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Characterization of a Novel High-Affinity Monoclonal Immunoglobulin G Antibody against the Ricin B Subunit

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There is an urgent need for the development of a passive immunotherapy against the category B select agent ricin, a lethal ribosome-inactivating toxin composed of an enzymatic A subunit (RTA) and a single binding B subunit (RTB). To date, only one monoclonal antibody (MAb), a mouse immunoglobulin G (IgG1) against RTA called R70, has been deemed sufficiently potent in animal models to warrant further testing in humans. In this study, we have identified and characterized MAb 24B11, a murine IgG1 directed against RTB. In a Vero cell cytotoxicity assay, 24B11 was approximately two times more effective at neutralizing ricin than was R70. The equilibrium dissociation constants of 24B11 ($K_D = 4.2 \times 10^{-9}$ M) and R70 ($K_D = 3.2 \times 10^{-9}$ M) were virtually identical, suggesting that the difference in neutralization activity between the two MAbs was not due to differing affinities for the toxin. 24B11 blocked ricin attachment to galactoside receptors on primary mouse splenocytes and on the apical surfaces of human mucosal epithelial cell monolayers. Surprisingly, R70 also effectively interfered with ricin attachment to receptors on cell surfaces. Using a phage-displayed peptide library, we determined that 24B11 binds an epitope on RTB adjacent to, but not within, one of the two galactose binding domains. Finally, we demonstrate that R70 and 24B11, when combined, function synergistically to neutralize ricin in vitro, raising the possibility that these two MAbs could serve as a novel immunotherapeutic in vivo.

The National Institutes of Health and the Centers for Disease Control and Prevention consider the toxin ricin to be a public health threat (32). Ricin is lethal to humans upon injection, inhalation, or ingestion (2, 26) and has already proven to be an effective agent of bioterrorism both internationally and domestically (25). In February 2004, for example, an envelope containing ricin was sent to the office of U.S. Senate Majority Leader Bill Frist, forcing the evacuation of Senate staff members and the closure of the Capitol for 2 days (17). Ricin has also been found in the possession of individuals in New York; Oregon; North Carolina; California; Paris, France; and London, United Kingdom (5, 21). The toxin is of particular concern as a bioterrorism agent, because it is easily purified from castor beans in large quantities with the use of rudimentary-grade chemistry equipment and by the fact that there is currently no treatment available for intoxicated individuals.

Ricin (molecular weight, 64,000) is a relatively simple toxin consisting of an enzymatic A subunit (RTA) and a binding B subunit (RTB) joined by a disulfide bond (36). RTB is a bivalent lectin with specificity for glycoproteins and glycolipids containing β (1-3)-linked galactose or *N*-acetylgalactosamine residues (1). Once bound to cell surfaces, the toxin is internalized by endocytosis and is trafficked via vesicular retrograde transport to the endoplasmic reticulum (ER) (22, 42). The toxin's enzymatic subunit is then transported across the ER membrane and into the cytosol by a process known as retrotranslocation. Once within the cytosol, RTA selectively depurinates the highly conserved adenine residue in the so-called sarcin/ricin loop of 28S rRNA, thereby inactivating the cell's ribosomes and arresting protein synthesis (11).

Despite a sophisticated understanding of ricin at the cellular and molecular levels, the development of an antidote has proven elusive. Chemical inhibitors targeting RTA's active site have been identified but have had limited application due to issues of specificity, deliverability, and toxicity (3, 30). Antibody-based therapies, on the other hand, are more promising. For example, several studies have demonstrated that polyclonal antiricin antisera can protect mice from lethal doses of toxin administered intravenously (20) or as an aerosol (16). Neutralizing monoclonal antibodies (MAbs) against RTA and RTB have also been identified, although only a few have been deemed suitable for human development (38). The most potent MAb identified to date is called UNIVAX 70/138, hereafter referred to as R70, which is a mouse immunoglobulin G1 (IgG1) directed against RTA (20, 38). R70 was produced from animals immunized with ricin toxoid and was identified as being capable of protecting mouse leukemia cells from the cytotoxic effects of ricin in vitro. The in vitro neutralization activity of R70 proved to be consistent with its in vivo activity. Even so, R70 is inferior to polyclonal antisera at neutralizing ricin, suggesting that an optimal immunotherapy based on R70 should include additional MAbs (20).

The goal of our study was to identify and characterize MAbs that could potentially be developed in conjunction with R70 as an immunotherapeutic for the prevention and treatment of ricin intoxication. We were particularly interested in MAbs that interfere with the earliest steps in ricin's cytotoxic pathway, namely, attachment to cell surfaces. We report the identification of a MAb directed against RTB that neutralizes ricin slightly more effectively than does R70, at least in vitro. The MAb, called 24B11, blocked ricin attachment to galactoside receptors on mouse splenocytes and human lung epithelial

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cells. The most striking finding of this study was the fact that R70 and 24B11, when combined, functioned synergistically to neutralize ricin in vitro, raising the possibility that these two MAbs could serve as a novel immunotherapeutic in vivo.

MATERIALS AND METHODS

Ricin and other chemical reagents. Unlabeled and labeled derivatives of ricin (also known as *Ricinus communis* agglutinin II), the 120-kDa nontoxic lectin *Ricinus communis* agglutinin I (RCA-I), and polyclonal goat antiricin antiserum were purchased from Vector Laboratories (Burlingame, CA). Tween 20, broad-range molecular weight markers, and polyacrylamide were obtained from Bio-Rad (Torrance, CA). Paraformaldehyde (16%) solution was purchased from Electron Microscopy Sciences (Fort Washington, PA) and diluted 1:4 into phosphate-buffered saline (PBS) prior to use. All other chemicals were obtained from the Sigma Company (St. Louis, MO), unless noted otherwise. Dialysis was performed using Slide-a-lysers from Pierce Chemical (Rockford, IL).

