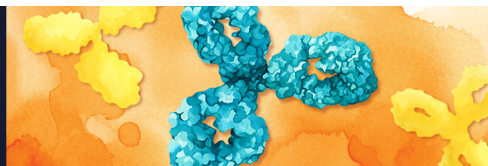


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## Immunological Characteristics Associated with the Protective Efficacy of Antibodies to Ricin

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# Immunological Characteristics Associated with the Protective Efficacy of Antibodies to Ricin<sup>1</sup>

Massimo Maddaloni,\* Corrie Cooke,\* Royce Wilkinson,\* Audrey V. Stout,\* Leta Eng,<sup>†</sup> and Seth H. Pincus<sup>2\*‡</sup>

A/B toxins, produced by bacteria and plants, are among the deadliest molecules known. The B chain binds the cell, whereas the A chain exerts the toxic effect. Both anti-A chain and anti-B chain Abs can neutralize toxins *in vivo* and *in vitro*. B chain Abs block binding of the toxin to the cell. It is not known how anti-A chain Abs function. Working with ricin toxin, we demonstrate that immunization with A chain induces greater protection than immunization with B chain. A panel of mAbs, binding to A chain, B chain, or both chains, has been produced and characterized. Immunologic characteristics evaluated include isotype, relative avidity, and epitope specificity. The ability to inhibit ricin enzymatic or cell binding activity was studied, as was the ability to block ricin-mediated cellular cytotoxicity on human and murine cell lines. Finally, the *in vivo* protective efficacy of the Abs in mice was studied. The Ab providing the greatest *in vivo* protective efficacy was directed against the A chain. It had the greatest relative avidity and the greatest ability to block enzymatic function and neutralize cytotoxicity. Interestingly, we also obtained an anti-A chain Ab that bound with high avidity, blocked enzymatic activity, did not neutralize cytotoxicity, and actually enhanced the *in vivo* toxicity of ricin. Anti-A chain Abs with moderate avidity had no *in vivo* effect, nor did any anti-B chain Abs. *The Journal of Immunology*, 2004, 172: 6221–6228.

Antibodies, induced by immunization or administered passively, have been used to protect against the effects of biological toxins for over a century, since the pioneering work of von Behring and Kitasato (1). The studies of Pappenheimer et al. (2) showed that one mechanism by which Abs can protect is by blocking the entry of toxins into cells. This view is frequently presented in textbooks (3–6), which ignores an important body of evidence demonstrating that Abs to the toxic A chain of A-B toxins have equal or greater neutralizing activity than anti-B chain Abs, which block toxin adherence (7–13).

Ricin toxin, derived from the castor bean *Ricinus communis*, is a prototypic A-B toxin in which the B chain binds to the target cell, and the A chain mediates the toxic activity. Ricin B chain (RBC)<sup>3</sup> is a lectin that binds to galactose residues found at the cell surface (e.g., on glycoproteins), whereas the A chain functions enzymatically as an RNA N-glycosidase that depurinates adenine 4324 in the 28S rRNA of the 60S ribosomal subunit (14–17). Ricin toxin is highly lethal, with an LD<sub>50</sub> of <2 µg/kg. Its use as a bioterrorist weapon is of considerable concern. However, ricin has also been used as a therapeutic agent, in the form of immunotoxins where the B chain is replaced by a specific targeting moiety (18, 19). In both cases, the immunogenicity of ricin is a matter of great interest; either active or passive immunity may protect against a bioterrorist

exposure, whereas the development of anti-ricin Abs is a major limitation for ricin-based immunotoxins.

In this manuscript we have studied the anti-ricin Ab response and correlated immunologic characteristics of Abs to their protective efficacy, both *in vitro* and *in vivo*. The data demonstrate that anti-A chain Abs have the greatest protective efficacy. Although Ab avidity is a major correlate of protection, there exist high avidity Abs that fail to protect *in vitro* and enhance toxicity *in vivo*. The mechanism(s) by which anti-A chain Abs protect against ricin intoxication are not fully understood.

## Materials and Methods

### Abs and reagents

Ricin holotoxin was obtained from Inland Laboratories (Austin, TX), and purified A and B chains were purchased from Sigma-Aldrich (St. Louis, MO). The IC<sub>50</sub>, as measured on SP2/0 cells, is 0.01 ng/ml for the holotoxin, 150 ng/ml for the A chain, and 2 ng/ml for the B chain. Alkaline phosphatase- and FITC-conjugated secondary Abs were obtained from Zymed Laboratories (South San Francisco, CA). Guinea pig anti-ricin A chain (RAC) Ab was prepared by immunization of guinea pigs with purified RAC in CFA and boosting in IFA. Protein G agarose was obtained from Sigma-Aldrich.

### Immunization with ricin and production of mAbs

BALB/c mice were immunized with 10 µg of either purified RAC or RBC in CFA s.c. Four weeks later they were boosted with 10 µg of the same Ag i.p. in IFA. When mice were to be used to make mAbs, they received an i.v. injection of 10 µg of the same Ag in saline 3 days before death. For the production of mAbs to the holotoxin, mice were immunized and boosted with RAC, they then received two booster immunizations of 10 µg of ricin holotoxin in IFA, 1 mo apart, and an i.v. boost of the holotoxin 3 days before death. mAbs were produced as described previously (20–22). Splenocytes (5 × 10<sup>7</sup>) and SP2/0 cells (10<sup>7</sup>) were fused with polyethylene glycol, selected in hypoxanthine/aminopterin/thymine medium, and cloned by limiting dilution. Hybridomas secreting anti-ricin mAbs were identified by ELISA using the appropriate Ag. In one fusion, intact ricin toxin (2 ng/ml) was added to select for Abs with ricin-neutralizing activity (23).

