Cellular Processing of a Ricin-Antibody Conjugate

A KINETIC ANALYSIS OF THE RATE-LIMITING STEP*

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Upon exposure to holoricin-antibody conjugates, target cells display a period of no measurable intoxication followed by a concentration-dependent exponential decline in protein synthesis. The rate of this decline is increased by conjugate structural variables such as addition of a second ricin to the antibody or introduction of a peptide spacer between the toxin and antibody. Additionally, the rate is enhanced by the addition of ammonia or monensin.

The relationship between immunotoxin concentration and the rate of protein synthesis inhibition is shown to obey typical Michaelis-Menten kinetics. By displacing bound immunotoxin with native antibody, it was demonstrated that the rate-limiting saturable process is not due to antibody-surface antigen interaction. Although addition of a second ricin as well as addition of ammonia elevated the measured rate of protein synthesis inhibition, curve-fitting techniques indicated that they did not elevate the maximal rate; however, introduction of a peptide spacer into the conjugate or addition of monensin increased the maximal rate of this saturable process. Additionally, the kinetic examination of the potentiation effects of ammonia and monensin indicates that they operate on different cellular processes involved with the intracellular routing toward intoxication. An equation model is developed which helps interpret the known features of the conjugate intoxication process.

Upon exposure to numerous protein toxins such as ricin or diphtheria toxin or their antibody conjugates, targeted cells display a period of no measurable intoxication, commonly called the lag period, followed by a toxin concentration-dependent exponential decline in protein synthesis. This decline can be made linear on a semilog plot, and the slope, typically given the units, logs/h, has been used to establish rate constants for the intoxication process. Although the loss of protein synthesis is (pseudo) first order with respect to the incubation media (8-11). We have used the model developed in this report to describe how these structural variables and modifiers can bring about changes in efficacy.

EXPERIMENTAL PROCEDURES

Ricin D was purified as described previously (12) from Ricinus communis beans grown in Japan and kindly provided by Dr. Gunki Funateu, Kyushu University, Fukuoka, Japan. m-Maleimidobenzoyl-N-hydroxysuccinimide ester and 2-iminothiolane were obtained from Pierce. RPMI 1640 and leucine-free RPMI 1640 culture media were from GIBCO. 1-1-[U-3H]Leucine (300 mCi/mmol) was from Du Pont-New England Nuclear. Monensin was purchased from Sigma.

Synthesis of Ricin Conjugates—Ricin-antibody conjugates were synthesized as described previously. Briefly, ricin and an anti-Thy, OX7, antibody were coupled through a modified insulin B chain in the peptide spacer conjugate (7); other conjugates were synthesized by thiolation of antibody with 2-iminothiolane followed by activation of antibody with m-maleimidobenzoyl-N-hydroxysuccinimide ester, resulting in a stoicohedral linkage between the two moieties (4).

Protein Synthesis Assay—The effect of immunotoxin on protein synthesis was measured as described previously (4) on the Thy1 AKR SL2 cell line. Typically, 10^6 cells in leucine-free RPMI 1640 medium containing 2 mM NaHCO_3, 25 mM Hepes, pH 7.4, and 0.1% bovine serum albumin were added to medium containing the desired level of immunotoxin and lactose (final concentration, 100 mM) so that the final volume was 0.1 ml. This was done in a 96-well round bottom plate. After the designated time, a 1/10 dilution of stock 1-[U-3H]
leucine (0.01 mCi/ml) into leucine-free RPMI was added in a 10-ml volume to the cells. After a 60-min incubation, the cells were harvested on glass fiber filters with a Titertek cell harvester (Flow Laboratories, Inc.) and then counted. Unless otherwise stated, all protein synthesis assay time points were done in quadruplicate. Data were derived as cpm with standard deviations of 10% or less of the mean value unless otherwise stated. Comparison studies presented with a single figure were done on identical cell preparations at the same time since we find day-to-day variations in sensitivity of cells to toxins and toxin conjugates. The timed additions of ammonia or monensin were achieved by addition of a 0.10 volume of a 10 X (of final desired concentration) solution of NH4Cl or monensin in RPMI 1640. Monensin was initially dissolved in ethanol at 10 mM then diluted further in RPMI medium. For all experiments involving the effect of the addition of ammonia or monensin on immunotoxin activity, control values were determined with similar potentiation additions.

