Kinetics of Protein Synthesis Inactivation in Human T-Lymphocytes by Selective Monoclonal Antibody-Ricin Conjugates

John E. Leonard, Quing-cheng Wang, Nathan O. Kaplan, and Ivor Royston

The Cancer Center (J. E. L., Q.-c. W., N. O. K., I. R.) and the Department of Chemistry (Q.-c. W., N. O. K.), The University of California, San Diego, La Jolla, California 92033, and Veterans Administration Medical Center, San Diego, California 92161 [I. R.]

ABSTRACT

Immunotoxins synthesized with the pan-T-cell monoclonal antibody T101 and ricin, acetylricin, or ricin A-chain have been compared. Native ricin was acetylated with N-acetylimidazole to block the galactose-binding site of the toxin B (binding)-chain. In the presence of lactose, both whole-ricin-containing immunotoxins were selectively cytotoxic but the ricin A-chain conjugate was less effective in blocking cellular protein synthesis. Immunotoxin-treated cells cultured in fresh growth medium exhibited no growth, declining viabilities, and no protein synthesis activity. Lymphocytes treated with T101:ricin or ricin did not form clusters or colonies when plated in 0.3 M Bacto-agar. Ammonium chloride markedly enhanced the efficacy of T101:ricin and T101:ricin A-chain. Our results suggest that: (a) all immunotoxins were selectively cytotoxic; (b) in the presence of ammonium chloride the effectiveness of the T101:ricin A-chain conjugate approached that of T101:ricin; and (c) the toxin B-chain may facilitate conjugate internalization and/or processing.

INTRODUCTION

Numerous recent studies describe the synthesis and efficacy of immunotoxins, antibodies conjugated to bacterial or plant toxins or their catalytically active polypeptides (10, 18). Because the toxins act catalytically rather than stoichiometrically, immunotoxins are more effective than are drug conjugates and require as few as one or two cytoplasmic toxin molecules for cell kill.

Conjugates consisting of the enzymatically active ricin A-chain are more specific than those containing the whole toxin but have exhibited variable in vivo effectiveness (10, 30, 36). While some researchers have shown that A-chain-containing conjugates were as effective as their whole-ricin counterparts, especially in the presence of ammonium chloride or carboxylic ionophores (3, 4), others have found that cytotoxicity of these immunotoxin conjugates was much less than that of the whole ricin conjugate (27, 30). However, Youle and Neville (36) showed that addition of ricin B-chain to cells previously exposed to ricin A-chain conjugate accelerated the protein synthesis inactivation compared to cells not treated with ricin B-chain. Further McIntosh et al. (14) showed that ricin B-chain could enhance A-chain conjugate efficacy even in the presence of 0.1 M lactose, suggesting that the B-chain need not bind to cellular galactose residues to increase A-chain conjugate effectiveness and that the observed enhancement may be due to annealing of the B-chain to the A-chain moiety of the immunotoxin conjugate.

While some whole-ricin conjugates are as effective as native ricin on a molar basis (18, 30, 35), these immunotoxins must be used in the presence of galactose or lactose to prevent nonspecific B-chain binding to cell surface galactose-containing receptors. This precludes their use in vivo. However, whole-ricin preparations could be used for in vitro removal of malignant cells from autologous bone marrow prior to transplantation (12, 27).

In this report, we compare the effectiveness of immunotoxins made with the pan-T-cell monoclonal antibody T101 (21) and native ricin, acetylated ricin, or ricin A-chain. Of the three conjugate preparations, T101:ricin was the most effective, approximating ricin in its cytotoxicity, followed by T101:acetylricin and T101:ricin A-chain. Based on this relative order of effectiveness, the data suggest that the B-chain may facilitate conjugate internalization and/or processing. In agreement with recently published work, our results also suggest that ammonium chloride increases immunotoxin effectiveness (3, 4).

