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Rate of internalization of an immunotoxin correlates with cytotoxic activity against human tumor cells

(endocytosis/disialoganglioside/ricin A-chain/immunotherapy)

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ABSTRACT **The relationship between the cellular internalization of an anti-ganglioside GD2 monoclonal antibody (14.G2a) and the toxic effect of its ricin A-chain immunotoxin (14.G2a–RA) was examined on GD2-bearing M21 human melanoma and T293 small cell lung carcinoma cell lines. The capacity for ligand uptake was determined by examining the parameters that contribute to this constant, including the number of cell-surface binding sites and the internalization rate constant (k_e). The maximum uptake of 14.G2a is 11-fold greater for M21 than for T293 cells, due to a 2.7-fold difference in binding sites and a 4-fold difference in the rate of antibody internalization. The capacity for ligand uptake correlates with the cytotoxic activity of the 14.G2a–RA immunotoxin against these two cell lines. Furthermore, we were able to demonstrate that the consequence of internalization of 14.G2a–RA is the intracellular release of undegraded ricin A-chain from the antibody. These studies indicate that the rate of internalization is a quantitative parameter that plays a key role in predicting the cytotoxic potency of this immunotoxin.**

Immunotoxins are cytotoxic agents constructed by covalently linking antibodies to potent plant or bacterial toxins (1, 2). These conjugates are potentially useful for the treatment of cancer, particularly by using monoclonal antibodies (mAbs) directed against tumor-associated antigens to target either single-chain toxins or the A-chain of heterodimer toxins to tumor cells (1–9). In this regard, it is known that the cytotoxic effect of ricin A-chain (RA) can be attributed to its ability to inhibit protein synthesis by catalytically inactivating ribosomes through the hydrolysis of an N-glycosidic bond adjacent to the α-sarcin site in 28S RNA (10). In the case of ricin holotoxin, the process is receptor-mediated endocytosis of the heterodimer with the ricin B-chain binding to cells via galactose-terminating glycoproteins and glycolipids, followed by internalization of the toxin via clathrin-coated pits and delivery to endosomes that translocate RA to their sites of action (11, 12).

Anti-tumor mAbs may substitute for ricin B-chain to mediate the specific binding to the tumor cell surface and together with RA produce an immunotoxin that internalizes into intracellular compartments where RA translocates to the cytosol. Several parameters such as epitope topography and density (4), antibody affinity (13), and rate of intracellular degradation (4) are critical in determining the cytotoxic efficacy of an immunotoxin. It has been proposed that receptor-mediated endocytosis of cell-surface antigen–immunotoxin complexes is essential for their effectiveness as cytotoxic agents (6–9), particularly since immunotoxins that fail to be internalized are unable to kill tumor cells (6). In this regard, in a recent article, Youle and Neville (7) have extensively reviewed the role of endocytosis and receptor recycling in ligand–toxin and antibody–toxin conjugate activity. However, at present there is relatively little information regarding the actual effect of the internalization rate per se on the efficiency of tumor cell killing by RA immunotoxins.

Therefore, we quantitatively examined the cytotoxicity of one such immunotoxin and determined whether there is a correlation between the internalization rate of an anti-GD2 ganglioside–RA immunotoxin and its *in vitro* cytotoxicity against human tumor cells.

MATERIALS AND METHODS

Cell Lines. The cultured human melanoma cell line UCLA-SO-M21 (M21) and the lung adenocarcinoma cell line UCLA-SO-P3 (UCLA-P3) were kindly provided by D. L. Morton (University of California, Los Angeles) and the T293 small cell lung carcinoma cell line was a gift from Y. Masui when he was at the University of California, San Diego.

mAbs. mAb 14.G2a (IgG2a), an isotype switch-variant of mAb 14.18 (IgG3), recognizes disialoganglioside GD2, and mAb 9.2.27 (IgG2a) is directed against a chondroitin sulfate proteoglycan preferentially expressed on human melanoma cells. Both antibodies are produced by our laboratory and have been extensively characterized (14–16).

Preparation of the 14.G2a–RA Conjugate. The method of Olsnes and Pihl (17) was used to isolate RA from purified ricin, which was kindly provided by D. J. Knauer (University of California, Irvine). The 14.G2a–RA conjugate was prepared as described (18). The *N*-succinimidyl-3(2-pyridyldithio)propionate-modified mAb 14.G2a (28 nmol) was admixed with 84 nmol of RA under nitrogen and incubated overnight at room temperature. The reaction mixture was purified on a Sephacryl S-300 column and by chromatography on Blue-Sepharose CL-6B (19). This purified disulfide-linked 14.G2a–RA conjugate contains an average number of one RA molecule per molecule of antibody, no detectable free RA, and <10% free antibody as determined by Coomassie blue staining on $NaDodSO_4$/PAGE.

