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Neutralizing Activity and Protective Immunity to Ricin Toxin Conferred by B Subunit (RTB)-Specific Fab Fragments

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Abstract

SylH3 and 24B11 are murine monoclonal antibodies directed against different epitopes on ricin toxin's binding (RTB) subunit that have been shown to passively protect mice against ricin challenge. Here we report that Fab fragments of SylH3 and 24B11 neutralize ricin in a cell based assay, and in a mouse challenge model as effectively as their respective full length parental IgGs. These data demonstrate that immunity to ricin can occur independent of Fc-mediated clearance.

Keywords

Toxin; Neutralizing Antibodies; Biodefense; Mouse

Ricin is a member of the A–B family of protein toxins, which includes cholera toxin, Shiga toxin (Stx), botulinum neurotoxins (BoNT) and anthrax toxin. Ricin's B subunit (RTB) is a galactose- and N-acetylgalactosamine (Gal/GalNac) lectin that promotes toxin attachment and entry into virtually all mammalian cell types (Rutenber et al., 1987; Sandvig et al., 1976). RTB also mediates the intracellular retrograde trafficking of ricin from the plasma membrane to the endoplasmic reticulum (ER). Ricin's A subunit (RTA) is an RNA N-glycosidase (RTA) whose sole substrate is a universally conserved adenosine residue within the so-called sarcin/ricin loop (SRL) of mammalian ribosomal RNA (Spooner and Lord, 2012). Hydrolysis of the SRL by RTA results in the cessation of cellular protein synthesis, activation of the ribotoxic stress response (RSR), and cell death via apoptosis (Jandhyala et al., 2012).

Structurally, RTB consists of two globular domains with identical folding topologies (Montfort et al., 1987). Each of the two domains (1 and 2) is comprised of three homologous sub-domains (, ,) that probably arose by gene duplication from a primordial carbohydrate recognition domain (CRD) (Montfort et al., 1987; Rutenber et al., 1987). However, only the external sub-domains, 1 and 2 , retain functional carbohydrate recognition activity (Rutenber et al., 1987; Swimmer et al., 1992). Subdomain 1 (residues

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17–59) is Gal-specific and is considered a “low affinity” CRD, whereas sub-domain 2 (residues 228–262) binds both Gal and GalNAc and is considered a “high affinity” CRD (Newton et al., 1992; Rutenber and Robertus, 1991; Zentz et al., 1978). Either sub-domain 1 or 2, which are separated by approximately 70 Angstroms, are sufficient to promote toxin attachment to cells (Montfort et al., 1987).

Our laboratory has recently produced and characterized a large collection of RTB-specific murine monoclonal antibodies (mAbs) (McGuinness and Mantis, 2006; Yermakova and Mantis, 2011; Yermakova et al., 2012). The majority of these mAbs fail to neutralize ricin, even though they bind ricin holotoxin with high affinities. For example, TFTB-1 has an affinity of $>5 \times 10^{-9}$ M, yet has no demonstrable capacity to inactivate ricin *in vitro* or *in vivo* (Yermakova and Mantis, 2011). To date we have identified only three RTB-specific mAbs (24B11, SylH3 and JB4) that are capable of neutralizing ricin *in vitro* and able to passively protect mice against a lethal toxin challenge. All three are IgG₁s and each bind ricin with nanomolar affinities (SylH3, 3.38×10^{-9} M; 24B11, 4.2×10^{-9} M; JB4, 2.01×10^{-10} M) (Yermakova and Mantis, 2011; Yermakova et al., 2012). 24B11's epitope has been tentatively localized within RTB's sub-domain 1 (McGuinness and Mantis, 2006). We speculate that SylH3 and JB4 bind a similar or overlapping epitope in RTB's sub-domain 2 (Yermakova et al., 2012).

We consider SylH3 and JB4 as being class I antibodies as they are very effective at blocking ricin binding to cell surfaces, suggesting they work by steric hindrance (Yermakova and Mantis, 2011). We consider 24B11 a class II antibody, as it, neutralizes ricin in cell-based assays as effectively as SylH3 and JB4 but only partially affects toxin attachment to cell surfaces or surrogate receptors like asialofetuin (ASF). We therefore postulate that 24B11 neutralizes ricin at a step downstream of attachment.