Kinetic Analysis — The level of protein synthesis for a toxin-treated cell is determined from the level of [3H]leucine incorporation as compared with a control not exposed to toxin and is expressed as percent of control. Graphically, this is represented on a semilogarithmic plot, and the resultant slopes were determined with Graphit (Graphit Software, Philadelphia). We have used the slope, given a positive value for ease of calculation, of this semilog plot (units = h⁻¹) to characterize the rate of inactivation of protein synthesis for any immunotoxin concentrations. To estimate kinetic parameters involved with the intoxication process, we have used both linear and graphic methods (Lineweaver-Burk and Hanes-Woolf plots; see Ref. 13) as well as nonlinear regression methods concerned with fitting data to the Michaelis-Menten equation. Graphic methods have been criticized since some linear forms of the Michaelis-Menten equation can place undue emphasis on certain data points (see Refs. 14 and 15 for recent discussions). For that reason, the parameters being compared in this report were determined from nonlinear regression fit to the Michaelis-Menten equation using the EZ-FIT software on an IBM-PC. The EZ-FIT program as written by Perrella (15) utilizes the Nelder-Mead simplex (16) and Marquardt (17) nonlinear regression algorithms sequentially. The estimated maximal rate (normally expressed as \( V_{\text{max}} \)) and the standard error are reported for each experiment. Although the parameters reported are derived as described above, they are depicted graphically and fitted by least squares to linear forms of the Michaelis-Menten equation, independently of the EZ-FIT analysis, with the Cricket Graph software. Figure legends include results from both the nonlinear and linear regressions.

In that the examined processes cannot be considered at this time to be enzymatic, we have purposefully avoided the typical enzymatic nomenclature to prevent the appearance of any undue chemical picture of the studied processes.

Unlike studying enzymatic rates, the intoxication process by immunotoxins can only be studied in a narrow concentration range. The concentration range resulting in statistically significant different rates is approximately 10-20 fold; in the absence of potentiators and under the conditions used here, this range is 0.2-2.0 nM. Lower levels result in very shallow declines in protein synthesis. Higher levels rates is approximately 10-20 fold; in the absence of potentiators and under the conditions used here, this range is 0.2-2.0 nM. Lower levels result in very shallow declines in protein synthesis. Higher levels.
squares) fit in the Hanes-Woolf plot (no competitor: open squares) or without (open squares) competing native OX7 antibody (500 ng/ml) under conditions described in Fig. 1. The derived r-squares fit in the Hanes-Woolf plot (no competitor: y = 1.19 + 1.26x, r = 1.00; plus competitor: y = 1.21 + 2.03x, r = 1.00). Nonlinear regression estimate of maximal rates: no competitor: Vmax = 0.80 ± 0.004; plus competitor: Vmax = 0.51 ± 0.037.

For the Hanes-Woolf plot, this is typical of uncompetitive inhibition and suggests that the native antibody is competing at a site other than the rate-limiting process. If the saturable process is not antibody binding, then the limiting process might be described as follows:

\[ \text{IT}_p + R \rightarrow \text{IT}_pR \rightarrow \frac{k_1}{k_{-1}} \text{IT}_p + P \rightarrow \text{IT}_pP \rightarrow \text{ribosomal inactivation} \]

where IT is the intracellular immunotoxin, P is the binding rate for the rate-limiting step, k1 and k_{-1} are the rate constants for the reversible binding, and k2 is the irreversible rate-limiting step that leads to cytosolic entry and ribosomal inactivation. The nature of P cannot be defined; it could be, for example, a processing enzyme or a receptor. Furthermore, it is essential to appreciate that the arrows of Equation 2 do not necessarily depict chemical events but could represent cellular processes or events that display Michaelis-Menten kinetics.

