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# Anti-Thy 1.2 monoclonal antibody linked to ricin is a potent cell-type-specific toxin

(hybrid proteins/cancer chemotherapy/immunotherapy/protein transport/cell membranes)

RICHARD J. YOULE AND DAVID M. NEVILLE, JR.

Section on Biophysical Chemistry, Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, Maryland 20205

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ABSTRACT **The cell-type specificity of the toxin ricin, which ordinarily binds, enters, and kills cells through receptors containing galactose, has been altered by covalently binding it to a monoclonal antibody and by reversibly binding it to lactose. The antibody, a monoclonal rat $IgG_{2b}$ directed against the Thy 1.2 antigen, provides ricin with a new binding site for the murine thymus cell surface. Addition of lactose saturates the galactose-binding site on ricin and inhibits ricin from binding and killing cells via the galactose-containing receptors. The antibody–ricin hybrid protein, anti-Thy 1.2–ricin, formed with a thioether linkage, has been purified by size exclusion and affinity chromatography. When assayed by inhibition of protein synthesis of EL-4 cells, which express the Thy 1.2 antigen, anti-Thy 1.2–ricin is equally as toxic as ricin on a molar basis. The hybrid protein toxicity is unchanged in the presence of 100 mM lactose, whereas unmodified ricin toxicity is reduced to 1% of its toxicity in the absence of lactose. This demonstrates the altered receptor specificity of the ricin hybrid. The cell-type specificity of the anti-Thy 1.2–ricin inhibition of protein synthesis correlates with the presence of the Thy 1.2 antigen. Anti-Thy 1.2–ricin at 4 μg/ml in the presence of lactose inhibits protein synthesis within 3.5 hr by 60–80% in EL-4 cells but does not affect protein synthesis in AKR T-cell lines from mice that carry the Thy 1.1 alloantigen and HeLa cells that lack the Thy 1 antigen. Anti-Thy 1.2–ricin in the presence of lactose selectively kills EL-4 cells at concentrations that do not kill AKR-K36 cells. This selectivity, expressed as the ratio of anti-Thy 1.2–ricin concentrations required to kill 40% of both cell types, is 700. Ricin–monoclonal antibody hybrids of this type combine a high degree of cell-type selectivity and toxicity and may have pharmacologic utility as antitumor reagents.**

Artificial hybrid proteins with the specificity of antibodies or hormones and the toxicity of ricin or diphtheria toxin have been proposed as a new class of pharmacological reagents (1–3). We have shown that it is possible to alter the receptor specificity of both ricin and diphtheria toxin and to maintain the high toxicity of these toxins (4, 5). We recently constructed a cell-type-specific toxin by coupling mannose 6-phosphate, the receptor recognition moiety of lysosomal hydrolases, to ricin. The ordinary route of ricin binding and uptake was inhibited with lactose (4). The hybrid toxin inhibited protein synthesis in human fibroblasts via the mannose 6-phosphate receptor but not in other cell types that lack this receptor. Importantly, the new receptor-mediated toxicity was half as active as the extremely potent galactose receptor-mediated toxicity of ricin.

We report here the successful modification of ricin's native receptor and cell-type specificity to that dictated by a covalently bound monoclonal antibody. Monoclonal anti-Thy 1.2 rat $IgG_{2b}$ was covalently coupled to ricin. This antibody binds to the murine T-cell surface differentiation antigen that occurs in most mouse strains with the exception of AKR and A strains (6, 7). When the hybrid anti-Thy 1.2–ricin was exposed to cells in the presence of lactose, the observed toxicity exhibited cell-type specificity of the antibody. Thus, anti-Thy 1.2–ricin plus lactose inhibits protein synthesis in murine T cells carrying Thy 1.2 but does not affect murine T cells of AKR background or a human cell line. Cells carrying Thy 1.2 are sensitive to 1/700th the concentration of Thy 1.2–ricin plus lactose than cells lacking Thy 1.2, as judged by cell killing.

Many cell-type-specific and tumor-specific monoclonal antibodies are being developed (7–16). The work reported here may lead to pharmacologically useful cell-type- and tumor-specific toxins.