### ELISA

Abs to RAC, RBC, or holotoxin were detected by ELISA (20–22). Microtiter wells (Immulon II, Dynatech, Chantilly, VA) were coated with 100

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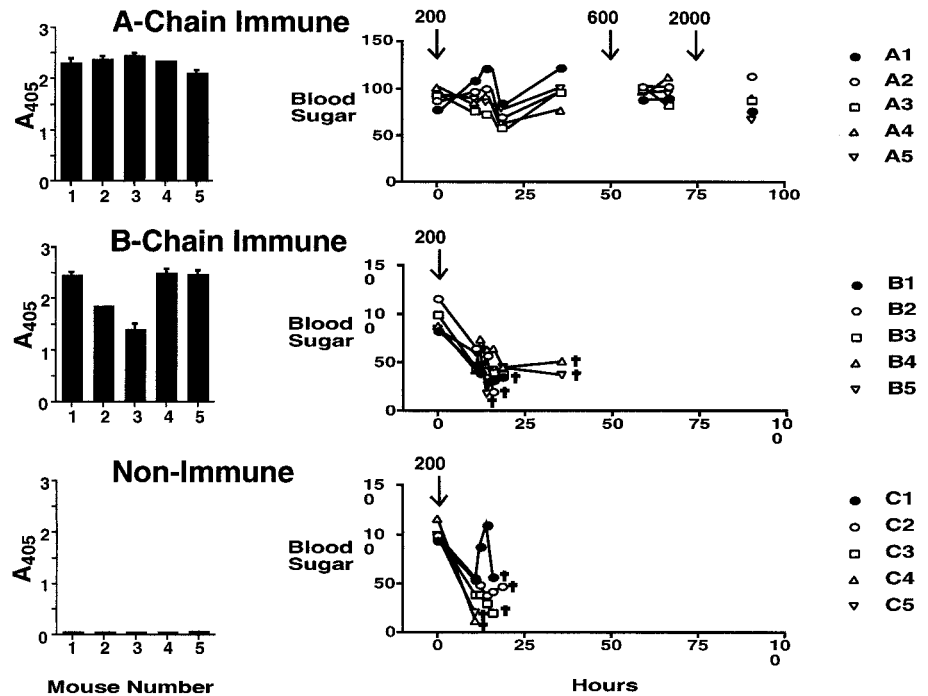
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<sup>3</sup> Abbreviations used in this paper: RBC, ricin B chain; RAC, ricin A chain.

**FIGURE 1.** Protection against ricin challenge by mice immunized with either RAC or RBC. Mice were immunized with RAC, RBC, or saline (10  $\mu\text{g}$  of A or B chain sc in CFA, followed 1 mo later by 10  $\mu\text{g}$  i.p. in IFA; challenge was performed 1 mo later). ELISA results are on the *left*, the response to challenge is on the *right*. ELISA was performed with a 1/5000 dilution of serum. Results are the mean and SEM of triplicate samples. The challenge dose in micrograms per kilogram is indicated above the arrow. Crosses indicate euthanized mice. No control mice or B chain-immune mice survived the initial challenge, whereas all A chain-immune animals survived three successive challenges with 200, 600, and 2000  $\mu\text{g}/\text{kg}$ . This is one of two different experiments yielding essentially the same observations.



$\mu\text{l}$  of Ag (0.5–3.0  $\mu\text{g}/\text{ml}$ ) in PBS overnight and then blocked for at least 2 h with 200  $\mu\text{l}$  of Blotto (10% skim milk/0.01% Tween 20; Sigma-Aldrich) in PBS. Because RBC is a lectin, and Ab is a glycoprotein, we have compared the use of Blotto, which contains 0.15 M lactose, to the use of PBS/1% BSA with or without 0.125 M lactose for its ability to block nonspecific lectin binding of Ab by RBC. The data (not shown) demonstrate that Blotto and 0.125 M lactose are equivalent at blocking the binding of irrelevant Abs by RBC and have little effect on the binding by A or B chain Abs to the holotoxin. Therefore, Blotto was used in all ELISA experiments. Test Ab, diluted in Blotto, was added to the coated wells in a volume of 100  $\mu\text{l}$  and incubated overnight at 4°C. The wells were washed six times in PBS containing 0.01% Tween 20 and alkaline phosphatase goat anti-mouse IgG, H+L chain, or IgG isotype-specific Ab was added and incubated for 4 h at room temperature. The plates were again washed, and the colorimetric substrate *p*-nitrophenyl phosphate (Sigma-Aldrich) was added at a concentration of 0.5 mg/ml in 10% diethanolamine buffer, pH 9.8. Absorbance at 405 nm was read 20–60 min later. In performing Ab competition ELISA, the ability of unconjugated mAbs to compete with an alkaline phosphatase-conjugated mAb for binding to Ag was measured. Anti-ricin mAbs were conjugated to calf intestine alkaline phosphatase (Sigma-Aldrich) by glutaraldehyde cross-linking (21). The unconjugated mAb was added to Ag-coated wells, followed 0.5 h later by the conjugated mAb. After overnight incubation the plates were washed, and substrate was added.

#### Epitope mapping

Peptide display phage libraries were selected for binding to mAbs as previously described (24, 25). Three different libraries were used: J404 (26, 27), which displays a linear nonamer and has a complexity of  $4 \times 10^8$ ; PhD-C7C (New England Biolabs, Beverly MA), displaying a cysteine-constrained, seven-amino acid loop and having a complexity of  $2 \times 10^9$  phage; and PhD-12 (New England Biolabs) displaying a 12-amino acid linear peptide with a complexity of  $2 \times 10^9$  phage. Ab was immobilized on cyanogen bromide-activated Sepharose (Sigma-Aldrich) at a concentration of 1 mg/ml beads. Phage ( $\sim 10^{12}$ ) were incubated with the Ab-coated beads and the unbound phage were washed away with TBS. Phage were eluted with 0.2 M glycine-HCl, pH 2.5, and reamplified to a titer of  $10^{12}$  and reapplied to fresh Ab-coated beads. Three or four successive rounds of amplification, adherence, and elution resulted in the identification of phage that bound uniquely to the selecting Abs and not to irrelevant controls. Binding was confirmed by both plaque lift immunoblots and ELISA (24, 25). Phage were sequenced by automated methods using M13 primers (Davis Sequencing, Davis, CA). Epitopes were mapped onto the three-dimensional structure of ricin using Insight II (Accelrys, San Diego, CA), and the figure was drawn using PyMOL version 0.82 (www.pymol.org).