MATERIALS AND METHODS

L-[U-3H]Leucine was obtained from New England Nuclear. Ficoll-Paque was obtained from Pharmacia Fine Chemicals, Inc. Electrophoretically pure ricin was prepared as described previously (11) or obtained from Calbiochem-Behring; ricin A-chain was prepared as described (16). Purified ricin A-chain was found to be nontoxic to the human T-cells CEM and 8402 at a concentration of 1 μM and yet effectively blocked protein synthesis (approximately 85% inhibition) in a cell-free reticulocyte lysate system (below) at a final concentration of 1.0 μM. Rabbit reticulocyte lysate and other protein synthesis materials were obtained from Promega Biotech. The pan-T-cell monoclonal antibody T101 (15, 20, 21) was purified from murine ascites fluid by ammonium sulfate precipitation and by chromatography on protein A-Sepharose (Pharmacia). The T101 antibody is an IgG2α κ made against the leukemic cell line 8402 (15); it reacts with a M, 65,000–67,000 antigen (T65) found on all peripheral blood T-cells, thymocytes, T-cell lines, and all cases of T-cell acute lymphocytic leukemia. This antigen is also found on chronic lymphocytic leukemia cells but not on normal B-cells, B-cell lines, or B-cell lymphomas or leukemias (20, 21). When incubated in the presence of T101, approximately 94% of T65 is lost from the surface of normal T-cells incubated at 37°C for 24 h. This modulation process is inhibited at 4°C suggesting that the process is energy dependent (26). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed the antibody to be greater than 95% homogeneous.

Conjugate Synthesis. Using the cleavable cross-linking reagent SPDP (Pharmacia) (2), purified ricin A-chain, ricin, or acetylricin were coupled to separate aliquots of purified T101. The antibody was treated with SPDP for 30 min at 23°C and the derivatized antibody (T101-SPDP) was used in the presence of lactose or lactate to prevent nonspecific B-chain binding to cell surface galactose-containing receptors. This precludes their use in vivo. However, whole-ricin preparations could be used for in vitro removal of malignant cells from autologous bone marrow prior to transplantation (12, 27).

In this report, we compare the effectiveness of immunotoxins made with the pan-T-cell monoclonal antibody T101 (21) and native ricin, acetylated ricin, or ricin A-chain. Of the three conjugate preparations, T101:ricin was the most effective, approximating ricin in its cytotoxicity, followed by T101:acetylricin and T101:ricin A-chain. Based on this relative order of effectiveness, the data suggest that the B-chain may facilitate conjugate internalization and/or processing. In agreement with recently published work, our results also suggest that ammonium chloride increases immunotoxin effectiveness (3, 4).

1 This work was supported in part by USPHS Training Grant 5 T32 HL07107-07 and NIH Grant CA 11683. John E. Leonard is the recipient of New Investigator Research Award 1R23 CA35692 from the National Cancer Institute.
2 To whom requests for reprints should be addressed.
3 Present address: Shanghai Institute of Biochemistry, Chinese Academy of Sciences, 320 Yo-Yang Road, Shanghai, China.
4 The abbreviations used are: SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate; T101-SPDP, the pyridylthiopropionate derivative of the monoclonal antibody T101; PBS, phosphate-buffered saline (0.01 M potassium phosphate buffer, pH 7.4, containing 0.14 M NaCl); ricin-PDP, the pyridylthiopropionate derivative of ricin; ID50, inhibitory conjugate concentration producing 50% inhibition of cell growth or protein synthesis.
T-LYMPHOCYTE-SPECIFIC ANTIBODY-RICIN IMMUNOTOXINS

was stored at 4°C until used; the 2-pyridyldisulfide content was determined as described (28). Approximately 2 mol of 3-(2-pyridyldithio)propionyl groups were introduced per mol of T101. As determined by indirect immunofluorescence, binding of T101-PDP to T-lymphocytes was identical to native immunoglobulin (33).

T101:Ricin A-Chain. Purified ricin A-chain was reduced at pH 5.5 by the addition of 1 m diithiothreitol to a final concentration of 20 mM. After incubation for 30 min at 23°C, the reduced A-chain was separated from the excess diithiothreitol and mixed overnight with T101-PDP at a ricin-A-chain:T101-PDP molar ratio of approximately 2:1. The conjugate was then purified by gel permeation chromatography using Sephadex G-150 equilibrated with PBS (Chart 1). The ricin A-chain conjugate typically eluted between fractions 26 and 31, unconjugated antibody between fractions 32 and 37, and free ricin A-chain at approximately fraction 50. The conjugate contained 1–2 mol of ricin A-chain per mol of antibody and blocked >85% of the protein synthesis activity in a cell-free lysate system (below). Similar results have been obtained with T101:ricin A-chain chromatographed on Sephacryl S-200.

