Kinetics of Cytotoxicity Induced by Immunotoxins
ENHANCEMENT BY LYSOSOMOTROPIC AMINES AND CARBOXYLIC IONOPHORES

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The kinetics of cytotoxicity induced by ricin and a series of immunotoxins consisting of ricin A-chain coupled to antibodies against cell-surface antigens has been studied. The inhibition of protein synthesis in cells treated with immunotoxins or ricin occurs after a lag period. The rate of protein synthesis decreases according to a mono-exponential function, indicating a first-order process. With increasing concentration of immunotoxin, a maximal rate of inhibition is reached. The inactivation rate induced by immunotoxins was much slower than that achieved with ricin, even when products were compared on a basis of an identical number of molecules bound per cell, demonstrating the real higher efficacy of ricin. The time required to reduce protein synthesis by 90%, denoted $T_{10}$, was 1.4–1.6 h with ricin, 60 h with anti-T65 immunotoxin on CEM human T leukemia cells (T65 positive), 65 h with anti-p97 immunotoxin on SK-NEL 28 human melanoma cells (p97 positive), and 20 h with an IgM anti-Thy 1.2 immunotoxin on WEHI-7 mouse T leukemia cells (Thy 1.2 positive). In this latter case, when the IgM antibody was replaced by an IgG anti-Thy 1.2, a 5-fold increase in the inactivation rate was obtained, demonstrating the importance of the binding moiety for the immunotoxins.

Lysosomotropic amines such as ammonium chloride, chloroquine, and methylamine and carboxylic ionophores such as monensin, which are known to interfere with the uptake of certain macromolecules, strongly increased the rate of protein synthesis inhibition by all immunotoxins tested and increased 4–50,000-fold the sensitivity of cells to the immunotoxin. Enhancement in the inactivation rate was as much as 7–10-fold when either of these compounds was added, generating $T_{10}$ values comparable to those of ricin.

A new strategy in cancer chemotherapy using hybrid molecules possessing dual functions of recognition and cytotoxicity towards tumor cells is presently under study in numerous laboratories. This mode of treatment was initially envisaged by Ehrlich, who conceived the notion of using agents consisting of a binding component (the haptophore) and a toxic part (the toxophore). In recent years, conjugates (referred to here as immunotoxins), in which the toxophore consists of an antibody molecule or fragment thereof and the toxophore consists of a toxin or a part of it, have been synthesized (see Refs. 1–6 for recent reviews). The toxic moiety which has been used in our laboratory is derived from the plant toxin ricin, which is a glycoprotein consisting of two chains, A and B. The A-chain is an inhibitor of protein synthesis, and the B-chain is a binding agent (7). The mode of action assumed for the whole toxin (A + B) implies that the B-piece binds to the surface of the cell and in some way facilitates the translocation of the A-piece through the plasma membrane to the cytoplasm. There, the A-chain enzymatically inactivates the 60 S ribosomal subunit with an extreme potency, in accordance with the widely held view that a single molecule is sufficient to kill a cell (7).

In previous studies, immunotoxins were constructed in which the A-piece of ricin was conjugated to antibodies specific for a variety of target antigens. The results obtained with these reagents demonstrated the expected specific in vitro cytotoxicity since more than 99.9% of target cells were eliminated, whereas cells not expressing the relevant antigens were unaffected (6, 8, 9). However, despite these encouraging data, immunotoxins are far less toxic than the parent toxin ricin, and in vivo experiments, complete eradication of tumor cells in animal models has not been possible with immunotoxin treatment (8, 10). In recent studies, we (9) and others (2, 11, 12) have pointed out that the rate of cell killing induced by immunotoxins is extremely slow, and this may account for the relative ineffectiveness of the immunotoxins in vivo. However, since the kinetics of cell killing by immunotoxins has so far been inadequately studied, a more detailed examination is warranted.

