INFECTION AND IMMUNITY, June 2006, p. 3455–3462 Vol. 74, No. 6
0019-9567/06/$08.00+0 doi:10.1128/IAI.02088-05

Immunoglobulin A Antibodies against Ricin A and B Subunits Protect Epithelial Cells from Ricin Intoxication

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Received 28 December 2005/Returned for modification 8 February 2006/Accepted 28 February 2006

Epithelial cells of the respiratory and gastrointestinal tracts are extremely vulnerable to the cytotoxic effects of ricin, a Shiga-like toxin with ribosome-inactivating properties. While mucosal immunity to ricin correlates with secretory immunoglobulin A (IgA) antibody levels in vivo, the potential of IgA to protect epithelial cells from ricin in vitro has not been examined due to the unavailability of well-defined antitoxin IgA antibodies. Here we report the characterization of four monoclonal IgA antibodies (IgA MAbs) produced from the Peyer's patches and mesenteric lymph nodes of BALB/c mice immunized intragastrically with ricin toxoid. Two IgA MAbs (33G2 and 35H6) were active against ricin's lectin subunit (RTB), and two (23D7 and 25A4) reacted with the toxin's enzymatic subunit (RTA). All four IgA MAbs neutralized ricin in a Vero cell cytotoxicity assay, blocked toxin-induced interleukin-8 release by the human monocyte/macrophage cell line 28SC, and protected polarized epithelial cell monolayers from ricin-mediated protein synthesis inhibition. 33G2 and 35H6 reduced ricin binding to the luminal surfaces of human intestinal epithelial cells to undetectable levels in tissue section overlay assays, whereas 23D7 had no effect on toxin attachment. 23D7 and 25A4 did, however, reduce ricin transcytosis across MDCK II cell monolayers, possibly by interfering with intracellular toxin transport. We conclude that IgA antibodies against RTA and RTB can protect mucosal epithelial cells from ricin intoxication.

Recent bioterrorism incidents in the United States and abroad have alerted public health officials to the need for vaccines and therapies against pathogens and toxins previously deemed to be of little concern (2, 7, 27). Ricin, for example, is an extremely potent toxin that is easily purified in high concentrations from its natural source, the castor bean (*Ricinus communis*). 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 (32). Its potency is attributed in part to the fact that RTB is a bivalent lectin with specificities for ubiquitous glycoproteins and glycolipids containing β(1-3)-linked galactose or *N*-acetylgalactosamine residues (4), which enable the toxin to bind and be internalized by all known cell types. The toxic properties of ricin are further compounded by RTA, which is an extremely efficient *N*-glycosidase specific for a highly conserved adenine residue in the so-called sarcin/ricin loop of eukaryotic 28S rRNA (9). Due to the absence of a specific vaccine or therapy, treatment of individuals intoxicated with ricin is strictly supportive (3).

As an agent of bioterrorism, it is feared that ricin will be disseminated by aerosol or food/water supplies, thereby potentially exposing humans via the respiratory or gastrointestinal tract. It is known from animal studies that both mucosal compartments are susceptible to ricin intoxication. Monkeys or rodents exposed to aerosolized ricin (~20 to 40 μg/kg) develop necrotizing airway lesions, interstitial edema, pulmonary inflammation, and widespread damage to the respiratory epithelium (6, 45). Rodents exposed to ricin (5 to 10 mg/kg) by the intragastric route develop widespread villus atrophy, mucus secretion, swelling of interepithelial spaces, separation of the epithelium from the lamina propria, and sloughing of enterocytes from the tips of villi (39; J. M. Yoder and N. Mantis, unpublished data). In both situations, it is clear that epithelial cells are a primary target for ricin.

Vaccinologists have long appreciated the need to stimulate mucosal immunity against ricin in both the respiratory and gastrointestinal tracts to confer protection against a mucosal toxin challenge (19, 21, 22, 27, 34, 46). This is best exemplified by the fact that mice vaccinated parenterally with ricin toxoid (RT) are immune to ricin administered systemically but are only partially protected against ricin administered as an aerosol (19, 34). Mucosal immunity to ricin was achieved in the respiratory tract when animals were immunized intranasally or intragastrically (i.g.), correlating with the production of secretory immunoglobulin A (S-IgA) antibodies in bronchoalveolar lavage fluids (15, 16, 22, 46). However, direct proof that S-IgA can safeguard mucosal epithelia from ricin intoxication is lacking.

In this paper, we describe the production and characterization of four monoclonal IgA antibodies (IgA MAbs) derived from the Peyer's patches and mesenteric lymph nodes of mice immunized intragastrically with RT. Two of the IgA MAbs were directed against RTA (23D7 and 25A4), and two were directed against RTB (33G2 and 35H6). Each of the four IgA MAbs was capable of protecting polarized epithelial cell monolayers from ricin intoxication, although apparently by different mechanisms. These data demonstrate for the first time that IgA antibodies against RTA and RTB can neutralize

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ricin in vitro, and they implicate this class of antibodies as a first line of defense against ricin in vivo.

MATERIALS AND METHODS

Chemicals and reagents. Unlabeled and biotinylated derivatives of ricin (*Ricinus communis* agglutinin II), as well as polyclonal goat anti-RCA-I/II antiserum, were purchased from Vector Laboratories (Burlingame, CA). Hanks balanced salt solution, HEPES, horseradish peroxidase (HRP) conjugated to avidin, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Tween 20 was obtained from Bio-Rad (Torrance, CA), and paraformaldehyde (16%) was purchased from Electron Microscopy Sciences (Fort Washington, PA). Mowiol was purchased from Calbiochem, and mounting medium containing 1,2-diazobicyclo-[2,2,2]-octane (DABCO; Sigma) was made as described previously (18). All media for cell culture were prepared by the Wadsworth Center media facility.

Production and screening of antiricin IgA MAbs. Female BALB/c mice of approximately 8 weeks of age were primed by intraperitoneal injection with RTB (50 μg) on day 0 and then immunized i.g. on days 17 and 27 with a cocktail (0.5 ml) containing RT (250 μg), RTB (50 μg), cholera toxin (5 μg), and a mixture of protease inhibitors consisting of 4-(2-aminoethyl)benzenesulfonylfluoride-HCL (500 μM), aprotinin (150 nM), E-64 (1 μM), EDTA (500 μM), and leupeptin (1 μM) (Calbiochem). On day 34, all mice had detectable antiricin IgG and IgA Abs in their sera and feces, as determined by an enzyme-linked immunosorbent assay (ELISA) (data not shown). Mice were boosted on day 37 and sacrificed on day 42. Total Peyer's patch and mesenteric lymph node lymphocytes from immunized animals were fused with the myeloma cell line P3X63.Ag8.653 (CRL-1580; American Type Culture Collection [ATCC], Manassas, VA), as done by others (1). Hybridomas (0.1 ml) were seeded into 96-well microtiter plates with a layer of irradiated MRC-5 cells (ATCC) (applied 24 h earlier) and were then fed daily for 10 days. Hybridoma supernatants were screened by ELISA for IgA and IgG antibodies that reacted with ricin holotoxin. Hybridomas from positive wells were cloned by limiting dilution a minimum of three times (17). Stable hybridomas were initially cultured in a 1:1 mixture of RPMI 1640 and NCTC-109 containing 10% fetal bovine serum plus penicillin-streptomycin and were eventually transitioned to CD Hybridoma serum-free, protein-free medium (Gibco-Invitrogen, Carlsbad, CA) without antibiotics. Three independent fusions with six mice each were performed for this study. RT was produced essentially as described by Yan and colleagues (46).

