et al* in 1987 (27). His group developed a mAb, 75/3B12 (IgG2a), from the mice immunized with purified RTB. This mAb appeared to bind to a galactose-binding domain of RTB. 75/3B12 antibody antigen-binding fragment (Fab) was evaluated for its efficacy against ricin challenge in AKR mice by co-incubation approach (mixing ricin with the antibody before administration of the mixture) through titrating the ricin challenge dose against a constant dose of 330 μg or 750 μg per mouse of the antibody. As shown in Table 1, when the mixture of ricin and antibody was delivered by an intravenous (i.v.) route with the antibody constant dose of 330 μg /mouse, the highest ricin challenge dose with complete protection from the antibody could be up to 270 μg/mouse. Moreover, in an i.p. delivery approach, when the antibody constant dose was 750 μg/mouse and the highest ricin challenge dose could be up to 2,400 μg/mouse with 100% survival of the mice (Table 2).

Table 1. Titration of ricin challenge dose against a constant dose of 330 μg/mouse of 75/3B12 Fab in AKR mice by an i.v. administration of the mixture of ricin and antibody

	Ricin (μg/mouse)	Survival
Non treatment control	135	none
	45	100%
75/3B12 Fab	270	100%
	135	100%

Table 2. Titration of ricin challenge dose against a constant dose of 750 μg/mouse of 75/3B12 Fab in AKR mice by an i.p. administration of the mixture of ricin and antibody

	Ricin (μg/mouse)	Survival
Non treatment control	800	none
	266	65%
	90	100%
75/3B12 Fab	2,400	none
	800	100%

Similarly, Lemley, *et al* (24) titrated a mAb, UNIVAX 70/138 (also named R70) developed from the mice immunized with ricin toxiod, against ricin challenge in CD-1 mice (body weight, 25-32 g) via an i.v. approach. This antibody was IgG1 and specific for RTA. When the ricin challenge dose was fixed to 18 μg/kg (>6×LD50), the minimum antibody dose, which could provide 100% protection to the mice against ricin challenge was 75 μg/mouse (2.7 mg/kg). One the other hand, when the antibody dose was held constant at 100 μg/mouse (3.6 mg/kg), the lowest ricin challenge dose with complete protection from the antibody was 25 μg/kg, >8×LD50. Later on, R70 was further demonstrated to be able to provide 100% of protection to the female Balb/c mice when it was administrated (i.p.) at 20 μg/mouse (1 mg/kg) 24 hr before i.p. injection of ricin (5×LD50, 50 μg/kg) (20, 22) as shown in Table 3.

Guo *et al* (12) developed that a mAb binding to a conformation epitope of RTB, 3E1. As shown in Table 3, when it was given to adult Balb/c mice (body weight ~20 g) (3 mice each group), 100 μg per mouse (5 mg/kg) (i.p. injection) 10 or 20 min post ricin challenge (i.p. injection) at 6×LD50 (60 μg/kg), all the mice survived. Non-treatment control mice died with 16 hr. When the ricin challenge dose was increased to 10×LD50 (100 μg/kg) and the time for injection of the antibody was delayed up to 20 min post ricin challenge, all the mice still survived. However, if the time of antibody administration was further delayed up to 30 min post ricin challenge, the mice could not survive, but the death was delayed to 95 hr compared to 14.3 hr of the non-treatment control group. One year later, Guo *et al* (33) developed another anti-ricin mAb, 4C13, which recognized a linear epitope of ricin. 4C13 could rescue all the Balb/ c mice (4 mice) when it was administered (i.p. injection) at the dose of 100 μg per mouse (5 mg/kg) 30 min post ricin challenge (10×LD50) (100 μg/kg) via an i.p. administration (Table 3).

