Influence of Cellular Trafficking on Protein Synthesis Inhibition of Immunotoxins Directed against the Transferrin Receptor

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ABSTRACT

Previously, a quantitative analysis that related protein synthesis inhibition of transferrin-toxin conjugates to the cellular trafficking of transferrin was proposed (P. T. Yazdi and R. M. Murphy, Cancer Res., 54: 6387–6394, 1994). Here, this work is extended to evaluate cellular trafficking of anti-transferrin receptor antibodies and protein synthesis inhibition kinetics of immunotoxins constructed from the same antibodies and the toxin gelonin. Cellular trafficking models for two monoclonal anti-transferrin receptor antibodies (5E9 and OKT9) in HeLa cells were developed. The two mAbs had similar trafficking parameters, which differed significantly from those for transferrin.

Protein synthesis inhibition kinetics for immunotoxins constructed from 5E9 or OKT9 and gelonin were measured. Analysis of the data using our previously proposed relationship between protein synthesis and cellular trafficking indicated that the relationship is also valid for these new systems. The protein synthesis inhibition constants for 5E9-gelonin and OKT9-gelonin conjugates were similar to those for the transferrin-gelonin conjugate. These results suggest that it may be possible to predict the efficacy of gelonin immunotoxins from knowledge of the trafficking of the corresponding targeting agent. A sensitivity analysis showed which cellular trafficking parameters have the greatest influence on immunotoxin efficacy and are, therefore, the most likely to be profitably manipulated.

INTRODUCTION

Immunotoxins are chemical conjugates of a targeting agent and a toxin. The targeting agent, which is generally an antibody or ligand, binds specifically to antigens or receptors on the target cell. The toxin then kills or damages the cell, typically by inhibiting protein synthesis. Toxins like ricin and gelonin act enzymatically and are capable of rapidly inactivating ribosomes (1, 2). It has been estimated that only one or a few toxin molecules in the cytosol are sufficient to completely shut off protein synthesis (3).

The cytotoxic activity of immunotoxins varies widely from one system to another, depending on the selected toxins, targeting agents, and target cells. Differences in immunotoxin efficacy have been variously attributed to affinity, antigen density, internalization rate, degradation rate, or intracellular routing (4). However, there are no generally accepted rules for relating these properties to cytotoxicity. In some studies, for example, sensitivity of target cells to immunotoxin efficacy increases with increasing surface antigen density (5–8), although this was not universally true (7, 9). It has been suggested that immunotoxin efficacy increases with increasing affinity (10, 11). May et al. (12), however, showed that ricin A chain immunotoxins constructed from six mAbs directed against different epitopes of the same antigen had significantly different toxicities, despite having similar binding affinities. No clear correlation between the rate of internalization and cytotoxicity has been found (13–15); it has been suggested that slower internalization may actually be advantageous (16). Increased intracellular accumulation correlated with increased sensitivity to immunotoxins in some studies (5, 13, 17). In most cases, more rapidly degraded immunotoxins were less effective (13, 18–20), although exceptions have been reported (9). These conflicting results argue that a more systematic investigation of the importance of these parameters in concert is necessary.

The human TIR (7) has been used frequently as a target for immunotoxins (7, 21–25). The receptor has been identified as a marker of rapid cell proliferation (26, 27) and is expressed at higher densities on tumor cells than on most normal cells (28). The TIR on tumor cells is likely to be less susceptible to modulation, shedding, or loss than other tumor-associated antigens, due to its critical role in supplying intracellular iron (7). Finally, the receptor is internalized, probably a necessary step in intoxication (29). Immunotoxins constructed from TF or anti-Tf mAb have shown considerable cytotoxicity in vitro against a wide variety of cells (5, 23, 25, 30).

Previously, we proposed a quantitative model that related protein synthesis inhibition to cellular trafficking of the corresponding targeting agent and showed that the model was valid for Tf conjugated to two different toxins and used in two different cell lines (31). In this report, kinetics of binding, uptake, and cellular processing of two mAbs directed against TIR expressed on HeLa cells were experimentally determined. mAbs 5E9 and OKT9 were chosen for this study because they bind to different sites on TIR and do not block binding of Tf (27, 32), and because effective immunotoxins have been prepared using 5E9 (5, 30, 33, 34) and OKT9 (35). Hence, the influence of targeting agent cellular trafficking on the efficacy of the corresponding immunotoxin could be studied by comparing the trafficking of 5E9 and OKT9 to Tf and the ability of the corresponding immunotoxins to inhibit protein synthesis.

A model was developed for the cellular trafficking of 5E9 and OKT9 and used to estimate trafficking parameters in HeLa cells. Kinetics of protein synthesis inhibition of conjugates of gelonin and 5E9 or OKT9 were measured. We show that the data for the anti-Tf mAb immunotoxins are nicely represented by our model for the relationship between cellular processing and inhibition of protein synthesis. Furthermore, k_{PS}, a constant that relates cellular trafficking to protein synthesis inhibition, is essentially the same for 5E9-gelonin, OKT9-gelonin, and Tf-gelonin, indicating that the result is independent of the targeting agent. Finally, we show how individual cellular trafficking parameters influence the kinetics of protein synthesis inhibition. This analysis helps to explain the widely disparate conclusions in the literature regarding the influence of cellular trafficking on cytotoxicity.