ELISAs. NUNC Maxisorb F96 microtiter plates (Krackeler Scientific, Albany, NY) were coated overnight at 4°C with 0.1 μg of ricin (or RTA or RTB) per well in a volume of 0.1 ml in phosphate-buffered saline (PBS; pH 7.4). Microtiter plates were washed with PBS-Tween 20 (PBS-T; 0.05% [vol/vol]), blocked with goat serum (2% [wt/vol] in PBS-T), and overlaid with serum or fecal extracts diluted in blocking solution. Secondary goat anti-mouse IgG- and IgA-specific antibodies labeled with HRP were obtained from Southern Biotech (Birmingham, AL). ELISA plates were developed with one-component TMB colorimetric substrate (Kirkegaard & Perry, Gaithersburg, MD) and were read using a SpectraMax 250 microtiter plate reader equipped with Softmax software (Molecular Devices, Union City, CA). Averages and standard errors (SE) between duplicate samples were calculated using Softmax and Excel 2003.

Western blot analysis. Ricin, RTA, or RTB was boiled for 10 min in Laemmli sample buffer (with or without 5% [vol/vol] β-mercaptoethanol), size fractionated in precast sodium dodecyl sulfate (SDS)-12% polyacrylamide gels (Bio-Rad), and transferred to nitrocellulose membranes (0.45-μm pore size; Bio-Rad) via semidry electroelution. Nitrocellulose membranes were blocked with goat serum or BSA (2% [wt/vol] in PBS-T) and were then incubated with IgA MAbs (~2 μg/ml) for 1 h at room temperature. Membranes were then probed with goat anti-mouse IgA conjugated to HRP (0.4 μg/ml; Southern Biotech), developed using an enhanced chemiluminescence (ECL) detection kit (Bio-Rad), and exposed to Kodak X-OMAT film (Fisher Scientific). Polyacrylamide gels were routinely stained with Gel Code Blue (Pierce) to visualize proteins.

Vero cell cytotoxicity assays. Vero cells (CCL-81; ATCC) were grown routinely in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and were maintained in a humidified incubator (37°C, 5% CO_2). For cytotoxicity assays, Vero cells were trypsinized, seeded into 96-well microtiter plates (~1×10^4 cells per well in 100 μl), and incubated for 18 h at 37°C. Vero cells in triplicate were then overlaid with ricin (1 ng) or ricin-MAb mixtures for 2 h, washed to remove unbound toxin, and scored 40 h later for viability using a nonradioactive 3-(4,5-dimethylthiazolyl-z)-2, 5-diphenyltetrazolium bromide proliferation assay kit (ATCC) (31).

IL-8 secretion by 28SC cells in response to ricin. The human monocyte/macrophage cell line 28SC (CRL-9855; ATCC) was propagated in Iscove's modified DMEM supplemented with 4 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.02 mM thymidine, 0.1 mM hypoxanthine, 0.05 mM β-mercaptoethanol, and FBS (10% [vol/vol]), as described previously (14). For interleukin-8 (IL-8) assays, semiconfluent monolayers of 28SC cells (1×10^6 cells per well) in six-well tissue culture plates were overlaid with ricin (400 ng) or ricin-MAb mixtures for 2 h, washed, and then incubated in fresh toxin-free medium for an additional 40 h. IL-8 levels in cell supernatants were determined using a human IL-8 ELISA kit from Bender Medsystems (San Bruno, CA).

Caco-2 and MDCK II cytotoxicity assays. Caco-2_{BBe1} (CRL-2102; ATCC) and MDCK II (CCL-34; ATCC) cells were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS. To establish confluent, polarized cell monolayers, we seeded MDCK II cells (1×10^5) on 0.33-cm^2 Transwell inserts (3.0-μm pore size; Costar, Cambridge, MA) and incubated them for approximately 4 days. Caco-2 cells were treated similarly, except that they were seeded onto Transwell inserts pretreated with rat tail collagen and then incubated for 10 to 14 days (12). Typically, transepithelial resistance values, as measured using an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL), exceeded 200 Ω for MDCK II monolayers and 400 Ω for Caco-2 monolayers at the time of use.

For protein synthesis inhibition assays, azide-free ricin (30 to 300 ng) was mixed with control or antiricin IgA MAbs (2 μg) in DMEM (0.2-ml final volume) for 1 h at room temperature and then applied to the upper chamber of a Transwell filter containing an MDCK II or Caco-2 cell monolayer at 37°C. The monolayers were washed with Hanks balanced salt solution 2 h later to remove unbound toxin and incubated overnight in fresh DMEM. Cells were then rinsed with methionine-cysteine-free DMEM and labeled for 2 h with [^{35}S]methionine-cysteine (10 μCi/ml) (Translabel; ICN). After repeated washings, the Transwell filters were cut out from the plastic inserts, immersed in liquid scintillation fluid, and subjected to scintillation counting. The results were expressed as percentages of the incorporation in control samples not treated with toxin.

Ricin binding to human duodenal tissue sections. Paraffin sections of human duodenal biopsies were obtained from the Department of Pathology at the Children's Hospital with prior approval from the Internal Review Board. Biotinylated ricin (10 μg/ml) was incubated with IgA MAbs (20 μg/ml) for 1 h at room temperature before being applied to deparaffinized tissue sections (5-μm thick) of human duodenum. After 1 h, sections were washed with PBS and labeled with streptavidin-fluorescein isothiocyanate (streptavidin-FITC) (50 μg/ml; Pierce). Sections were fixed with 4% paraformaldehyde for 10 min, mounted with glass coverslips, and visualized by fluorescence microscopy.

BSA and ASF binding assays. Nunc Maxisorb F96 microtiter plates were coated with galactosylated 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-T (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 then washed to remove unbound toxin, labeled with avidin-HRP (0.4 μg/ml), and developed using TMB, as described above.

Ricin transepithelial transport assays. We measured the transepithelial transport of ricin across MDCK II cell monolayers by using a modification of a previously described protocol (43). Biotinylated ricin (0.2 μg) and IgA MAbs (4 μg) were diluted in DMEM to a final volume of 0.2 ml and applied to the apical polarized surfaces of MDCK II cell monolayers grown on porous Transwell inserts (0.33 cm^2). Eighteen hours later, biotinylated ricin in the lower chambers of the Transwell inserts (i.e., basolateral supernatants) was recovered using avidin-agarose beads (Pierce Chemical). The beads were boiled in Laemmli sample buffer with β-mercaptoethanol (5% [vol/vol]), subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (0.45-μm pore size; Bio-Rad) via semidry electroelution. Biotinylated ricin bound to the nitrocellulose membranes was detected by blotting with avidin-HRP followed by ECL, as described above.