T101:Ricin/Acetylricin. Native ricin, suspended in PBS containing 50 mM lactose, was reacted with SPDP as described above. Following overnight dialysis, a portion of the derivatized toxin was reacted with W-ITIM lactose, was reacted with SPDP as described above. Following dialysis, acetylricin was added, to the dialyzed, reduced ricin derivative T101-PDP and T101:ricin. The plates were incubated at 37°C under 10% humidified CO2 for 7 days; clusters (aggregates of 20–40 cells) and colonies (>40 cells per aggregate) were determined at that time.

Mice. Female BALB/c nude (nu/nu) mice, 8–10 weeks old, were obtained from the University of California, San Diego, nude mouse facility. They were bred under pathogen-free conditions and housed in sterile, covered cages kept in a laminar flow room.

RESULTS

Evaluation of Toxins and Immunotoxins. Ricin binding to various cell types, as determined by indirect immunofluorescence in the absence of lactose, has been shown previously to vary markedly (12). While CEM bound the most ricin of all cells tested, the T-cell line HPB-ALL and the B-cell line WIL-2 exhibited intermediate binding capacities and the B-cell line 8392 bound the least amount of ricin.

Binding of T101:ricin, T101:acetylricin, and T101:ricin A-chain to antigen-positive CEM cells incubated at 4°C was demonstrated by indirect immunofluorescence (33); whole-ricin conjugates were assayed in the presence of lactose. In each instance, the percentage of positive cells was 82% or greater and the mean intensity of fluorescence values were in excess of 116; mean intensities of fluorescence for cells treated with irrelevant IgG2a (RPC-5, an adjuvant-induced plasmacytoma) varied between 2 and 7 (data not shown). No increase in fluorescence intensity or percentage of positive cells was observed following treatment with 5 mM ammonium chloride (data not shown).

The concentration dependence of protein synthesis inactiva-
tion in CEM cells by ricin A-chain and T101:ricin A-chain is shown in Chart 2. Cells incubated with T101-PDP or ricin A-chain showed essentially no inhibition of protein synthesis. Assays with T101:ricin A-chain showed it to be only moderately effective; the ID₅₀ was approximately 5 nM. When assayed in the presence of 5 mM ammonium chloride, the ricin A-chain conjugate was nearly 10-fold more effective in blocking protein synthesis, with an ID₅₀ of approximately 0.7 nM. The use of 10 mM ammonium chloride produced an additional 2-fold improvement in conjugate effectiveness (not shown).

Protein synthesis inhibition by ricin, acetylricin, T101:acetylricin, and T101:ricin is shown in Chart 3. When assayed in the presence of 0.1 mM lactose, ricin blocked approximately 90% of the protein synthesis in CEM cells only at concentrations above 10 nM; the ID₅₀ was approximately 3 nM. Under similar conditions, the ID₅₀ for acetylricin was approximately 9 nM (data not shown). In the absence of lactose, protein synthesis activity was inhibited by more than 90% at 0.1 nM ricin (Chart 3). Acetylricin was approximately 1 log less effective than native ricin whether assayed in the absence or presence of lactose. While the ID₅₀ for ricin (no lactose) was approximately 0.03 nM, that for acetylricin was roughly 0.35 nM. Further when ricin and acetylricin were tested at a final concentration of 1 nM in a time course experiment using a cell-free reticulocyte lysate assay system, ricin and acetylricin blocked protein synthesis by 97 and 94%, respectively (data not shown). When ricin and acetylricin were injected i.p. into athymic mice, the LD₅₀ was approximately 5 nM (data not shown).

A comparison of the protein synthesis inhibition produced by T101:ricin and T101:ricin A-chain (Charts 2 and 3) shows that the whole-ricin conjugate is approximately 35-fold more effective than its A-chain counterpart. While the ID₅₀ for T101:ricin is approximately 0.15 nM, that for T101:ricin A-chain is approximately 5 nM. When T101:ricin and T101:acetylricin are assayed together, the ID₅₀ was approximately 0.2 nM, indicating that the conjugate is 10-fold more effective in blocking protein synthesis than native ricin. The ID₅₀ for acetylricin was approximately 1 log less effective than native ricin whether assayed in the absence or presence of lactose.

Effects of Whole-Ricin Conjugates on Cell Growth, Viability, and Protein Synthesis. Because the whole-ricin conjugates were more effective than was T101:ricin A-chain, they were selected for further study.