#### Inhibition of ricin enzymatic and cell-binding activity

The ability of Abs to inhibit ricin's enzymatic activity was measured in a cell-free *in vitro* translation assay using rabbit reticulocyte lysates (Ambion, Austin, TX) as both the source of mRNA and ribosomes. Ab and ricin A chain were mixed, added to the lysate in the presence of [ $^{35}\text{S}$ ]methionine, and incubated for 2 h at 37°C. Protein was precipitated with trichloroacetic acid, and the amount of label incorporated was determined by liquid scintillation counting. The ability of Abs to block the binding of ricin to cells was measured by indirect immunofluorescence and flow cytometry. Anti-B chain Abs were mixed with ricin holotoxin and incubated for 30 min at 24°C, then the mixture was incubated with H9 lymphoma cells for 60 min at 4°C. The cells were washed three times with 1% BSA/PBS/0.01% sodium azide, followed by incubation with guinea pig anti-RAC Abs for 60 min at 4°C. Again the cells were washed and then incubated with FITC-conjugated rabbit anti-guinea pig IgG. The FITC-conjugated Ab had previously been tested for cross-reaction with mouse IgG or for binding to cells in the absence of ricin holotoxin and was found to be negative for both. After a final wash, the cells were analyzed by flow cytometry (FAC-Scan; BD Biosciences, San Jose, CA). Results are reported as the mean fluorescence of 5000 cells.

#### Neutralization of ricin-mediated cytotoxicity

The ability of Ab to protect against the cytotoxic effect of ricin was measured using an MTT dye reduction assay. Two forms of neutralization were tested. In the first, Ab and ricin holotoxin were mixed and then added to target cells ( $2 \times 10^3$ ) in 96-well, flat-bottom plates. In the second, ricin was mixed with hybridoma cell lines secreting anti-ricin Abs, and the survival of the hybridoma cells was measured. Three days later, a mixture of MTS-tetrazolium salt (Promega, Madison, WI) and phenazine methosulfate (Sigma-Aldrich) was added to final concentrations of 0.4 and 0.01 mg/ml, respectively. The cells were incubated for 2.5 h at 37°C, and the absorbance was read at 490 nm.

#### *In vivo* protective effects

We used a newly described surrogate assay to measure the ability of Ab to protect mice from the toxic effects of ricin (28). In this assay, hypoglycemia, rather than lethality, was measured. Ricin-immune mice were injected with ricin holotoxin *i.p.* Passive immunity produced by mAbs was measured by premixing ricin and mAb, then injecting mice with the mixture. Outbred CD-1 mice were injected with ricin or ricin/Ab, and blood sugar was measured at various times thereafter via saphenous venipuncture and a hand-held glucometer. Mice were euthanized when blood sugar levels

Table I. Subclass and specificity of anti-ricin Abs<sup>a</sup>

	Isotype	A Chain	Holotoxin	B Chain	BSA	Western Blot Reactivity
Fusion 1: immunize with A chain, select for A chain binding						
RAC mAb 1	IgG2b	0.33	1.00	0.11	0.02	+
RAC mAb 3	IgG1	2.26	2.29	0.07	0.03	+
RAC mAb 4	IgG1	0.36	0.24	0.03	0.01	+
RAC mAb 5	IgG1	0.22	0.95	0.02	0.01	+
RAC mAb 6	IgG2a	0.15	0.18	0.02	0.03	+
RAC mAb 8	IgG1	2.53	2.43	0.09	0.02	+
RAC mAb 9	IgG2b	2.30	2.40	0.58	0.08	+
RAC mAb 10	IgG1	0.83	1.51	0.02	0.03	+
RAC mAb 11	IgG1	0.79	0.54	0.03	0.03	+
RAC mAb 12	IgG2b	1.10	0.99	0.45	0.04	+/-
RAC mAb 14	IgG1	0.42	1.04	0.02	0.02	+
RAC mAb 16	IgG1	2.30	2.27	0.06	0.03	+
RAC mAb 17	IgG1	2.38	2.33	0.13	0.05	+
RAC mAb 18	IgG2a	2.29	2.56	0.06	0.03	+
RAC mAb 19	IgG1	0.61	1.41	0.03	0.02	+/-
RAC mAb 20	IgG2b	0.59	1.35	0.05	0.01	+
RAC mAb 21	IgG1	0.18	0.43	0.04	0.03	-
RAC mAb 22	IgG1	2.54	2.45	0.11	0.05	+
RAC mAb 23	IgG1	2.19	2.38	0.04	0.03	+
Fusion 2: immunize with A chain, then holotoxin, select for survival in ricin						
RAC mAb 24	IgG2a	0.74	1.51	0.03	0.01	+/-
Holo mAb 1	IgG1	0.18	0.57	0.02	0.01	-
HAB mAb 1	IgG1	0.85	1.13	0.93	0.01	-
Fusion 3: immunize with B chain, select for B chain binding						
RBC mAb 1	IgG1	0.14	0.99	1.17	0.06	-
RBC mAb 2	IgG1	0.01	0.44	0.35	0.03	-
RBC mAb 3	IgG1	0.01	0.07	0.25	0.03	-
RBC mAb 4	IgG2a	0.48	1.95	2.14	0.03	+
RBC mAb 5	IgG1	0.19	1.50	1.64	0.03	+
RBC mAb 6	IgG1	0.02	0.48	0.93	0.04	-
RBC mAb 7	IgG2b	0.10	0.99	1.37	0.05	+
RBC mAb 8	IgG2b	0.01	1.73	1.82	0.02	-
RBC mAb 9	IgG2a	0.00	0.07	0.14	0.02	-
RBC mAb 10	IgG2b	0.58	1.96	2.02	0.03	+
RBC mAb 11	IgG2b	0.50	2.09	2.03	0.04	+
RBC mAb 12	IgG2a	0.00	0.22	0.37	0.08	-
RBC mAb 13	IgG1	0.01	0.16	0.16	0.04	-
RBC mAb 14	IgG2b	0.49	1.96	2.15	0.03	+
RBC mAb 15	IgG2a	0.18	1.76	1.60	0.08	+
RBC mAb 16	IgG2a	0.02	0.88	1.35	0.05	+
RBC mAb 17	IgG1	0.40	1.90	1.79	0.01	+
RBC mAb 18	IgG1	0.17	1.21	1.35	0.01	-
Medium		0.02	0.04	0.01	0.01	
Irrel mAb		0.04	0.07	0.02	0.02	

<sup>a</sup> The specificity of each Ab was tested in two to nine different experiments, subclass in one to three. Data shown are representative of those experiments.

were 25 mg/dl or the animals became highly lethargic. For statistical purposes, blood sugar levels of euthanized animals were assumed to be 25 mg/dl (the lower limit of detection). All experiments were approved by the institutional animal care and use committee of Montana State University.