RESULTS

Production of IgA MAbs against RTA and RTB. In a separate study, we showed that i.g. immunization of mice with RT induces antiricin IgA antibodies in serum and fecal extracts and confers protection against subsequent i.g. ricin challenge (J. M. Yoder, O. Sonuyi, and N. Mantis, unpublished data). To test whether mucosal IgA antibodies elicited following RT immunization are capable of protecting epithelial cells from

TABLE 1. Monoclonal IgA Abs used in this study

Antibody	Specificity[a]	Source
23D7	RTA	This study
25A4	RTA	This study
33G2	RTB	This study
35H6	RTB	This study
MOPC-315	DNP	Sigma
TEPC-15	PC	Sigma

[a] DNP, dinitrophenyl; PC, phosphorylcholine.

ricin intoxication, we generated a collection of B-cell hybridomas derived from the Peyer's patches and mesenteric lymph nodes of RT-vaccinated BALB/c mice. Of the several hundred hybridomas that we screened from three independent fusions, we identified 24 that secreted IgAs reactive with ricin. We also identified 36 hybridomas secreting antiricin IgGs capable of neutralizing ricin (C. McGuinness and N. Mantis, unpublished data). Based on a number of criteria (e.g., subunit specificity, neutralization activity, level of antibody production, and sustained growth in culture), we chose four IgA MAbs for further study: two (23D7 and 25A4) are specific for RTA, and two (33G2 and 35H6) are specific for RTB (Table 1).

Antiricin IgA MAbs recognize distinct epitopes on RTA and RTB. IgA MAbs 23D7, 25A4, 33G2, and 35H6 bound to ricin with virtually identical profiles, as determined by ELISA (Fig. 1A). By using plates coated with purified ricin subunits, we determined that 23D7 and 25A4 are specific for RTA, whereas 33G2 and 35H6 are specific RTB (Fig. 1B and C). 23D7 bound RTA less well than did 25A4, even though the two MAbs recognized the holotoxin with similar affinities (Fig. 1B). We speculate that 23D7 recognizes an epitope on RTA that is affected by the presence of the toxin's B subunit. While 33G2 and 35H6 were indistinguishable from one another by ELISA, Western blot analysis revealed that the two MAbs are in fact distinct from each other. 35H6 recognized RTB in its reduced form, whereas 33G2 did not (Fig. 2).

Anti-RTA and anti-RTB IgA MAbs neutralize ricin in vitro. The ability of 23D7, 25A4, 33G2, and 35H6 to neutralize ricin was tested in a Vero cell cytotoxicity assay. We chose Vero cells as a reporter cell line because they are extremely sensitive to Shiga-like toxins, including ricin (23, 44). Ricin (10 ng/ml) was mixed with antiricin IgAs or TEPC-15, a control murine IgA MAb with irrelevant specificity (Table 1), at a range of concentrations and then applied in triplicate to Vero cell monolayers grown in 96-well microtiter plates. The viability of the Vero cell monolayers was determined 40 h later by means of the MTT assay (10, 31). We observed that all four antiricin IgA MAbs, 23D7, 25A4, 33G2, and 35H6, were capable of neutralizing ricin, albeit with various degrees of efficacy (Fig. 3). In contrast, TEPC-15 had no detectable neutralization activity at any of the concentrations tested. 25A4 (anti-RTA) and 35H6 (anti-RTB) were the most potent neutralizing MAbs, with each having an estimated 50% inhibitory concentration of 80 ng/ml.

We recently reported that the human monocyte/macrophage cell line 28SC secretes IL-8 when exposed to ricin (14). To test whether 23D7, 25A4, 33G2, and 35H6 could suppress the toxin-induced production of proinflammatory cytokines, ricin (200

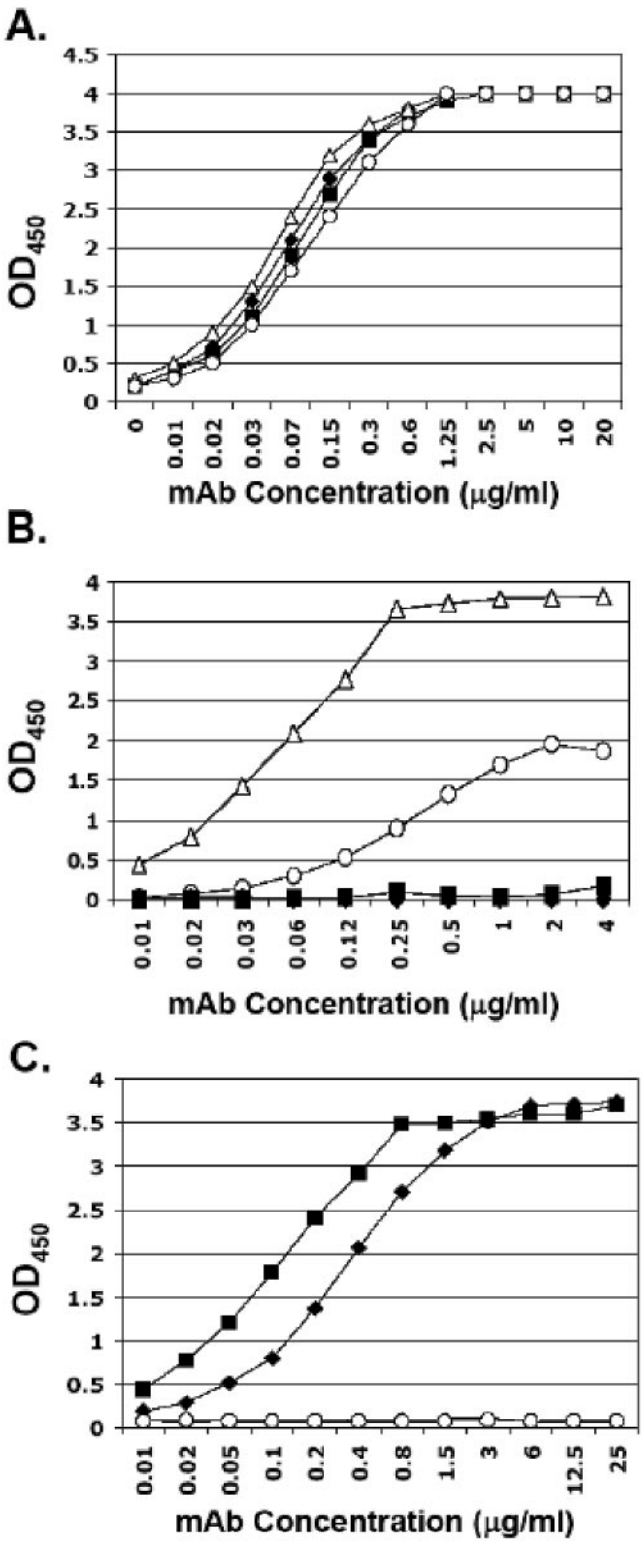

FIG. 1. Specificities of IgA MAbs for ricin and ricin subunits. ELISAs were performed using microtiter plates coated with purified (A) ricin, (B) RTA, or (C) RTB, and plates were probed with the indicated concentrations of IgA MAbs. The following IgA MAbs were tested: 25A4 (open triangles), 23D7 (open circles), 35H6 (filled diamonds), and 33G2 (filled squares). OD_{450}, optical density at 450 nm.