## MATERIALS AND METHODS

Ricin was purified from seeds of *Ricinus communis*, kindly supplied by Anthony Huang, according to Nicolson and Blaustein (17) as described (5). L-[*U*-$^{14}$C]Leucine in 10 mM HCl (290 Ci/mol; 1 Ci = $3.7 \times 10^{10}$ becquerels) was from New England Nuclear and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was from Pierce. Mouse thymic leukemia cells, EL-4, of B6 background (18) were kindly supplied by Kyung-Jin Kim (National Institute of Allergy and Infectious Diseases, Bethesda, MD); AKR-K36 thymic leukemia cells (18) by Robert Nowinski (Fred Hutchinson Research Center, Seattle, WA); and AKT8, a spontaneous thymoma from AKR mice, by S. P. Staal (Johns Hopkins University, Baltimore, MD). Monoclonal antibody specific for Thy 1.2 mouse T-cell antigen, clone 30-H12 derived by Ledbetter and Herzenberg (7), was purchased from Becton Dickinson (Mountain View, CA). The hybridoma (19) was generated by fusion of the mouse myeloma line NS1 with spleen cells of LOU/Ws1/M rats immunized with SJL (Thy 1.2) mouse spleen cells. This xenogeneic antibody is highly reactive against mouse cells carrying the Thy 1.2 alloantigen, but not reactive against thymocytes of AKR mice carrying the Thy 1.1 allele (7).

**Protein Synthesis Assay.** EL-4, AKR-K36, and AKT8 cells were resuspended in fresh RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum at $1.2 \times 10^6$ cells per ml and dispensed in 1-ml aliquots into sterile 1.5-ml Eppendorf centrifuge tubes. For assays with 100 mM lactose present, 100 μl of medium made 1 M with lactose was added. Toxins or hybrids were added to the tubes in 50 μl of medium or 10 mM $Na_2HPO_4$, pH 7.4/0.85% NaCl with or without 100 mM lactose. Controls showed that addition of buffer or medium had no effect on protein synthesis or toxin action. The cells were incubated at 37°C in 5% $CO_2$ for 3 hr and again for 1.5 hr after

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Abbreviations: Anti-Thy 1.2–ricin, anti-Thy 1.2 monoclonal antibody–ricin; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester.

addition of 100 μl of medium containing 0.9 μCi of L-[U-$^{14}$C]leucine per ml. The cells were centrifuged for 3 min at 8000 × g and the medium was aspirated. The cells were washed twice with 5% (wt/vol) trichloroacetic acid and solubilized with 1 ml of 50 mM $Na_2CO_3$/2% (wt/vol) $NaDodSO_4$/10 mM EDTA. Radioactivity of the samples was measured in toluene/Fluoralloy/Biosolve (Beckman) by using a narrow $^{14}$C window.

HeLa cells were assayed in monolayers as described (5).

**Cell Killing Assay.** EL-4 and AKR-K36 cells were suspended in fresh medium (between 1 and 5 × $10^4$ cells per ml) and dispensed in 100-μl aliquots into 6.4-mm-diameter wells of Costar 96-well plates. Toxins or hybrids were added to the wells in 10 μl of medium and lactose was added to 50 mM concentration in 10 μl of medium. The cells were incubated at 37°C in 5% $CO_2$ for 3 days. Trypan blue, 4 μl of a 0.4% solution, was added to each well and then mixed. All cells and trypan blue-excluding cells were counted in a 0.49-$mm^2$ grid. Triplicate counts were taken and averaged.

**Hybrid Synthesis.** The bifunctional crosslinking agent MBS (20) was used to link ricin to IgG by a method similar to that used to link ricin to diphtheria toxin A chain (5). Ricin, 4 mg of protein in 400 μl of 10 mM $Na_2HPO_4$ (pH 7.5), was mixed while on a Vortex mixer with 13 μl of dimethylformamide containing 32 μg of MBS, giving a molar ratio of MBS to ricin of 1.7. The mixture was incubated at room temperature for 30 min.

The monoclonal IgG against Thy 1.2 was prepared for crosslinking by partial reduction of disulfide bonds. IgG, 0.6 mg in 0.6 ml of buffered saline (as supplied by Becton Dickinson), was mixed with 30 μl of 1 M dithiothreitol and incubated at room temperature for 30 min. The IgG in 300-μl aliquots was passed over a Sephadex G-25 column (1.4 × 5 cm) in 6.7 mM $Na_2HPO_4$, pH 7.4/14 mM NaCl and the peak fractions were pooled. The final volume of the pooled IgG fractions was about 3 ml, containing 0.6 mg of protein. The 3 ml of partially reduced IgG was mixed with 400 μl of MBS–ricin and incubated at room temperature for 2 hr. The mole ratio of ricin to IgG was 18:1.

## RESULTS

**Hybrid Synthesis and Purification.** Artificial hybrid conjugates of ricin and monoclonal antibody against the Thy 1.2 cell surface component of mouse thymus cells were synthesized with the bifunctional crosslinking agent MBS. In later preparations, the reaction between MBS–ricin and IgG was stopped after 2 hr by making the mixture 6.6 mM in *N*-ethylmaleimide.