Radioiodination. mAb 14.G2a, its conjugate with RA (14.G2a–RA), and ricin holotoxin were labeled with $Na^{125}I$ by the Iodo-Gen method described (20). The specific activity was 15,000–25,000 cpm per ng of 14.G2a or 14.G2a–RA and 40,000 cpm per ng of ricin holotoxin.

Scatchard Plot Analysis. The binding of ^{125}I-labeled 14.G2a (^{125}I-14.G2a) (25–1300 ng/ml) to cells was determined during 2 hr on 8×10^3 M21 cells or 8.5×10^3 T293 cells suspended in ice-cold RPMI 1640 medium/1% bovine serum albumin. Nonspecific binding was assessed in the presence of 500-fold excess of unlabeled 14.G2a and was typically 5% of the total bound radiolabel. Duplicate data points were analyzed according to the method of Scatchard (21).

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Abbreviations: mAb, monoclonal antibody; RA, ricin A-chain.

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Determination of the Internalization Rate Constant (k_e). One hundred nanograms of ^{125}I-14.G2a or ^{125}I-14.G2a–RA together with 0.1 M lactose was added to the tumor cells, and every 4 min cell-surface ligand was distinguished from internalized ligand by treating the cells for 8 min on ice with a 1-ml solution of 0.2 M acetic acid (pH 2.5) containing 0.5 M NaCl to remove cell-surface-bound ^{125}I-14.G2a (22). After two washes, the remaining cell-associated radioactivity was removed by dissolving the cells in boiling 1 M NaOH. Internalized and cell-associated ^{125}I-14.G2a were determined by γ counting. We found that this treatment removes >97% of surface-bound 14.G2a from M21 and T293 cells at 4°C. Nonspecific binding was 5–10% of total binding and was subtracted from each data point. The k_e of 14.G2a, which defines the probability of an occupied receptor (antigen) being internalized in 1 min at 37°C (20), was determined according to Wiley and Cunningham (20), with modifications suggested by Opresko and Wiley (23). This was done by plotting the integral of the surface-associated ^{125}I-14.G2a at any time (t_i) versus the amount internalized at the same time point (23)—i.e., $[LR]_i = k_e \int_0^t [LR]_s dt$, where $[LR]_i$ is the amount of ligand complex inside the cell and $[LR]_s$ is the concentration at the cell surface.

For analysis by NaDodSO$_4$/PAGE, cultured cells were harvested by addition of 200 μl of boiling sample buffer (24) and subsequent scraping of the monolayers. Samples were analyzed on 5% and 12.5% polyacrylamide gels. The gels were stained, dried, and exposed on Kodak X-Omat AR film at −70°C.

Cytotoxicity Assay. The cytotoxicity of the immunotoxins was determined by the incorporation of [^{3}H]thymidine as described (6).

RESULTS

Binding and Cytotoxicity of the 14.G2a–RA Conjugate. The immunoreactivity and specificity of the antibody component of 14.G2a–RA conjugate was tested by its ability to bind to GD2 antigen-positive M21 and T293 cells versus GD2 antigen-negative UCLA-P3 cells. In an ELISA, the immunotoxin maintains its antibody binding reactivity and behaves identically to mAb 14.G2a in its ability to bind to GD2-bearing M21 and T293 cells (data not shown). Nonspecific binding of 14.G2a and its immunoconjugate in UCLA-P3 cells was observed at concentrations of >0.1 μM. In contrast, specific binding occurred at concentrations of >10 pM.

Since 14.G2a–RA binds only to cells expressing GD2, we determined whether this conjugate also has cytotoxic specificity. M21, T293, and UCLA-P3 cells were exposed for 24 hr to either ricin holotoxin-free RA, mAb 14.G2a, or 14.G2a–RA conjugate. As shown in Fig. 1, native ricin is equally cytotoxic for these three cell lines, with an IC_{50} of 20 pM. The same is true for RA; however, the corresponding IC_{50} is 30 nM for these same cell lines. This ability of RA to block protein synthesis at high concentration is most likely because of its nonspecific internalization via fluid-phase pinocytosis (24). The 14.G2a–RA conjugate effectively kills M21 cells with an IC_{50} of 0.15 nM—i.e., it is 10-fold less potent than intact ricin holotoxin. In contrast, a 460-fold greater conjugate concentration is required (70 nM) to achieve IC_{50} with the antigen-negative cell line UCLA-P3.

