Endocytosis of Immunotoxin-791T/36-RTA by Tumor Cells in Relation to Its Cytotoxic Action

Vera S. Byers, Izabella Z. A. Pawluczyk, Doreen S. W. Hooi, Michael R. Price, Steve Carroll, Michael J. Embleton, Martin C. Garnett, Nicola Berry, R. Adrian Robins, and Robert W. Baldwin


ABSTRACT

Ricin A chain immunotoxin constructed with monoclonal antibody 791T/36, which recognizes a tumor associated glycoprotein M, 72,000 antigen present on sarcomas and colon and ovarian cancer cells, is cytotoxic for cell lines from tumors expressing this antigen. Incubation of sarcoma 791T cells with immunotoxin for only 5 min is sufficient to produce >95% inhibition of tumor cell growth. Papain treatment of these cells to remove immunotoxin from the cell surface indicated that the cell surface acts as a reservoir for continued internalization of immunotoxin over several hours, but even so, 50% inhibition of cell survival was produced over a 2- to 3-h period.

Analysis of the rate of endocytosis demonstrated that 30–50% of cell bound immunotoxin was internalized over a 180-min period. This was primarily dictated by the antibody moiety, regardless of the degree of conjugation to ricin A chain. This rate is much slower than that of other cell surface ligands such as transferrin. Cell cytosol acidification experiments were performed to determine whether this immunotoxin was internalized by clathrin coated pits, which is relatively rapid, or by smooth pits, which is slower, and the results indicated the latter mechanism is almost exclusively used. Intracellular trafficking of antibody 791T/36, conjugated to human serum albumin-tetramethylrhodamine was investigated by flow cytometry. The movement of the conjugate into the lysosomal compartment was delayed so that degradation products were only detected after a lag phase of 30–60 min. The lack of potentiatior dependence of 791T/36 immunotoxin is in keeping with these findings.

INTRODUCTION

Immunotoxin 791T/36-RTA, constructed by conjugating RTA1 to the murine monoclonal antibody 791T/36, is specifically cytotoxic for a variety of tumor cells expressing the gp72 antigen which is recognized by this antibody (1). These include osteogenic sarcoma, colorectal, gastric, and ovarian carcinoma cells (2, 3) and the immunotoxin is being evaluated for the treatment of colorectal cancer (4).

In order to initiate a cytotoxic response, immunotoxins must bind effectively to the target cell antigen through the antibody moiety (5). The cell bound immunotoxin is then endocytosed and, by analogy with whole ricin, a part of the endocytosed product is trafficked to the trans-Golgi network where it penetrates the cytosol and blocks protein synthesis (6–8).

The importance of endocytosis of ricin A chain immunotoxins in eliciting cytotoxicity is illustrated by studies on a series of RTA conjugates with antibodies binding to CD (Cluster of Differentiation) structures on human lymphocytes (9). Anti-CD7 antibodies were effectively internalized, whereas anti-CD8 antibodies were hardly internalized, and these findings correlated with the cytotoxicity of their respective immunotoxins. Intracellular trafficking is also important in determining the cytotoxicity of immunotoxins. This is highlighted by the influence of potentiaters such as lysosomotropic amines or carbonyc ionophores on the cytotoxicity of ricin A chain conjugates with the anti-CD5 monoclonal antibody T101 (10).

In the present studies, the cytotoxic potential of cell bound 791T/36-RTA immunotoxin has been examined in relation to the kinetics of endocytosis. The rates of endocytosis and lysosomal trafficking of 791T/36-RTA were also investigated as well as the effects of a lysosomotropic amine (ammonium chloride) and carbonyc ionophore (monensin) on cytotoxicity. In this way, we attempted to elucidate the pathway(s) of cellular uptake of an anti-tumor immunotoxin which led to the eventual destruction of the tumor target.

MATERIALS AND METHODS

Monoclonal Antibody 791T/36 and 791T/36-RTA. Immunotoxin. Monoclonal antibody 791T/36 (IgG2b) was produced in ascites form in Balb/c mice. Ascites fluid was fractionated by Sepharose protein A chromatography to yield purified antibody. Immunotoxin 791T/36-RTA was prepared by conjugating RTA to antibody using the N-succinimidyl-3-(2-pyridyldithio)propionate linker (1). Conjugates were also prepared using RTA alone, a naturally occurring form of RTA that contains reduced amounts of carbohydrate (11).