Maddaloni, *et al* (19) developed 44 mAbs against ricin by immunization of Balb/c mice with purified RTA or RTB. These bound to either RTA (13), or RTB (6), or both of them (33). They were evaluated in outbred CD-1 mice by measuring antibody ability against ricin-induced hypoglycemia, rather than lethality (3). Fifteen μg/kg ricin and 0.8 mg/kg of mAb were premixed and then the mixture was injected to Balb/c mice. Blood sugar was measured at 18 and 34 hr post administration, and mortality was assessed at 34 hr. Only anti-RTA mAbs were protective. Among them, mAb RAC18 was the best in terms of preventing the induced hypoglycemia. Roche, *et al* further evaluated these anti-RTA mAbs in male C57BL/6 mice (body weight 22-24 g) (34). When mice were given 20 μg per mouse (0.9 mg/kg) of a single mAb, RAC 17 or RAC 18 by an i.v. injection, 6 hr after ricin challenge (i.p. injection) (40 μg/kg, equivalent to the LD100), all the mice survived and the non-treatment control died within 4-5 days (Table 3). In addition, 90% of the mice could be protected when administered with a cocktail of anti-RTA mAbs, including 20 μg/mouse (0.9 mg/kg) of each mAb, RAC 17, RAC 18, and RAC23, 10 hr after the ricin challenge. RAC 18 was also evaluated in a ricin lung challenge model using an oropharyngeal aspiration delivery of both ricin and mAb (35). Male Balb/c mice (body weight 20-25 g) were challenged with 3~5×LD50 (16 μg/kg), the non-treatment control died with 5 days. By contrast, the administration of 50 μg per mouse (2.5 mg/kg)

of RAC 18 mAb at 4, 18, and 24 hr after ricin challenge resulted in 100%, 60% and 50% protection respectively, while an anti-RTA polyclonal mouse antibody (50 μg per mouse, 2.5 mg/kg) still showed 100% protection to the mice when the delay of antibody administration for up to 18 hr post ricin challenge.

Mantis's group developed a couple of protective mAbs from female Balb/c mice immunized with ricin toxiod or Rivax. In a pre-exposure prophylaxis setting, Balb/c mice (body weight ~20 g) were administration (i.p. injection) of 5 to 40 μg/mouse (0.25 to 2 mg/kg) of a mAb, such as GD12 (IgG1, RTA specific) (20), R70 (IgG1, RTA specific) (20, 22), 24B11 (IgG1, RTA specific) (22), SylH3 (IgG1, RTB specific) (22), SyH7 (IgG1, RTA specific) (28), or PB10 (IgG2b, RTA specific) (28). Twenty-four hr later, the mice were challenged (i.p. injection) with ricin 5×LD50 (50 μg/kg). All the mice survived over a 3-day period without significant difference of blood glucose level between the treatment group and non-toxin control group within 76 hr post ricin challenge (Table 3). All the non-treatment control mice died within 2 days.

Prigent, *et al* demonstrated that a combination of three anti-ricin mAbs, 2 anti-RTB (RB34 and RB37) and 1 anti-RTA (RA36) protected 90% of the female CD-1 mice (22-25 g) when the three mAbs (5 mg/kg, 110 to 125 μg/mouse) were administered by an i.v. injection within 7.5 hr after intranasal challenge of ricin at 5×LD50 (7.5 μg/kg) (21) as shown in Table 3. Thus, it appears that Prigent *et al*. demonstrated a proof of concept for effective post-exposure prophylaxis to lethal-dose intranasal challenge to ricin.

Dai, *et al* (18) developed a group of anti-RTA mAbs by immunization of Balb/c mice with recombinant RTA and tested them both *in vitro* and *in vivo*. 6C2 (IgG1) and 6G3 (IgG1), binding to an alpha-helix comprising the residues 99-106 in RTA, showed the best efficacy against ricin challenge *in vivo.* The i.p administration of 5 μg mAb (0.25 mg/kg) of 6C2 or 6G3 per mouse could protect 100% of the adult Balb/c mice at 2 hr post challenge with ricin (50 μg/kg, i.p. injection).