We wished to investigate the role of the fragment crystallizable (Fc) components of RTB-specific class I and class II Abs. *In vitro*, the Fc components of class I Abs, by virtue of their ability to obstruct one or both of RTB CRDs, may be important in steric hindrance and interference with toxin attachment to cell surfaces (McGuinness and Mantis, 2006). *In vivo*, the Fc-determinants of both class I and class II Abs could assist in toxin clearance via Fc receptor (Fc R)-dependent mechanisms. In the case of anthrax toxin and BoNT, there is evidence that Fc R-mediated clearance is critical in Ab-mediated toxin immunity (Mukherjee et al., 2012; Sepulveda et al., 2010; Verma et al., 2009; Vitale et al., 2006).

To investigate the role of Fc-determinants in ricin neutralization *in vitro* and *in vivo*, we prepared Fab fragments of SylH3 (class I) and 24B11 (class II). As a control, we also produced Fab fragments of the non-neutralizing mAb TFTB-1. Fab fragments of SylH3, 24B11 and TFTB-1 were produced using a commercially available ficin-based, mouse IgG₁ Fab preparation kit (ThermoScientific, Rockford, IL). SDS-PAGE and SureBlue (ThermoScientific) staining confirmed that each mAb was digested to completion (Fig. S1). Furthermore, the purified Fabs each retained their specificity for ricin and RTB, although reactivity of 24B11 Fab with RTB (but not ricin holotoxin) was slightly diminished, as compared to the full length IgG (Fig. S2).

To determine whether the Fc domains of class I Abs are involved in interfering with ricin from accessing cellular receptors, we compared full length SylH3 with its respective Fab in a ricin-ASF attachment assay. For comparison, 24B11 and TFTB-1 full length IgGs and Fabs were subjected to the same assay. Nunc Maxisorb F96 microtiter ELISA plates (ThermoFisher Scientific) were coated with ASF (0.4 µg/well; EY Laboratories, San Mateo, CA) in PBS for 18 h at 4°C, and then probed with pre-incubated mixtures of biotinylated

ricin (Vector Laboratories, Burlingame, CA) and mAbs or Fabs at a range of concentrations (Fig. 1).

As expected, full length SylH3 IgG inhibited, in a dose-dependent manner, the attachment of ricin to ASF. Fifty percent inhibition (IC_{50}) was achieved with ~ 1.0 nM SylH3, while $>90\%$ inhibition (IC_{90}) was achieved with ~ 15 nM SylH3 (Fig. 1A). Although SylH3 Fab fragments also inhibited ricin attachment to ASF at high concentrations, they were 2–4 fold less effective at lower concentrations than the full length SylH3 IgG. These data suggest that the Fc component of SylH3 is in fact important in inhibiting ricin attachment to ASF. By comparison, 24B11 and 24B11 Fab fragments only partially interfered with ricin attachment to ASF, while TFTB-1 and TFTB-1 Fab fragments had no effect on toxin binding (Fig. 1B).

We next employed a Vero cell (ATCC, Manassas, VA) cytotoxicity assay in order to determine to what degree SylH3 and 24B11 Fab fragments retained the capacity to neutralize ricin *in vitro*. SylH3 neutralized ricin in a dose-dependent manner, with an IC_{50} of ~ 15 nM SylH3 (Fig. 2A). With an IC_{50} of 30 nM, SylH3 Fab fragments were ~ 2 -fold less effective at neutralizing ricin than the respective full length mAb, again arguing for a role of the Fc domain in toxin neutralization by SylH3. In contrast, Fab fragments of 24B11 were equally effective as full-length 24B11 at toxin neutralization (Fig. 2B), thereby confirming that the Fc domain of 24B11 is not required for toxin neutralizing activity *in vitro*. Finally, neither TFTB-1 nor TFTB-1 Fab fragments had any demonstrable neutralizing activity, as expected (Fig. 2C).