Radioiodinated Preparations. 791T/36 and 791T/36-RTA were wholly labeled with iodine-125 to a specific activity of approximately 1 μCi/μg using Iodogen as the oxidizing agent (12). 791T/36-RTA conjugate containing [125I]labeled RTA [791T/36-(RTA-125I)] was prepared as previously described (12).

Cytotoxicity Assay. The cytotoxicity of immunotoxins was assessed using a cell survival assay ([125I]-Selenomethionine incorporation) or in a tumor cell colony formation assay (1, 13). In the [125I]-Selenomethionine incorporation assay (13), immunotoxins were incubated continuously for 24 h with target cells in 96 well microtiter plates. After washing cells were then pulsed with [125I]-Selenomethionine (0.1 μCi/well) for 16 h and cell incorporated radioactivity was determined. The percentage of cytotoxicity was calculated with reference to control cells treated with medium. Dose response curves were constructed and the IC50 was determined.

In the clonogenic assay, the capacity of immunotoxins to suppress tumor cell colony formation in 30-mm tissue culture dishes was determined (1). Tumor cells were incubated continuously with immunotoxins for 5–6 days of culture and surviving colonies counted.

Endocytosis of Radiolabeled Antibody 791T/36 and Immunotoxin 791T/36-RTA. To determine the endocytosis of 125I-labeled antibody or immunotoxin, osteogenic sarcoma 791T cells were incubated with saturating doses (1 μg/2 × 105 cells) of radiolabeled product at 0°C for 30 min. Target cells were then washed three times in medium (Dulbecco’s minimum essential medium + 2% newborn calf serum) to remove unbound product. At this stage, and dependent upon the specific activity of the radiolabeled preparation used, between 10,000 and 30,000 cpm/sample (2 × 106 cells) remained cell surface bound. Incubation at 37°C was then continued for various periods of time to allow endocytosis to occur. Cells were centrifuged (10,000 rpm for 10 s) and supernatants were removed. Cells were then washed three times in medium (Dulbecco’s minimum essential medium + 2% newborn calf serum) to remove unbound product. At this stage, and dependent upon the specific activity of the radiolabeled preparation used, between 10,000 and 30,000 cpm/sample (2 × 106 cells) remained cell surface bound. Incubation at 37°C was then continued for various periods of time to allow endocytosis to occur.
removed. Nonendocytosed cell surface bound immunotoxin was removed from cells by treatment with activated papain (15 units/ml in 20 mM cysteine) for 60 min at 0°C. Cells were again centrifuged (10,000 rpm for 10 s) and the radioactivity in cell pellet and supernatant determined. From these determinations endocytosis (E_e) was calculated as:

\[ \% E_e = \frac{P_t - S_t - P_o}{P_t + S_t} \times 100 \]

where \( E_e \) = endocytosis at time \( t \), \( P_t \) = counts/min in cell pellet at time \( t \), \( S_t \) = counts/min in supernatant at time \( t \), \( P_o \) = counts/min in cell pellet at time 0, and \( S_o \) = counts/min in supernatant at time 0.

The component \( (S_t - P_o)/S_o \) in the numerator corresponds to the radioactivity which is not extracted from the surface by papain treatment (a process which has been shown to be between 80 and 90% efficient), and hence this value must be subtracted from the value for pellet associated radioactivity. Thus, a corrected value for internalized (i.e., endocytosed) radioactivity is computed.

Endocytosis of Radiolabeled Transferrin. Human transferrin (10 mg) was saturated with ferric ammonium citrate in phosphate buffered saline (pH 7.3), incubated at room temperature for 3 h, and dialyzed overnight at 4°C against 2 changes of phosphate buffered saline (pH 7.3). Iron saturated transferrin was radiiodinated using the chloramine-T procedure using 1 mg/100 \( \mu \)g protein. The endocytic uptake of radiolabeled transferrin by 791T cells was thereafter measured as described for antibody and immunonjugate (vide supra).

Acidification of Tumor Cell 79IT Cytosol. The cytosol of 791T cells was acidified by the method of Sandvig et al. (14). Tumor cells were incubated at 4 \times 10^5 cells/ml for 30 min at 37°C in N-(2-hydroxyethyl)piperazine-N'-[(2-ethanesulfonic acid) medium with or without 25 mM NH_4Cl. Cells were then sedimented and the supernatant was removed and with medium containing 0.14 M KCl-2 mM CaCl_2-1 mM MgCl_2-20 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), pH 7.0. After incubation for 5 min radiolabeled immunotoxin was added and endocytosis assayed.