Interestingly, 16-fold more immunotoxin is required to kill T293 cells (IC_{50} = 2.5 nM) than M21 cells (Fig. 1). These data indicate that the killing efficiency of the immunotoxin varies between these two phenotypically distinct human tumor cell lines, although intact ricin kills them with equal effectiveness. The cytotoxic effect of the immunotoxin is a direct result of conjugate binding to GD2, because coincubation of M21 cells with immunotoxin in the presence of excess unconjugated 14.G2a is completely inhibitory (Fig. 2). This competitive

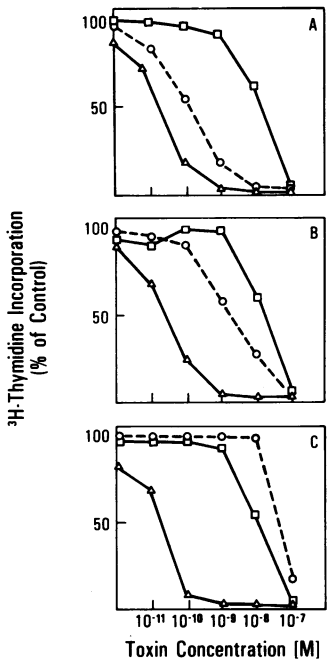

FIG. 1. Inhibition of DNA synthesis. The inhibition of DNA synthesis in M21 (*A*), T293 (*B*), and UCLA-P3 (*C*) cells was measured after 24 hr of exposure to ricin (△), RA (□), and 14.G2a–RA immunotoxin (○). Incorporation of [^{3}H]thymidine is expressed as percent of that obtained with control medium. The immunotoxin concentration is expressed as molarity of the RA equivalent.

inhibition is a specific antigen–antibody interaction since mAb 9.2.27, directed against a cell-surface proteoglycan expressed on M21 cells (13), fails to inhibit the immunotoxin's cytotoxic effect (Fig. 2).

Additional experiments demonstrated that the cytotoxic effect of the immunotoxin is not attributable to any trace contamination of intact ricin holotoxin since simultaneous incubation of cells with 20 mM lactose with either ricin or immunotoxin for 48 hr blocks the toxicity of ricin but has no effect on the cytotoxic effect of the 14.G2a–RA immunotoxin (data not shown).

Scatchard Plot Analysis of mAb 14.G2a Binding Data. The number of GD2 binding sites per M21 cell is 1.3×10^7 while 4.7×10^6 such sites are expressed per T293 cell. The relative binding affinities as judged from the dissociation constant (K_d) are essentially identical—i.e., 6.7 nM for M21 and 6.2 nM for T293 cells. Since there are only 2.7 times more GD2 binding sites on M21 than on T293 cells, it is apparent that the difference in the number of binding sites can account for only a portion of the 16-fold difference in cytotoxicity achieved by 14.G2a–RA on M21 and T293 cells, respectively. These data indicate that the level of cytotoxicity produced by 14.G2a–RA is not solely dependent on the number of GD2 binding sites expressed on these two cell types.

Rate of Internalization of mAb 14.G2a and 14.G2a–RA Immunotoxin. Since the immunotoxin differs in its ability to

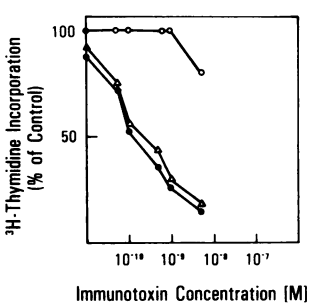

FIG. 2. Specific blocking of the cytotoxic effect of 14.G2a–RA by mAb 14.G2a. Inhibition of DNA synthesis was measured in M21 cells after 24 hr of exposure to 14.G2a–RA (●) or 14.G2a–RA in the presence of either 1 μM 14.G2a (○) or 1 μM 9.2.27 (△).