As outlined in the Introduction, the way in which a toxin-antibody conjugate is constructed can affect its efficacy; for example, the addition of a second ricin to an antibody results in a 4-fold increase in efficacy (4). When an anti-Thy antibody possessing one ricin (Fig. 3, triangles) is compared in a Line-weaver-Burk plot with a conjugate preparation containing two ricins per antibody (open squares), we find that at all concentrations examined the conjugate containing a second ricin was more efficacious; however, at saturating levels, the multiricin conjugate would result in an elevated level of internalized ricin over the monoricin conjugate, and if antigen binding was rate-limiting, it would lead to greater efficacy. In terms of Equation 2, the presence of a second ricin may result in an elevated concentration of the IT;P complex either due to an increase in the IT;P concentration or through changes in k1/k_{-1}. Since the theoretical maximal rates as determined by nonlinear regression (see “Experimental Procedures”) are not significantly different (monoricin conjugate, Vmax = 0.63 h⁻¹ ± 0.01; multiricin conjugate, Vmax = 0.58 ± 0.03), evidence for alteration in the rate-limiting k2 is lacking.

Another structural feature that increases the rate of protein synthesis inhibition of a target cell is the introduction of a flexible peptide spacer between the ricin and the antibody moiety (7). When this peptide spacer conjugate is compared with a nonspacer conjugate on a double-reciprocal plot (Fig. 3, filled squares), the spacer conjugate displays a higher rate at all measured concentrations. The estimated value of the maximal rate as determined by nonlinear regression was found to be 0.80 h⁻¹ ± 0.013 for the peptide spacer conjugate. Mechanistically, this structural alteration increases measurable efficacy in a way different from that seen with the addition of a second ricin, and in terms of Equation 2, the pronounced alteration would appear to be at k3, the designated rate-limiting process in this model. Although other alterations within the scheme such as at k4, can bring about increases in efficacy at nonsaturating concentrations, the increase in the maximal rate would be due to an increased k3.

In addition to immunotoxin structural elements, lipophilic amines such as ammonia and carboxylic ionophores such as Nigericin and monensin increase the toxicity of ricin and ricin conjugates (8–11). The addition of 10 mM NH₄Cl to an immunotoxin incubation remarkably increases the efficacy of the anti-Thy-conjugate; however, at saturating levels of conjugate, the ammonia-containing incubation (Fig. 4a, filled squares) does not appear to possess an elevated maximal rate relative to its ammonia-free control (open squares). The nonlinear regression analysis suggests that the ammonia-treated cells possess a depressed maximal inhibition rate (Vmax = 0.52 ± 0.16), approximately 70% of that seen with cells not treated with ammonia (Vmax = 0.71 ± 0.046). The finding that measured rates are elevated in the presence of ammonia is similar to the comparison of mono- and biricin conjugates, and in terms of Equation 2, the presence of ammonia presumably results in elevated levels of the IT;P complex when compared with the incubations lacking ammonia. Again, there is no implied alteration of the rate-limiting step at k2.
The addition of the second type of potentiator, monensin, affected the system differently. The addition of this carboxylic ionophore at a final concentration of $5 \times 10^{-8}$ M resulted in increased efficacy at all concentrations examined (see Fig. 4b); additionally, the extrapolate maximal rate value was greater than the maximal rate for the same conjugate in the absence of monensin. Like the incorporation of the peptide spacer, the effect induced by monensin implies an increase in $k_2$ of Equation 2.

In order to generate a broad range of statistically significant inhibition rates (see "Experimental Procedures"), the stock dilutions of conjugate used to examine the effect of ammonia or monensin were different (more dilute) than those used to examine the decrease in protein synthesis by the immunotoxin in the absence of either potentiator. To demonstrate further the difference between ammonia and monensin, identical conjugate dilutions were compared in the presence of ammonia or monensin. As anticipated, the maximal rate of monensin-treated cells was more than 2-fold greater than ammonia-treated cells (see Fig. 5). This finding confirms that monensin can enhance a cellular-toxin process that is unmodified or affected differently by ammonia. Although monensin, like ammonia, may increase the $IT_7P$ concentration, it also alters the rate-limiting process characterized in Equation 2 by an elevated $k_2$. Note that the monensin concentration is 0.71 by an elevated ionophore at a final concentration of $5 \times 10^{-7}$ M. For monensin, the extrapolate maximal rate value was $10^{-3}$, more than 2-fold greater than the maximal rate for the same conjugate in the absence of monensin. Like the incorporation of the peptide spacer, the effect induced by monensin implies an increase in $k_2$ of Equation 2.