Endocytosis of 791T/36-HSA-TRITC Conjugate. The kinetics of endocytosis by tumor cells of a conjugate containing antibody 791T/36 linked to HSA-TRITC was determined by flow cytometry (15, 16). The 791T/36-HSA-TRITC conjugate was prepared as previously described, first conjugating TRITC to HSA and then coupling HSA-TRITC to iodoacetylated 791T/36 (15). Tumor cells were reacted at 0°C with 791T/36-HSA-TRITC and then adjusted to 37°C to allow endocytosis and intracellular release of tetramethylrhodamine to proceed. Intracellular rhodamine fluorescence was determined by fluorescence activated cell sorter flow cytometry following excitation from an argon laser. The cell suspension was sampled at intervals following incubation and a temperature controlled sheath was used to maintain cell suspension temperature. Changes in cell fluorescence were related to baseline control values for cells incubated with conjugate at 0°C.

RESULTS

Cytotoxicity of 791T/36-RTA for Tumor 791T Cells: Influence of Time of Exposure. Immunotoxin 791T/36-RTA is cytotoxic for tumor cells, its activity being directly proportional to the density of the target gp72 antigen (1). To determine the duration of exposure necessary for cytotoxicity, 791T cells were incubated with immunotoxin for various periods of time up to 8 h. Aliquots of cells were removed at intervals, washed to remove unbound immunotoxin, and then cultured in fresh medium and cell survival was assayed by colony formation (Fig. 1). Incubation with immunotoxin for only 30 min resulted in a reduction of the number of colonies from 80 to 9 (i.e., 89% cytotoxicity) with little increase in response over a further 7.5 h of exposure (maximum cytotoxicity at 8 h, 94%). This suggests either that sufficient immunotoxin is endocytosed by tumor cells within 30 min of exposure or that immunotoxin remains bound to cell surface antigen and this serves as a reservoir of product for continued endocytosis. Duplicate aliquots of tumor cells were removed after incubation with immunotoxin and residual cell surface membrane bound product was removed by papain treatment. This procedure removes 84–86% of cell bound immunotoxin without damaging the capacity of tumor cells to form colonies (data not shown). Exposure of 791T cells to immunotoxin for 30 min followed by papain stripping of treated cells resulted in the survival of 51 colonies compared with 84 colonies surviving in cells which were not treated with immunotoxin (i.e., a 39% inhibition of colony formation), this representing the cytotoxic response by endocytosed product over this period of time (Fig. 1). Further reductions in colony survival were observed over the 8-h period of incubation.

The influence of time of immunotoxin-tumor cell interaction on cytotoxic response was further explored by determining the dose of 791T/36-RTA required to produce IC_50 at different times of exposure using a [\(^{75}\)Se]selenomethionine incorporation assay (Table 1). Thus the IC_50 of 791T/36-RTA following tumor cell exposure for 30 min and then papain stripping of residual cell bound product before further cell incubation was 199 ng/ml. As the period of incubation of tumor cells with immunotoxin increased, the IC_50 values progressively decreased, being 72 ng/ml and 16 ng/ml after 4 and 18 h incubation, respectively.

Influence of Potentiators on 791T/36-RTA Cytotoxicity. Lysosomotropic amines, e.g., ammonium chloride and carboxylic...
ionophores such as monensin, greatly enhance the cytotoxicity of certain immunotoxins (10). These findings have been interpreted as indicating that the activity of endocytosed immunotoxins is influenced by inhibition of trafficking to a lysosomal compartment. The cytotoxicity of 791T/36-RTA for 791T tumor cells was not greatly influenced, however, by the presence of ammonium chloride in the medium (Table 2). At the highest concentration of ammonium chloride tested (20 mM) there was an increased cytotoxic response to 791T/36-RTA, but even greater enhancement was observed with RTA alone. Monensin also produced a small increase in 791T/36-RTA cytotoxicity, but again similarly enhanced cytotoxicity was observed with RTA alone. These experiments suggest that, after endocytosed, sufficient immunotoxin is able to traffic intracellularly by pathways leading to localization at a site (trans-Golgi network) and then to its cytoplasmic release and for 791T/36-RTA, this trafficking is not influenced by potentiators.