## Results

### Protection against challenge after immunization with RAC or RBC

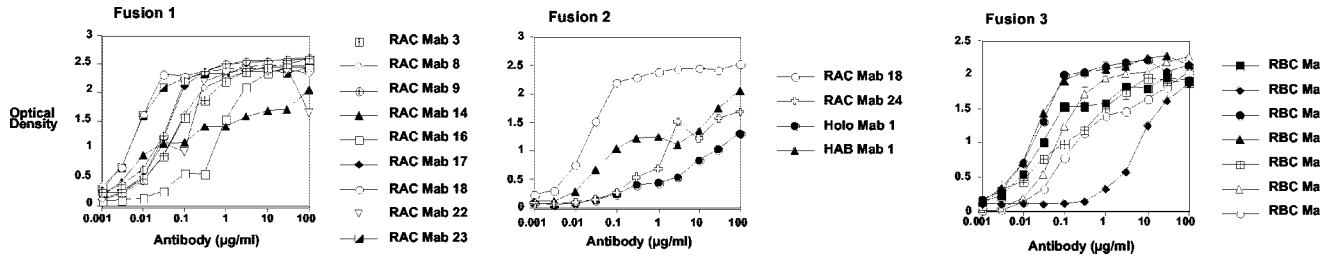
To determine whether immunization with RAC could elicit the same degree of protection as immunization with RBC, mice were immunized with either the purified A chain or the purified B chain. After a prime and then a booster immunization, animals were bled, Ab levels were determined, and ricin challenge was performed (Fig. 1). Ab to the intact toxin was measured and was somewhat higher in RAC-immune animals (ELISA results on the left). Mice were then challenged with 200  $\mu$ g/kg of ricin. Animals were sacrificed when blood sugar reached 25 or when they became comatose. Immunization with RAC resulted in complete protection against this challenge as well as challenges at 600 and 2000  $\mu$ g/kg. Only partial protection was seen in B chain-immune mice at 200

$\mu$ g/kg, and no protection was observed in control animals. These results clearly demonstrate that Ab to RAC is highly protective.

### Production of mAbs to RAC and RBC

To further explore the neutralizing and protective capabilities of Abs to ricin, we have produced a panel of mAbs that bind determinants on the A chain, B chain, or compound determinants present on both A and B chains. Mice were immunized with purified A chain, purified B chain, or purified A chain, followed by ricin holotoxin. Primary immunization with either holotoxin or a mixture of purified A and B chains was lethal in immunogenic doses; the latter group in our study was protected against the lethal effects by the pre-existing anti-A chain Abs. Hybridoma cells were either selected on the basis of binding to ricin or in fusion 2 for their ability to survive in ricin (2 ng/ml). The latter method can be used to select for Abs with neutralizing activity (23). We have identified 20 mAbs specific for RAC, 16 RBC mAbs, and two that recognize compound determinants present on the holotoxin (HoloMab and HAB mAb, depending upon whether individual chains





**FIGURE 2.** Relative avidity of anti-ricin Abs. Anti-ricin mAbs were purified on protein G. Purified Abs were titrated in ELISA for binding to intact ricin holotoxin (50 ng/well). Abs with the highest binding at the lowest concentrations have the greatest relative avidity for ricin. Similar titration curves were performed two to five times for each Ab; the data are representative of those experiments.

or only the holotoxin are bound). Ig subclass, ELISA binding results of supernatant, and Western blot reactivity of relevant Abs are shown in Table I.

#### Immunological characterization of mAbs

mAbs were purified by protein G affinity chromatography. The relative avidity of binding was determined by ELISA (Fig. 2). Serial dilutions of Ab were incubated in microtiter wells coated with ricin holotoxin. The wells were washed and incubated with alkaline phosphatase-conjugated anti-mouse IgG (H+L) and then with substrate. Binding is reported as  $A_{405}$ . There was a considerable range of binding activity demonstrated among the Abs. Among the anti-A chain Abs, RAC mAb 18 and RAC mAb 23 demonstrated the greatest relative avidity for intact ricin, whereas RBC mAb 10 and RBC mAb 11 did so for the anti-B chain mAbs. Surprisingly, all the mAbs selected in the presence of ricin toxin (fusion 2) had low avidity.

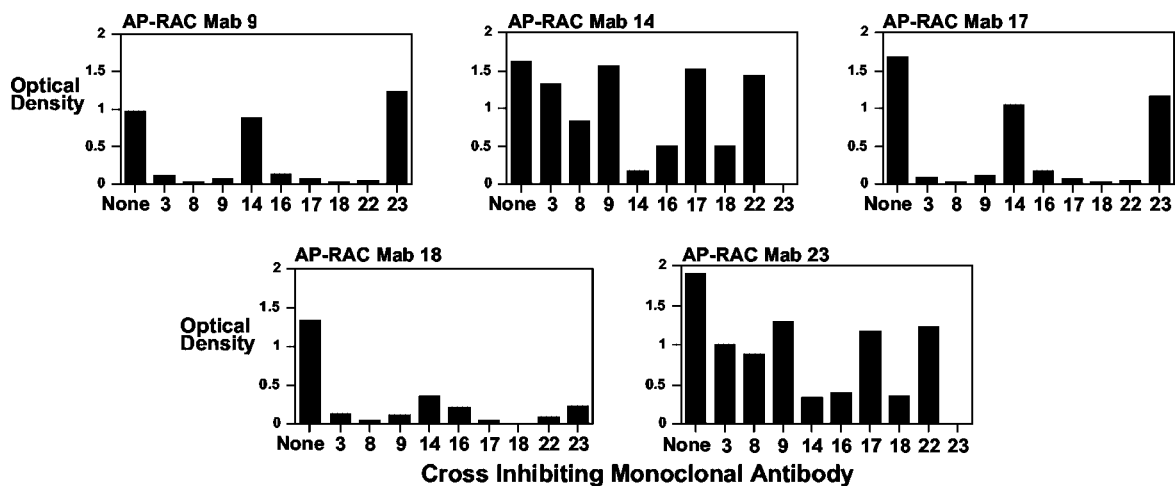
As a preliminary form of epitope mapping, we performed Ab cross-inhibition assays among the RAC mAbs, in which the binding of an alkaline phosphatase-conjugated RAC mAb was inhibited by unconjugated Abs (Fig. 3). RAC mAbs 9 and 17 appear to have similar epitope specificity, in that they were inhibited by all Abs tested, except 14 and 23. In the same manner, 14 and 23 had comparable patterns of inhibition, blocking the binding of each other, with good inhibition by 16 and 18, some inhibition with 3 and 8, and little by 9 and 17. Interestingly, RAC mAb 18, which had the highest relative avidity, could be blocked by all other Abs. No RBC mAbs inhibited the binding of RAC mAbs (data not