To establish that the conjugate killed the CEM cells, separate CEM cultures were treated with PBS, 1.0 nM T101-PDP, 1.0 nM T101:acetylricin (plus lactose), or 1.0 nM ricin (Chart 4). During the 48-h treatment phase of the experiment, control cultures exhibited increases in viable cell number and the expected levels of viability and protein synthesis (Chart 4, A–C). The immunotoxin- and ricin-treated cultures showed no growth, declining cell viability, and low levels of protein synthesis. Following the 48-h incubation, equal numbers of viable cells were suspended in reagent-free growth medium. Viable cells from the control cultures exhibited normal growth and protein synthesis (Chart 4, A–C).
T-LYMPHOCYTE-SPECIFIC ANTIBODY-RICIN IMMUNOTOXINS

Chart 4. Cell growth, viability, and protein synthesis during and after treatment with T101:acetylrin or ricin. Mid-log phase CEM cells were treated with either PBS, T101-PDP, T101:acetylrin, or ricin for 48 h at 37°C (treatment phase; A–C) (T101:acetylrin was used in the presence of lactose). Following treatment, the lymphocyte cultures were centrifuged through Ficoll and the live cells were removed; washed three times with RPMI 1640 containing 2% human serum, 10% calf serum, and 0.3% glutamate; and finally resuspended in the wash medium at cell densities between 1 and 5 x 10^6 cells/ml. The washed cells were subsequently incubated at 37°C in 5% humidified CO_2 (posttreatment phase) and the cell number (D), viability (E), and protein synthesis activities (F) were determined at the indicated times. O, PBS-treated CEM cells; Δ, lymphocytes treated with 1 nM T101-PDP; ○, cells treated with 1 nM T101:acetylrin; ●, lymphocytes treated with 1 nM native ricin. The ricin-treated cultures were terminated because of inadequate cells. The data presented are the mean values of triplicate determinations; bars, SD (bars shown unless smaller than the data point). The actual cell number values may be obtained by multiplying the mean values shown in A and D by a factor of 10^6.

D–F) while apparently “viable” cells (based upon trypan blue exclusion) from the conjugate- and toxin-treated cultures showed no growth, declining viabilities, and no protein synthesis activity. Similar viability data were obtained by cytofluorography using a mixture of ethidium bromide and fluorescein diacetate (29).

When CEM cells were treated with 0.2, 0.4, or 1 nM T101:ricin (plus lactose) plus varying concentrations of free T101, no effect on protein synthesis inhibition was observed until the free antibody concentration was present at a 5-fold molar excess over the immunocomplex concentration. At or above this concentration, the free antibody reduced significantly the inhibition of protein synthesis produced by the conjugate (data not shown).

Results similar to those shown in Chart 4 were obtained with line 8402 cells treated with 1.0 nM T101:ricin for 24 h at 37°C. When cultured for 7 days in 0.3% Bacto-agar, CEM or 8402 cells treated for 30 min at 37°C with 1.0 nM ricin or T101:ricin (plus lactose) produced no clusters or colonies. CEM or 8402 cells treated similarly with 0.1 nM ricin in the presence of 0.1 m lactose showed no inhibition of colony formation; 1 nM ricin (plus lactose) produced an approximately 60% inhibition of colony formation. Cells treated with 0.1 m lactose, washed, and plated in 0.3% agar exhibited normal growth (data not shown). These results show that conjugate-treated cells are indeed killed, as judged by the lack of protein synthesis activity and the absence of colonies despite the presence of “viable” cells.

Enhancement of Conjugate Cytotoxicity by Ammonium Chloride. The effects of lysosomotropic amines on whole-toxin internalization and processing have been characterized (9, 22), but little is known about its effects on immunoglobulin or immunotoxin internalization and processing (4, 8). As shown in Chart 5, the presence of ammonium ion markedly enhanced the efficacy of T101:ricin. Chart 5A shows that the rate of protein synthesis inhibition in CEM cells increased with immunotoxin concentration. Inactivation profiles were biphasic; following a lag period of 2–3 h, there was an apparent first order decline in protein synthesis activity. Further the lag period decreased with increasing immunotoxin concentration, and the first order rate of protein synthesis inhibition for 1 nM T101:ricin (Chart 5, Line 6) approached that of 1 nM ricin (Chart 5, Line 7). Using 1 nM T101:ricin, approximately 7 h are required to inhibit protein synthesis by 99%.