MATERIALS AND METHODS

mAb Purification. mAbs 5E9 and OKT9 were purified from culture supernatants of hybridoma cell line HB21 and CRL8021, respectively (American Type Culture Collection, Rockville, MD) by protein A affinity chromatography. Purity was checked by SDS-PAGE using the pHastGel system (Pharmacia, Piscataway, NJ). The subclass of mAbs 5E9 and OKT9 was determined to.
be IgG1,κ by ELISA. mAbs were labeled with 125I (DuPont-New England Nuclear, Boston, MA) using Iodo-beads (Pierce, Rockford, IL), following the manufacturer’s directions. The concentration was determined by absorbance at 280 nm, using an extinction coefficient of 1.40 ml mg⁻¹ cm⁻¹. The specific radioactivity of 125I-labeled mAb was 4–9 × 10⁶ dpm/μg.

Cell Growth Kinetics. HeLa cells (American Type Culture Collection) were cultured as monolayers in MEM supplemented with 10% fetal bovine serum, 3.6 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Gibco, Gaithersburg, MD). Cells were harvested by a wash with 5% DMSO in PBS [0.01 M phosphate buffer (pH 7.2) containing 0.14 M NaCl] containing 0.53 mM EDTA, followed by a brief treatment (30 s) with 0.25% trypsin in PBS. Cells were resuspended in culture medium at 40,000 cells/ml. From this suspension, 6.6 ml was placed in each 60-mm tissue culture dish. After 24 h, medium was removed, and cell monolayers were incubated in leucine-free medium containing 32% (v/v) PBS at 37°C for various periods of time. Cells were harvested, and cell concentration was determined using a hemacytometer. Two measurements of cell concentration were taken in each of two plates. Data were fit to:

\[ x = \frac{x_0 e^{\mu t}}{1 - e^{\mu (1 - e^{\beta t})}} \]  

where \( x_0 \) is initial cell concentration, \( x \) is cell concentration, \( t \) is time, \( \beta \) is the stationary cell concentration, and \( k \) is a fitted constant (36).

Steady-State Binding and Internalization. Cells were freshly plated onto culture dishes as described. After 24 h, medium was removed, and 3.3 ml of a solution containing various concentrations of 125I-labeled mAb in 32% (v/v) PBS and 68% (v/v) leucine-free medium were added to each dish. Cells were incubated at 37°C for 8–8.5 h. Preliminary experiments showed that the system reached steady state by this time. The concentration of mAb in the supernatant was determined in a gamma counter. Total cell-associated, surface, and internalized mAbs were determined as described previously (31), using the cell concentration as determined from Eq. A. Nonspecific binding and internalization of 125I-labeled mAbs were measured in the presence of excess (100-fold) unlabeled mAb.

Binding and Intracellular Processing Kinetics. Monolayer samples were prepared as described above and incubated at 37°C in the presence of a constant concentration of 125I-labeled 5E9 (1.3 × 10⁻⁸ M) or 125I-labeled OKT9 (2.0 × 10⁻⁸ M) for varying times. Samples were then processed as described above to determine total, surface-bound, and internal concentrations. In some assays, cells were incubated in the presence of 0.1 mg/ml cycloheximide for 1 h prior to the addition of 125I-labeled mAb, and total, surface-bound, and internal concentrations were measured as a function of time.

The kinetics of mAb degradation were determined by measuring TCA-soluble radioactivity in cell culture supernatants as a function of time. Medium (100 μl) was removed at various time intervals and mixed with 400 μl of 10 mg/ml BSA and 500 μl of 10% (w/v) TCA. After 10 min, the mixture was centrifuged, the supernatant was removed, and soluble radioactivity was measured. TCA-soluble radioactivity measured in control samples, which were prepared and treated similarly in the absence of cells, was subtracted. Degraded material was not significantly accumulated intracellularly since less than 5% of total-associated radioactivity was TCA soluble (data not shown).

Pulse-Chase Kinetics. Monolayer samples were prepared as described above. After 24 h, medium, was removed, and 2 ml of fresh leucine-free medium containing 32% (v/v) PBS and 2.8 × 10⁻⁸ M 125I-labeled 5E9 were added to each dish. Dishes were incubated at 4°C for 3–3.2 h to allow surface binding. Medium was removed, and monolayers were washed once with cold medium. Cells were then incubated at 37°C in 3.25 ml of leucine-free medium containing 32% (v/v) PBS and 2.8 × 10⁻⁸ M unlabeled 5E9. At appropriate time intervals, cell monolayers were washed once with cold PBS, treated with 0.3% (w/v) Pronase, and processed as described above to determine total, surface-bound, and internal mAbs/cell.

Cellular Trafficking Model. Fig. 1 depicts a general schematic for anti-TfR mAb cellular processing. A model for anti-TfR mAb binding, internalization, and intracellular processing was developed based on this schematic and experimental results:

\[ \text{Ab} + \text{TFR} \rightarrow \text{Ab}_{\text{u}} \]  

\[ \text{Ab} + \text{m} \rightarrow \text{Ab}_{\text{n}} \]  

\[ \text{Ab}_{\text{n}} \rightarrow \text{Ab}_{\text{i}} + \text{TFR} \]  

\[ \text{Ab}_{\text{i}} \rightarrow \text{Ab}_{\text{d}} \]  

\[ \text{Ab}_1 \rightarrow \text{Ab}_2 + \text{TFR} \]  

where \( \text{Ab} \) represents the concentration of mAbs in the medium. \( \text{Ab}_{\text{u}} \) and \( \text{Ab}_{\text{n}} \) denote specifically and nonspecifically bound surface antibody, respectively. \( \text{TFR} \) represents the unoccupied antibody-binding sites on the cell surface. \( \text{Ab}_{\text{i}} \) and \( \text{Ab}_1 \) denote intracellular and degraded antibody, respectively. \( m \) represents nonspecific binding sites on the cell membrane. Both specific and nonspecific binding are included, but only specifically bound mAbs are internalized. Bivalency of mAb and TFR is neglected. It is possible that multiple recycling pathways are available to mAb-TFR complexes (37, 38); for simplicity, we assume that a single rate constant for exocytosis is sufficient. Dissociation of a fraction of internalized mAb from TFR, routing to the lysosome, degradation, and fragment secretion, are incorporated into a single step described by the rate constant \( k_d \).