shown). Thus, there appear to be at least three different Ab binding sites on the RAC.

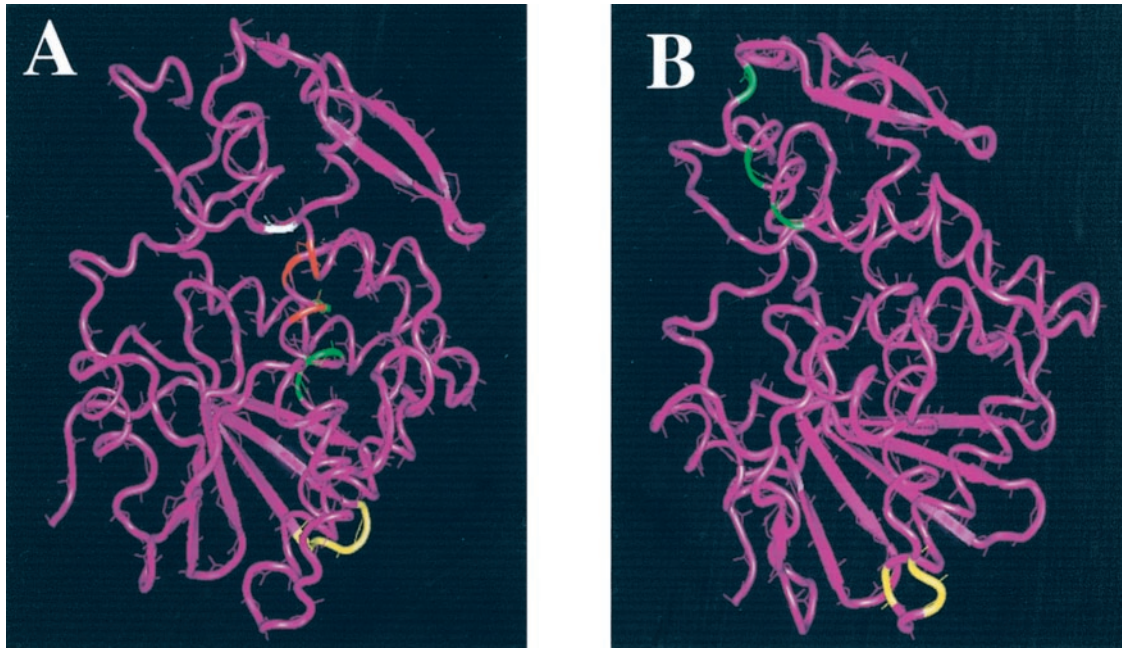
The epitopes to which the anti-A chain mAbs bind were mapped with random peptide phage display libraries (24, 25, 29). RAC mAbs 3, 8, 9, 17, and 22 bind to the amino acid sequence HAEL, which corresponds exactly to residues 66–69 of RAC. Phage selected by RAC mAb 18 all have a common motif of QXXWXXA. Overlaying this sequence on the three-dimensional structure of RAC (30, 31) provides a best fit with Q173, A178, W211, which fold into part of the enzyme active site (32). Fig. 4A shows the mapping of these epitopes onto the structure of ricin. Mapping RAC mAb 23 with the random peptide library yielded a consensus sequence of GTXS. A synthetic peptide, GTHSWDHPHHSYC, corresponding to one of the phage, was able to bind RAC mAb 23 and block binding of the Ab to RAC (not shown). There are at least two possible fits for the RAC mAb 23 consensus sequence on RAC (Fig. 4B): 1) the epitope shown in green consists of G213, T217, and S222, which are brought into juxtaposition by the turns of the helix; and 2) the epitope in yellow is G159, T160, and S156, with the loop bring S156 closer to the other AAs. We were unable to map the epitope of RAC mAb 14.

#### Inhibition of ricin enzymatic and binding activities

Abs that bind to RAC or to the holotoxin were tested for their ability to inhibit ricin enzymatic activity, which was measured as the inhibition of protein synthesis ( $[^{35}\text{S}]\text{Met}$  incorporation) in a cell-free system using rabbit reticulocyte lysates. RAC mAbs 14, 18, and 23 gave the best inhibition of ricin activity, with HAB



**FIGURE 3.** Cross-inhibition among RAC mAbs. Microtiter plates were coated with 50 ng/well of ricin holotoxin. Unconjugated RAC mAbs (5  $\mu\text{g}/\text{ml}$ ), indicated on the horizontal axis, were added to the coated microtiter wells and incubated for 60 min, the alkaline phosphatase-conjugated Ab indicated at the top of the graph was added, and the mixture was incubated for 4 h. The plates were washed, and colorimetric substrate was added. Abs were tested in up to four different experiments, of which this is representative.