At all immunotoxin concentrations, 2 mM ammonium chloride increased immunotoxin efficacy (Chart 5A). Although the profiles remain biphasic at lower immunotoxin concentrations, the lag period was uniformly reduced to 2 h. The same effect was observed with ricin (Chart 5, Line 5). At T101:ricin concentrations of 0.5 and 1 nM (Chart 5, Lines 6 and 7), rates of protein synthesis inhibition were linear and more rapid than in the absence of ammonium chloride (Chart 5A). In the presence of 5 mM ammonium chloride (Chart 5C), the lag time was reduced to approximately 1 h for immunotoxin at concentrations of 0.01, 0.1, and 0.2 mM. While the time required to reduce cellular protein synthesis to 1% of the level determined for control cells was decreased to approximately 4.5 h for T101:ricin at both 0.5 and 1 nM (Chart

![Chart 5](chart5.jpg)
with 1 nM T101: ricin (plus lactose) and 10 mM ammonium chloride produced no inhibition of protein synthesis. When assayed in the presence of 0.1 M lactose, the effect of ammonium chloride on ricin toxicity was the same as that noted above. These results suggest that ammonium chloride enhances specific immunotoxin effectiveness but has little effect on ricin toxicity.

Cells from the human T-cell line 8402 were more sensitive to T101: ricin in the absence of ammonium chloride than were CEM cells (33) and the lag periods were shorter (Chart 6). The conjugate was more effective in the presence of 2 mM ammonium chloride (Chart 6B), and the biphasic inhibition curves were similar to those obtained with CEM cells assayed in the presence of 10 mM ammonium chloride (Chart 5D). At a concentration of 10 mM ammonium chloride, the lag period was completely abolished at all immunotoxin concentrations, and the maximum rate of protein synthesis inhibition was obtained at T101: ricin concentrations of 0.2 nM and above. As noted for CEM cells, the presence of ammonium chloride ultimately abolished the lag period associated with ricin intoxication of line 8402 cells but only slightly enhanced ricin cytotoxicity.

Cultures of CEM or 8402 cells grown for 32 h in the presence of ammonium chloride at concentrations of up to 10 mM exhibited no effects on cell growth, viability, or protein synthesis (data not shown). Treatment of 8392 cells with 1 nM T101: ricin in the presence of 0.1 M lactose and 10 mM ammonium chloride produced no inhibition of protein synthesis. Similar results were also obtained with the promyelocytic cell line HL-60 (data not shown). Thus the presence of ammonium chloride specifically increased immunotoxin effectiveness without altering ricin toxicity.

Effects of Ammonium Chloride on Conjugate Processing. Lag periods similar to those depicted above have been observed with plant and bacterial toxins in both cell-free and whole-cell assays (17, 22, 32). In assays using whole cells, these lag periods were ascribed to the time required for toxin binding and internalization and escape of toxin A-chain from intracellular vesicles (17). Further Olsnes et al. (17) showed that a plot of the time required for 50% inhibition of protein synthesis, f50, versus the inverse of the square root of the bound toxin concentration yielded a straight line passing through the origin only if free ricin A-chain polypeptides inactivate cellular ribosomes and that ammonium chloride alters the way in which the conjugate is processed by the cell.

To further support the contention that the ricin A-chain must first dissociate from the immunotoxin before ribosomal inactivation can occur, the t50 values were plotted versus the inverse of the effective immunotoxin concentrations (17). If intact immunotoxin were responsible for the inhibition of cellular protein synthesis, these plots should have produced straight lines. The profiles for CEM and 8402 cells at all ammonium ion concentrations required for toxin binding and internalization and dissociation of the A- and B-chains.

For the profiles shown in Chart 7, the data from Charts 5 and 6 are plotted as described above; regression lines were calculated by the method of least squares (r² > 0.94). In the absence of ammonium chloride, a plot of data for CEM cells (Chart 7A) intersected the ordinate at a value of approximately 1.5 h; the presence of ammonium ion altered the data such that the lines passed through the origin and their slopes decreased with increasing ammonium chloride concentrations. A plot of the data for 8402 cells (Chart 7B) assayed in the absence of ammonium ion passed through the origin. Data from assays conducted in the presence of ammonium chloride produced lines which intersected near the ordinate at a value of approximately 0.2 h and the slopes of which decreased with increasing concentrations of ammonium ion.