Hybridomas and MAbs. Hybridoma 24B11 was derived from Peyer's patch and mesenteric lymph node lymphocytes from BALB/c mice immunized intragastrically with a mixture of ricin toxoid and RTB, as described in a separate study (27). Hybridoma 24B11 was cloned three times by limiting dilution. Initially cultured in a 1:1 mixture of RPMI medium and NCTC medium (Sigma) containing 10% fetal calf serum and penicillin-streptomycin, hybridoma 24B11 was eventually transitioned to CD Hybridoma serum-free, protein-free, antibiotic-free medium (Gibco-Invitrogen, Carsbad, CA). The hybridomas R70, originally described by Lemley and colleagues (20), and TFTB-1, originally described by Fulton and colleagues (12), were purchased from the ATCC (Manassas, VA) and were maintained in CD Hybridoma medium, as described above. Hybridoma 35H6 secretes a monoclonal IgA specific for RTB and is described in a separate study (27). The MAb MOPC-21, a murine IgG1 specific for phosphoryl choline, was purchased from Sigma.

Purification of MAbs 24B11 and R70. 24B11 and R70 IgGs were purified from serum-free, protein-free hybridoma supernatants by means of a HiTrap protein G-Sepharose column (GE Healthcare Life Sciences, Piscataway, NJ). Purity of the MAb preparations was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of each purified MAb was determined by absorbance spectroscopy (13). Antibody preparations were endotoxin free, as determined by the *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Enzyme-linked immunosorbent assays (ELISAs). Nunc Maxisorb F96 microtiter plates (Fisher) were coated with ricin, RTA, RTB, or RCA-1 (0.1 μg/well) in PBS (pH 7.4) overnight at 4°C, washed three times with PBS-Tween 20 (PBS-T; 0.05%, vol/vol), and blocked with goat serum (2%, wt/vol, in PBS-T) for 1 h, before being probed with primary Abs. Primary Abs were detected using horseradish peroxidase (HRP)-labeled goat anti-mouse IgG-specific polyclonal secondary antibodies (Southern Biotech) and TMB (3,3′,5,5′-tetramethylbenzidine) colorimetric substrate (Kirkegaard & Perry, Gaithersburg, MD). Microtiter plates were analyzed with a SpectroMax 250 microtiter spectrophotometer (Molecular Devices), interfaced with a personal computer running Softmax software. Averages and standard errors between replicate samples were calculated using Excel 2002 (Microsoft, Redmond, WA).

Ricin cytotoxicity assays. Vero cells (CCL-81; ATCC) were grown on 100-mm^2 petri dishes in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and were maintained in a humidified incubator (37°C, 5% CO_2). For cytotoxicity assays, Vero cells were removed from petri dishes by treatment with trypsin, collected by centrifugation, and then suspended in DMEM at a concentration of $\sim 1 \times 10^5$ cells per ml. The cell suspensions were dispensed into 96-well microtiter plates (100 μl/well) and incubated for 18 h at 37°C before being used for cytotoxicity assays. Cells were treated with ricin (1 ng/well) or ricin-MAb mixtures for 2 h, after which they were washed to remove unbound toxin and returned to the incubator for approximately 40 h. At the completion of the experiment, we determined the viability of the Vero cell population in each well using the nonradioactive MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) proliferation assay kit obtained from ATCC (30-1010K) (31). Cytotoxicity assays using J774A.1 (TIB-67; ATCC) and A549 (CCL-185; ATCC) cells were done essentially as described for the Vero cells, with the exception that J774A.1 cells were collected using a cell scraper, rather than being treated with trypsin.

Determination of MAb affinities by surface plasmon resonance. IgG binding kinetics were determined by measuring surface plasmon resonance in a BIA3000 unit (Biacore International AB, Uppsala, Sweden). Approximately 600 response units of ricin (5.0 μg/ml in 10 mM acetate buffer, pH 4.0) were coupled to a CM5 research grade chip using *N*-hydroxysuccinimide-*N*-ethyl-*N*-dimethylaminopropyl-carbodiimide chemistry. For collection of binding data, the chips were equilibrated for 3 min in running buffer 0.01 M HEPES (pH 7.4)–0.15 M NaCl–3 mM EDTA–0.005% surfactant P20 prior to injection of MAbs (concentrations of 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.125 nM, and 1.56 nM) at a constant flow rate of 30 μl/min. Dissociation was monitored over a period of 10 min, before the chips were regenerated with 10 mM glycine (pH 1.5) at a flow rate of 50 μl/min. The association and dissociation rate constants were calculated as done by others (34).

Ricin binding to polarized lung epithelial cell monolayers and splenocytes. For lung epithelial cell binding assays, biotinylated ricin (1 μg) was applied at 4°C to the apical sides of polarized A549 cell monolayers grown on 0.33-cm^2 Transwell inserts (3.0-μm pore size; Costar, Cambridge, MA). The experiments were performed at 4°C to permit toxin binding but not endocytosis. After 1 h of incubation, the monolayers were washed with cold Hanks balanced salt solution to remove unbound toxin, fixed with paraformaldehyde (4%, vol/vol, in PBS), blocked with 2% goat serum, and labeled with streptavidin-fluorescein isothiocyanate (FITC; 2 μg/ml; Pierce). Transwell filters were removed from the inserts by means of a razor blade and were placed right side up on glass microscope slides. Coverslips were mounted on the slides using VectaShield (Vector Labs). The cells were visualized using a Zeiss Axioskop II microscope equipped with epifluorescence. Images were captured using a charge-coupled device camera and then cropped and framed using Photoshop (Adobe Systems, Inc.).