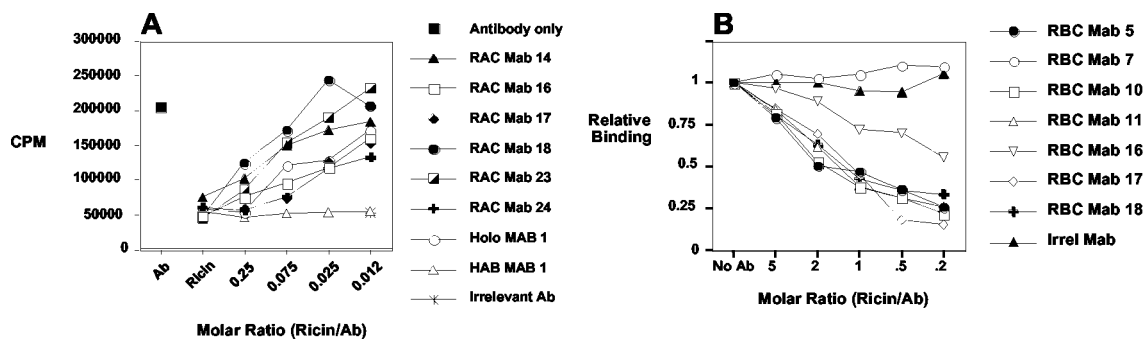
**FIGURE 4.** Epitope mapping of RAC mAbs. Epitopes defined by mapping using peptide display phage libraries were plotted onto the three-dimensional structure of ricin. *A*, Yellow indicates RAC mAb 17 (AA66–69 HAEL), green RAC mAb 18 (Q173, A178), red enzyme active site (E177, R180, N209), and white residue present in the active site and bound by RAC mAb 18 (W211). *B*, The two potential sites for RAC mAb 23 binding: green, G213, T217, and S222; and yellow, S156, G159, and T160. The molecule has been rotated on all three axes from *A* to *B* to allow best visualization of the epitopes.

mAb 1, which binds both chains, giving none (Fig. 5A). Flow cytometry was used to measure the ability of RBC mAbs to block ricin’s binding to cells. RBC mAb and the holotoxin were incubated together and then mixed with H9 tissue culture lymphoma cells. Binding of ricin to the cells was detected using a guinea pig anti-RAC polyclonal and FITC-conjugated anti-guinea pig anti-serum. The ability of RAC-mAbs to interfere with binding could not be measured using this assay, because they might compete with the guinea pig serum for binding to RAC. However, studies with FITC-conjugated ricin have shown that RAC mAb 18, the highest avidity RAC mAb, does not inhibit the binding of ricin to cells (not shown). The results demonstrate that all Abs, except RBC mAbs 7 and 16, were able to efficiently block binding of ricin to cells. RBC mAb 7 had no blocking activity, whereas 16 had marginal activity (Fig. 5B).

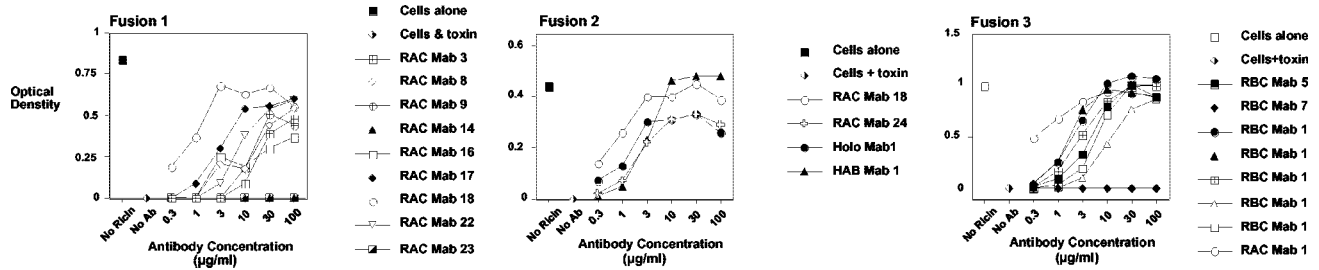
*Neutralization of ricin cytotoxicity*

We next asked which mAbs had the best ability to neutralize the cytotoxicity of ricin. Abs were tested in two forms: the protection

afforded H9 cells by free mAbs, and the protection of hybridoma cells by the Abs they were secreting. Preliminary studies demonstrated the minimum LD<sub>100</sub> of ricin on SP2/0 cells was 0.4 ng/ml; we tested the ability of free Abs to block cytotoxicity at a ricin concentration of 2 ng/ml. The viability of the cells was measured using the MTT dye reduction assay. Results are shown in Fig. 6. Of the RAC mAbs resulting from fusion 1, the best neutralizing activity was seen with Ab 18, followed by 17. Interestingly, Abs 14 and 23 had no effect, even though both were excellent at blocking ricin enzymatic activity, and RAC mAb 23 had high avidity for ricin toxin. RAC mAb 14 bound to ricin with low avidity (Fig. 3), which probably accounts for its failure to neutralize cytotoxicity. RAC mAb 16 also has weak avidity, enzymatic blocking activity, and neutralization. Cell lines from fusion 2 were selected in the presence of ricin and had only weak neutralizing activity. Anti-RBC mAbs (fusion 3) were all less effective than RAC-mAb 18. RBC mAb 7 was ineffective, which corresponds to its inability to block the binding of ricin to cells and its low avidity. RBC mAb 16 was able to neutralize as well as any of the RBC mAbs despite



**FIGURE 5.** Inhibition of ricin enzymatic and binding activities by Abs. *A*, A fixed concentration of ricin holotoxin was mixed with varying concentrations of Ab. *A*, The ability of Abs to block ricin’s inhibition of cell-free protein synthesis was measured. *B*, Flow cytometry was used to measure binding of ricin to cells. The results are reported as the ratio of binding (mean fluorescence) in the presence of Ab/binding in the absence of Ab. Each Ab was tested one to three times in the different assays; these are the data from the most complete experiment of each.