The hybrids were separated from unreacted ricin by high-pressure liquid chromatography on a 7.5 × 600 mm G3000 SW TSK gel permeation column (21) (Toyo Soda USA, New York). The column was run at a flow rate of 1 ml/min with 100 mM $Na_2HPO_4$ (pH 7.2). Anti-Thy 1.2 IgG eluted from the column after 13.4 ml; ricin eluted after 19 ml. Chromatography of the hybrid mixture of partially reduced IgG and MBS–ricin showed a peak at 12.4 ml and another at 19 ml. Ricin eluted later than expected on the basis of molecular size, presumably due to interaction with the column matrix.

The peak of hybrid protein, at an elution volume between 10 and 14 ml, was collected from 17 runs and concentrated to 17 ml by dialysis against 60% (wt/vol) sucrose in 6.7 mM $Na_2HPO_4$, pH 7.4/150 mM NaCl. The final concentration of protein was between 34 and 85 μg/ml.

The hybrid protein was bound to the galactose residues of Sepharose-4B and thereby separated from unreacted IgG. The pooled hybrid protein and IgG fraction from the TSK column was applied to a 2-ml Sepharose-4B column and washed with 6.7 mM $Na_2HPO_4$, pH 7.4/150 mM NaCl, and the ricin–IgG hybrids were eluted in a sharp peak with 100 mM lactose in the same buffer. The final yield of hybrid protein was about 95 μg of protein containing 30% of the initial IgG protein.

**Hybrid Activity.** The hybrid proteins, ricin, and antibody were assayed for inhibition of protein synthesis in T-cell leukemic EL-4 cells, which contain the Thy 1.2 antigen (18). The antibody alone at 4000 ng/ml did not inhibit protein synthesis in EL-4 cells (data not shown). Fig. 1 shows the dose–response of ricin and anti-Thy 1.2–ricin in EL-4 cells. The hybrid toxin on a weight basis was 25% as potent as ricin, and, therefore, on a mole basis anti-Thy 1.2–ricin equals or exceeds ricin toxicity (Fig. 1). Lactose, which competitively blocks ricin binding to cells, inhibited toxicity of ricin at least 200-fold in EL-4 cells (Fig. 1). In contrast, the hybrid was inhibited less than 2-fold by lactose. Thus, in the presence of 100 mM lactose, EL-4 cells were more than 100-fold more sensitive to the hybrid protein than to ricin. Mixtures of equal amounts of anti-Thy 1.2 antibody and ricin behaved like ricin (data not shown).

Thymus cells from AKR mice carrying the Thy 1.1 allele do not bind the clone 30-H12 Thy 1.2-specific monoclonal antibody (7). HeLa cells lack the Thy 1 antigen altogether. Two thymic cell lines from AKR mice and HeLa cells were assayed for sensitivity to anti-Thy 1.2–ricin. In Fig. 2 the sensitivity of these cell lines to anti-Thy 1.2–ricin in the presence of 100 mM lactose is shown. No inhibition of protein synthesis was seen at the highest concentration of hybrid protein. In contrast, three different preparations of anti-Thy 1.2–ricin showed marked inhibition of protein synthesis in Thy 1.2 EL-4 cells. AKR-K36, AKT8, and HeLa cells were more sensitive to ricin and anti-Thy 1.2–ricin in the absence of lactose than the EL-4 cells were (data not shown). Thus, lactose showed a large (100- to 1000-fold) protection of anti-Thy 1.2-ricin in cells lacking Thy 1.2. Lactose inhibited anti-Thy 1.2–ricin in AKR and HeLa cell lines to the same extent as it inhibited unmodified ricin in AKR, EL-4, and HeLa cells (data not shown). Thus, no antibody-mediated toxicity of anti-Thy 1.2–ricin was detected in these cell lines lacking Thy 1.2.

A cell killing assay was developed to determine the selectivity between sensitive and nonsensitive cell lines to anti-Thy 1.2–ricin. EL-4 and AKR-K36 cells were plated in the presence of varying amounts of either anti-Thy 1.2–ricin or ricin with or