The model for Tf processing is similar, with \( \text{Tf} \) replacing \( \text{Ab} \) in Eqs. B, C, and D. Degradation (Eq. E) is neglected, and Eq. F is replaced with:

\[ \text{Tf}_d \rightarrow \alpha \text{Tf}_d = \text{TfR} \]  

where \( \alpha \text{Tf}_d \) represents apotransferrin released from the cell, and \( k_d \) lumps together rate constants for recycling of the apotTf-TFR complex to the surface and dissociation of apoTf from the receptor (31).

Kinetic experiments showed that the total, surface-bound, and internalized mAbs/cell initially increased and then remained constant over 24 hours. To account for this observation, it was assumed that all internalized transferrin receptors recycle back to the cell surface, although a fraction of the internalized antibodies are directed to lysosomes. A numerically equivalent model would include degradation of TFRs, which is exactly compensated for by new surface expression of TFRs (either by new synthesis or by expression from an intracellular pool of receptors). The addition of 0.1 mg/ml cycloheximide, which completely eliminated protein synthesis, had no effect on mAb binding and internalization kinetics for up to 3 h or on the total, internal, or surface number of mAbs/cell after 24 h (data not shown). The proposed model accounts for the experimental observations that the total, surface, and internal number of occupied receptors/cell reaches a constant value in the presence or absence of...
protein synthesis and that this number does not decrease with time over a 24-h period.

Non-specific binding was independent of time, and non-specifically bound mAb did not internalize (data not shown). Therefore, non-specific binding was assumed to be at equilibrium:

\[
[Ab_n] = \frac{N}{C} \frac{k'}{k_{-2}} [Ab]
\]  

where \( k' = k_{-2} \) is a pseudo-first order rate constant, \( C \) denotes cell concentration in cells/liter, and \( N \) is Avogadro’s number. The following differential equations were derived based on the model presented above:

\[
\frac{d[Ab]}{dt} = \frac{C}{N} (-k[A]([TfR] - [Ab_0]) + k_1[Ab_n])
\]  

\[
\frac{d[Ab_1]}{dt} = k_2[A](TfR) - [Ab_0] - (k_{-1} + k_4)[Ab] + k_2[Ab]
\]  

\[
\frac{d[Ab_2]}{dt} = k[A] - (k_{-2} + k_3)[Ab]
\]  

\[
\frac{d[Ab_3]}{dt} = k[A] - (k_{-2} + k_4)[Ab]
\]  

where \([TfR] = [Tf] + [Ab_0]\) is the total number of binding sites on the cell surface. The total number of mAb molecules/cell, \([Ab_1]\), is the sum of surface-bound and internal antibodies:

\[
[Ab] = [Ab_0] + [Ab_n] + [Ab]
\]  

All variables are expressed in molecules/cell, with the exception of \([Ab]\), which is in m. The following equations are applicable at steady state:

\[
[Ab] = [Ab_0] + [Ab_n] + [Ab]
\]  

\[
[Ab_0] = [Ab] + k_{-1}/k_4 + (k_2/[k_4] + k_3)
\]  

\[
[Ab_1] = \frac{k_1}{k_2 + k_4} [Ab_n]
\]  

Differential and steady-state equations for Tf processing were given previously (31). Parameter estimation was performed using the general purpose regression program GREG (39, 40) in the multiresponse mode. Differential equations were solved using DDASAC (41).

**Immunotoxin Preparation.** Purified mAb (10 mg/ml in PBS) was modified with 0.02 M SPDP in DMSO for 30 min at room temperature at a 2:1 molar ratio of SPDP:antibody. Unreacted SPDP was removed on a Sephadex G-25 chromatography column equilibrated with 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1 M NaCl. The modification resulted in the introduction of 0.6–0.8 2-pyridyl disulfide groups/mAb, determined using methods in the literature (42).

Gelonin was modified with 2-iminothiolane and conjugation of mAb to gelonin was carried out as described previously (31). The conjugation mixture was diluted by a factor of 4 in water and passed through a Blue Sepharose CL-6B (Pharmacia) column equilibrated with 0.01 M sodium phosphate buffer (pH 7.2) containing 0.05 M NaCl. Unconjugated antibody did not bind to Blue Sepharose under these conditions. mAb-Gel was eluted from the column by increasing the mobile phase NaCl concentration to 0.3 M. The eluted fraction was concentrated and passed through a Blue Sepharose G-200 Superfine column (Pharmacia) equilibrated with PBS to remove high molecular weight aggregates and unconjugated gelonin. Analysis by SDS-PAGE showed that the final preparation did not contain any detectable species with molecular weights higher than that of 1:1 mAb-gelonin conjugate. The concentration was determined by absorbance at 280 nm using an extinction coefficient of 1.28 ml mg\(^{-1}\) cm\(^{-1}\). The toxicity of 5E9-Gel, OKT9-Gel, and intact gelonin was measured in a cell-free translation assay as described previously (31). The protein synthesis inhibitory activity of the immunotoxins was similar to that of intact gelonin (data not shown). The binding affinity of immunotoxins was compared to that of \(^{125}\)I-labeled mAb in a competitive inhibition assay. Immunotoxins had binding affinities similar to those of the corresponding \(^{125}\)I-labeled mAb (data not shown).