kill the phenotypically distinct M21 and T293 GD2-positive human tumor cell lines, we used this model to examine whether differences in the rate of internalization of mAb 14.G2a and immunotoxin 14.G2a–RA are responsible for this phenomenon. Initially, we applied the In/Sur (ratio of internalized to surface-bound 14.G2a) plot technique (20) to determine the internalization rate constant, k_e. This plot provides a general indication of the relationship between the internalization rate of mAb 14.G2a on both cell lines and demonstrates that the internalization rate of mAb 14.G2a is faster for M21 than T293 cells (Fig. 3). Because of the relatively slow rate with which 14.G2a reaches a steady state of cell-surface binding, we used the method of Opresko and Wiley (23) to determine k_e. As shown in Fig. 4, the slope of the line expressing the internalization rate constant (k_e) of mAb 14.G2a is 6.1×10^{-3} min^{-1} for M21 melanoma cells and 1.5×10^{-3} min^{-1} for T293 small cell lung carcinoma cells. The immunotoxin has essentially the same internalization rate as native mAb 14.G2a in either the presence or absence of 20 mM lactose (data not shown). In other words, the rate of uptake of surface-bound mAb 14.G2a is 4 times more rapid for M21 than for T293 cells.

As shown in Table 1, the calculated theoretical maximal net rate of ligand internalization [$V_{e(max)}$] of mAb 14.G2a is 8×10^4 molecules·min^{-1} for M21 cells and 7×10^3 molecules·min^{-1} for T293 cells. Since the K_d of 14.G2a is the same for both cell lines, this relationship will also hold for concentrations of immunotoxin below saturation. From this calculation, the predicted maximal ligand uptake is 11-fold greater for M21 cells than for T293 cells, and these values correlate well with the difference in cytotoxicity of the immunotoxin observed against the two cell lines.

Intracellular Release of RA from Immunotoxin. In an attempt to further characterize 14.G2a–RA, we determined its intracellular fate. This appeared to be especially pertinent since it was reported that immunotoxins delivered rapidly to the lysosomes are less effective, presumably because the RA is being rapidly degraded (4). We found that when ^{125}I-labeled mAb 14.G2a is exposed to M21 or T293 cells at 37°C, it is not degraded or released in such form into the culture medium over a period of 24 hr. This was assessed by NaDodSO$_4$/PAGE analysis of both the cell lysate and culture medium and was confirmed quantitatively by trichloroacetic acid precipitation (data not shown). In assessing the degree of intracellular cleavage of immunotoxin 14.G2a–RA, we observed that 70% of the radioactivity is associated with the immunotoxin represented by the 180-kDa component at the zero time point—i.e., 2 hr after incubation at 37°C. The other 30% of radioactivity is found in a 150-kDa component that represents the 14.G2a antibody from which the 30-kDa RA has already been cleaved (Fig. 5). After 22 hr of incubation at 37°C, only 30% of the total radioactivity remains associated with a 180-kDa component, as determined by counting gel slices. These data indicate that 70% of the immunotoxin is cleaved intracellularly during this period of time. To assess whether the intracellular loss of the 180-kDa component is indeed due to the cleavage of RA from the antibody, we constructed an immunotoxin with ^{125}I-RA and unlabeled mAb 14.G2a. It should be emphasized that in this preparation only the RA is radiolabeled. After 10 hr of exposure of cells to immunotoxin, two radiolabeled components with molecular masses of 30 and 32 kDa are evident (Fig. 5), corresponding exactly to the molecular mass of the initially purified RA (A_1 and A_2) (26). Since only the RA portion of the immunotoxin is radiolabeled in this experiment, these data clearly demonstrate that the immunotoxin is cleaved intracellularly, resulting in a release of intact RA over this time period. Other degradation products of RA are not visualized (Fig. 5).