Splenocytes were isolated following standard techniques from BALB/c mice killed by CO_2 asphyxiation (15). All experiments involving animals were approved by the Wadsworth Center Institutional Animal Care and Use Committee. Freshly isolated splenocytes were washed with serum-free DMEM, adjusted to $\sim 1 \times 10^5$ cells/ml, and then exposed to FITC-labeled ricin or a FITC-labeled ricin-MAb mixture for 1 h on ice. The cells were washed three times, fixed with 4% paraformaldehyde for 10 min, and then subjected to flow cytometry using a Becton Dickinson FACSCalibur located in the Wadsworth Center Immunology Core facility. A minimum of 10,000 cells were analyzed per sample.

Galactose and ASF binding assays. Nunc Maxisorb F96 microtiter plates were coated with galactosylated bovine serum albumin (BSA; 0.1 μg/well; EY Laboratories, San Mateo, CA) or asialofetuin (ASF; 0.4 μg/well) in PBS (pH 7.4) for 18 h at 4°C. Plates were washed with PBS-Tween 20 (0.05%, vol/vol), blocked with BSA (2%, wt/vol, in PBS-T), and then overlaid with biotinylated ricin (8.0 ng/ml) and IgA MAbs (20 μg/ml) for 1 h. The plates were washed to remove unbound toxin, labeled with avidin-HRP (0.4 μg/ml), and developed using TMB, as described above for ELISAs.

Epitope mapping using a random peptide phage-displayed library. The Ph.D.-7 phage-displayed peptide library (New England Biolabs [NEB], Beverly, MA) was used as recommended by the manufacturer. To determine the epitope specificity of MAb 24B11, we subjected the phage library ($\sim 2 \times 10^{11}$ phage) to three rounds of immunoaffinity selection (i.e., "panning") on polystyrene petri dishes (60 by 15 mm) coated with 24B11. Phage was eluted from the petri dishes by incubation with RTB (100 μg/ml) and was amplified by infection of *Escherichia coli* strain ER2738 (NEB). Purified phage was subjected to automated dideoxy DNA sequencing using an Applied Biosystems model 3700 DNA analyzer at the Molecular Genetics Core facility at the Wadsworth Center. All DNA sequencing reactions were initiated using the −96 gIII primer (5′-CCC TCA TAG TTA GCG TAA CG-3′) provided by NEB.

Western blot analysis. Ricin or RTB was solubilized in Laemmli sample buffer with or without β-mercaptoethanol (5%, vol/vol), boiled for 10 min, and then size-fractionated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes (0.45-μm pore size; Bio-Rad) via semidry electroelution. The membranes were washed with PBS-T, blocked for 1 h with BSA (2%, wt/vol, in PBS-T), and then incubated with monoclonal antibodies (2 μg/ml) for 1 h at room temperature. Membranes were probed with goat anti-mouse IgG or IgA conjugated to HRP (0.4 μg/ml) and were developed using the ECL kit (Amersham Pharmacia) and exposed to Kodak X-OMAT film (Fisher Scientific). When necessary, polyacrylamide gels were stained with Gel Code Blue (Pierce) to visualize proteins.

Production of Fab fragments. Fab fragments of MAbs R70 and 24B11 were produced using the ImmunoPure IgG1 Fab and $F(ab')_2$ preparation kit from Pierce Chemical. Fab preparations were dialyzed against PBS and analyzed for purity by SDS-PAGE. Fab preparations were further verified to be free of Fc contamination by Western blotting and ELISAs using goat anti-mouse Fc-specific antisera. The concentrations of Fab samples were determined using absorbance spectroscopy (13).

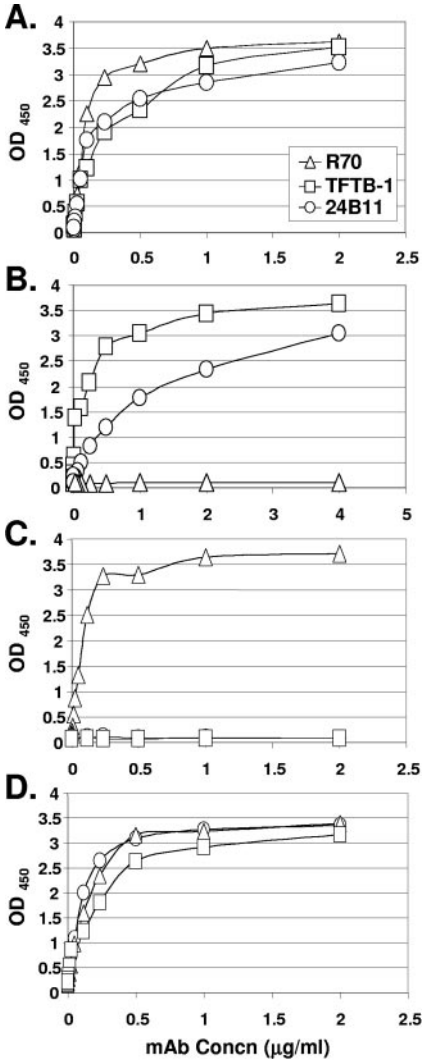

FIG. 1. Reactivity of 24B11 with ricin, RTB, RTA, and RCA-1 by ELISA. Microtiter plates were coated with (A) ricin, (B) RTB, (C) RTA, or (D) RCA-1 and then probed with MAb R70, TFTB-1, or 24B11 at the indicated concentrations, in a standard ELISA. Each datum point is the average from three wells.