Recently, four mAbs bound to conformational epitopes of ricin toxin B (RTB) with high affinity were developed in our laboratory (29). The four mAbs were found to have potent

ricin-neutralizing capacities and synergistic effects among them as determined by *in vitro* neutralization assay. *In vivo* post-exposure protection assay demonstrated that all the four mAbs had strong efficacy against ricin challenges *in vivo*. As shown in Table 3, D9 was found to be exceptionally effective. Administration of D9 (i.p. injection) at a dose of 5 μg per mouse (0.25 mg/kg), 6 hr after an i.p. challenge with 5×LD50 (50 μg/kg) of ricin was found to rescue 100% of the female Balb/c mice (6 week old, body weight 20-25 g). D9 was further evaluated for pre-exposure prophylaxis against ricin challenge *in vivo*, and 5 μg per mouse (0.25 mg/kg) delivered by the i.p. route 6 weeks before 5×LD50 (50 μg/kg) ricin challenge (i.p. injection) protected 100% of the mice.

8. Chimerization and humanization

Murine antibodies cannot directly be used in humans. Although murine antibodies are structurally similar to human's, the antibody sequence difference between them is sufficient to invoke an immune response in humans when murine antibodies were directly injected into humans. The immune response would result in a rapid removal of murine antibodies from the human blood, systemic inflammatory effects, and possible anaphylaxis, which can sometimes be fatal (36). To overcome this hurdle, two murine anti-ricin mAbs (C4C13 and GD12) were chimerized by genetically fusing murine antibody variable regions to human antibody constant regions to generate antibody molecules with ~70 % human content (37, 38). The chimeric GD12 (cGD12) was further evaluated in both pre-exposure prophylaxis and post-exposure rescue settings. As shown in Table 3, the cGD12 could provide 100% protection to the female mice (8-12 week old) against 5×LD50 (50 μg/kg) of ricin challenge (i.p. injection) when i.p. administration of 10 μg per mouse (0.5 mg/kg) of the cGD12 24 hr pre-ricin challenge. Administration of 100 μg per mouse (5 mg/kg) of cGD12 at 4 hr post ricin challenge could completely protect the mice against 10×LD50 (100 μg/kg) of ricin challenge (i.p. injection). Administration of 100 μg per mouse (5 mg/kg) of cGD12 at 6 hr post ricin challenge conferred partial protection (2/5 survived) and extended the mean time to death to 96 hr.

Chimeric antibodies successfully retained the mouse parental antibody antigen-binding specificity and diminished its immunogenicity. However, chimeric antibodies could still elicit an undesirable anti-antibody variable region response (39). As molecular biology technology developed, it became possible to further reduce the immunogenicity of the chimeric antibodies by replacing murine variable region frameworks with those of the selected human antibodies using an approach called “complementarity-determined region grafting” (40). The resulting “humanized” antibodies contain 85-95% human sequences. Numerous clinical studies have confirmed that humanized antibodies are less immunogenic and more therapeutic than murine or chimeric antibodies in humans (41, 42). A potent anti-ricin neutralizing antibody, D9 was successfully humanized in our laboratory (43). The humanized D9 (hD9) exhibited high efficacy *in vivo*. In a female Balb/c mouse model, a dose of 5 μg hD9 per mouse (0.25 mg/kg) could rescue 100% of the mice (6 week old, body weight 20-25 g) up to 4 hr and 50% of the mice up to 6 hr after 5×LD50 (50 μg/kg) ricin challenge (Table 3).

Table 3. Prophylactic and therapeutic windows of protective mAbs against ricin intoxication