Endocytosis of Immunotoxin 791T/36-RTA. Tumor cell cytotoxicity requires internalization and intracellular trafficking of immunotoxins to appropriate compartments (6–8). Experiments were carried out, therefore, to determine the kinetics of endocytosis and trafficking of 791T/36-RTA in tumor 791T cells. Endocytosis may be influenced by antibody bound ligands; therefore, the endocytosis of 791T/36-RTA was compared to that of the unconjugated 791T/36 antibody (Fig. 2). Both products showed comparable rates of endocytosis with an initial rapid uptake over the first 60 min of tumor cell exposure, followed by a slower rate of uptake over the subsequent 180 min when the level of uptake in this experiment (Fig. 2) was 23–31% of cell bound radioactivity.

The percentage of endocytosis for any data set was calculated as defined in “Materials and Methods.” Expression of the results in this way allows direct comparison of the uptake of different antibodies or immunotoxins, which have been radio-labeled to (or have decayed to) different specific activities.

A major focus of these experiments was to determine the rate of endocytosis of the RTA moiety in the immunoconjugate. Accordingly, a further series of experiments were carried out using 791T/36-RTA immunotoxin prepared with radiolabeled RTA [791T/36-(RTA-125I)]. These demonstrate that the RTA moiety in the immunoconjugate is endocytosed effectively by tumor 791T cells with >45% of cell bound immunotoxin being internalized over a 180-min incubation period (Table 3).

Endocytosis of cell bound ligands is an energy dependent process which is considerably reduced at low temperature. As illustrated in Fig. 3 endocytosis of 791T/36-RTA occurred when tumor cells were reacted with immunotoxin at 4°C and then incubated at 37°C. No endocytosis resulted when tumor cells were maintained at 4°C.

Immunotoxins have been constructed by conjugation of 791T/36 antibody to ricin A chain and to a subfraction (RTA30) which has a lower carbohydrate content (11). Both types of immunotoxin were endocytosed at comparable rates (Table 4). Also immunotoxins constructed with RTA30 with antibody:RTA molar ratios of 1:2.2–1:1 had comparable rates of endocytosis. These findings indicate that the endocytosis of 791T/36-RTA conjugates is governed primarily by the rate of endocytosis of the 791T/36 monoclonal antibody.

Rate of Movement of 791T/36 Antibody into Lysosomal Vacuoles. The demonstration that ammonium chloride or monensin produces only minimal enhancement of cytotoxicity (Table 2) suggests that following endocytosis the immunotoxin is preferentially trafficked to the Golgi network and not directly to lysosomal vacuoles with their acidic environment. To explore this possibility further, studies were carried out on the intracellular localization of 791T/36 antibody linked to TRITC. Because of the high level of TRITC labeling of HSA, the fluorescence signal of the conjugate following excitation is quenched (15, 16). After endocytosis and lysosomal degradation of 791T/36-HSA-TRITC, however, intracellular fluorescence becomes detectable. Thus, the movement of the antibody conjugate into the lysosomal compartment can be monitored by measuring cell fluorescence by fluorescence activated cell sorter flow cytometry. These measurements, taken up to 4 h after treatment of tumor 791T cells with 791T/36-HSA-TRITC, increased in a dose related manner (range, 5–40 μg/2.5 × 10⁵ cells) and were blocked when tumor cells were also treated with a 10-fold excess of unconjugated 791T/36 antibody (data not shown). The kinetics of increase in fluorescence of 791T and 788T cells (both expressing gp72 antigen) and the gp72 negative

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### Table 2 Effect of potentiators on the cytotoxicity of immunotoxin 791T/36-RTA and free RTA for tumor 791T cells

<table>
<thead>
<tr>
<th>Potentiator</th>
<th>Concentration (mM)</th>
<th>791T/36-RTA (m × 10⁻⁸)</th>
<th>RTA (m × 10⁻⁸)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium chloride</td>
<td>0</td>
<td>0.8</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.1</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.3</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Monensin</td>
<td>25</td>
<td>0.3</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