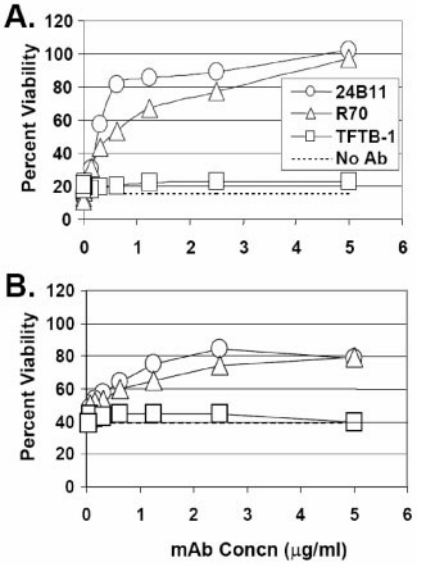

FIG. 2. 24B11 protects cells from the cytotoxic effects of ricin. Each of the MAbs 24B11, R70, and TFTB-1, at the indicated total concentrations, was incubated with ricin for 1 h and then applied in triplicate to Vero cells (A) or A549 cells (B) grown in 96-well microtiter plates. Forty hours later, the viability of the cells was determined by means of the MTT assay (see Materials and Methods). As a reference, the concentration of ricin required to induce 50% cytotoxicity (i.e., CD_{50}) of Vero cell cultures ranged between 0.1 to 1.0 ng/ml, whereas the CD_{50} for A549 cells exceeded 10 ng/ml. One hundred percent viability was defined as the MTT value obtained from cells not treated with ricin. Each datum point represents the average from three wells.

RESULTS

24B11: a monoclonal IgG specific for RTB. To identify neutralizing IgG MAbs against ricin, we generated B-cell hybridomas from the Peyer's patches and mesenteric lymph nodes of BALB/c mice immunized intragastrically with ricin toxoid and RTB, as described in a separate study (27). Of the several hundred hybridomas that we screened, approximately 40 secreted IgGs against ricin (data not shown). Based on a number of criteria that will be discussed below (e.g., subunit specificity, neutralization activity, MAb affinity) we chose hybridoma 24B11 for further investigation.

By ELISA, MAb 24B11 reacted with ricin holotoxin and purified RTB, but not RTA (Fig. 1A to C), thus demonstrating the specificity of 24B11 for the ricin B subunit. In parallel, we confirmed that R70 is specific for the toxin's enzymatic subunit. The MAb TFTB-1, originally described by Fulton and colleagues (12), bound ricin and RTB with a profile similar to that of 24B11. TFTB-1 is a nonneutralizing MAb, which we used as a negative control in the study. All three MAbs, 24B11, R70, and TFTB-1, also reacted with RCA-1, a nontoxic lectin from castor beans that shares significant amino acid identify with ricin (Fig. 1D) (39), thus demonstrating that the epitopes recognized by these MAbs are conserved between the two proteins. Antibody isotyping indicated that MAb 24B11 is an IgG1 antibody (data not shown).

24B11 neutralizes ricin more effectively than R70. We compared the abilities of 24B11 and R70 to neutralize ricin using a Vero cell cytotoxicity assay (18). Ricin (10 ng/ml) was incubated for 1 h with 24B11, R70, or TFTB-1, at a range of concentrations, and then applied in triplicate to Vero cells grown in 96-well microtiter plates. The viability of Vero cells was determined 40 h later by means of the MTT assay. 24B11 protected Vero cells from the cytotoxic effects of ricin in a dose-dependent manner, with an estimated 50% inhibitory concentration (IC_{50}) of 300 ng/ml (Fig. 2A). When the MAbs were compared directly, 24B11 was approximately two times more effective at neutralizing ricin than was R70, which had an estimated IC_{50} of 600 ng/ml. As expected, TFTB-1 failed to protect Vero cells from ricin intoxication, irrespective of MAb concentrations. 24B11 also protected A549 cells (Fig. 2B), a human lung epithelial cell-derived cell line, and J774 cells (data not shown), a mouse macrophage cell line, from the cytotoxic effects of ricin slightly better than R70 did. We conclude that 24B11 is at least as effective as R70 in neutralizing ricin in vitro.

TABLE 1. Association and dissociation rate constants and equilibrium dissociation constants for 24B11 and R70

MAb	K_D (M^{-1})	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})
24B11	4.2×10^{-9}	1.2×10^{5}	5.2×10^{-4}
R70	3.2×10^{-9}	1.1×10^{5}	3.5×10^{-4}

24B11 and R70 have similar affinities for ricin. We used surface plasmon resonance in a Biacore unit (see Materials and Methods) to determine the affinities of 24B11 and R70 for ricin. Biacore sensorgrams indicated that 24B11 and R70 had virtually identical association (k_{on}) and dissociation rates (k_{off}), as shown in Table 1. Correspondingly, the K_D values, calculated as k_{off}/k_{on}, were determined to be 3.2×10^{-9} M for R70 and 4.2×10^{-9} M for 24B11. These data suggest that the difference in neutralization activity between the two MAbs is not due to differences in their affinities for the toxin.

Ricin attachment to host cell surfaces is blocked by 24B11 (and R70). To examine whether 24B11 interfered with the ability of ricin to attach to host cell surfaces, we mixed FITC-labeled toxin with various concentrations of 24B11, R70, or MOPC-315 and then incubated the mixtures on ice with freshly isolated mouse splenocytes for 1 h. The cells were then washed and analyzed by flow cytometry to measure the amount of toxin bound to the cell surfaces. MOPC-315 is an IgG1 against phosphoryl choline and was used as a negative control before TFTB-1 was available to us. We observed that 24B11 reduces ricin binding to mouse splenocytes in a dose-dependent manner, whereas MOPC-315 at the same concentrations has no effect of toxin binding (Table 2). Unexpectedly, R70 inhibited ricin binding to splenocytes as effectively as did 24B11. These data demonstrate that both 24B11 and surprisingly R70 interfere with ricin attachment to cell surfaces.