**RESULTS**

**Pulse-Chase Kinetics.** Results from pulse-chase experiments of mAb 5E9 processing by HeLa cells are depicted in Fig. 2. Total cell-associated mAb decreased slowly, with more than 60% still remaining with the cell after 4 h of chase. This is in sharp contrast to similar experiments with Tf, in which ~95% was chased out of the cell within 30 min (data not shown) and consistent with other reports of pulse-chase experiments with anti-TfR mAb (43). Surface-bound mAb decreased by 60% in 5 min upon increasing the temperature to 37°C. Thereafter, surface-bound mAb decreased only by 5% of the initial value/hour. The initial rapid decrease in surface-bound mAb coincided with a sharp rise in internal mAb. Upon reaching a maximum after approximately 15 min, internal mAb decreased at the same rate as surface-bound mAb. Data were fit to Eqs. J, K, and M, with the assumption that re-association of dissociated radiolabeled mAb was insignificant due to the presence of excess unlabeled mAb in the medium. Results of model fitting are shown by the solid lines in Fig. 2. Kinetic parameters determined from this fit were \( k_1 = 0.23 ± 0.06 \) min\(^{-1}\), \( k_2 = 0.10 ± 0.04 \) min\(^{-1}\), and \( k_4 = 2.5 ± 0.6 \times 10^{-3} \) min\(^{-1}\). The dissociation rate constant \( k_d \) was indeterminate.

**Degradation Kinetics.** The kinetics of mAb degradation are shown in Fig. 3. After an initial period during which very little degraded material was detectable, degraded mAb in the medium increased linearly. The degradation rate constant \( k_d \) was calculated by dividing the slope of this plot in the steady-state region (>4 h) of the data by the steady-state concentration of intracellular mAb (Eq. L). The degradation rate constant \( k_d \) was estimated to be

![Fig. 2. Pulse-chase kinetics. Cells were incubated with 2.8 × 10⁻⁸ M \(^{125}\)I-labeled 5E9 at 4°C to load surface, then placed in fresh medium containing excess unlabeled 5E9. Total (○), surface (□), and internal (●) cell-associated mAbs were measured as a function of time. Lines show fits of model to data.](https://cancerres.aacrjournals.org)
Fig. 3. Degradation kinetics. Total TCA-soluble radioactivity was measured as a function of time for 5E9 (●) and OKT9 (○). Line, linear regression fit of the integrated form of Eq. L for ≥ 4 h, assuming [Ab] is at its steady-state value.

2.0 ± 0.1 × 10⁻³ min⁻¹ for both 5E9 and OKT9, in agreement with k_d determined from pulse-chase data for 5E9.

**Steady-State Binding and Internalization.** The total steady-state cell-associated, surface-bound, and internalized mAbs/cell as a function of mAb concentration in the medium is shown in Fig. 4. Eqs. H, M, N, and O were fit to the total cell-associated, surface-bound, and nonspecifically bound mAb data. [Ab] was determined by measuring the radioactivity of the culture medium of each sample at the end of the incubation period. Antibodies that were degraded by cells and released into the medium were assumed to amount to an insignificant fraction of radioactive material present in the medium. Simulations indicated that at a mAb concentration of 1.2 × 10⁻⁸ M, 2% of radioactivity present in the medium after 8.3 h of incubation was due to degraded antibodies, thus justifying this assumption.

The model fits are shown in Fig. 4. Parameter values determined from the fits are listed in Table 1, along with previously determined parameters for Tf (31). The number of surface TfRs/cell is equal for the two mAbs and about 25% higher for Tf than for mAb. The parameter group \( \{k_{-1}/k_t + k/k_t(1/k_t + k_d)\} \) is an indicator of the relationship between surface-bound and medium concentration of mAb (Eq. N). This value was slightly greater for OKT9 than for 5E9. The value of this parameter group is nearly an order-of-magnitude lower for mAbs than for Tf, indicating that at low extracellular concentrations, more mAb than Tf will be bound. The parameter group \( k/(k_t + k_d) \) is the ratio of internal to surface receptor-bound mAb at steady state (Eq. O). For both OKT9 and 5E9, approximately two-thirds of the cell-associated mAbs are intracellular at steady state. This is similar to the result obtained for Tf in HeLa cells.

**Binding and Internalization Kinetics.** Total cell-associated, surface-bound, and internal mAbs as a function of time are shown in Fig. 5. Total, surface, and internal mAbs increased slowly over 3–4 hours, then leveled out at a constant number, which was maintained for at least 24 h. The addition of cycloheximide to stop protein synthesis did not alter the kinetics or plateau values on a per cell basis (data not shown). Taken together, these results indicate that the slow binding and internalization kinetics of mAb were due to slow antibody-receptor association and not to antibody-induced new receptor synthesis or increased surface receptor expression from intracellular pools.

Eqs. A, H, J, K, and M were simultaneously fit to data for total and surface cell-associated mAb. The concentration of radioactive material in the medium (corresponding to intact and degraded ¹²⁵I-labeled mAb) was constant within experimental error, indicating that any

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![Graph](image-url)  
**Fig. 4. Steady-state binding and internalization.** Total (●), surface (□), and internal (○) cell-associated mAb at equilibrium as a function of mAb concentration in the medium. Data include specific and nonspecifically bound mAb. Lines, fits of model to the data. A, 5E9; B, OKT9.