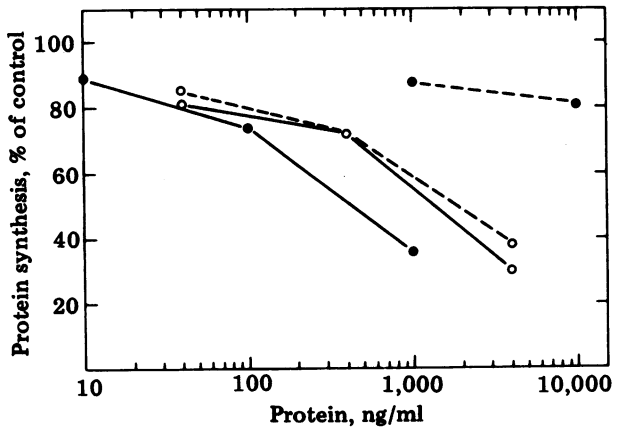


FIG. 1. Dose–response curves of ricin and anti-Thy 1.2–ricin hybrids on protein synthesis of Thy 1.2 EL-4 cells. Protein synthesis was assayed by incorporation of L-[U-$^{14}$C]leucine and is referenced to controls without toxins with or without lactose. Controls with lactose exhibited 70% of the protein synthesis of controls without lactose. Points are the means ± 22% (SD) of triplicates. ●—●, Ricin; ●---●, ricin plus lactose; ○—○, anti-Thy 1.2–ricin; ○---○, anti-Thy 1.2–ricin plus lactose.

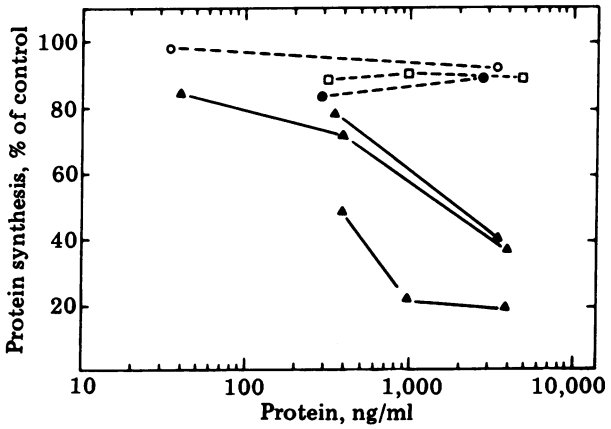


FIG. 2. Dose–response of anti-Thy 1.2–ricin in the presence of 100 mM lactose on Thy 1.2 EL-4 cells (▲—▲), Thy 1.1 AKR-K36 cells (○---○), Thy 1.1 AKT8 cells (□---□), and HeLa cells (●---●). Protein synthesis was assayed by incorporation of L-[$U$-$^{14}$C]leucine. Points are means ±22% (SD) of triplicates. Three different preparations of anti-Thy 1.2–ricin are shown for the EL-4 cells; the same preparations were used for the other cell lines.

without 50 mM lactose, and the number of viable and nonviable cells was counted 3 days later. Under these conditions, EL-4 cells and AKR cells underwent approximately three and five cell division cycles, respectively, in the presence of lactose alone. Anti-Thy 1.2–ricin and ricin had two effects: cell killing and inhibition of cell division.

The 3-day assay of cell death (Fig. 3) was 20-fold more sensitive than the 3-hr assay of protein synthesis (Figs. 1 and 2). The Thy 1.2 EL-4 cell line was 700-fold more sensitive to cell killing by anti-Thy 1.2–ricin in the presence of lactose than the AKR cell line. When viable cell number was plotted (data not shown) instead of the percent viable cells shown in Fig. 3$A$, a 700-fold differential also existed between EL-4 and AKR cells. In one experiment, the initial seeding of viable cells was 1000 for AKR-K36 and 5000 for EL-4. After 3 days of incubation with 2000 ng of anti-Thy 1.2–ricin per ml, the number of viable AKR-K36 cells per well was 37,000 ± 10,000 and the number of viable EL-4 cells was 300 ± 60. The number of cells in control wells made 50 mM in lactose was 32,000 for AKR-K36 and 29,000 for EL-4 cells. Ricin and the protein hybrid at sublethal doses stimulated cell division, as shown by the increased cell