To test whether 24B11 and/or R70 can prevent ricin attachment to mucosal epithelial cells, we applied MAb-toxin mixtures to the apical surfaces of polarized A549 cell monolayers grown on permeable Transwell inserts. These binding assays were performed at 4°C to permit toxin attachment but not endocytosis. In the absence of antibody, ricin labeled individual epithelial cells and clusters of cells with various degrees of intensity (Fig. 3A). Such a mosaic pattern of staining is likely due to a variation in glycosylation levels among individual cells within the monolayer. Preincubation of ricin with 24B11, on the other hand, reduced toxin binding to A549 cell monolayers to nearly undetectable levels (Fig. 3B). R70 also inhibited ricin binding (Fig. 3C), although to a slightly lesser extent than 24B11. Neither TFTB-1 nor MOPC-21 interfered with ricin attachment (Fig. 3D and E). We conclude that 24B11 (and R70) can inhibit ricin binding to mucosal epithelial cells.

24B11 recognizes an epitope within domain 1α of RTB. We used a phage-displayed peptide library to identify the epitope on RTB that is recognized by 24B11. As described in the Materials and Methods, a commercial library consisting of $>10^9$ random 7-mers displayed as fusion proteins on the surface of the filamentous phage M13 was subjected to three successive rounds of panning on 24B11. From the final elution step, we randomly selected and purified 20 phage. DNA sequence analysis of the phage-encoded peptides revealed a six-amino-acid consensus sequence consisting of P-X-X-S-X-T, where X is any amino acid (Fig. 4A). This consensus sequence aligned with RTB residues PCKSNT (38 to 43), located within the low-affinity galactose binding site of domain 1α (Fig. 4B). The P-X-X-S-X-T motif is also present in RCA-1, a fact consistent with our observation that 24B11 reacts with RCA-1 by ELISA (Fig. 1).

TABLE 2. 24B11 and R70 reduce ricin binding to mouse splenocytes

MAb concn (μg/ml)	Geometric mean fluorescence[a] for:		
	24B11	R70	MOPC-21
2.5	80	120	71
5.0	41	60	80
10	20	27	65
20	12	14	76

[a] Freshly isolated mouse splenocytes were incubated with FITC-labeled ricin on ice for 1 h, washed, and then subjected to flow cytometry. Shown are the geometric mean fluorescence values for 10,000 events.

The crystal structure of RTB indicates that the conformation of 24B11's putative epitope is constrained by a disulfide bond between cysteine residues 20 and 42 (41). We predicted, therefore, that treatment of RTB with reducing agents would abolish the binding of 24B11. To test this prediction, ricin and RTB were subjected to SDS-PAGE in the presence or absence of β-mercaptoethanol, transferred to nitrocellulose, and probed with 24B11. We observed that 24B11 bound both ricin and RTB under nonreducing conditions, but it failed to recognize ricin or RTB that had been treated with β-mercaptoethanol (Fig. 5). A control MAb, 35H6, bound RTB in its reduced and nonreduced forms (Fig. 5). These data demonstrate that 24B11 binds a linear epitope, most likely residues 38 to 43 within domain 1α, that is constrained by a disulfide bond.

The proximity of the putative 24B11 epitope to domain 1α's galactose binding site prompted us to examine whether the binding of 24B11 to RTB could be competitively inhibited with galactosides. Microtiter plates were coated with ricin and then preincubated with saturating concentrations of galactose (5 mg/ml), lactose (5 mg/ml), galactosylated BSA (1 mg/ml), or ASF (1 mg/ml) prior to the addition of 24B11. None of the ligands tested had any effect on MAb binding to ricin (data not shown). Similar results were obtained when the ligands were applied simultaneously with 24B11. We conclude that 24B11 recognizes an epitope adjacent to, but not within, the galactose binding site of domain 1α, and we propose that 24B11 blocks ricin attachment to cell surfaces by steric hindrance.

24B11 and R70 Fab fragments block ricin attachment to galactose. To examine whether the Fc domain of 24B11 is important in steric hindrance, we produced 24B11 Fab fragments and tested them for the ability to block ricin attachment to immobilized glycoproteins (Fig. 6A). In parallel, we also produced R70 Fabs, expecting them to be severely attenuated in their ability to interfere with ricin attachment to glycoprotein ligands. Microtiter plates were coated with the serum glycoprotein ASF and then probed with ricin or a ricin-MAb mixture. In agreement with our previous results, we observed that both 24B11 IgG and R70 IgG reduced ricin attachment to ASF in a dose-dependent manner with equal efficacies (Fig. 6B). Surprisingly, 24B11 and R70 Fabs were only slightly less effective than were the parental IgGs in blocking ricin attach-