| Parameter | 5E9 | OKT9 | Tf  
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<tr>
<td>[TfR]₀, surface binding sites/cell</td>
<td>3.9 ± 0.7 × 10⁵</td>
<td>4.2 ± 0.5 × 10⁵</td>
<td>5.4 ± 0.6 × 10⁵</td>
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<tr>
<td>( \left{ k_{-1}/k_t + k/k_t(1/k_t + k_d) \right} )</td>
<td>4 ± 2 × 10⁻⁹</td>
<td>9 ± 3 × 10⁻⁹</td>
<td>6 ± 2 × 10⁻⁸</td>
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</tr>
<tr>
<td>or ( \left{ k_{-1}/k_t + k/k_t \right} ((M⁻¹)) )</td>
<td>2.3 ± 0.3</td>
<td>1.9 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td></td>
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<tr>
<td>( k_t )</td>
<td>8 ± 6 × 10⁻⁴</td>
<td>2.5 ± 0.2 × 10⁻⁴</td>
<td>1.4 ± 0.1 × 10⁻³</td>
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<tr>
<td>( k_{-1} )</td>
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* Values taken from Yazdi and Murphy (31).
* Reported range is 95% confidence interval.
TRAFFICKING AND CYTOTOXICITY OF IMMUNOTOXINS

Fig. 5. Kinetics of binding and internalization. Total (●), surface (▲), and internal (○) cell-associated mAbs as a function of time. Data include specific and nonspecifically bound mAbs. A, 5E9, at 1.3 × 10⁻⁸ M initial concentration in medium. B, OKT9, at 2 × 10⁻⁹ M initial concentration in medium. Inset, total, surface, and internal cell-associated mAbs reach constant values at long times.

Table 2 Anti-TfR mAb kinetic parameters

<table>
<thead>
<tr>
<th>Parameter*</th>
<th>5E9</th>
<th>OKT9</th>
<th>Tf</th>
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<tbody>
<tr>
<td>k₁ (s⁻¹)</td>
<td>3.6 ± 0.6 × 10⁻⁶</td>
<td>2.5 ± 0.2 × 10⁻⁶</td>
<td>4 ± 1 × 10⁻⁶</td>
</tr>
<tr>
<td>k₂ (min⁻¹)</td>
<td>7 ± 4 × 10⁻⁵</td>
<td>7 ± 2 × 10⁻⁵</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>k₃ (min⁻¹)</td>
<td>0.08 ± 0.03</td>
<td>0.2 ± 0.1</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>k₄ (min⁻¹)</td>
<td>0.04 ± 0.02</td>
<td>0.09 ± 0.07</td>
<td>0.18 ± 0.02</td>
</tr>
</tbody>
</table>

* Values taken from Yazdi and Murphy (31).

Reported range is 95% confidence interval.

Results of the fit are shown in Fig. 5. Parameter values are listed in Table 2, along with previously determined values for Tf. The association rate constant k₁ was nominally higher for 5E9 than for OKT9, whereas the dissociation rate constants k₋₁ were similar. Both k₁ and especially k₋₁ were substantially smaller for mAb than for Tf. The internalization rate constant k₀ and recycling rate constant kᵣ for 5E9 determined from kinetic experiments were approximately 3-fold lower than parameter values determined from pulse-chase studies, although the ratio kᵣ/k₀ was the same. Estimates of internalization and recycling rate constants from pulse-chase experiments are subject to some uncertainty due to coated pit formation during rapid warmup (44) and microtubule repolymerization (45). Therefore, internalization and recycling rate constants from kinetic experiments were used for further calculations. Both k₀ and kᵣ were 2- to 5-fold lower for 5E9 than for OKT9, although the difference is probably not significant due to the large uncertainty in these parameters for OKT9. Both k₀ and kᵣ were 2- to 5-fold lower for anti-TfR mAb than for Tf. For both pulse-chase and kinetic data, model fitting to the parameters k₀ and kᵣ produced a covariance close to one. Reparameterization to k₀ and K = kᵣ/k₀ reduced the covariance to an acceptable value without changing the estimated parameter values. The error in k₀ is primarily due to the error in kᵣ; the uncertainty in the ratio kᵣ/k₀ is considerably smaller than in either of the individual rate constants (k₀/kᵣ = 0.46 ± 0.04 for 5E9 and 0.53 ± 0.03 for OKT9). The parameter groups \{k₀/kᵣ + kᵣ/(kᵣ + kᵣ)} and \{k₀(kᵣ/kᵣ + kᵣ)} were calculated from the kinetic rate constants. These values agreed with those obtained as steady-state parameters (Table 1) within the confidence interval.

Protein Synthesis Inhibition. The kinetics of protein synthesis inhibition by 5E9-Gel and OKT9-Gel was measured in HeLa cells for immunotoxin concentrations between 10⁻⁹ M and 10⁻⁷ M (Fig. 6). At the lowest concentration studied (1 × 10⁻⁹ M), little or no significant...
protein synthesis inhibition was observed. Increasing the concentration to $1 \times 10^{-8}$ M resulted in substantially increased inhibition of protein synthesis. Increases in concentration above $10^{-8}$ M resulted in only minor additional reductions in protein synthesis. Concentration-dependent lag times in initiation of protein synthesis inhibition were evident, with lag times ranging from approximately 5 h at the highest concentration to approximately 14 h at the lowest concentration. The concentration needed to achieve 50% protein synthesis inhibition after 24 h exposure was estimated at $5 \times 10^{-8}$ M for 5E9-Gel and $2 \times 10^{-9}$ M for OKT9-Gel. These values are significantly lower than the concentration of $2 \times 10^{-8}$ M for Tf-Gel to achieve 50% protein synthesis inhibition in 24 h (31).