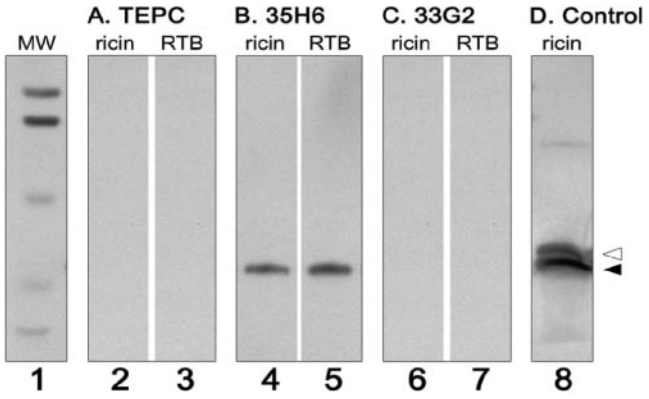

FIG. 2. Differential reactivities of 35H6 and 33G2 with RTB by Western blotting. Ricin and RTB were solubilized in SDS sample buffer containing β-mercaptoethanol, size fractionated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were probed with one of the IgA MAbs (10 μg/ml) or with polyclonal goat antiricin antisera (1:2,000) and were developed with appropriate HRP-labeled secondary reagents. Lanes: 1, molecular weight markers; 2 and 3, TEPC-15; 4 and 5, 35H6; 6 and 7, 33G2; and 8, goat antiricin. The arrowheads to the right of lane 8 indicate the positions of RTA (open) and RTB (filled).

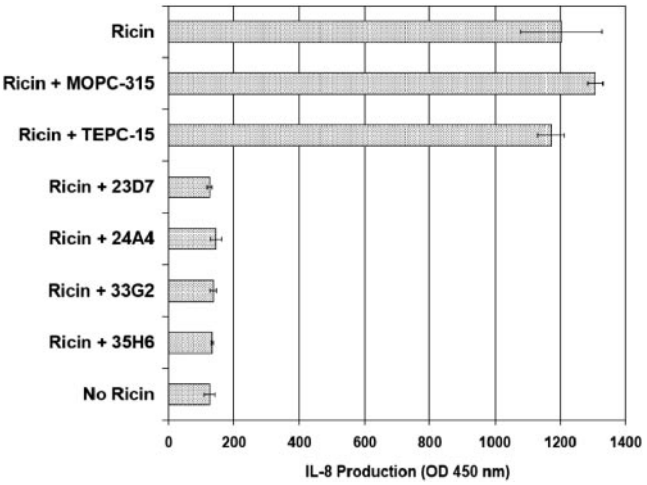

FIG. 4. Antiricin IgA MAbs suppress ricin-induced IL-8 secretion by human monocytes/macrophages. Ricin (200 ng/ml) was incubated with control (TEPC-15 and MOPC-315) or antiricin (20 μg/ml) IgA MAbs for 1 h and then applied in triplicate to 28SC cells grown in six-well microtiter plates. Approximately 36 h later, the cell supernatants were collected and assayed for IL-8 by ELISA. Each bar represents the average, with SE, of triplicate samples.

ng) was mixed with each of the antiricin IgA MAbs (20 μg) or each of two control IgA MAbs (TEPC-15 and MOPC-315) (Table 1) and then applied to 28SC cells grown in six-well tissue culture plates. IL-8 levels were measured by ELISA in cell supernatants 40 h later. We detected a 10-fold increase in IL-8 level in the supernatants of cells treated with ricin compared to the levels in untreated control cells (Fig. 4). A similar increase was observed for cells treated with ricin and TEPC-15 or MOPC-315. In contrast, there was no detectable increase in IL-8 level over the background level in cell supernatants exposed concomitantly to ricin and 23D7, 25A4, 33G2, or 35H6. These data indicate that all four of the antiricin IgA MAbs are capable of preventing toxin-induced cytokine release by 28SC cells.

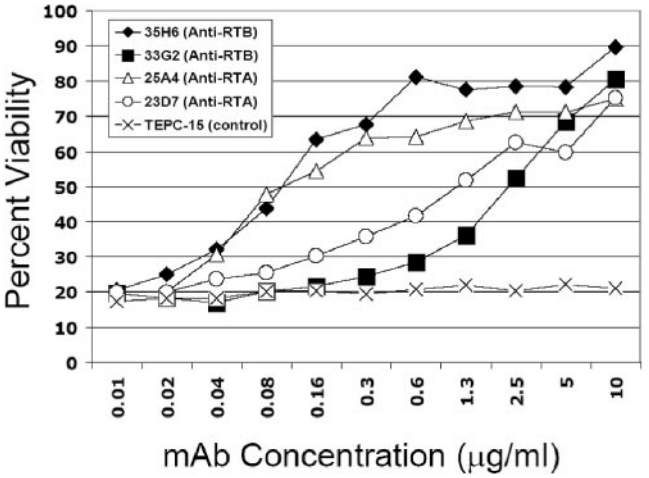

FIG. 3. Neutralization activities of IgA MAbs in a Vero cell cytotoxicity assay. Ricin (10 ng/ml) was incubated at room temperature for 1 h with IgA MAbs at the indicated concentrations and then applied in triplicate to Vero cells ($\sim 2 \times 10^5$) grown in 96-well microtiter plates. Cell viability was measured 40 h later by the MTT assay. Cells grown in parallel but not treated with ricin were used to define 100% viability. Each datum point on the graph represents the average of triplicate samples.

Anti-RTA and anti-RTB IgA MAbs neutralize ricin on the apical surfaces of polarized epithelial cell monolayers. Application of ricin to the apical surfaces of polarized epithelial cell monolayers (e.g., MDCK and Caco-2 cells) results in internalization of the toxin by multiple endocytic mechanisms and in an arrest in host cell protein synthesis (38, 43). To test whether anti-RTA and/or anti-RTB IgA MAbs can protect polarized epithelial cells from ricin-induced cytotoxicity, we incubated ricin at three different concentrations (1.0, 0.3, and 0.1 μg/ml) with 23D7, 25A4, 33G2, 35H6, or the control MAb TEPC-15 (10 μg/ml) for 1 h and then applied the mixture to the apical surfaces of monolayers grown on permeable Transwell filters. The cytotoxic effect of ricin was determined by measuring the amount of incorporation of exogenous ^{35}S-labeled cysteine-methionine into total cellular protein 18 h later.

Each of the four antiricin IgA MAbs protected polarized MDCK II cell monolayers against ricin intoxication in a dose-dependent manner (Fig. 5). For example, at the highest dose of toxin tested (1.0 μg/ml), protein synthesis levels in monolayers treated with an anti-RTA (23D7 or 25A4) or an anti-RTB (33G2 or 35H6) IgA MAb were reduced 10 to 35% compared to non-toxin-treated controls, whereas protein synthesis levels in monolayers treated with TEPC-15 were reduced >95%. When challenged with one-third less toxin (0.3 μg/ml), monolayers treated with any of the four antiricin IgA MAbs had normal levels of protein synthesis, whereas levels of protein synthesis in TEPC-15-treated cells were reduced by ~80%. We obtained similar results when these studies were performed using monolayers of Caco-2 cells, a human colon carcinoma-derived epithelial cell line used widely as a model of absorptive enterocytes (33; data not shown). We conclude that IgA MAbs

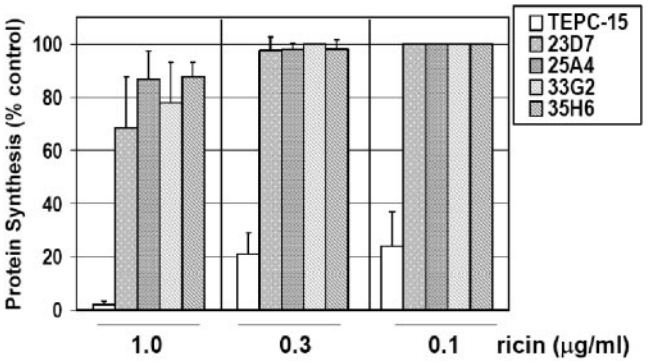

FIG. 5. Antiricin IgA MAbs protect epithelial cells from ricin intoxication. Ricin at each of the indicated concentrations was incubated with IgA MAbs (20 μg/ml) for 1 h at room temperature and then applied to the apical surfaces of MDCK II cell monolayers grown on permeable Transwell filters. Eighteen hours later, the monolayers were pulsed with [^{35}S]Met-Cys. The amount of radioactivity incorporated into cell monolayers was plotted as a percentage of that taken up by control (no ricin treatment) monolayers. Each bar represents the average, with SE, of three separate experiments done in duplicate.