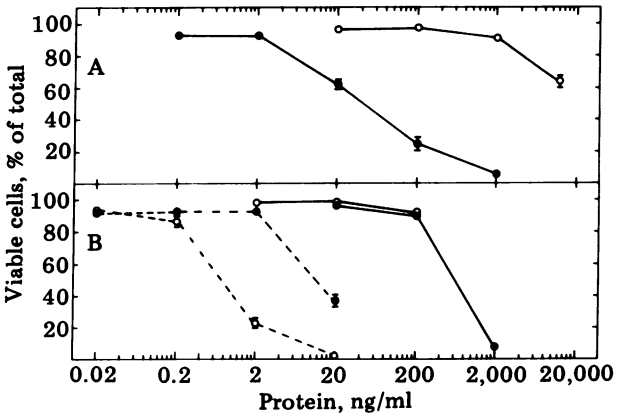


FIG. 3. Dose–response of ricin and anti-Thy 1.2–ricin on EL-4 and AKR cell viability. Cell viability was assayed by trypan blue exclusion after a 3-day exposure to the toxins. Points are means of triplicates; SD is shown when greater than the size of the symbol. ($A$) Anti-Thy 1.2–ricin plus 50 mM lactose: ●—●, on El-4 cells; ○—○, on AKR-K36 cells. ($B$) Ricin plus 50 mM lactose: ●—●, on EL-4 cells; ○—○, on AKR-K36 cells. Ricin alone: ●---●, on EL-4 cells; ○---○, on AKR-K36 cells.

number relative to controls. In the presence of lactose, 100 ng of anti-Thy 1.2 antibody alone per ml, as well as 100 ng of antibody and 20 ng of ricin per ml mixed but not covalently linked, failed to kill EL-4 cells. The sensitivity of EL-4 and AKR cells to ricin in the absence and presence of 50 mM lactose was compared. As in the protein synthesis assay, AKR cells were more sensitive to ricin than EL-4 cells (Fig. 3$B$). The two cell lines had similar sensitivity to ricin in the presence of lactose (Fig. 3$B$).

## DISCUSSION

We have demonstrated that potent cell-type-specific toxins can be made by the proper alteration of toxin-binding specificity. This report shows that a monoclonal antibody can be used as the receptor-binding moiety of a hybrid toxin and will define the hybrid cell-type specificity. By coupling Thy 1.2-specific monoclonal antibody to ricin and blocking the ricin galactose-binding site with lactose we alter the receptor specificity of ricin. Under these conditions the new reagent, anti-Thy 1.2–ricin, is 700 times more toxic to T cells that contain the Thy 1.2 surface antigen than to cells lacking it. This degree of selectivity can allow one to kill antigen-bearing cells and not other cells in tissue culture. This differential of toxicity may be sufficient to selectively kill antigen-bearing cells *in vivo*.

Several studies of toxins or their subunits linked to polyclonal antibodies (1, 22–26), hormones (2, 27–29), and lectins (30, 31) have been reported. These hybrids, though generally showing altered receptor specificity of the toxins, have, with one exception (24), very low potency compared to the native toxins. The monophosphotetramannosyl-1-deoxymannitoyl–ricin and anti-Thy 1.2–ricin hybrids prepared in this laboratory maintain the high potency of the toxin. Our mild and specific coupling methods undoubtedly play a role in maintaining high toxin potency. More important perhaps is the inclusion of the ricin B chain within the hybrid. We have previously presented evidence that can be interpreted as indicating that the ricin B chain performs an entry function that is independent of ordinary ricin binding to surface galactose receptors (4). If a wide variety of high-potency monoclonal antibody hybrids can be made irrespective of the antigen receptor specificity, our hypothesis of the entry function of the ricin B chain will be strengthened. Alternatively, it is possible that the high degree of selective cytotoxicity elicited by anti-Thy 1.2–ricin is dependent on unique features inherent in the Thy 1.2 antigen. It will be necessary to construct a variety of monoclonal antibody–toxin hybrids of varying specificities to distinguish between these possibilities.

Because the monophosphotetramannosyl-1-deoxymannitoyl–ricin and anti-Thy 1.2–ricin hybrids contain the ricin B chain, these hybrids require the presence of lactose to block the ricin B chain binding in order to achieve cell-type specificity. This limits the presently achievable selectivity betweeen cell types to between 30- and 700-fold. Naturally occurring toxins that use receptor-mediated protein-transport systems can exhibit cell-type selectivities up to 10,000-fold (32, 33). This degree of selectivity could, in principle, be also reached by antibody–toxin hybrids of the proper construction. The currently available selectivity, however, is more than ample for the use of hybrids as selective agents for the isolation of mutant cell lines that lack receptor. It may be possible to efficiently select for variants that lack any cell surface components toward which an antibody can be raised. This approach avoids use of complement-dependent cell lysis.

The use of monoclonal antibody as the cell recognition moiety of toxin hybrids greatly expands the possible uses of antibody–toxin hybrids. Several cell-type-specific (7, 8, 10, 12,

15) and tumor-specific or tumor-associated (9, 11, 13, 14, 16) monoclonal antibodies have been produced. Hybrids of ricin with these antibodies would likely kill the antigen-bearing cells selectively. We believe there is considerable scientific and pharmacologic potential for these potent monoclonal antibody–ricin hybrids as cell-type and tumor-specific toxins.

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