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Fig. 2. Endocytosis of 125I-labeled 791T/36 MAb (top) and 791T/36-RTA (bottom) by tumor 791T cells. Tumor cells were incubated with saturating amounts (1 μg/10⁶ cells) of 791T/36 MAb or immunotoxin at 0°C and excess reactants removed by washing (radioactivity bound, 10797 and 24025 cpm/2 × 10⁶ cells in antibody and immunotoxin treated cells, respectively). Tumor cells were then incubated at 37°C for up to 240 min. At intervals aliquots of cells were removed and treated with activated papain (15 units/ml in 20 mM cysteine) to remove residual cell surface bound product. Endocytosed product was then determined from radioactivity remaining in cell pellets and the results are expressed as the difference in radioactivity in cell pellets obtained at time t and time 0. Bars, SD; points, means.
Over which endocytosis by 79IT cells has been investigated. In acidification were examined over the complete period of 4 h (Fig. 5, top and bottom, respectively). The effects of cytosol docytosis through smooth pits to proceed unimpeded but inhib 36-RTA by 79IT Cells. At least two methods of internalization involving clathrin coated pits and the other smooth pits (17, 18). These two mechanisms have been differentiated using a technique whereby acidification of the cell cytosol allows en

Involvement of transferrin in the endocytosis of Iodinated 791T/36-RTA was done to determine the possibility of receptor-mediated endocytosis (14, 19, 20). When endocytosis of transferrin was measured in acidified cells, the initial rapid rate of uptake exceeded via qualitatively different routes.

Further studies have shown that contact time of no more than 5 min for cytotoxicity to be elaborated. Sarcoma 79IT cells express approximately 2 x 10^5-1 x 10^6 copies of the gp72 antigen at the cell surface membrane antigens. These products must then undergo endocytosis and appropriate intracellular trafficking across intracellular organelle membranes to the trans-Golgi network are essential steps for RTA to produce cell death (6-8). Adequate amounts of immunotoxin must first react with the cell surface membrane antigens. These products must then undergo endocytosis and appropriate intracellular trafficking for cytotoxicity to be elaborated. Sarcoma 79IT cells express approximately 2 x 10^5-1 x 10^6 copies of the gp72 antigen at the cell surface (1, 21, 22) and interaction with 791T/36-RTA antibody and 791T/36-RTA is extremely efficient (1). Previous studies have shown that contact time of no more than 5 min between 791T and 791T/36-RTA is sufficient to produce >90% inhibition of tumor cell growth (1). These results are confirmed and extended in the present investigation in which the inhibiting effect of immunotoxin on tumor cell colony formation was essentially the same regardless of whether exposure was for 30

Control tests using 51Cr-labeled 791T cells, it was shown that the acidification procedure did not modify cell viability over the period of the assay. In positive control tests, the endocytosis of radiolabeled transferrin by 791T cells was much more rapid, reaching maximal values within 5-10 min (Fig. 6). This ligand is known to be endocytosed by receptor mediated endocytosis via coated pits (14, 19, 20). When endocytosis of transferrin was measured in acidified cells, the initial rapid rate of uptake over the first 5-10 min could be reduced by up to 50% (Fig. 6). The findings are consistent with the proposal that the uptake of transferrin and 791T/36 antibody immunonoconjugates proceeds via qualitatively different routes.

**DISCUSSION**

Endocytosis of cell bound immunotoxin and appropriate intracellular processing to allow translocation of ricin A chain across intracellular organelle membranes to the trans-Golgi network are essential steps for RTA to produce cell death (6-8). Adequate amounts of immunotoxin must first react with the cell surface membrane antigens. These products must then undergo endocytosis and appropriate intracellular trafficking for cytotoxicity to be elaborated. Sarcoma 79IT cells express approximately 2 x 10^5-1 x 10^6 copies of the gp72 antigen at the cell surface (1, 21, 22) and interaction with 791T/36-RTA antibody and 791T/36-RTA is extremely efficient (1). Previous studies have shown that contact time of no more than 5 min between 791T and 791T/36-RTA is sufficient to produce >90% inhibition of tumor cell growth (1). These results are confirmed and extended in the present investigation in which the inhibiting effect of immunotoxin on tumor cell colony formation was essentially the same regardless of whether exposure was for 30