Correlation between Cellular Trafficking and Protein Synthesis. Previously (31), we showed that the cellular trafficking kinetics of Tf could be correlated with protein synthesis inhibition kinetics of Tf-Gel and Tf-CRM107 conjugates for both HeLa and SK-MEL-2 cells using the equation:

$$PS = \exp(-K_{PS}CTV)$$  \hspace{1cm} (Q)$$

where

$$CTV = \int_0^t \int_0^t [TA_t] dt dt'$$  \hspace{1cm} (R)$$

and

$$K_{PS} = \frac{k_{cat}K_m}{k_e}$$  \hspace{1cm} (S)$$

$PS$ is the fraction of protein synthesis relative to control, measured after an exposure time $t_i$ to the immunotoxin. $[TA_t]$ is the intracellular concentration (molecules/cell) of the targeting agent (mAb or ligand), $k_{cat}$ and $K_m$ are Michaelis-Menten constants for the enzymatic rate of substrate inactivation by the toxin, $k_e$ is a first-order translocation rate constant for the rate of translocation of toxin from an intracellular vesicle to the cytosol, and $t'$ is a dummy variable. $CTV$ (cellular trafficking variable) is a function of targeting agent trafficking properties only.

To investigate the validity of this equation for gelonin immunotoxins made from anti-TfR mAb, $[Ab]$ for 5E9 and OKT9 as a function of time was calculated from the trafficking models using Eqs. H-K, $k_2/k_1$ from Table 1, rate constants listed in Table 2, $k_d$ from degradation kinetic experiments, and the number of surface mAb-binding sites from Table 1. The conditions of the protein synthesis inhibition assay, including cell concentration, immunotoxin incubation period, and initial immunotoxin concentration, were used in these calculations. $CTV$ was calculated by numerically integrating $[Ab]$ twice over time per Eq. R. Protein synthesis in the presence of 5E9-Gel or OKT9-Gel was then plotted versus $CTV$, and Eq. Q was fitted to the data by nonlinear least-squares regression, treating $K_{PS}$ as a parameter. Results (Fig. 7) indicate that the model represents the experimental data well and adequately describes the time- and concentration-dependence of protein synthesis inhibition by the gelonin immunotoxins.

$K_{PS}$ was estimated to be $2 \times 10^{-12}$ and $5 \times 10^{-12}$ cells/molecule-min$^2$ for 5E9-Gel and OKT9-Gel, respectively. These values are nearly identical to that determined previously for Tf-Gel ($K_{PS} = 4 \times 10^{-12}$) but are smaller by several orders of magnitude than $K_{PS} = 9 \times 10^{-9}$ for Tf conjugated to the diphtheria toxin mutant CRM107 (31).

Sensitivity Analysis. According to Eq. Q, the sensitivity of a cell line to a given immunotoxin is related to the cellular trafficking of the corresponding targeting agent. The contribution of cellular trafficking to immunotoxin efficacy can be divided into two parts: (a) factors affecting the accumulation of targeting agent intracellu-
Then the extracellular concentration of Tf or mAb was assumed to be high compared to the total concentration of binding sites \((CN)\) \([TfR\text{,}]\). Therefore, any measurable decrease in extracellular mAb is due only to degradation, or, from Eqs. L, P, and T:

\[
- \frac{d[Ab]}{dr} = \left( \frac{C}{N} \right) \frac{d([Ab])]_{tr}}{dr} = \left( \frac{C}{N} \right) k_{1}[Ab]_t
\]

and any decrease in extracellular Tf is due only to conversion to apoTf, or:

\[
- \frac{d[Tf]}{dr} = \frac{d(aTf)}{dr} = \left( \frac{C}{N} \right) k_{1}[Tf] = \left( \frac{C}{N} \right) k_{1}[Tf]_{tr}
\]

and any decrease in intracellular Tf is due only to conversion to apoTf, or:

\[
- \frac{d[Tf]}{dr} = \frac{d(aTf)}{dr} = \left( \frac{C}{N} \right) k_{1}[Tf] = \left( \frac{C}{N} \right) k_{1}[Tf]_{tr}
\]

Again, Eqs. V and W are similar in form if \(k_{1} \gg k_{r}\). It is likely that apoTf can, in fact, scavenge available iron from cell culture medium (46, 47). However, the rate and extent to which iron is donated to apoTf is highly variable (48). We assume for these calculations that apoTf is not reconverted to Tf in the medium.

For maximum immunotoxin efficacy, low depletion rates and high intracellular concentrations are needed. These approximate equations show that, at constant cell concentration, the key parameters are \([TfR]_{tr}\), \(f_{R}\) (which is a function of mAb or Tf concentration in the medium and several rate constants), \(k_{r}\), and \((k_{r}/k_{d})\) for Tf, and \([TfR]_{tr}\), \(k_{r}\) \((k_{r}/k_{d})\) and \((k_{r}/k_{d}+k_{d})\) for mAb. \(k_{d}/(k_{r}+k_{d})\) represents the ratio of internalized mAb that is routed for degradation to the total internalized mAb processed by the cell.

Protein synthesis relative to control in HeLa cells treated with Tf-Gel or 5E9-Gel for 24 h was calculated by numerically solving model equations for cellular trafficking of Tf or 5E9 along with Eqs. Q and R. KPS values estimated for the two immunotoxins, and the cell concentration in protein synthesis inhibition assays, were used in these calculations. The complete set of differential equations, not the approximate equations, were used. Values for \(k_{r}\), \([TfR]_{tr}\), or \((k_{r}/k_{d})\) (for Tf), and of \(k_{r}\), \([TfR]_{tr}\), \(k_{r}/k_{d}\), and \((k_{r}/k_{d}+k_{d})\) (for 5E9) were then adjusted up or down by a factor of 10 to determine the sensitivity of protein synthesis to the numerical value of these parameters as a function of receptor saturation \(f_{R}\). In general, \(f_{R}\) is a function of time; for these calculations, initial mAb or Tf extracellular concentration and the assumed parameter values were used to calculate \(f_{R}\).