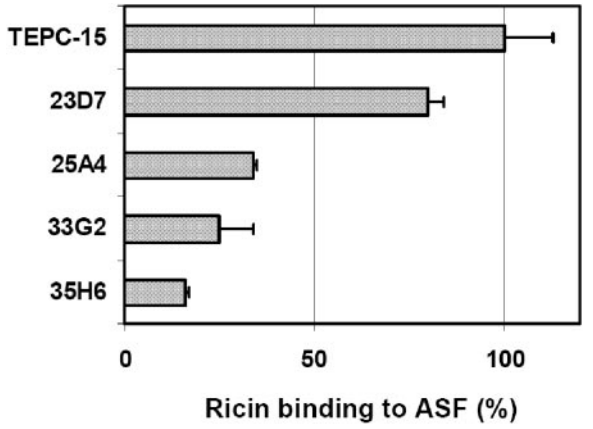

FIG. 6. Anti-RTB IgA MAbs block ricin attachment to ASF. Biotinylated ricin (8 ng/ml) was mixed with each of the indicated MAbs at a saturating concentration (10 μg/ml) and overlaid in triplicate onto ELISA plates coated with ASF. The plates were then washed and developed using avidin-HRP and TMB, as described in Materials and Methods. One hundred percent binding was defined as the value obtained for wells probed with ricin in the absence of antibody. Each bar represents the average, with SE, of two samples performed in duplicate.

against either RTA or RTB, when applied concomitantly with ricin, are capable of protecting polarized epithelial cell monolayers from the cytotoxic effects of ricin.

33G2 and 35H6 block toxin attachment to galactosides. We hypothesized that IgA MAbs directed against RTB may neutralize ricin by interfering with toxin attachment to cell surfaces. We used an in vitro binding assay to test this hypothesis. Biotin-labeled ricin was mixed with 33G2 or 35H6 and then applied to 96-well microtiter plates coated with ASF, a serum glycoprotein with terminal galactose residues that serve as high-affinity receptors for ricin (4). In parallel, we also examined the effects of 23D7, 25A4, and TEPC-15 on ricin binding in this assay. As expected, TEPC-15 had no effect on ricin binding to ASF (Fig. 6). 33G2 and 35H6, on the other hand, reduced ricin binding to ASF by 75% and 84%, respectively (Fig. 6). Surprisingly, 25A4, which is an IgA against RTA, reduced ricin binding to ASF almost as efficiently as the IgA MAbs against RTB, whereas 23D7 had a minimal effect on ricin attachment.

Based on these experiments, we postulated that 33G2 and 35H6 block toxin attachment to galactose residues by one of two mechanisms, either receptor interference (i.e., binding to an epitope located in one or possibly both of RTB's carbohydrate recognition domains, thereby physically excluding mono- or oligosaccharide occupancy) or steric hindrance (i.e., binding to an epitope on a face of RTB that positions the antibody so as to obstruct RTB's contact with cell surfaces). We performed competitive inhibition assays to distinguish between these possibilities. Microtiter plates coated with ricin were incubated with high concentrations of galactose (5 mg/ml), lactose (5 mg/ml), galactosylated BSA (1 mg/ml), and ASF (1 mg/ml) prior to being probed with 33G2 or 35H6. None of the ligands tested interfered with binding of 33G2 and 35H56 to ricin (data not shown). Similar results were obtained when the ligands and MAbs were added simultaneously. We concluded from these studies that 33G2 and 35H6 likely neutralize ricin by steric hindrance rather than by receptor interference.

Anti-RTB, but not anti-RTA, IgA MAbs reduce ricin binding to luminal surfaces of human duodenum. The luminal surfaces of intestinal enterocytes in vivo are coated with a thick, glycoprotein-rich filamentous brush border glycocalyx, which does not fully develop on epithelial cell lines grown in culture (11, 13). Because the filamentous brush border glycocalyx represents a potential high-avidity binding site for ricin, we tested whether antiricin IgA MAbs were capable of reducing (or preventing) ricin attachment to the luminal surfaces of human duodenum. Biotinylated ricin was incubated with individual MAbs for 1 h and then overlaid onto deparaffinized tissue sections of human duodenum. The sections were then stained with avidin-FITC and visualized by fluorescence microscopy. As observed previously (28), ricin uniformly labeled the luminal surfaces of intestinal villi (Fig. 7A). Treatment of ricin with the monoclonal antibody 33G2 reduced toxin binding to the luminal surfaces to nearly undetectable levels (Fig. 7B), consistent with what we observed on Caco-2 cell monolayers (data not shown). Treatment of ricin with 35H6 prior to its application to tissue sections resulted in a visible reduction in the amount of toxin attached to epithelial surfaces (Fig. 7C). However, 35H6 promoted the formation of large Ab-toxin complexes that were imaged as fluorescent speckles evenly distributed over the surfaces of the microscope slides; we were unable to remove these by repeated washings. 23D7 (Fig. 7D) had no discernible effect on toxin attachment to the human tissue sections compared to slides treated with ricin alone or with ricin plus TEPC-15 (Fig. 7E). We concluded that 33G2 and 35H6 can block ricin attachment to the luminal surfaces of human intestinal epithelial cells.

Anti-RTA IgA MAbs reduce ricin transepithelial transport. Because MAb 23D7 had minimal effects on ricin attachment to epithelial cells, we hypothesized that it neutralizes ricin intracellularly, as proposed for other MAbs (26). Similarly, we postulated that 23D7 may also potentially interfere with intracel-