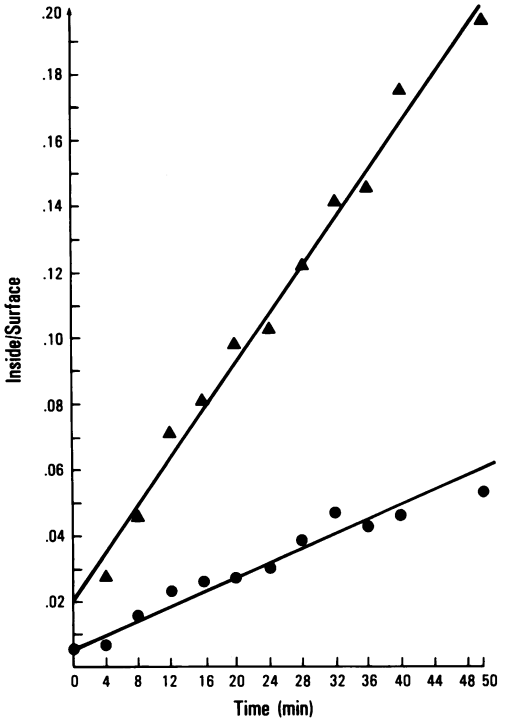

FIG. 3. Ratio of surface-bound vs. internalized mAb 14.G2a on M21 and T293 cells. Confluent cultures of M21 cells (▲) and T293 cells (●) were incubated for various times at 37°C with 100 ng of ^{125}I-14.G2a. At the time points indicated, the cells were rinsed and the surface-bound or internalized ligand was determined. At each time point, the ratio of internalized to surface-bound 14.G2a is plotted versus time.

DISCUSSION

The use of monoclonal antibodies as specific vehicles for the delivery of toxins and drugs has been widely explored (1–9). In theory, a toxin covalently linked to a site-specific mAb should deliver this cytotoxic agent to cells bearing antigenic determinants specifically recognized by this ligand. Since immunotoxins must enter cells to be effective cytotoxic agents, the rate of their cellular uptake should be a critical parameter for predicting their effectiveness.

The net rate of ligand uptake (V_e) is the product of two parameters; the concentration of occupied cell-surface receptors $[LR]_s$ and the internalization rate constant (k_e) (20). Since immunotoxins must enter the cell, we reasoned that the determination of V_e for specific immunotoxins would aid in predicting their efficacy. In this report, we show that V_e accurately predicts the toxic effect of our antibody–RA conjugate against two distinct tumor cell lines. The differences in cytotoxicity and V_e for this immunotoxin can be attributed to differences in receptor number and the internalization rate constant. We observed that two tumor cell lines expressing GD2 on the cell surface are differentially affected by the 14.G2a–RA immunotoxin. The IC_{50} of 14.G2a–RA for M21 cells is 0.15 nM but 2.5 nM on T293 cells—i.e., a 16-fold difference. This cell killing is exclusively due to the immunotoxin, since an excess of free 14.G2a blocks its effect. In an effort to determine why the difference in cytotoxicity exists between M21 and T293 cells, we examined the parameters that constitute V_e—i.e., the number of cell-surface binding sites and the affinity of the antibody for GD2 on the cell surface. M21 cells express 1.3×10^7 cell-surface binding sites, but T293 cells have only 4.7×10^6 such sites. The dissociation constant of mAb 14.G2a for both cell lines is virtually identical.

The number of 14.G2a binding sites differs by a factor of 2.7 between the two cell lines, thus accounting for some but not