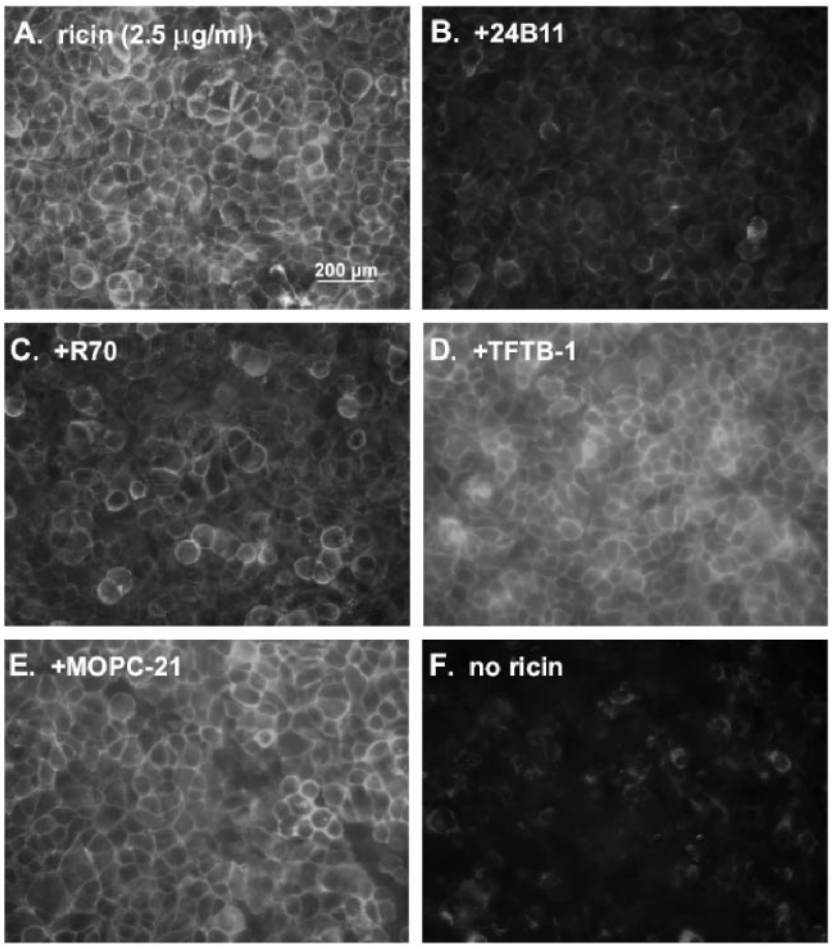

FIG. 3. 24B11 and R70 block ricin attachment to polarized human lung epithelial cells. Fluorescein-labeled ricin (2.5 μg/ml) was mixed with the indicated MAb and then applied at 4°C to the apical surface of A459 cells grown on permeable Transwell filters. After 1 h, the monolayers were washed, fixed with paraformaldehyde, and visualized en face using a Zeiss Axioskop II fluorescence microscope. A459 cell monolayers treated with (A) ricin in the absence of MAbs, (B) ricin plus 24B11, (C) ricin plus R70, (D) ricin plus TFTB-1, or (E) ricin plus MOPC-21, an IgG1 of irrelevant antigen specificity, or (F) received no ricin treatment. The scale bar (A) applies to all panels.

ment. These data demonstrate that 24B11 Fabs and R70 Fabs are sufficient to block ricin attachment to galactosides.

24B11 and R70 function synergistically to neutralize ricin. Because R70 and 24B11 recognize different toxin subunits, we rationalized that a combination of the two MAbs could be more effective than either of the individual MAbs in neutralizing ricin. To examine this possibility, we tested equal concentrations of 24B11, R70, or a 1:1 mixture of 24B11-R70 IgGs in a Vero cell cytotoxicity assay. We observed that the 1:1 mixture of 24B11-R70 IgGs consistently neutralized ricin 30 to 50% more effectively than did either unmixed 24B11 or R70 IgGs at the same concentration (Fig. 7A). For example, the viability of Vero cells treated with 0.6 μg/ml of 24B11-R70 was approximately 80%, whereas the viabilities of cells treated with the same total concentration of 24B11 or R70 were reduced to 50% and 30%, respectively. The synergism was also evident when we tested a 1:1 mixture of 24B11-R70 Fabs (Fig. 7B). Combining 24B11 or R70 with other antiricin MAbs in our collection did not result in enhanced neutralization activity (data not shown), demonstrating that the synergistic activity observed between 24B11 and R70 is not simply a consequence of mixing MAbs directed against RTA and RTB.

DISCUSSION

Recent bioterrorism incidents in the United States and abroad have alerted public health officials to the need to develop vaccines and therapies against pathogens and toxins previously deemed to be of little concern (38). The development of an effective immunotherapy against ricin has proven surprisingly elusive, despite the fact that dozens of MAbs against RTA and RTB have been described over the past 2 decades (6, 9, 10, 14, 20, 24). It is becoming apparent from the study of other toxins, notably botulinum and anthrax toxins, that the most effective antidotes will likely consist of oligoclonal combinations of high-affinity MAbs (or Fabs) capable of functioning cooperatively (23, 28). In this study we have characterized 24B11, a novel neutralizing mouse MAb that binds the ricin B subunit with high affinity. The MAb protected cells from ricin by interfering with the earliest step in the intoxication process:

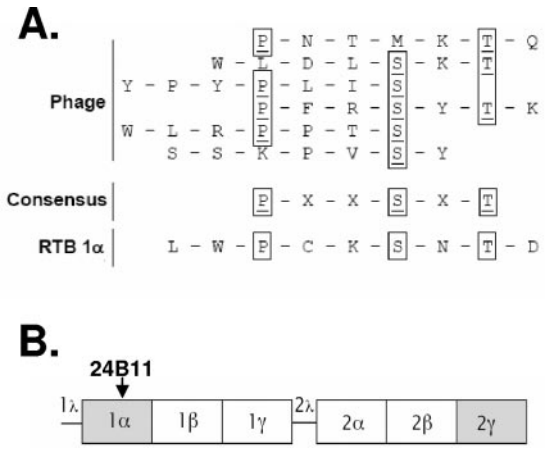

FIG. 4. 24B11 binds an epitope within domain 1α of RTB. (A) Alignment of representative amino acid sequences derived from a random phage-displayed peptide library panned on 24B11, as described in Materials and Methods. The consensus sequence (X-T-X-S-X-X-P) aligned with the sequence D-T-N-S-K-C-P from domain 1α of RTB. (B) Schematic of RTB, highlighting the protein's subdomains, as defined by Rutenber and Robertus (40). RTB is composed of two homologous domains (1 and 2), each divided into three "primordial" galactose binding elements (α, β, and γ). Only domains 1α and 2γ (shaded boxes) are able to bind galactose.