Results are summarized in Fig. 8, A and B. For Tf, increasing receptor density \([TfR]_{tr}\) had only a small favorable effect on inhibiting protein synthesis, with the greatest effect at high receptor occupancy (large \(f_{R}\)). At low \(f_{R}\) (which corresponds to low Tf concentrations in the medium), the increased depletion of Tf due to the greater antigen density partially offset the increase in steady-state intracellular concentration. On the other hand, reducing \([TfR]_{tr}\) markedly decreased protein synthesis inhibition. Changes in the internalization rate constant \(k_{r}\) had only minor effects on protein synthesis, with the greatest effect at small \(f_{R}\) (not shown). Reducing \(k_{r}\) had a small favorable impact on inhibiting protein synthesis at lower receptor occupancy due to the influence of \(k_{r}\) on depletion from the extracellular fluid (not shown). Protein synthesis was most sensitive to changes in the ratio \(k_{d}/k_{r}\); increasing \(k_{d}/k_{r}\) (by decreasing \(k_{d}\)) led to a marked increase in accumulation of intracellular Tf, with no adverse effect on Tf depletion from the medium.

Sensitivity to cellular processing parameters for anti-TfR mAb-gelonin conjugates differs considerably. Protein synthesis inhibition by 5E9-gelonin conjugates is greatly enhanced by an increase in \([TfR]_{tr}\). Depletion from the medium is slower for 5E9 than for Tf; therefore, the increase in degradation due to a higher antigen density is insufficient to overcome the strong increase in intracellular levels with increasing antigen density. Changes in the internalization rate constant \(k_{r}\) in either direction had very little effect (data not shown). Decreasing the degradation rate, at constant internalization rate and constant fraction degraded (i.e., \(k_{d}\) and \(k_{r}/(k_{r}+k_{d}) = \) constant), had a significant impact on enhancing efficacy. As can be seen from Eq. T, this effect is due primarily to an increase in intracellular mAb. Interestingly, the system was most responsive to an increase in the fraction of intracellular mAb degraded by the cell \(k_{d}/(k_{r}+k_{d})\) at constant degradation rate constant \(k_{d}\). This is achieved by reducing the recycling rate constant \(k_{r}\) and, hence, retaining mAb intracellularly.

The effect of cell concentration or \(K_{PS1}\) on protein synthesis was investigated (Fig. 8, C and D). Reducing cell concentration had little effect on protein synthesis for either Tf or 5E9. Increasing the cell concentration reduced protein synthesis relative to the base case for both Tf and 5E9, with the greater effect on Tf. The greater sensitivity to cell concentration of Tf is due to the greater rate of depletion of Tf from the medium (Eq. V or W). For both Tf and 5E9, protein synthesis was very sensitive to 10-fold changes in \(K_{PS1}\), as expected from Eq. Q.

**DISCUSSION**

The cellular trafficking model of mAb directed against the TfR represents the data well. The consistency of the estimates of \(k_{d}\) between pulse-chase and degradation experiments, and the consistency of ratios of parameters determined from pulse-chase, kinetic,
and steady-state experiments, argue for reasonable confidence in the trafficking model equations and parameters.

Pirker et al. (5) investigated the binding of 5E9 to three different carcinoma cell lines and reported affinity constants and antigen densities that agree reasonably well with our results for HeLa cells. In that study, up to three h incubation was required to reach a plateau in cell-associated mAb, consistent with the slow association that we see. Accumulation kinetics were reported in terms of initial uptake rate over the first hour; these values are similar to or slightly higher than ours.

Weissman et al. (49) investigated OKT9 processing by K562 cells. TfR half-life decreased by a factor of two in the presence of OKT9, presumably due to degradation of OKT9-TfR complexes, and there was an increase in the biosynthesis rate of new TfR, which partially compensated for the increased TfR degradation rate. The number of Tf binding sites decreased after pretreatment with OKT9. In the same vein, Pirker et al. (5) reported that binding of 5E9 to several cell lines declined after reaching a maximum. These results are not entirely consistent with our data. Despite a greater degradation rate for anti-Tf mAb than Tf, in agreement with Weissman et al. (49), we do not see a drop in surface expression of TfR with time. Discrepancies between our data and that of Pirker et al. (5) and Weissman et al. (49) could be due to differences in intracellular processing in different cell lines, such as small differences in the pH sensitivity of the mAb-TfR interaction. The decrease reported by Pirker et al. (5) could conceivably be due to a decrease in extracellular mAb because of degradation at the greater cell density used in those experiments.

To our knowledge, cellular processing kinetic parameters for anti-Tf mAb have not been reported previously. The most striking difference between mAb and Tf is the markedly reduced dissociation rate for mAb. Of interest also is that the two mAbs, but not Tf, degraded measurably over the experimental time frame. Routing of mAb-TfR complexes to lysosomes could be caused by mAb cross-linking of receptors (50). Alternatively, greater pH sensitivity of mAb-TfR compared to Tf-TfR binding could lead to partial dissociation of complexes in the endosome and delivery of mAb but not Tf to lysosomes (50). Internalization and exocytosis rate constants were somewhat lower for mAbs than for Tf. The reason for these differences is not known; it is possible that cross-linking of TfR by mAb slows down transport of mAb-TfR complexes through tubules, or that a larger fraction of mAb-TfR complexes are routed through the slow recycling pathway. As described previously, slow and fast intracellular routing paths are lumped together in our model.