If the effective (bound) concentrations of T101: ricin are assumed to be one-half those of the initial conjugate concentrations, similar plots are generated for both cell lines (data not shown). Because both plots produced straight lines, the data suggest that only free ricin A-chain polypeptides inactivate cellular ribosomes and that ammonium chloride alters the way in which the conjugate is processed by the cell.

To further support the contention that the ricin A-chain must first dissociate from the immunotoxin before ribosomal inactivation can occur, the t50 values were plotted versus the inverse of the effective immunotoxin concentrations (17). If intact immunotoxin were responsible for the inhibition of cellular protein synthesis, these plots should have produced straight lines. The profiles for CEM and 8402 cells at all ammonium ion concentrations required for toxin binding and internalization and dissociation of the A- and B-chains.

For the profiles shown in Chart 7, the data from Charts 5 and 6 are plotted as described above; regression lines were calculated by the method of least squares (r² > 0.94). In the absence of ammonium chloride, a plot of data for CEM cells (Chart 7A) intersected the ordinate at a value of approximately 1.5 h; the presence of ammonium ion altered the data such that the lines passed through the origin and their slopes decreased with increasing ammonium chloride concentrations. A plot of the data for 8402 cells (Chart 7B) assayed in the absence of ammonium ion passed through the origin. Data from assays conducted in the presence of ammonium chloride produced lines which intersected near the ordinate at a value of approximately 0.2 h and the slopes of which decreased with increasing concentrations of ammonium ion.

If the effective (bound) concentrations of T101: ricin are assumed to be one-half those of the initial conjugate concentrations, similar plots are generated for both cell lines (data not shown). Because both plots produced straight lines, the data suggest that only free ricin A-chain polypeptides inactivate cellular ribosomes and that ammonium chloride alters the way in which the conjugate is processed by the cell.

To further support the contention that the ricin A-chain must first dissociate from the immunotoxin before ribosomal inactivation can occur, the t50 values were plotted versus the inverse of the effective immunotoxin concentrations (17). If intact immunotoxin were responsible for the inhibition of cellular protein synthesis, these plots should have produced straight lines. The profiles for CEM and 8402 cells at all ammonium ion concentrations required for toxin binding and internalization and dissociation of the A- and B-chains.

For the profiles shown in Chart 7, the data from Charts 5 and 6 are plotted as described above; regression lines were calculated by the method of least squares (r² > 0.94). In the absence of ammonium chloride, a plot of data for CEM cells (Chart 7A) intersected the ordinate at a value of approximately 1.5 h; the presence of ammonium ion altered the data such that the lines passed through the origin and their slopes decreased with increasing ammonium chloride concentrations. A plot of the data for 8402 cells (Chart 7B) assayed in the absence of ammonium ion passed through the origin. Data from assays conducted in the presence of ammonium chloride produced lines which intersected near the ordinate at a value of approximately 0.2 h and the slopes of which decreased with increasing concentrations of ammonium ion.

If the effective (bound) concentrations of T101: ricin are assumed to be one-half those of the initial conjugate concentrations, similar plots are generated for both cell lines (data not shown). Because both plots produced straight lines, the data suggest that only free ricin A-chain polypeptides inactivate cellular ribosomes and that ammonium chloride alters the way in which the conjugate is processed by the cell.

To further support the contention that the ricin A-chain must first dissociate from the immunotoxin before ribosomal inactivation can occur, the t50 values were plotted versus the inverse of the effective immunotoxin concentrations (17). If intact immunotoxin were responsible for the inhibition of cellular protein synthesis, these plots should have produced straight lines. The profiles for CEM and 8402 cells at all ammonium ion concentrations required for toxin binding and internalization and dissociation of the A- and B-chains.

For the profiles shown in Chart 7, the data from Charts 5 and 6 are plotted as described above; regression lines were calculated by the method of least squares (r² > 0.94). In the absence of ammonium chloride, a plot of data for CEM cells (Chart 7A) intersected the ordinate at a value of approximately 1.5 h; the presence of ammonium ion altered the data such that the lines passed through the origin and their slopes decreased with increasing ammonium chloride concentrations. A plot of the data for 8402 cells (Chart 7B) assayed in the absence of ammonium ion passed through the origin. Data from assays conducted in the presence of ammonium chloride produced lines which intersected near the ordinate at a value of approximately 0.2 h and the slopes of which decreased with increasing concentrations of ammonium ion.