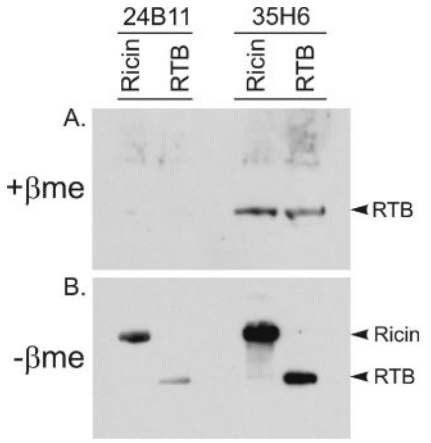

FIG. 5. 24B11 recognizes an epitope on RTB that is sensitive to reducing agents. Ricin or RTB was solubilized in Laemmli sample buffer either with (A) or without (B) β-mercaptoethanol (βme) and then subjected to SDS-PAGE and Western blot analysis. 24B11 reacted with nonreduced forms of ricin and RTB but not reduced forms. The control MAb 35H6 reacted with ricin and RTB in the presence or absence of β-mercaptoethanol.

attachment of ricin to cell surfaces. More importantly, we observed that 24B11 and R70, when combined, functioned synergistically to neutralize ricin in vitro, thus raising the possibility that these two MAbs could serve as a novel immunotherapeutic in vivo. Although comprehensive animal studies are beyond the scope of the present study, we are beginning to examine the ability of 24B11, both singly and in combination with R70, to neutralize ricin in mouse models of systemic and mucosal intoxication.

Prior to this study, the only MAb against RTB that has been characterized in any detail is 75/3B12 (8). While 75/3B12 and 24B11 show some similarities, it is clear that they are distinct from one another. For example, both 75/3B12 and 24B11 effectively block toxin attachment to galactosides. They differ in that the binding of 75/3B12 to RTB is competitively inhibited by the addition of lactose, galactose, or galactose-containing glycoproteins, whereas the binding of 24B11 to RTB is not. It was proposed that 75/3B12 recognizes an epitope that is within one (or possibly both) of the galactose recognition domains on RTB (8). In contrast, we postulate that 24B11 associates with residues adjacent to, but not directly involved in, sugar binding. As further evidence that 24B11 and 75/3B12 are distinct, we have demonstrated here that 24B11 functions synergistically with R70 to neutralize ricin, at least in vitro. In contrast, Lemley and colleagues failed to observe any cooperative activity between 75/3B12 and R70 (20). Although other MAbs against RTB have been described, they have not been characterized in sufficient detail for us to determine whether any of them are similar (or identical) to 24B11 (24).

We identified the epitope recognized by 24B11 as a small solvent-exposed six-amino-acid loop within domain 1α, adjacent to one of RTB's two galactose binding domains. Based on this information, we propose that 24B11 neutralizes ricin by steric hindrance (i.e., by physically obstructing the access by ricin to glycolipids and glycoproteins on cell surfaces). However, the exact mechanism by which steric hindrance is achieved remains unclear. RTB is composed of six primordial galactoside binding motif repeats, of which only two, 1α and 2γ, remain functional (Fig. 4B). Selective ablation of domains 1α and 2γ by genetic or biochemical methods has revealed that both domains must be inactivated for RTB's ability to attach to cells to be abolished (43, 44). Mutant proteins with only one functional galactose binding domain remain cytotoxic. Because domains1α and 2γ are on the extreme ends of RTB and are separated by approximately 75Å, it is unlikely that a single MAb (or Fab) can obstruct both sites simultaneously. Rather, we propose that 24B11 binds to RTB in an orientation such that it occupies the entire "face" of the toxin that would normally make contact with cell surfaces. Through this interaction, 24B11 could serve as a physical barrier between ricin and the cell surfaces, thereby reducing the likelihood of receptor engagement and repressing the efficiency of uptake to below cytotoxic levels.

It is interesting that TFTB-1 did not interfere with ricin attachment to cell surfaces, despite the fact that TFTB-1 bound to RTB as effectively as did 24B11, as judged by ELISA (Fig. 1). Considering that the molecular mass of IgG (~150,000 kDa) is five times greater than the mass of RTB (~29,000 kDa), we might expect that the association of any MAb with RTB would suffice to interfere with toxin attachment. This clearly was not the case. Furthermore, the inability of TFTB-1 to protect Vero cells from ricin intoxication indicates that TFTB-1 has no adverse effects on the retrograde transport of ricin holotoxin to the ER. This finding is somewhat surprising, considering that RTB is proposed to play an active role in intracellular trafficking of ricin (35). We have tentatively mapped the epitope recognized by TFTB-1 to RTB's domain 2γ (C. McGuinness and N. Mantis, unpublished results), a region that is involved in galactose binding. These data suggest that rather subtle differences in epitope specificity may dictate whether a MAb is neutralizing or not.