The lower concentration required for 50% protein synthesis inhibition at 24 h by mAb-Gel (2-5 × 10⁻⁹ m) versus Tf-Gel (2 × 10⁻⁹ m) is due to a combination of factors: (a) the two mAbs have higher receptor saturation than Tf at extracellular concentrations below ~10⁻⁷ m. This can be seen by comparing the values for the parameter group \(\{k_{-1}/k_1 + (k_A/k_1)\} + \{k_{-1}/k_1\} + \{k_{-1}/k_k\}\) for mAb to the value for the parameter group \(\{k_{-1}/k_1\}\) for Tf. The major reason for the difference in these values is the difference in affinity between mAb and Tf; and (b) the decrease in cell-associated mAb by dissociation from the surface or degradation is considerably slower than the corresponding process for Tf. The half-life for dissociation of mAb is approximately 100 min, compared to a half-life for Tf dissociation or apoTf exocytosis of 0.5 min or 4 min, respectively. Since internalization rate constants for mAb and Tf are of similar order-of-magnitude, the net effect is that mAb stays associated with the cell for a significantly longer period of time than Tf and is depleted from the medium at a much slower rate than is Tf. Both of these effects can be demonstrated semiquantitatively by the approximate Eqs. T-W.

The differences in trafficking between mAb and Tf may have clinical significance. Both mAbs tested have greater affinities than Tf, so receptor saturation can be reached at lower plasma concentrations. The slow association rate of mAb may be a drawback in a clinical setting, where the half-life for association with the tumor may be of similar magnitude to the half-life for removal of immunotoxin from the circulation (30). On the other hand, once bound, mAb stays associated with target cells for a significantly longer period of time than Tf, which will lead to persistence of mAb at the tumor site. Nearly every Tf-toxin conjugate internalized by the cell is converted to apoTf-toxin. If the apo form of the conjugate cannot quickly scavenge iron, it will no longer be able to rebind efficiently to target cells and will be removed from the circulation. In contrast, the degradation and dissociation rates for mAbs are considerably slower than the recycling rate, so that each mAb makes several cycles through the cell prior to degradation or dissociation. Once dissociated, mAb is still capable of rebinding or binding to a nearby cell. Lysosomal degradation may be preferable to conversion to a nonbinding form (like apoTf); since the toxin would likely also be degraded, any secreted fragments would not retain nonspecific toxicity directed against nontarget cells. Finally, because of the greater depletion rate for Tf compared to mAb, mAb-gelonin conjugates will retain effectiveness better than Tf-gelonin conjugates as the tumor burden (cell concentration) increases.

The kinetics of protein synthesis inhibition for mAb-Gel were related to the cellular trafficking of mAb using our previously proposed model. Fig. 7 shows that Eq. Q holds for trafficking agents other than Tf, providing additional support for the general utility of this approach. Furthermore, \(K_{ps2}\) determined from the two systems investigated here (5E9-Gel and HeLa, OKT9-Gel and HeLa) and the two systems investigated previously (Tf-Gel and HeLa, Tf-Gel and SK-MEL-2) were very similar, and significantly different than that determined previously for immunotoxins of Tf and the diptheria toxin mutant CRM107 (\(K_p\) = 9 × 10⁻⁹; Ref. 31). This indicates that, at least for gelonin immunotoxins targeted against the transferrin receptor, \(K_{ps2}\) is virtually independent of the cell line or the targeting agent. This result shows that it may be possible to predict cytotoxicity of anti-Tf-gelonin immunotoxins from knowledge of the cellular trafficking characteristics of the antibody used. Extension of the predictive capability of the model to other toxins and other antigens/receptors requires additional experimental confirmation and model validation.

The sensitivity analysis shows that the primary considerations in understanding cytotoxicity of immunotoxins as it is related to cellular trafficking of the targeting agent are: (a) rate of depletion of extracellular material; and (b) concentration of intracellular immunotoxin. This analysis shows that investigating the effect of a single parameter is insufficient for understanding the relationship between cytotoxicity and cellular processing. The sensitivity of the system to a single parameter depends on the numerical value of other parameters. Key parameters or groups of parameters were identified in this analysis, which may be useful in interpreting some of the literature data. For example, affinity influences the concentration at which high receptor saturation can be obtained, but other kinetic parameters also contribute to \(f_{ps}\). As another example, our analysis showed that lower \(k_{-1}\) but higher \(k_A/k_1\) ratios enhance cytotoxicity of Tf-Gel. Thus, increasing \(k_{-1}\) (without increasing \(k_A\)) could have positive, negative, or no influence on cytotoxicity under differing circumstances. As a third example, antigen density may have marked or minimal influence, depending on factors such as the degree of receptor saturation and the rate of depletion. The influence of antigen density on the sensitivity of cells to immunotoxins is particularly important for cases where discrimination between normal and neoplastic cells depends on differences in number rather than kind of antigen. As a final example, higher degradation rates would be disadvantageous, but increasing the frac-
tion of internalized immunotoxin degraded without increasing the degradation rate could markedly improve cytotoxicity.

The approximate Eqs. T-W are useful for determining the key parameter groups in predicting protein synthesis inhibition by immunotoxins. They may also be quite useful in rapid screening of mAbs that are candidate targeting agents. Cellular trafficking models and kinetic parameters would not have to be derived. Degradation kinetics and steady-state measurements would be sufficient to determine, to a first approximation, the essential parameters or parameter groups for estimation of cytotoxicity of a gelonin conjugate constructed from this mAb.

Based on this analysis, the most effective means to enhance efficacy of immunotoxins by manipulating cellular trafficking would be to devise methods to retain the immunotoxin inside the cell longer (i.e., decrease \( k_d \) or \( k_r \)) in order to increase \( CTV \). As clearly shown by Eqs. Q and S, methods that enhance rates of translocation (increase \( k_{trans} \), or, equivalently, \( k_{pro} \)) would be of equal importance.

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Influence of Cellular Trafficking on Protein Synthesis Inhibition of Immunotoxins Directed against the Transferrin Receptor

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