Having confirmed that SylH3 and 24B11 Fab fragments retain some or all of their ricin-neutralizing activity *in vitro*, we next sought to examine their ability to passively protect mice against a $10 \times LD_{50}$ toxin challenge. To control for the fact that Fab fragments generally have a shorter half-life in humans and animal models (<20 h) than full-length IgG (~ 9 – 30 days) (Bazin-Redureau et al., 1997; Jones et al., 2006; Kim et al., 2008; Ujhelyi and Robert, 1995), mAbs and Fabs were co-administered with ricin toxin ($10 \times LD_{50}$) at the time of challenge. Furthermore, to ensure that the molar ratio of toxin to the antibody variable domain (Fv regions) was the same, mice received $60 \mu\text{g}$ full-length mAbs and $40 \mu\text{g}$ of Fab fragments. Following challenge, mice were monitored for the onset of hypoglycemia, a well-established surrogate marker of ricin intoxication, as well as mean time to death (Pincus et al., 2002).

The ricin-only group of mice succumbed to intoxication within 24 h, as did the animals treated with ricin and TFTB-1 or TFTB-1 Fab fragments (Table 1; Fig. 3A; Fig. S3). In contrast, mice treated with ricin and concomitantly with SylH3 or SylH3 Fab fragments survived toxin challenge and did not experience a significant drop in blood glucose levels, thereby demonstrating that, at the concentrations tested, both the full-length and Fab fragments were equally effective at neutralizing ricin *in vivo*. Examination of 24B11-treated mice revealed that 24B11 conferred only partial protection against a $10 \times LD_{50}$ ricin challenge, as evidenced by the fact that one mouse in the 24B11-treated succumbed to ricin intoxication while the surviving mice experienced a significant reduction in blood glucose levels at 24 hr post challenge (Table 1; Fig. 3B,C; Fig. S3). The Fc-domain of 24B11 is apparently not involved in neutralizing ricin *in vivo*, as 24B11 Fab fragments were as effective as the full length IgG at protecting mice against $10 \times LD_{50}$ ricin challenge. These data demonstrate that the capacities of SylH3 and 24B11 to neutralize ricin *in vivo* are both Fc-independent.

The fact that Fab fragments of SylH3 and 24B11 were capable of protecting mice against a lethal dose toxin challenge demonstrates that ricin neutralization, at least by these two RTB-specific mAbs, is Fc-independent. While we cannot exclude the possibility that antibody

(Ab) constant regions may influence the half-life or tissue distribution of toxin-immune complexes, our data are in accordance with other studies suggesting that ricin neutralization is primarily dictated by Fv-specificity (Vance and Mantis, 2012; Yermakova and Mantis, 2011; Yermakova et al., 2012). For example, non-neutralizing, high-affinity mAbs against RTA or RTB (e.g., TFTB-1) afford no protection against toxin challenge in a mouse model. Nor do oligoclonal mixtures of non-neutralizing mAbs provide any degree of protection (A. Yermakova and N. Mantis, unpublished results). This is in contrast to what has been observed in the case of BoNT where Fc receptor-mediated clearance is important in counteracting high-dose toxin exposure (Nowakowski et al., 2002; Sepulveda et al., 2010) and in the case of anthrax toxin where protection is modulated by IgG subclass and Fc R utilization (Abboud et al., 2010; Harvill et al., 2008; Mabry et al., 2005; Maynard et al., 2002; Wild et al., 2003).

One limitation of this study is that we did not examine RTA-specific mAbs in parallel. Nonetheless, such experiments would be highly informative, as more than a dozen RTA-specific toxin neutralizing mAbs have been described (O'Hara et al., 2010; O'Hara et al., 2012b). A number of these RTA-specific mAbs have been shown to be highly effective at protecting mice when administered prior to, concomitantly, or even as much as 6 h after ricin challenge (O'Hara et al., 2010; O'Hara et al., 2012a; Roche et al., 2008). In general, mAbs directed against RTA have little effect on ricin's ability to associate with host cell receptors, suggesting that like 24B11 they may neutralize ricin at a step downstream of attachment (Maddaloni et al., 2004; Neal et al., 2010; O'Hara et al., 2010).

