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Short communication

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.

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 (α, β, γ) 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, 1α and 2γ, retain functional carbohydrate recognition activity (Rutenber et al., 1987; Swimmer et al., 1992). Sub-domain 1α (residues 17–59) is Gal-specific and is considered a "low affinity" CRD, whereas sub-domain 2γ (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 1α or 2γ, which are separated by approximately 70 Å, 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

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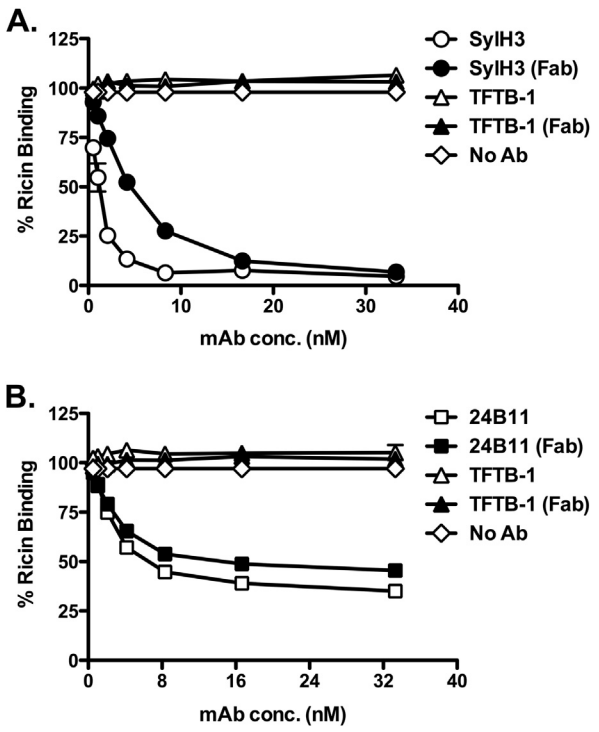

Fig. 1. Inhibition of ricin-receptor interactions by SylH3 and 24B11 Fabs. Biotin labeled ricin (770 pM) was mixed with SylH3 (**A**), 24B11 (**B**) or TFTB-1 (**A, B**) mAbs and their respective Fab fragments at the indicated concentrations (*x*-axis) and then applied to 96-well microtiter plates coated 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 OD_{450} values of each treatment/OD_{450} of no Ab control × 100. Each symbol (with SEM) represents the average of at least two replicate wells.

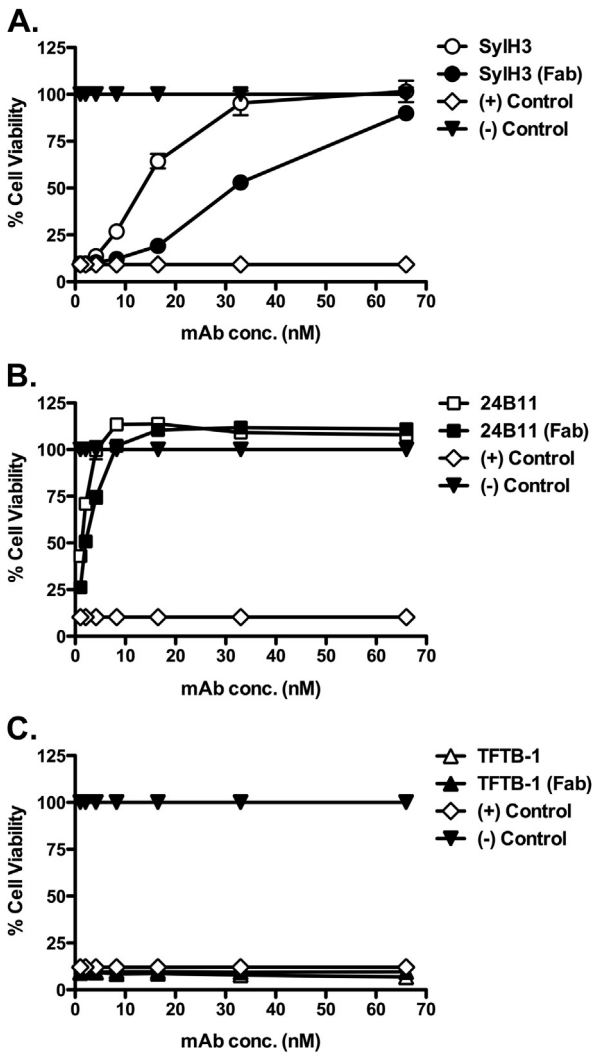

Fig. 2. Neutralizing activity of SylH3, 24B11 and TFTB-1 Fabs. Fab fragments of SylH3 (**A**), 24B11 (**B**) and TFTB-1 (**C**) were assessed for their capacity to protect Vero cells from the cytotoxic effects of ricin and compared to their respective full length IgGs. Vero cells (5×10^4 cells/ml) were 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.

mice against a lethal toxin challenge. All three are IgG_1s 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 1α (McGuinness and Mantis, 2006). We speculate that SylH3 and JB4 bind a similar or overlapping epitope in RTB's sub-domain 2γ (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 Fcγ receptor (FcγR)-dependent mechanisms. In the case of anthrax toxin and BoNT, there is evidence that FcγR-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 IgG_1 Fab preparation kit (ThermoScientific, Rockford, IL).