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below that which is necessary to alter intracellular pH measurable (19–21).

The previous experimental results describe the kinetic nature of immunotoxin processing and do not indicate the nature of the saturable single site process. Neither do they fully describe the temporal location of this step in the overall pathway. From the sequence of steps of Equation 2, one might anticipate that the enhancing effect of ammonia (acting prior to the \( k_0 \) step) occurs at an earlier step in the intoxication process than monensin (at \( k_0 \)). To examine this feature, we have implemented the reversal of a method used previously with diphtheria toxin (21) and diphtheria toxin conjugates (23, 24) to time the passage of the first toxin molecules completing the acid-requiring step. Diphtheria toxin requires vesicular acidification, and the addition of ammonia at the time of toxin exposure results in protection from intoxication (22). If the ammonia is added to the cell population at time points after exposure to diphtheria toxin, then as the time of toxin preexposure increases, the addition of ammonia offers decreasing protection. With ricin and ricin conjugates, the addition of ammonia and monensin does not decrease toxicity but rather increases it. By choosing a ricin conjugate concentration (50 ng/ml) which displays little protein synthesis inhibition in the absence of potentiators, the effect of the time of potentiation addition can be studied. Aliquots of cells were incubated with the antibody-ricin conjugate at 4 °C for 1 h, then warmed to 37 °C, incubated further, and followed by a 60-min 4°C-leucine pulse. In the absence of potentiaters, there was minimal inhibition of protein synthesis; however, the addition of 10 mM ammonium chloride or 2 × 10^{-7} M monensin at the time of immunotoxin addition resulted in 70% and greater than 90% inhibition, respectively. Addition of the potentiators 1 h prior to the addition of the immunotoxin resulted in no greater increase in efficacy (data not shown); however, if the potentiators were added at times after the temperature elevation to 37 °C (time 0, Fig. 6, a and b), the enhancing effects of the agents were unchanged for approximately 5 min for ammonia and for nearly 30 min for monensin. After these times, the toxicity of the conjugates diminished. The inflection point identifies the time at which the first conjugates move through the cellular compartments (or biochemical processes) that are altered by these agents and thus are no longer subject to full potentiation by the presence of them. When different levels of immunotoxin were examined, the level of intoxication was changed but had no effect on the time of the inflection point (data not shown; see "Discussion"). Although the increase in toxicity induced by monensin is greater than that of ammonia, the concentration of monensin is not effective in rapidly neutralizing acidic vesicles (19–21).

The above findings demonstrate both a temporal and functional difference for toxin potentiation by monensin and ammonia and are consistent with the proposed effects on the rate-limiting process (\( k_0 \)) and the internal concentration of productive toxin conjugate (\( IT \cdot P \)).

**DISCUSSION**

This report describes the relationship of toxin-conjugate concentration to the concentration-dependent inhibition rate of protein synthesis in the classical terms of Michaelis-Menten kinetics. The model derived from this type of analysis describes the rate-limiting process of intoxication by an anti-Thy-holoricin conjugate as intracellular, affected by the chemical means of conjugation, and enhanced by the addition of monensin. Interestingly, this process is not affected by ammonia, indicating that these potentiators operate at different steps and possibly different cellular compartments.

The experimental findings in this report only describe the kinetic nature of immunotoxin processing and do not describe the nature of the saturable single site process, which, for example, could be binding to a receptor or an enzymatic site. Additionally, it should be noted that the measurements used in this study are the loss of cellular capacity to incorporate label and that the actual inactivation of ribosomes, the only known enzymatic step in intoxication, may not be the rate-limiting feature; that is, what we have characterized as the rate-limiting step may be distal to the process we actually measure. Although the process of intoxication by these conjugates may differ from native toxin, it should be noted that much is known about the rate-limiting step for native toxins, which appears to be the toxin entry into the relevant cytosolic compartment and not the enzymatic inactivation of protein synthesis machinery (for review see Ref. 25).