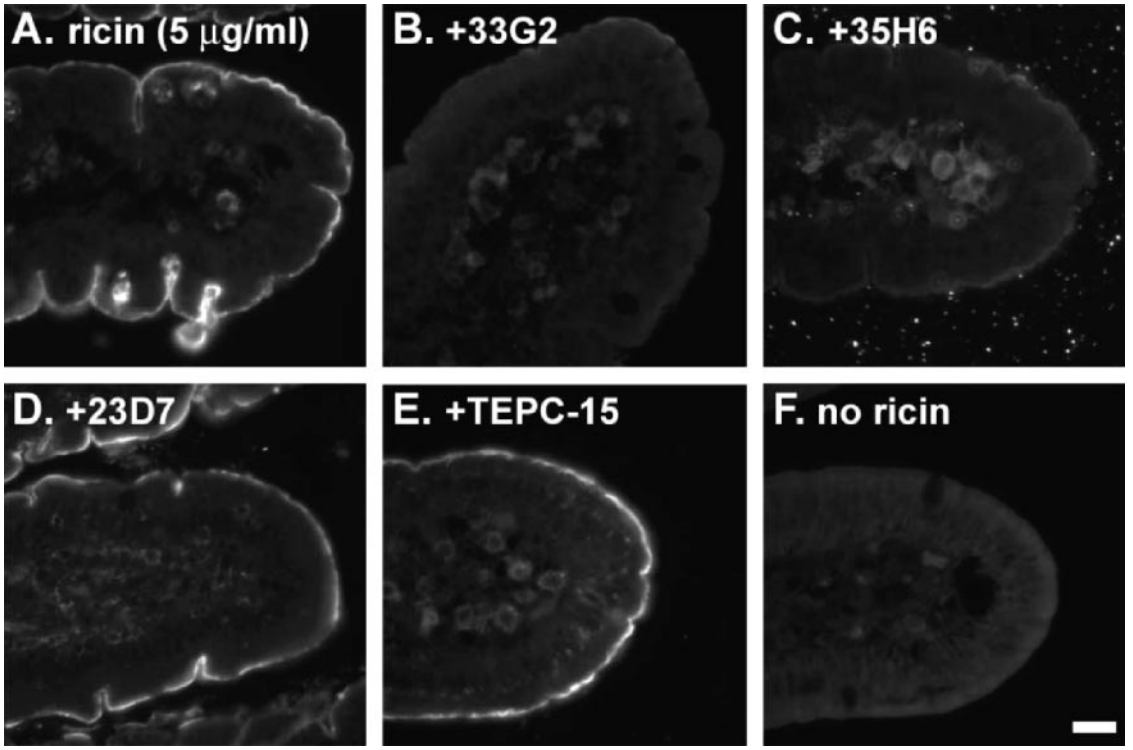

FIG. 7. Anti-RTB IgA MAbs inhibit ricin binding to the luminal surfaces of the human duodenum. Biotinylated ricin (5 μg/ml) was incubated with each of the indicated IgA MAbs (20 μg/ml) and then overlaid for 1 h onto deparaffinized tissue sections of human duodenum. The sections were washed, labeled with streptavidin-FITC, and visualized by fluorescence microscopy. (A) Ricin uniformly labeled the luminal surfaces of human intestinal villi. Occasional goblet cells were also labeled (bottom center). (B) 33G2 reduced ricin binding to luminal surfaces to undetectable levels. (C) 35H6 reduced toxin binding to luminal surfaces, but residual staining was observed on the surrounding glass surfaces. Neither 23D7 (D) nor TEPC-15 (E) had an effect on ricin attachment to tissue sections compared to sections treated with ricin only. (F) Tissue section not labeled with ricin. Bar, 20 μm.

lular toxin transport, including transcytosis. We developed a nonradioactive transcytosis assay, using biotinylated ricin and polarized MDCK II cell monolayers, to test this hypothesis (43). Biotinylated ricin was mixed with 23D7, 25A4, or TEPC-15 and then applied to the apical surfaces of polarized MDCK II cell monolayers grown on permeable Transwell filters. We collected ricin from the basolateral supernatants 18 h later by using avidin-agarose beads, which were subsequently subjected to reducing SDS-PAGE and Western blot analysis. Approximately 10% of the total amount of toxin that had been applied to the apical surface of MDCK II cell monolayers was detected in the basolateral compartment 18 h later (Fig. 8, lane 1). Substantially less toxin was detected in the basolateral supernatants from cultures to which ricin had been applied concurrently with 23D7 (Fig. 8, lane 4). As expected, 25A4 also interfered with transepithelial transport due to its ability to reduce toxin attachment to cell surfaces (Fig. 8, lane 5), whereas TEPC-15 did not.

DISCUSSION

Several studies have demonstrated a positive correlation between mucosal immunity to ricin and levels of antiricin IgA antibodies in mucosal secretions (15, 16, 22, 46). However, discerning the role of S-IgA in protecting epithelial cells from ricin in vivo has been confounded by a number of factors, including the presence of antitoxin serum IgG antibodies and the difficulty in accurately measuring "local" IgA concentrations and specificities. For this study, we addressed this issue in vitro by using well-defined polarized epithelial cell monolayers and monoclonal IgA antibodies with known specificities. Our

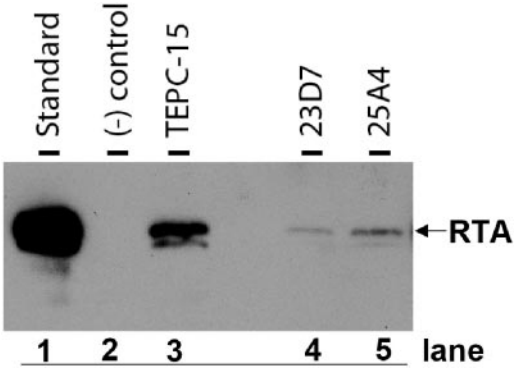

FIG. 8. Anti-RTA IgA MAbs reduce transcytosis of ricin across polarized epithelial cell monolayers. Biotinylated ricin (200 ng) was incubated with the indicated IgA MAbs (2 μg in DMEM) in a volume of 0.2 ml for 1 h and then applied to the apical surfaces of polarized MDCK II cell monolayers. Eighteen hours later, ricin in the basolateral compartments was collected using avidin-agarose beads, which were then subjected to SDS-PAGE and Western blot analysis, as described in Materials and Methods. As positive and negative controls, samples of DMEM containing ricin (200 ng) (lane labeled "standard") or no ricin (negative control) were treated in parallel.

study shows conclusively that IgA antibodies against RTA or RTB, when present on the apical surfaces of epithelial cells, are capable of fully neutralizing ricin. As the predominant class of antibody found in the secretions of the upper respiratory tract and the entire length of the gastrointestinal tract, S-IgA is undoubtedly an important first line of defense against the promiscuous toxin ricin. Our future studies will take advantage of the so-called "backpack tumor" model (20) to test in vivo whether IgA alone is capable of protecting animals from a lethal dose of ricin administered by inhalation or ingestion.

33G2 represents a class of neutralizing IgA MAbs we expected to identify from mice immunized with RT. Like neutralizing IgAs against cholera toxin (1), 33G2 targets the toxin binding subunit of ricin and blocks toxin attachment to galactoside receptors on cell surfaces. Evidence presented in this study suggests that 33G2 functions by steric hindrance. It is somewhat perplexing how this is achieved, considering that RTB has two galactose binding sites (a low-affinity site and a high-affinity site) that work independently and are separated by 70 Å (37). Both galactose binding sites must be obstructed to block ricin attachment to cell surfaces (41). We have tentatively determined that 33G2 recognizes an epitope adjacent to the high-affinity galactose binding site (C. McGuinness, T. Dembo, and N. Mantis, unpublished results), which suggests that the antibody's Fv region blocks one sugar binding site, while the Fc region blocks the other.