In conclusion, the demonstration that Fab fragments of single specificity are sufficient to neutralize ricin *in vivo* raises the possibility that single chain Abs like camelid Nanobodies (VHHs) may have therapeutic potential. While single chain antibodies have much shorter half-lives than full length human or chimerized mAbs, they do have the advantage of greater tissue penetration and longer shelf-lives (Sepulveda et al., 2010). Thus, future studies will be aimed at evaluating the use of RTB-specific Fabs or single chain Abs as post exposure therapeutics for ricin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. **B.**

Fig. 1. Inhibition of ricin-receptor interactions by SylH3 and 24B11 Fabs

Biotin labeled ricin (770 pM) was mixed with SylH3 (A), 24B11 (B) or TFTB-1 (A, B) mAbs and their respective Fab fragments at the indicated concentrations (x-axis) and then applied to 96-well microtiter plates coated with ASF (83 nM). Biotin-ricin binding to ASF (y-axis) was detected by incubation with avidin-HRP (Sigma-Aldrich Co.) and TMB substrate (KPL, Gaithersburg, MD). Percent ricin binding was defined as OD₄₅₀ values of each treatment/OD₄₅₀ of no Ab control × 100. Each symbol (with SEM) represents the average of at least two replicate wells.

Fig. 2. Neutralizing activity of SylH3, 24B11 and TFTB-1 Fabs

Fab fragments of SylH3 (A), 24B11 (B) and TFTB-1 (C) were assessed for their capacity to protect Vero cells from the cytotoxic effects of ricin and compared to their respective full length IgGs. Vero cells (5×10^4 cells/ml) were seeded (0.1 ml per well) into white-bottomed 96-well tissue culture plates (Corning Life Sciences, Corning, NY) and allowed to adhere overnight. Cells were then treated with a mixture of 154 pM ricin and varying dilutions of mAb or Fab (starting at 66 nM) for 2 h (x-axis). The cells were then washed and incubated for 48 h before being analyzed for cell viability (y-axis) using CellTiter-GLO (Promega, Madison, WI). Each symbol (with SEM) represents the average of at least two replicate wells.

Fig. 3. Passive protection conferred by SylH3 and 24B11 Fab fragments

Groups of 10 week old female BALB/c mice (Taconic Labs, Germantown, NY) were passively immunized with Fabs (4 μ M or 40 μ g Fab/animal) or full IgG mAbs (2 μ M or 60 μ g mAb/animal) by intraperitoneal injection and then challenged 24 h later with 10xLD₅₀s of ricin. **(Panel A)** Kaplan-Meier survival plot with SylH3, 2B11 and TFTB-1 mAbs and Fab fragments. **(Panels B, C)** Hypoglycemia was used as a surrogate marker of ricin intoxication, as described previously (Yermakova and Mantis, 2011). Blood glucose levels were measured at just prior to or 24 h after ricin challenge: **(Panel B)** SylH3, 24B11, TFTB-1 IgGs, **(Panel C)** SylH3, 24B11, TFTB-1 Fabs, **(Panel D)** ricin challenge control. Each group contained n = 4 mice. Both 24B11 and SylH3 were tested more than four times in passive protection studies. The Fab fragments were tested for neutralizing activity in a series of in vitro and in vivo pilot studies with 2–3 mice per group and were then compared directly to their respective full length IgGs in the study shown in this figure. P values were obtained using a two-tailed paired t test with a 95% confidence interval.

Table 1

Onset and recovery from ricin intoxication following challenge with SyIH3 and 24B11 IgG and Fabs.

Time ^a	SyIH3		24B11		TFTB-1		Control
	IgG	Fab	IgG	Fab	IgG	Fab	
0	127 $\pm$ 7	131 $\pm$ 6	118 $\pm$ 4	130 $\pm$ 9	119 $\pm$ 1	132 $\pm$ 2	110 $\pm$ 2
24	76 $\pm$ 2	81 $\pm$ 1	60 $\pm$ 4	67 $\pm$ 8	x	x	n.d.
48	92 $\pm$ 1	106 $\pm$ 6	61 $\pm$ 14	70 $\pm$ 25	x	x	n.d.
72	99 $\pm$ 1	103 $\pm$ 5	70 $\pm$ 23	86 $\pm$ 7	x	x	n.d.
96	127 $\pm$ 7	123 $\pm$ 6	102 $\pm$ 2	106 $\pm$ 4	x	x	n.d.

^aTime is hours post ricin challenge.

^bAverage blood glucose (mg/dl) of mice per group, per time point with standard errors. X, indicates that there were no survivors at these time points.