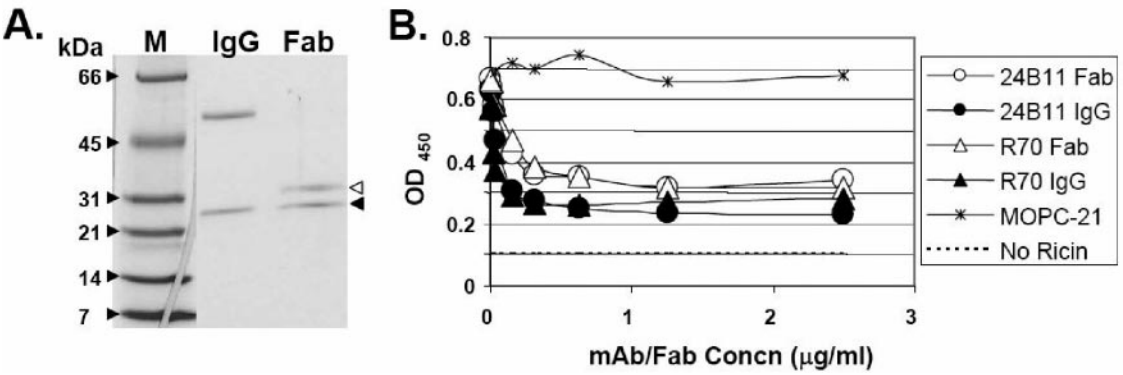

FIG. 6. 24B11 and R70 IgGs and Fab fragments block ricin attachment to ASF. (A) Purity of the 24B11 IgG and Fab preparations. 24B11 IgG and Fab preparations were solubilized in Laemmli sample buffer with β-mercaptoethanol and then subjected to SDS-PAGE. The IgG preparation migrated as two bands of approximately 50 and 25 kDa, corresponding to the antibody heavy and light (solid arrowhead) chains, respectively. The Fab preparation migrated as two bands of approximately 30 and 25 kDa. The 25-kDa band corresponded to the Fab light chains, whereas the 30-kDa band (open arrowhead) agrees with the expected size of the truncated heavy chains. (B) 24B11 and R70 IgGs and Fabs block ricin attachment to the galactose-containing glycoprotein ASF. Biotinylated ricin was mixed with IgGs or Fab fragments at the indicated concentrations and then applied to microtiter plates coated with ASF. The plates were washed and probed with avidin-HRP and TMB to detect the ricin bound to the wells. MOPC-21, an IgG of irrelevant specificity, was used as a negative control. The horizontal dashed line indicates the amount of activity detected in wells not treated with ricin. OD_{450}, optical density at 450 nm.

Although R70 was identified more than a decade ago, the mechanism by which this MAb neutralizes ricin remains unknown (20, 38). The major B-cell-neutralizing epitopes on RTA have been tentatively mapped to two regions that are conserved within the family of ribosome-inactivating proteins: residues 95 to 100 (19) and residues 161 to 175 (4, 9, 24). R70 presumably binds to the former (19), although this information provides few clues as to the mechanism by which the MAb inactivates ricin. It is generally assumed that antibodies against RTA exert their neutralizing activity intracellularly, after ricin has bound to cell surface galactosides (24). Contrary to this assumption, we observed that R70 blocked ricin binding to ASF, mouse splenocytes, and human lung epithelial cells as efficiently as did 24B11. The ability of an antibody against RTA to block ricin attachment to cell surfaces is not unique to R70, as we have recently identified three other anti-RTA MAbs with this property (C. McGuinness, J. Park, and N. Mantis, unpublished data). From our studies it is unclear whether R70's potency as a neutralizing MAb is solely due to its ability to interfere with toxin attachment or whether the MAb has additional, as yet undiscovered functions.

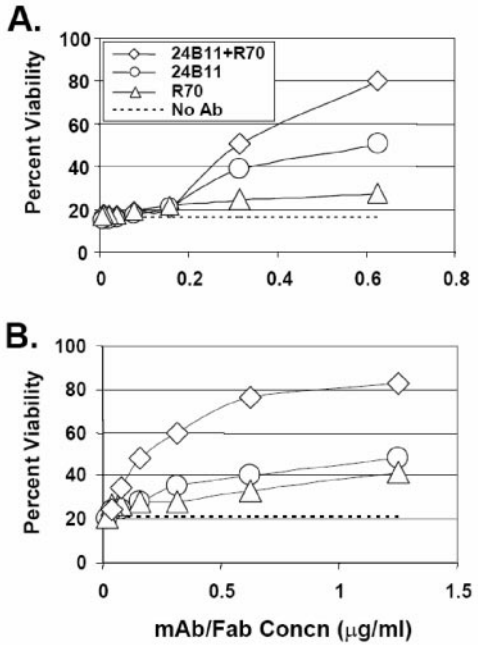

FIG. 7. 24B11 and R70 function synergistically to neutralize ricin in vitro. 24B11, R70, or a 1:1 mixture of 24B11-R70 IgGs (A) or Fabs (B) at the indicated total concentrations was incubated with ricin for 1 h and then applied in triplicate to Vero cells grown in 96-well microtiter plates. Forty hours later, cell viability was measured by MTT assay.

In the absence of comprehensive animal studies, it is somewhat premature to speculate on the potential use of the combination of R70 and 24B11 as a human immunotherapeutic to prevent or treat ricin intoxication. Nonetheless, the demonstration that R70 and 24B11 can function synergistically to neutralize ricin in vitro provides a rational for the examination of oligoclonal antibody combinations in vivo. Indeed, precedent-setting work by Marks and colleagues (28, 34) exemplifies the benefit of using double- and triple-antibody mixtures over monoclonal preparations, at least in the case of botulinum neurotoxin. The fact that 24B11 and R70 Fab fragments retain neutralization activity makes them particularly amenable to future development as therapeutics. For example, recent advances in the display of functional single-chain Fvs and Fabs on the surface of filamentous bacteriophage have revolutionized the field of antibody engineering, to the extent that is it now possible to increase the affinity of an antibody to its target by 10- to 100-fold (7, 29, 33, 37). Fab fragments are also preferable to IgGs as antidotes against toxins, in that they may have an increased therapeutic index in vivo due to greater tissue access, their faster clearance rates, and their incapacity to activate potentially harmful complement-driven inflammatory cascades.

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