The methods of analysis used here with an antibody-ricin conjugate cannot be directly applied to study native ricin activities. These appear to be composed of multiple cellular binding sites with multiple affinities (1) and with potentially different resultant efficacies for the individually bound ricin molecules. The affinity constant of the anti-Thy OX7 antibody is on the order of 10^{16} M^{-1}; under the conditions used here, binding is essentially irreversible; the concentration of added conjugate is directly proportional to the amount bound (4). Furthermore, the concentration of added immunotoxin must be directly proportional to the internal concentration of conjugate (\( IT \)) for the developed relationship of external immunotoxin concentration to the rate of protein synthesis inhibition to hold. We suspect, however, that this study also describes processes involved with cellular intoxication by the native toxin. The analysis may be more simplified with conjugates in that they have high affinity single plasma membrane-binding sites. All experiments were carried out in 100 mM lactose to minimize ricin moiety interaction with the outside of the cell.

In that the rate-limiting process is intracellular, we have no control, or for that matter, knowledge, of the state of the reaction in Equation 2. We cannot, however, demonstrate that a steady state condition exists; however, the ability to fit the generated data to Michaelis-Menten-type kinetics suggests that the process simulates steady state. More complicated equations could be developed to characterize this intoxication process, but the rather straightforward methodology used here puts the relationship in terms comprehensible to many; additionally, this mathematical description successfully illustrates how the process is altered differently by immunotoxin construction and the presence of potentiaters.

It is of interest that the theoretical maximal rate can be changed by variables in immunotoxin construction and that these resultant maximal rates are less than the experimentally measurable rates of native toxins. For example, ricin can result in a greater than 5 h^{-1} inhibition rate on AKR cells with extrapolated values of 8 h^{-1} at full receptor occupancy (1). Now that the maximal rate (true efficacy) can be determined, one can more accurately evaluate the effects of conjugate construction efficacy.

The inflexion points of the potentiation by ammonia or monensin (Fig. 6) reflect the time at which the first toxin moieties bypass the cellular compartment, or biochemical process, that is affected by these agents. This method has been used previously to examine antisera, ammonia, and temperature protection from intoxication by modeccin (26) as well as diphtheria toxin (22, 27) and diphtheria toxin

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\(^2\)J. W. Marsh, unpublished results.
monensin-sensitive step would be greater than the measured where effects on intracellular transport and Golgi function were evident the effect of monensin at lower concentrations have demonstrated little or no effect on vesicular pH (19–21), whereas effects on intracellular transport and Golgi function were evident (31–34).

Thus, the ability of ammonia to neutralize acidic vesicles is rapid. Addition of ammonia (5 mM) or monensin (10 μM) raises the pH (from under 5 to above 6) of endocytic vesicles in 1–2 min in 3T3 cells (29) and under 5 min in murine peritoneal macrophages (30). It has been shown previously that the first diphtheria toxin molecules complete the ammonia-inhibiting acid-requiring step at 3–5 min, similar to the time for enhancement of ricin moiety toxicity shown here. The effect of ammonia on diphtheria toxin is known to occur in an early endosomal (prelysosomal) vesicle (22). Studies on the effect of monensin at lower concentrations (10^−4 M or less) have demonstrated little or no effect on vesicular pH (19–21), whereas effects on intracellular transport and Golgi function were evident (31–34).

The sequence of the processing events affected by ammonia and monensin was described indirectly by kinetic analysis (see Equation 2) as well as directly examined by the experiment of Fig. 6. Although both increase potency of the ricin moiety, only monensin increases efficacy. Besides affecting the rate-limiting process, monensin also differs from ammonia in that the monensin-enhanced process occurs at a later time in the routing of the toxin moiety through the cell. As mentioned above, monensin can alter Golgi function. It is of interest that ricin (36) and ricin conjugates are relatively ineffective in cellular intoxication at temperatures below 20 °C. Routing of various ligands through the Golgi cisternae as well as into lysosomes is diminished at similarly reduced temperatures (37–39). Evidence that ricin intoxication involves the biosynthetic/secretory pathway has been supplied through demonstration of innate protection afforded a hybridoma cell line that is synthesizing an anti-ricin monoclonal antibody (40). Thus, the differential effects on ricin intoxication by ammonia and monensin as reported here in terms of a kinetic model are consistent with previous findings and suggest that the rate-limiting step for the ricin conjugates used in this report occurs in the Golgi apparatus or associated compartment.

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REFERENCES