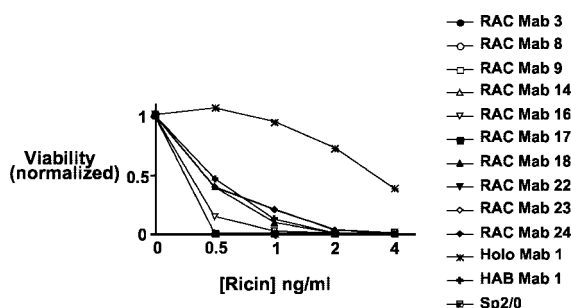


**FIGURE 6.** Ab-mediated inhibition of ricin cytotoxicity. H9 cells were incubated with ricin (2 ng/ml) and the indicated Ab. Three days later cell viability was assayed by MTT dye reduction. Viability was also measured in the absence of ricin and in the presence of ricin without Ab. Some Abs were tested in protection assays up to 15 times, but at least twice. The data are representative of these experiments.

its weak ability to block binding. We have performed experiments in which we mixed Abs to either the same chain or both A and B chains and tested for neutralization. We saw additive, but not synergistic, effects (data not shown). We next tested the ability of hybridomas secreting Abs that bind the A chain to survive in the presence of ricin (Fig. 7). It has previously been shown that intracellular neutralization of ricin occurs in hybridomas (23, 33). The mAbs selected in the presence of ricin (Holo mAb 1, RAC mAb 24, and HAB mAb 1) gave the highest degrees of protection. Of the remaining Abs, only RAC mAb 18 provided any protection. Thus, there appear to be marked differences in the ability of Ab to neutralize ricin in the extracellular milieu and within the cell secreting the Ab.

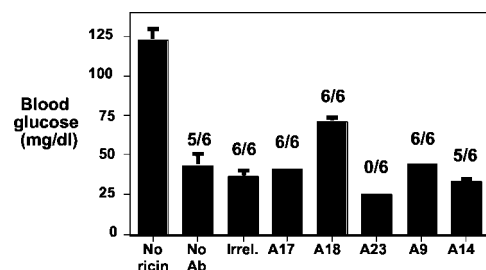
#### *In vivo protection by mAbs*

Using our *in vivo* model of ricin intoxication (28), we have studied the protective efficacy of anti-ricin mAbs in mice. In the first experiment we tested a panel of RAC mAbs. Mice were injected with a mixture of 30  $\mu\text{g}/\text{kg}$  of ricin and 0.8 mg/kg of Ab, and their blood sugar levels were determined 18 h later. The dose of Ab was chosen on the basis of prior dosing experiments (not shown); lower doses of Ab did not produce statistically significant differences between treated and control groups. If mice were moribund, they were killed, and their blood sugar value was set at 25 mg/dl for statistical purposes. Fig. 8 shows the mean and SEM of blood sugar determinations and mortality. RAC mAb 18 showed significantly ( $p < 0.001$ , by Student's *t* test) higher blood sugar levels, i.e., protection, compared with the irrelevant Ab. No other Ab protected. RAC mAb 23 had significantly greater mortality ( $p = 0.004$ , by  $\chi^2$  test), thus appearing to potentiate the toxicity of ricin. We repeated the Ab protection studies, but this time using a lower dose of ricin (15  $\mu\text{g}/\text{kg}$ ) and the same dose of Ab (0.8 mg/kg).



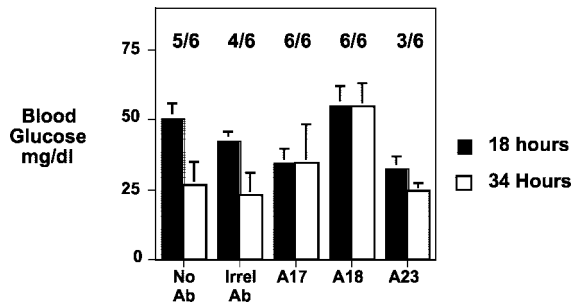
**FIGURE 7.** Protection of hybridoma cells from ricin-mediated toxicity by secreted Ab. Hybridoma cell lines secreting the indicated Ab (or the nonsecretor parental fusion partner SP2/0) were incubated in the presence of various concentrations of ricin for 3 days, and viability was tested by MTT dye reduction. Cell lines were tested two or three times, and representative data are shown.

Blood sugar was measured at 18 and 34 h, and mortality was assessed at 34 h (Fig. 9). At 18 h, compared with the irrelevant Ab, RAC mAb 18 provided significant protection ( $p = 0.0018$ ), and RAC mAb 23 provided significant enhancement ( $p = 0.0006$ ) of ricin toxicity. At 34 h, protection by RAC mAb 18 was more pronounced; enhancement of toxicity by RAC mAb 23 could not be assessed by blood sugar because of the low values found in control groups. In the next experiment we further tested the ability of RAC mAb 23 to enhance the toxicity of ricin. Mice were injected with 30  $\mu\text{g}/\text{kg}$  of ricin mixed with either 0.8 or 1.6 mg/kg of RAC mAb 23, saline, or 1.6 mg/kg of an irrelevant isotype-matched Ab. Blood sugar was measured 13 h later (when all mice were still alive), and the number of surviving mice was determined at 18 h. Fig. 10 shows a dose-related enhancement of toxicity by RAC mAb 23. At both doses of RAC mAb 23, there was a statistically significant depression of blood sugar ( $p = 0.03$  for the low dose,  $p = 0.0009$  for the high dose) and enhanced mortality ( $p = 0.0192$  for the low dose,  $p = 0.004$  for the high dose) compared with irrelevant Ab. Although there was a lower blood sugar and greater mortality in the high dose group compared with the low dose group, the differences were not significant. We have determined that endotoxin contamination of RAC mAb 23 does not account for the enhancement of toxicity. Measuring the amount of endotoxin using *Limulus* lysate (Sigma-Aldrich) showed there were trace amounts of endotoxin in many Ab preparations that could not be removed by absorption on polymyxin B. We deliberately added LPS to RAC mAbs to equalize the amount and found that this had no influence on the *in vivo* effect (data not shown). Further, nonspecific lectin binding of RAC mAb 23 is similar to that of the neutralizing Ab RAC mAb 18. Anti-B chain mAbs had no protective efficacy (data not shown).