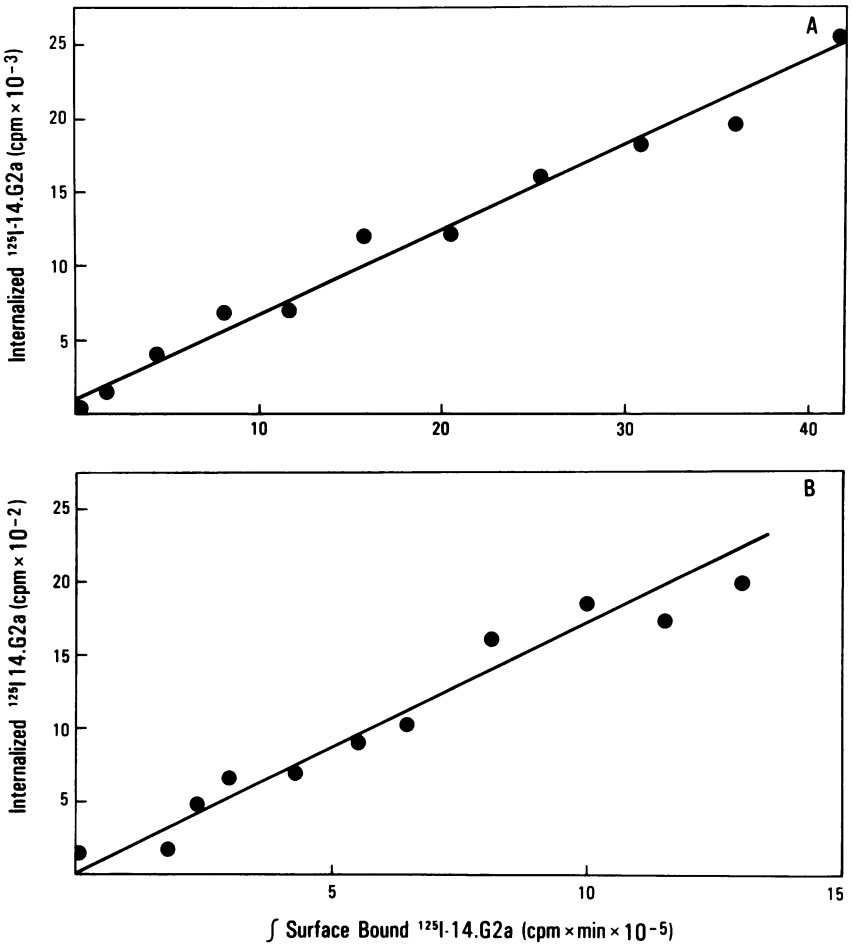

FIG. 4. Analysis of the internalization rate constant (k_e) for ^{125}I-14.G2a determined with M21 and T293 cells. M21 (*A*) and T293 (*B*) cells were incubated with 14.G2a as described in Fig. 3. The integral of surface-associated ligand is plotted against the internalized ligand at each time point. The slope of the corresponding straight line indicates a k_e of 6.1×10^{-3} min^{-1} for M21 cells and 1.5×10^{-3} min^{-1} for T293 cells.

all of the 16-fold difference in cytotoxicity. Therefore, we also examined the internalization rate constant of mAb 14.G2a by both cell lines, which was found to be similar to that of vitellogenin (23) but quite slow when compared to epidermal growth factor (20). M21 cells have a k_e of 6.1×10^{-3} min^{-1} and T293 cells show a k_e of 1.5×10^{-3} min^{-1}—i.e., a 4-fold difference. According to these results, the net rate of antibody uptake (V_e) by the two cell lines should differ by a factor of 11 because $\Delta V_e = \Delta k_e \times \Delta[LR]_s$. In fact, this difference in ligand uptake correlates well with the observed difference in cytotoxicity. We also demonstrated that the immunotoxin essentially binds the same number of binding sites and has essentially the same internalization rate as the native antibody—i.e., the V_e of free antibody is equivalent to the V_e of the immunotoxin.

Table 1. Binding and internalization parameters for 14.G2a and cytotoxicity of 14.G2a–RA immunotoxin

	Cell line	
	M21	T293
Binding sites per cell	1.3×10^7	4.7×10^6
K_d, M	6.2×10^{-9}	6.7×10^{-9}
k_e, min^{-1}	6.1×10^{-3}	1.5×10^{-3}
$V_{e(max)}$	8.0×10^4	7.0×10^3
IC_{50}, M	1.5×10^{-10}	2.5×10^{-9}

All binding measurements of 14.G2a on M21 melanoma cells and T293 small cell lung carcinoma cells were done at 4°C. The number of binding sites per cell and the relative affinity of binding (K_d) were determined by Scatchard plot analysis of the binding data. The internalization rate constant (k_e) was calculated by the method of Opresko and Wiley (23). The maximal net rate ligand internalization at saturation [$V_{e(max)}$] was calculated as $[LR]_{s(max)} \times k_e$ for each cell line. The cytotoxicity of the 14.G2a–RA immunotoxin is expressed by its IC_{50} value, indicating the molar concentration required to obtain 50% inhibition of [^{3}H]thymidine incorporation.

It is interesting that the same GD2 antigenic determinants expressed on two distinct tumor cells show a different rate of internalization subsequent to antibody binding. The reason for this difference is still under investigation. One can speculate that the lateral diffusion of the occupied GD2 receptors in the plasma membrane may be a rate-limiting factor for internalization. A decrease in the lateral diffusion coefficient may thus cause a change in the internalization rate. As reviewed by Wiley (27), some of the possible reasons for slower internalization rates include exclusion of the receptor from coated pits, low lateral diffusion coefficients, immobilization at distal areas, or lack of preferential association with internalization sites. Regardless of the molecular mechanism responsible for the difference in the internalization rate of mAb 14.G2a with different cell lines, its k_e can be used as a quantitative parameter to predict its potential as a carrier for a cytotoxic agent.