If the effective (bound) concentrations of T101: ricin are assumed to be one-half those of the initial conjugate concentrations, similar plots are generated for both cell lines (data not shown). Because both plots produced straight lines, the data suggest that only free ricin A-chain polypeptides inactivate cellular ribosomes and that ammonium chloride alters the way in which the conjugate is processed by the cell.

To further support the contention that the ricin A-chain must first dissociate from the immunotoxin before ribosomal inactivation can occur, the t50 values were plotted versus the inverse of the effective immunotoxin concentrations (17). If intact immunotoxin were responsible for the inhibition of cellular protein synthesis, these plots should have produced straight lines. The profiles for CEM and 8402 cells at all ammonium ion concentrations required for toxin binding and internalization and dissociation of the A- and B-chains.

For the profiles shown in Chart 7, the data from Charts 5 and 6 are plotted as described above; regression lines were calculated by the method of least squares (r² > 0.94). In the absence of ammonium chloride, a plot of data for CEM cells (Chart 7A) intersected the ordinate at a value of approximately 1.5 h; the presence of ammonium ion altered the data such that the lines passed through the origin and their slopes decreased with increasing ammonium chloride concentrations. A plot of the data for 8402 cells (Chart 7B) assayed in the absence of ammonium ion passed through the origin. Data from assays conducted in the presence of ammonium chloride produced lines which intersected near the ordinate at a value of approximately 0.2 h and the slopes of which decreased with increasing concentrations of ammonium ion.

If the effective (bound) concentrations of T101: ricin are assumed to be one-half those of the initial conjugate concentrations, similar plots are generated for both cell lines (data not shown). Because both plots produced straight lines, the data suggest that only free ricin A-chain polypeptides inactivate cellular ribosomes and that ammonium chloride alters the way in which the conjugate is processed by the cell.

To further support the contention that the ricin A-chain must first dissociate from the immunotoxin before ribosomal inactivation can occur, the t50 values were plotted versus the inverse of the effective immunotoxin concentrations (17). If intact immunotoxin were responsible for the inhibition of cellular protein synthesis, these plots should have produced straight lines. The profiles for CEM and 8402 cells at all ammonium ion concentrations required for toxin binding and internalization and dissociation of the A- and B-chains.
tions were curved and passed through the origin (data not shown), suggesting that the observed lag period represents more than the time required for conjugate internalization, that the intact immunotoxin is apparently not responsible for inactivation of protein synthesis, and that free ricin A-chain is apparently the true effector.

DISCUSSION

In this report, we assessed the efficacy of antibody:ricin conjugates synthesized with the reducible cross-linking reagent SPDP. Immunotoxins were constructed between the pan-T-cell antibody T101 and native ricin, acetylated whole ricin, or ricin A-chain. Their relative order of effectiveness was: T101:ricin > T101:acetylrucin > T101:ricin A-chain (Charts 2 and 3).

Acetylation of whole ricin with N-acetylimidazole reduced B-chain binding but not A-chain catalytic activity (24, 34); acetylrucin was approximately 21% as toxic to nude mice as was the native toxin. When assayed in vitro using T-lymphocytes (Chart 3), acetylrucin was approximately 10-fold less effective than native ricin in blocking cellular protein synthesis. Further equimolar concentrations of acetylrucin and ricin were equally effective in blocking protein synthesis in a cell-free reticulocyte lysate system. While these data suggest that the ricin toxin was not inactivated by acetylation, they do not rule out the possibility that the acetylrucin may be a mixture of acetylated and native toxin.

Indirect immunofluorescence demonstrated that while both T101:ricin and T101:acetylrucin were bound to antigen-positive T-lymphocytes (data not shown), the acetylrucin conjugate was approximately 10-fold less effective than the native ricin immunotoxin in both the presence and the absence of lactose. These observations suggest that the modified toxin B-chain may be less able to facilitate conjugate internalization and/or escape of the ricin A-chain into the cytosol (34). Neither immunotoxin was effective in blocking protein synthesis in the antigen-negative B-cell line 8392 when assayed with lactose.