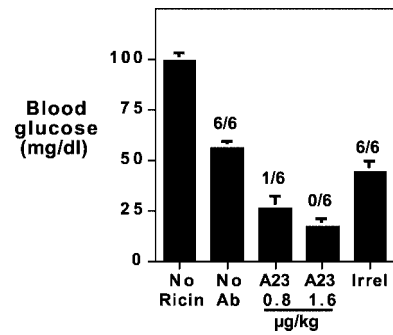


**FIGURE 8.** *In vivo* effect of RAC mAbs on ricin toxicity. Mice were injected with 30  $\mu\text{g}/\text{kg}$  of ricin holotoxin and 0.8 mg/kg of the indicated Ab. Blood sugar was determined 18 h later. Mice were sacrificed if they became moribund or if their blood sugar level fell below 25 mg/dl. Mean blood sugar levels ( $\pm$ SEM) and survival are shown.





**FIGURE 9.** In vivo effects of Abs to ricin A chain. Mice were injected with 15  $\mu\text{g}/\text{kg}$  of ricin and 0.8 mg/kg of Ab. Blood sugar was determined 18 and 34 h later. Mean ( $\pm$ SEM) blood sugar levels and survival are shown.



**FIGURE 10.** Potentiation of ricin toxicity by RAC mAb 23. Mice were injected with 30  $\mu\text{g}/\text{kg}$  of ricin and the indicated dose of RAC mAb 23 or 1.6 mg/kg of an irrelevant isotype-matched Ab. Blood sugar was determined 13 h later, and survival was determined at 18 h.

## Discussion

The interaction of Ab with ricin is of interest because it is both a potential weapon of bioterrorism for which antidotes and preventatives could be necessary and a therapeutic agent whose immunogenicity may be a limit to its use. In this manuscript we have examined the ability of anti-ricin Abs to neutralize ricin cytotoxicity in vitro and protect against toxicosis in vivo and have correlated this with immunologic characteristics of the Abs. Anti-A chain Abs appear to be more protective than anti-B chain Abs after both active and passive immunizations. Among the anti-A chain Abs, neutralization ability correlates with avidity for Ag, with one notable exception. The exception is RAC mAb 23, which is a high avidity Ab that inhibits the enzyme activity of RAC, but fails to demonstrate any protection in vitro against the cytotoxic effect of ricin. Moreover, RAC mAb 23 actually enhances the in vivo toxicity of ricin. To our knowledge, this is the first description of in vivo Ab-mediated enhancement of toxin activity. The sole difference between RAC mAb 23 and other RAC mAbs is epitope specificity.

We have used a newly defined in vivo assay to measure the ability of Ab to protect against ricin toxicity (28). This assay was developed in response to concerns expressed by an institutional animal care and use committee regarding conventional, lethal challenge assay. To identify a surrogate end point, we periodically performed blood chemistry analyses after parenteral administration of a lethal dose of ricin toxin. Much to our surprise, the only metabolic abnormality detected was a profound drop in blood sugar, which is likely to be the cause of death. Blood sugars may be easily, inexpensively, and repetitively monitored on small volumes of blood (25–50  $\mu\text{l}$ ), and we have found this assay to be a useful replacement for the lethal challenge.

Ab may neutralize biological toxins in multiple ways. It may prevent the toxin from reaching the target cell. Binding of the toxin to the target cell may be blocked by Ab. The intracellular processing of the toxin necessary for it to reach its site of action may be altered, or the intracellular degradation of the toxin may be enhanced. Ab may block the enzymatic activity that results in the toxic effect. In an intact animal, Ab may enhance the clearance of the toxin from the circulation by Fc-mediated mechanisms. For an optimal antitoxin response, as many of these processes should be harnessed as possible.

In 1890, von Behring and Kitasato (1) first showed that the mechanism of serum-mediated (i.e., Ab) protection from the lethal effects of diphtheria and tetanus was due to toxin neutralization. That finding has served as the basis for immunization of generations of individuals against these two diseases. However, it was not until the elegant studies by Pappenheimer (34) described the struc-

ture/function relationships within the diphtheria toxin that it was possible to begin to understand the mechanism by which Ab neutralized the activity of the toxin. In a classic paper (2), he demonstrated that Abs to the cell-binding domain were able to neutralize diphtheria toxin, whereas Abs to the toxic domain were not. This finding was extended when it was shown that Abs to the A chain could protect cells if they were injected intracellularly, but not if they were present in the medium surrounding the cells (35). These studies provided a simple and understandable mechanism to explain Ab neutralization: Ab would attach to the cell-binding domain of a toxin and prevent the toxin from binding to the target cell. This explanation was so attractive that it is often presented in texts as the sole mechanism of Ab-mediated protection (3–6).

Despite this textbook explanation of the mechanism of Ab-mediated neutralization of toxins in which only anti-B chain Abs are effective, immunologists in the field have long known that the story is more complex. In fact, it was Pappenheimer who with coworkers demonstrated in 1974 (7) that Abs to either chain could neutralize toxins other than diphtheria (7). Using hybrid toxins consisting of RAC and the B chain of abrin, and vice versa, it was shown that anti-ricin or anti-abrin Abs, which would not cross-protect against the other holotoxin, provided both in vivo and in vitro protection against both hybrid toxins. Further, absorptions showed that A or B chain-specific antisera could provide protection against the appropriate holotoxin or hybrid toxin. In the discussion, the difference between these results and those with diphtheria toxin were commented upon. It was argued that perhaps the reason that anti-A chain Abs were ineffective in protecting against diphtheria toxin is that critical antigenic determinants were blocked from access to the Ab. In the years since, a number of investigators have used both polyclonal and mAbs and have clearly demonstrated the in vitro neutralizing and in vivo protective effects of Abs to the A chain of multiple different toxins, including diphtheria (8–13). Although it has been amply demonstrated that anti-A chain Abs can protect cells in vitro or animals in vivo, the mechanism(s) of this effect is not fully understood.

The role of Ab affinity in protection against toxins is well studied. In the evaluation of Ab responses to toxoid vaccines, the correlation between the affinity of Ab and serum neutralization is established (36, 37). More recently, a particularly elegant and thorough study using recombinant Abs to the protective Ag of anthrax toxin and surface plasmon resonance firmly demonstrated that the ability of Ab to protect macrophages from cytotoxic effect and rats from lethality was directly correlated with Ab affinity (38). No matter what the mechanism(s) by which Ab protects against intoxications, it is not difficult to understand why high affinity Abs



function better. Our results are generally consistent with this observation.

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