Since 14.G2a–RA is highly cytotoxic, we can hypothesize that this immunotoxin, like intact ricin (11), must be cleaved intracellularly to gain access to the cytosol and to exert the cytotoxic effect of RA. Regarding the route that RA takes to reach the ribosomes after the internalization of the immunotoxin, electron microscopy studies on one such immunotoxin have shown that its RA component reached the cytosol and appeared to remain associated with the antibody (28). Furthermore, Raso (5) reported that the disulfide linkage of ^{125}I-labeled transferrin–RA conjugate remained intact within cells. However, the immunotoxin 14.G2a–RA described here appears to be sensitive to reduction and release of free RA after internalization. This was demonstrated by constructing an immunotoxin in which only the RA portion is radiolabeled.

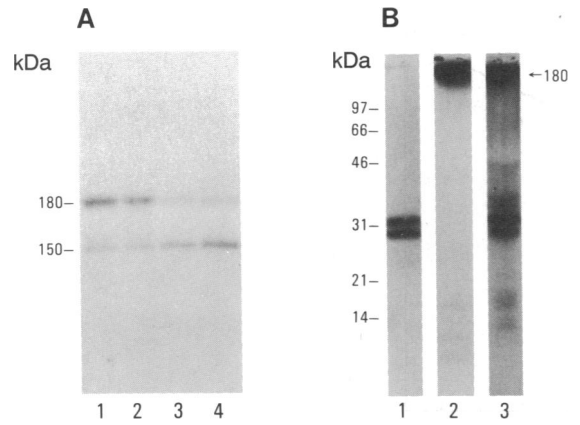

FIG. 5. Intracellular release of RA from immunotoxin. (*A*) M21 cells were exposed to 100 ng of ^{125}I-immunotoxin to generate an internal pool. After 2 hr, cells were placed on ice and cell-surface-associated immunotoxin was removed by incubating cell cultures with 1000-fold excess of unlabeled 14.G2a, followed by washing and incubation of cells at 37°C. At various time points, cells were lysed with boiling $NaDodSO_4$ sample buffer and analyzed by $NaDodSO_4$/PAGE, followed by autoradiography. Lanes: 1, 0 hr; 2, 1 hr; 3, 12 hr; 4, 24 hr of incubation at 37°C. (*B*) M21 cells were incubated for 10 hr in the presence of 0.1 M lactose with 100 ng of immunotoxin, constructed by conjugating mAb 14.G2a with ^{125}I-labeled RA. Cell-surface-associated radioactivity was removed by acetic acid treatment. Cultures containing only internalized radioactivity were lysed in boiling $NaDodSO_4$ sample buffer and analyzed by $NaDodSO_4$/PAGE, followed by autoradiography. Lanes: 1, purified ^{125}I-RA (A_1 + A_2); 2, purified ^{125}I-14.G2a–RA containing only radiolabeled RA; 3, intracellular 14.G2a–RA after 10 hr of exposure to M21 cells. Arrow indicates the 180-kDa component representing the 14.G2a–RA immunotoxin.

After this immunotoxin is internalized by M21 cells, the release of free RA is visualized as the 30- and 32-kDa radiolabeled components corresponding to RA on nonreducing polyacrylamide gels. Degradation of free 14.G2a or RA is not observed after internalization. Monensin, which specifically increases the pH of the lysosomal compartments (25), has no effect on the cytotoxicity of this conjugate (data not shown). Thus, it seems reasonable to propose that the intracellular routing of the 14.G2a–RA may have bypassed the lysosomal compartment where extensive degradation of immunotoxin would occur (4). The intracellular cleavage observed for this immunotoxin is consistent with the hypothesis that intact RA is released and translocated to the cytosol where it inhibits protein synthesis (11).

Our finding that the rate of internalization of 14.G2a–RA is the critical factor in predicting cytotoxicity differs from that recently reported by Press *et al.* (4). However, differences in interactions between the immunotoxin and the cell-surface antigen mentioned in both reports are consistent with two modes of immunotoxin delivery. In our experiments, the 14.G2a–RA conjugate is slowly internalized and once internalized its RA moiety is released intact. Neither the antibody nor the RA is degraded. In contrast, the RA–anti-CD2 conjugates described by Press *et al.* (4) are rapidly internalized and degraded in the lysosomes. Consequently, in this case, the rapid rate of degradation was the critical factor in determining cytotoxic efficacy of the immunotoxins. In conclusion, we suggest that in the absence of intracellular degradation, the rate of internalization of an immunotoxin is a key factor in determining its cytotoxic potency.

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