The T101:ricin A-chain conjugate, while exhibiting normal binding to antigen-positive cells, was approximately 35-fold less effective than was T101:ricin. The presence of 5 mM ammonium chloride enhanced T101:ricin A-chain effectiveness nearly 10-fold. Thus the antibody may not be able to functionally replace the toxin B-chain in some instances, contributing to the widely varying activities observed for ricin A-chain immunotoxins (30, 34, 36).

Despite the apparent inconsistency between the rapid inhibition of protein synthesis by T101:acetylrucin and the slow loss of cell viability (Chart 4), the conjugate was effective in blocking cell growth. The continued decline in viability of conjugate-treated cells is similar to the results with normal lymphocytes obtained by Bernheim et al. (1) and suggests there are at least two lymphocyte populations. While one exhibits a rapid rate of cell death, the other population is less sensitive to the conjugate and loses cell viability more slowly. Despite the presence of apparently viable cells, our results show that CEM cells briefly (e.g., 15–30 min) exposed to ricin or T101:ricin are indeed killed, as they did not form clusters or colonies in agar colony assays (data not shown).

Lysosomotrophic amines previously have been shown to raise the pH of intracellular vesicles and to interfere with the normal cellular processing of toxins (5). When the rate of protein synthesis inhibition by T101:ricin in malignant T-lymphocytes was studied in the absence of ammonium chloride, biphasic inactivation profiles were obtained with both CEM and 8402 cells (Charts 5 and 6). The initial slope of each plot was dependent upon the immunotoxin concentration and may represent the time required for intracellular conjugate processing and for an intracellular, steady-state concentration of the immunotoxin to be obtained (32). The second phase was concentration dependent and may represent a first-order decline in protein synthesis. Under these conditions, the rate of immunotoxin processing or ribosomal inactivation may be rate limiting. These results are similar to those obtained for diphtheria toxin and for abrin and ricin (17, 32). In the presence of ammonium chloride, the biphasic character was progressively lost, suggesting that ammonium ion may alter intracellular conjugate processing.

The data in Chart 7 tentatively suggest that once the conjugate is internalized the ricin A-chain polypeptide must dissociate from the remainder of the conjugate before ribosomal inactivation can occur. This conclusion is similar to one derived by Olsnes et al. (17) in experiments with toxin-treated HeLa cells.

The observation that CEM and 8402 cells apparently process immunotoxin molecules differently was surprising. In the absence of ammonium ion, the lag period associated with the protein synthesis inactivation profiles for CEM cells (Chart 5) may not be entirely due to the dissociation of the A-chain from immunotoxin since the plot of the fso data (Chart 7A) did not pass through the origin. Because the corresponding plot for 8402 cells passed through the origin, the data suggest that the A-chain dissociation step accounts for the lag periods observed with this cell line (Chart 7B). The fso data for protein synthesis inactivation in CEM cells in the presence of ammonium chloride suggest that ammonium ion alters some step in conjugate processing, perhaps blocking the transfer of conjugate molecules from lysosome-like vesicles to lysosomes and thereby preventing their degradation (23, 31). This would account for the apparent increase in immunotoxin efficacy under these conditions. While the lag periods observed with 8402 cells assayed in the presence of ammonium ion may represent more than the time required for the release of the A-chain, the data show that the presence of ammonium ion increases immunotoxin effectiveness in these cells as well.

In this communication, we present data comparing the efficacy of three T101 immunotoxins. Preparations of T101:ricin A-chain were less effective against T-lymphocytes than was either T101:ricin or T101:acetylrucin, suggesting that this conjugate was less well internalized. Acetylation of native ricin prior to conjugation to T101 reduced the in vitro toxicity of the conjugate whether assayed in the presence or the absence of lactose. Thus the reduced effectiveness of T101:acetylrucin compared to T101:ricin may be attributed to the reduced function of the toxin B-chain (34). T-lymphocytes incubated with T101:ricin in the presence of ammonium chloride exhibited apparent alterations in immunotoxin processing and more rapid inactivation of cellular protein synthesis.

ACKNOWLEDGMENTS

We would like to thank Douglas A. Lappi for the preparation of ricin and Dr. Raymond Taetle for his careful reading of the manuscript.
REFERENCES


Kinetics of Protein Synthesis Inactivation in Human T-Lymphocytes by Selective Monoclonal Antibody-Ricin Conjugates


*Cancer Res* 1985;45:5263-5269.

Updated version Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/45/11_Part_1/5263](http://cancerres.aacrjournals.org/content/45/11_Part_1/5263)

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.