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**Table 3 Endocytosis of 79IT/36-RTA by tumor 79IT cells**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Ligand</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>791T/36-(RTA-131I)</td>
<td>21.9 ± 3.2</td>
<td>41.6 ± 5.3</td>
<td>40.4 ± 6.4</td>
<td>45.3 ± 15.2</td>
</tr>
<tr>
<td>2</td>
<td>791T/36-(RTA-131I)</td>
<td>15.5 ± 5.7</td>
<td>34.7 ± 4.0</td>
<td>48.4 ± 2.3</td>
<td>47.3 ± 2.3</td>
</tr>
<tr>
<td>3</td>
<td>791T/36-(RTA-131I)</td>
<td>ND</td>
<td>12.6 ± 2.8</td>
<td>49.3 ± 6.4</td>
<td>58.5 ± 2.3</td>
</tr>
<tr>
<td>4</td>
<td>791T/36-(RTA-131I)</td>
<td>27.8 ± 6.3</td>
<td>30.1 ± 4.0</td>
<td>48.3 ± 5.5</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>791/36-(RTA-131I)</td>
<td>18.7 ± 1.7</td>
<td>28.4 ± 6.4</td>
<td>32.4 ± 1.1</td>
<td>36.1 ± 7.1</td>
</tr>
<tr>
<td>6</td>
<td>791T/36-(RTA-131I)</td>
<td>13.9 ± 5.3</td>
<td>23.6 ± 1.6</td>
<td>27.2 ± 3.6</td>
<td>35.8 ± 0.3</td>
</tr>
</tbody>
</table>

* Mean ± SD.

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**Table 4 Endocytosis of immunotoxin 791T/36-RTA by tumor 79IT cells**

<table>
<thead>
<tr>
<th>Immunotoxin</th>
<th>Antibody:RTA ratio</th>
<th>Endocytosis (%) after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60 min</td>
</tr>
<tr>
<td>791T/36-RTA</td>
<td>1:2.6</td>
<td>17.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.1 ± 3.7</td>
</tr>
<tr>
<td>791T/36-RTA</td>
<td>1:2.3</td>
<td>13.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.5 ± 1.7</td>
</tr>
<tr>
<td>791T/36-RTA</td>
<td>1:1.0</td>
<td>16.0 ± 5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.5 ± 1.1</td>
</tr>
</tbody>
</table>

* Mean ± SD.
min. The rate of internalization of this monoclonal antibody was not influenced by conjugation of ricin A chain or the less glycosylated RTA\textsubscript{30}, suggesting that the rate of internalization of the entire complex is directed by the \( M, 72,000 \) antigen to which it binds. Overall, the extent and rate of endocytosis of 791T/36-RTA is somewhat lower than that reported for ligands participating in receptor mediated endocytosis. However, given the extreme cytotoxicity of RTA when properly moved to the appropriate intracellular site, bulk transfer of this product is probably less important than the route of intracellular trafficking and translocation of the RTA moiety to the cytosol.

The potentiation of some immunotoxins by ammonium chloride (10) and conversely the potent inhibition of antigen processing by this agent (23) has helped define their routes of intracellular trafficking. Some toxins such as \textit{Pseudomonas} exotoxin and diphtheria toxin (24, 25) require an intracellular acidic environment for activation since they are inhibited by agents such as ammonium chloride. In contrast ricin cytotoxicity is potentiated by ammonium chloride, suggesting that an acidic compartment may be harmful to it (14). \textit{In vitro} exposure to low pH levels does not affect ricin cytotoxicity,\textsuperscript{4} but intracellularly low pH environment could influence the movement of the endosomes or affect adversely the ability of RTA to penetrate membranes of the vacuole and movement to the Golgi (26). In general, molecules endocytosed through coated pits rapidly move to lysosomal vacuoles. The suggestion, therefore, is that molecules endocytosed by smooth vacuoles internalized more slowly and are delayed in their location of these sites. This has been shown in these studies where in which pits are primarily utilized for internalization of 791T/36-RTA and cytotoxicity is independent of potentiators. Movement of antibody conjugated to HSA-TRITC into lysosomal vacuoles is slow, being first seen approximately 60 min after incubation with tumor cells.

These studies demonstrate that intracellular drug trafficking is a very important parameter in selecting antibodies as drug carriers and this is primarily dictated by the cell surface molecule selected as the antigen target. In the case of the 791T/36 defined gp72 antigen, binding of the antibody and its immunon conjugate results in intracellular uptake of cell surface bound product via a pathway other than receptor mediated endocytosis. Thereafter, as shown in this study with ricin A chain immunotoxins, the intracellular trafficking favors localization of product to compartments which permit release of toxin molecules and their translocation to the cytosol, rather than via the lysosomal route leading to toxin inactivation.

### REFERENCES


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