The mechanism by which 35H6 neutralizes ricin is different from that of 33G2. 35H6 induced the formation of what we postulate are toxin-MAb aggregates, which demonstrated a propensity to stick to glass surfaces. We hypothesize that these aggregates may be the result of 35H6 cross-linking ricin, although this remains to be tested. To promote cross-linking, 35H6 would need to recognize a protein or carbohydrate epitope on RTB that is present in multiple copies. We have ruled out the latter possibility, as the removal of RTB's two high-mannose type N-linked oligosaccharide side chains did not alter 35H6 binding to ricin or abolish the formation of toxin-MAb complexes (McGuinness and Mantis, unpublished results). It is therefore more likely that 35H6 recognizes a repeated peptide epitope. This is entirely possible considering that RTB consists of six 40-amino-acid peptide repeat elements that arose via gene duplication (37). We are especially interested in understanding how 35H6 functions because it neutralizes ricin better than the IgG that currently serves as the "gold standard" in the field (24, 35).

IgA MAbs against RTA, namely, 23D7 and 25A4, were as effective as IgA MAbs against RTB at protecting epithelial cells from ricin intoxication. While this was not an unexpected finding considering that neutralizing IgGs directed against RTA have been described previously (8, 24, 26, 47), the mechanism(s) by which these MAbs function remains unknown. It is generally accepted that anti-RTA MAbs exert their effects intracellularly. Some data presented in this study support this model. 23D7, for example, did not visibly reduce ricin attachment to the human duodenum, although the MAb protected MDCK II cell monolayers from toxin-mediated protein synthesis inhibition. On the other hand, 25A4 did reduce ricin binding to ASF in a microtiter plate assay, demonstrating that antibodies against RTA can function extracellularly by interfering with ricin attachment to galactosides. Indeed, 25A4 is not unique, as we have identified other IgAs and IgGs that act similarly (McGuinness and Mantis, unpublished data).

23D7 could neutralize ricin intracellularly by one of several mechanisms. Biochemical and genetic evidence indicates that ricin undergoes vesicular retrograde transport to the endoplasmic reticulum (ER), where RTA is unfolded and delivered across the ER membrane by the Sec61 complex (25, 36). Theoretically, 23D7 could interfere with vesicular transport from the early endosomes to the ER or with retrotranslocation of RTA across the ER membrane. 23D7 could serve as a paradigm for understanding how MAbs against enzymatic subunits of A-B toxins neutralize them intracellularly. Shiga toxin exploits a similar intracellular retrograde transport pathway, and neutralizing humanized MAbs against the A subunit are being considered for clinical trials (42).

The transport of ricin across polarized epithelial cell monolayers was first reported by van Deurs and colleagues (43), who used MDCK II cells grown on permeable filters. We confirmed in this study that ricin is capable of breaching MDCK II cell monolayers and demonstrated for the first time that MAbs directed against RTA can reduce toxin transepithelial transport. The amount of ricin that is transcytosed by intestinal epithelial cells in vivo is unknown, despite the fact that this information has implications for vaccine and therapy design. For example, if ricin is rapidly and effectively transported across the epithelial barrier, then a postexposure therapy administered systemically would be more effective than a therapy administered mucosally. On the other hand, if ricin remains localized within epithelial cells, then an oral therapy would be expected to be more beneficial than a systemic therapy.

The results from this study may have implications for the delivery of ricin vaccines to humans (29, 40). Our data suggest that stimulation of S-IgA against RTA or RTB in secretions will confer protection against ricin intoxication of mucosal epithelia. However, it is well established that S-IgA Abs are induced following mucosal vaccination, not systemic vaccination (5, 27, 30). Considering that both the respiratory and gastrointestinal tracts are susceptible to ricin intoxication, the efficacies of the vaccines currently under development should be evaluated following both intranasal and i.g. delivery. Intranasal immunization is a particularly attractive route for mucosal vaccine delivery, given that it stimulates S-IgA antibody secretions and IgG in serum (46) and avoids the harsh, protease-rich environment of the gastrointestinal tract.

ACKNOWLEDGMENTS

We thank Tessa-Vera Gonzalez for assistance with the IL-8 assays, Julie Pack for performing the ricin-asialofetuin binding studies, and Jessica Wagner for assistance with microscopy. We also acknowledge Ken Class (Wadsworth Center Immunology Core Facility) for technical assistance with flow cytometry.

This work was supported by a grant to N.J.M. from the National Institutes of Health (R21 AI-058147).

REFERENCES

1. **Apter, F. M., W. I. Lencer, R. A. Finkelstein, J. J. Mekalanos, and M. R. Neutra.** 1993. Monoclonal immunoglobulin A antibodies directed against cholera toxin prevent the toxin-induced chloride secretory response and block toxin binding to intestinal epithelial cells in vitro. Infect. Immun. **61:**5271–5278.
2. **Atlas, R. M.** 2002. Bioterrorism: from threat to reality. Annu. Rev. Microbiol. **56:**167–185.
3. **Audi, J., M. Belson, M. Patel, J. Schier, and J. Osterloh.** 2005. Ricin poisoning: a comprehensive review. JAMA **294:**2342–2351.