mAb	isotype	specificity	mice	Ricin poisoning	mAb administration	Survival	reference
3E1		RTB	Balb/c	100 μg/kg, i.p.	5 mg/kg, i.p. +20 min (1)	100%	Guo, 2005
4C13		RTA & RTB	Balb/c	100 μg/kg, i.p.	5 mg/kg, i.p. +30 min	100%	Guo, 2006
RAC17	IgG1	RTA	C57BL/6	40 μg/kg, i.p.	0.9 mg/kg, i.v. +6 hr	100%	Roche, 2008
RAC18	IgG1	RTA	C57BL/6	40 μg/kg, i.p.	0.9 mg/kg, i.v. +6 hr	100%	Roche, 2008
			Balb/c	16 μg/kg, i.n.	2.5 mg/kg, i.n. +4 hr	100%	Timothy, 2007
					2.5 mg/kg, i.n. +18 hr	60%	
					2.5 mg/kg, i.n. +24 hr	50%	
Cocktail 1(2)	IgG1	RTA	C57BL/6	40 μg/kg, i.p.	2.7 mg/kg, i.v. +10 hr	90%	Roche, 2008
GD12	IgG1	RTA	Balb/c	50 μg/kg, i.p.	0.25 mg/kg, i.p. -24 hr (3)	100%	Neal, 2009
					0.5 mg/kg, i.p. +6 hr	100%	O'Hara, 2012
cGD12	IgG1	RTA	Balb/c	100 μg/kg, i.p.	5 mg/kg, i.p. +4 hr	100%	O'Hara, 2012
					5 mg/kg, i.p. +6 hr	40%	
R70	IgG1	RTA	Balb/c	50 μg/kg, i.p.	1 mg/kg, i.p. -24 hr	100%	Neal, 2009
24B11	IgG1	RTB	Balb/c	50 μg/kg, i.p.	1 mg/kg, i.p. -24 hr	100%	Yermakova, 2011
SylH3	IgG1	RTB	Balb/c	50 μg/kg, i.p.	1 mg/kg, i.p. -24 hr	100%	Yermakova,
SyH7	IgG1	RTA	Balb/c	50 μg/kg, i.p.	2 mg/kg, i.p. -24 hr	100%	O'Hara, 2010

PB10 O'Hara, 2010	IgG2b	RTA	Balb/c	50 μg/kg, i.p.	2 mg/kg, i.p. -24 hr	100%	
Cocktail 2 (4) Prigent, 2011		RTA& RTB	CD-1	7.5 μg/kg, i.n.	5 mg/kg, i.v. +7.5 hr	90%	
6C2 Dai, 2011	IgG1	RTA	Balb/c	50 μg/kg, i.p.	0.25 mg/kg, i.p. +2 hr	100%	
6C3 Dai, 2011	IgG1	RTA	Balb/c	50 μg/kg, i.p.	0.25 mg/kg, i.p. +2 hr	100%	
D9 2013	IgG1	RTB	Balb/c	50 μg/kg, i.p.	0.25 mg/kg, i.p. +6 hr	100%	Hu,
					0.25 mg/kg, i.p. -6 w	100%	
hD9 Hu, 2012	IgG1	RTB	Balb/c	50 μg/kg, i.p.	0.25 mg/kg, i.p. +6 hr	100%	
					0.25 mg/kg, i.p. +6 hr	50%	

Note: (1) +: Administration of mAb after ricin poisoning;
(2) A cocktail of RAC 17, 18, and 23;
(3) -: Administration of mAb before ricin poisoning;
(4) A cocktail of RB 34, 36, and 37.

9. Conclusions

Continuous development of biotechnology (such as antibody cloning, screening, engineering, expression technologies, and humanization) and understanding of ricin toxicosis will play a pivotal role for development of anti-ricin antibody-based antidotes. There are some challenges for passive antibody therapy in medical countermeasure against ricin intoxication. One logistical challenge is the large dose of antibodies. Currently, most of antibodies have to be administered by intravenous administrations via hr-long infusions and repeated over a long period of time in a specific hospital environment due to a large dose required. Consequently, this approach would be impractical when large populations are exposed to a ricin biothreat. A second challenge is the cost. Therapeutic antibodies are undoubtedly among the most expensive drugs used in clinical practice.

These challenges might be overcome by developing potent anti-ricin antibodies, of which only a small dosage is required for a full protection. This small dose can be delivered with an auto-injector on the field by the victim himself. Meanwhile, the cost for production of antibodies is also dramatically reduced.

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