4. **Baenziger, J. U., and D. Fiete.** 1979. Structural determinants of Ricinus communis agglutinin and toxin specificity for oligosaccharides. J. Biol. Chem. **254:**9795–9799.
5. **Brandtzaeg, P.** 2003. Role of secretory antibodies in the defence against infections. Int. J. Med. Microbiol. **293:**3–15.
6. **Brown, R. F., and D. E. White.** 1997. Ultrastructure of rat lung following inhalation of ricin aerosol. Int. J. Exp. Pathol. **78:**267–276.
7. **Casadevall, A.** 2002. Passive antibody administration (immediate immunity) as a specific defense against biological weapons. Emerg. Infect. Dis. **8:**833–841.
8. **Colombatti, M., A. Pezzini, and A. Colombatti.** 1986. Monoclonal antibodies against ricin: effects on toxin function. Hybridoma **5:**9–19.
9. **Endo, Y., K. Mitsui, M. Motizuki, and K. Tsurugi.** 1987. The mechanism of action of ricin and related toxins on eukaryotic ribosomes. J. Biol. Chem. **262:**5908–5912.
10. **Ferrari, M., M. C. Fornasiero, and A. M. Isetta.** 1990. MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. J. Immunol. Methods **131:**165–172.
11. **Frey, A., K. T. Giannasca, R. Weltsin, P. J. Giannasca, H. Reggio, W. I. Lencer, and M. R. Neutra.** 1996. Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and vaccine targeting. J. Exp. Med. **184:**1045–1059.
12. **Giannasca, K. T., P. J. Giannasca, and M. R. Neutra.** 1996. Adherence of *Salmonella typhimurium* to Caco-2 cells: identification of a glycoconjugate receptor. Infect. Immun. **64:**135–145.
13. **Giannasca, P. J., K. T. Giannasca, P. Falk, J. I. Gordon, and M. R. Neutra.** 1994. Regional differences in glycoconjugates of intestinal M cells in mice: potential targets for mucosal vaccines. Am. J. Physiol. **267:**G1108–G1121.
14. **Gonzalez, T. V., S. A. Farrant, and N. J. Mantis.** 2006. Ricin induces IL-8 secretion in human monocyte/macrophages by activating the p38 MAP kinase pathway. Mol. Immunol. **43:**1920–1923.
15. **Griffiths, G. D., S. C. Bailey, J. L. Hambrook, M. Keyte, P. Jayasekera, J. Miles, and E. Williamson.** 1997. Liposomally-encapsulated ricin toxoid vaccine delivered intratracheally elicits a good immune response and protects against a lethal pulmonary dose of ricin toxin. Vaccine **15:**1933–1939.
16. **Griffiths, G. D., G. J. Phillips, and S. C. Bailey.** 1999. Comparison of the quality of protection elicited by toxoid and peptide liposomal vaccine formulations against ricin as assessed by markers of inflammation. Vaccine **17:**2562–2568.
17. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Harlow, E., and D. Lane.** 1999. Using antibodies. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Hewetson, J. F., V. R. Rivera, D. A. Creasia, P. V. Lemley, M. K. Rippy, and M. A. Poli.** 1993. Protection of mice from inhaled ricin by vaccination with ricin or by passive treatment with heterologous antibody. Vaccine **11:**743–746.
20. **Hutchings, A. B., A. Helander, K. J. Silvey, K. Chandran, W. T. Lucas, M. L. Nibert, and M. R. Neutra.** 2004. Secretory immunoglobulin A antibodies against the sigma 1 outer capsid protein of reovirus type 1 Lang prevent infection of mouse Peyer's patches. J. Virol. **78:**947–957.
21. **Kende, M., G. D. Giudice, N. Rivera, and J. Hewetson.** 2006. Enhancement of intranasal vaccination in mice with deglycosylated chain A ricin by LTR72, a novel mucosal adjuvant. Vaccine **24:**2213–2221.
22. **Kende, M., C. Yan, J. Hewetson, M. A. Frick, W. L. Rill, and R. Tammariello.** 2002. Oral immunization of mice with ricin toxoid vaccine encapsulated in polymeric microspheres against aerosol challenge. Vaccine **20:**1681–1691.
23. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18:**775–779.
24. **Lemley, P. V., P. Amanatides, and D. C. Wright.** 1994. Identification and characterization of a monoclonal antibody that neutralizes ricin toxicity in vitro and in vivo. Hybridoma **13:**417–421.
25. **Lord, J. M., E. Deeks, C. J. Marsden, K. Moore, C. Pateman, D. C. Smith, R. A. Spooner, P. Watson, and L. M. Roberts.** 2003. Retrograde transport of toxins across the endoplasmic reticulum membrane. Biochem. Soc. Trans. **31:**1260–1262.
26. **Maddaloni, M., C. Cooke, R. Wilkinson, A. V. Stout, L. Eng, and S. H. Pincus.** 2004. Immunological characteristics associated with the protective efficacy of antibodies to ricin. J. Immunol. **172:**6221–6228.
27. **Mantis, N. J.** 2005. Vaccines against the category B toxins: staphylococcal enterotoxin B, epsilon toxin and ricin. Adv. Drug Deliv. Rev. **57:**1424–1439.
28. **Mantis, N. J., S. A. Farrant, and S. Mehta.** 2004. Oligosaccharide side chains on human secretory IgA serve as receptors for ricin. J. Immunol. **172:**6838–6845.
29. **McHugh, C. A., R. F. Tammariello, C. B. Millard, and J. H. Carra.** 2004. Improved stability of a protein vaccine through elimination of a partially unfolded state. Protein Sci. **13:**2736–2743.
30. **Mestecky, J., M. W. Russell, and C. O. Elson.** 1999. Intestinal IgA: novel views on its function in the defence of the largest mucosal surface. Gut **44:**2–5.
31. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
32. **Olsnes, S., A. M. Pappenheimer, Jr., and R. Meren.** 1974. Lectins from Abrus precatorius and Ricinus communis. II. Hybrid toxins and their interaction with chain-specific antibodies. J. Immunol. **113:**842–847.
33. **Peterson, M. D., and M. S. Mooseker.** 1992. Characterization of the enterocyte-like brush border cytoskeleton of the Caco-2BBe clone of the human intestinal cell line Caco-2. J. Cell Sci. **102:**581–600.
34. **Poli, M. A., V. R. Rivera, M. L. Pitt, and P. Vogel.** 1996. Aerosolized specific antibody protects mice from lung injury associated with aerosolized ricin exposure. Toxicon **34:**1037–1044.
35. **Rainey, G. J., and J. A. Young.** 2004. Antitoxins: novel strategies to target agents of bioterrorism. Nat. Rev. Microbiol. **2:**721–726.
36. **Rapak, A., P. O. Falnes, and S. Olsnes.** 1997. Retrograde transport of mutant ricin to the endoplasmic reticulum with subsequent translocation to cytosol. Proc. Natl. Acad. Sci. USA **94:**3783–3788.
37. **Rutenber, E., M. Ready, and J. D. Robertus.** 1987. Structure and evolution of ricin B chain. Nature **326:**624–626.
38. **Sandvig, K., S. Grimmer, S. U. Lauvrak, M. L. Torgersen, G. Skretting, B. van Deurs, and T. G. Iversen.** 2002. Pathways followed by ricin and Shiga toxin into cells. Histochem. Cell Biol. **117:**131–141.
39. **Sekine, I., Y. Kawase, I. Nishimori, M. Mitarai, H. Harada, M. Ishiguro, and M. Kikutani.** 1986. Pathological study on mucosal changes in small intestine of rat by oral administration of ricin. I. Microscopical observation. Acta Pathol. Jpn. **36:**1205–1212.
40. **Smallshaw, J. E., J. A. Richardson, S. Pincus, J. Schindler, and E. S. Vitetta.** 2005. Preclinical toxicity and efficacy testing of RiVax, a recombinant protein vaccine against ricin. Vaccine **23:**4775–4784.
41. **Sphyris, N., J. M. Lord, R. Wales, and L. M. Roberts.** 1995. Mutational analysis of the Ricinus lectin B-chains. Galactose-binding ability of the 2 gamma subdomain of Ricinus communis agglutinin B-chain. J. Biol. Chem. **270:**20292–20297.
42. **Tzipori, S., A. Sheoran, D. Akiyoshi, A. Donohue-Rolfe, and H. Trachtman.** 2004. Antibody therapy in the management of Shiga toxin-induced hemolytic-uremic syndrome. Clin. Microbiol. Rev. **17:**926–941.
43. **van Deurs, B., S. H. Hansen, O. W. Petersen, E. L. Melby, and K. Sandvig.** 1990. Endocytosis, intracellular transport and transcytosis of the toxic protein ricin by a polarized epithelium. Eur. J. Cell Biol. **51:**96–109.
44. **van Deurs, B., T. I. Tonnessen, O. W. Petersen, K. Sandvig, and S. Olsnes.** 1986. Routing of internalized ricin and ricin conjugates to the Golgi complex. J. Cell Biol. **102:**37–47.
45. **Wilhelmsen, C. L., and M. L. Pitt.** 1996. Lesions of acute inhaled lethal ricin intoxication in rhesus monkeys. Vet. Pathol. **33:**296–302.
46. **Yan, C., W. L. Rill, R. Malli, J. Hewetson, H. Naseem, R. Tammariello, and M. Kende.** 1996. Intranasal stimulation of long-lasting immunity against aerosol ricin challenge with ricin toxoid vaccine encapsulated in polymeric microspheres. Vaccine **14:**1031–1038.
47. **Youle, R. J., and M. Colombatti.** 1987. Hybridoma cells containing intracellular antiricin antibodies show ricin meets secretory antibody before entering the cytosol. J. Biol. Chem. **262:**4676–4682.

Editor: A. D. O'Brien