We show here, from a kinetic analysis of a number of immunotoxins, that the time required to kill target cells can vary within a wide range depending on the model used and that two variables affected the rate of cell killing: the number of immunotoxin molecules bound per cell and the class of the antibody moiety. In addition, the previously described enhancement of target-cell killing in the presence of ammonium chloride (9) is here extended to include a variety of lysosomotropic amines and a group of carboxylic ionophores. These compounds, which improved both activity and specificity of immunotoxins, could have important therapeutic implications.

MATERIALS AND METHODS AND RESULTS
Kinetics of Cytotoxicity Induced by Immunotoxins and Ricin at a Comparable Amount of A-Chain Molecules Bound per

* Portions of this paper (including "Materials and Methods," part of "Results," Fig. 1–4 and 6, and Tables II–IV) are presented in miniprint at the end of this paper. The abbreviations used are: IT, immunotoxin; PBS, phosphate-buffered saline. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1041, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
We demonstrated that immunotoxins exhibited inactivation rates which varied to a large extent (3-fold) and which were significantly slower than that of ricin on the same cells (Fig. 2).

Next we examined if the differences between ricin and immunotoxins could be explained by differences of the number of molecules bound per cell. We found, from binding analysis, that at the doses tested in Fig. 1, the number of A-chain molecules bound per cell was around $2 \times 10^6$ and $7 \times 10^6$ with ricin on WEHI-7 and CEM cells, respectively, whereas it was $1.5 \times 10^6$ with the anti-Thy 1.2 immunotoxin (IgM) on WEHI-7 cells, $4 \times 10^6$ with the anti-T65 immunotoxin on CEM cells, and $4.5 \times 10^6$ with the anti-p97 immunotoxin on SK-MEL 28 cells. In order to compare the ability of the bound toxin and immunotoxins to intoxicate cells, we performed kinetic experiments at concentrations ensuring an identical number of A-chain molecules bound per cell. The results are given in Table I. Although the rate of protein synthesis inactivation induced by ricin slightly decreased when the number of ricin molecules bound per cell diminished, ricin achieved 30-fold and 12-fold faster inactivation rates compared to anti-T65 immunotoxin and anti-Thy 1.2 immunotoxin (IgM), respectively, demonstrating the higher efficacy of ricin versus immunotoxins. However, the differences between the two toxins could vary dramatically with the immunotoxin examined, as illustrated with the anti-Thy 1.2 immunotoxin (IgG). The rate of protein synthesis inactivation induced by anti-Thy 1.2 immunotoxin (IgG) on WEHI-7 cells was only 2.3-fold longer than that obtained with ricin (Table I).

In addition, this example demonstrated the importance of the antibody-moiety class for the immunotoxin efficacy since the simple substitution of the anti-Thy 1.2 IgM by an IgG of same specificity improved by a factor of 5 the rate of cell killing measured under comparable conditions.

It should be noticed that the IgG molecules could bind to the WEHI-7 cells to a higher extent than could IgM (Fig. 4). With higher immunotoxin concentrations, the binding could be even greater than $7 \times 10^6$, and this resulted in more rapid kinetics (data not shown). Binding differences could be due to the steric hindrance of the IgM molecule.

**Influence of Lysosomotropic Amines and Carboxylic Iono-
phores on the Cytotoxicity of Immunotoxins: Enhancement of Cytotoxicity**—Cytotoxicity was determined quantitatively using cells treated for 24 h with the relevant immunotoxin in the presence of lysosomotropic amines or ionophores. The results are presented in Table III. A considerable activation of the anti-T65 immunotoxin cytotoxicity on CEM cells was obtained with these drugs. The 50% inhibiting dose (IC$_{50}$) was reduced by a factor of 1.180 with 1 mM amantadine, 2.500 with 100 μM chloroquine, 6.700 with 10 mM NH$_4$Cl, and even 13,300 with 10 mM methylamine. However, when methyl-

| Cell line | Toxin | A-chain/Toxin molecule$^a$ | Dose$^b$ | Toxin molecules bound per cell | A-chain molecules bound per cell | $T_{1/2}$ | $T_{90}$ |
|-----------|-------|----------------------------|---------|-------------------------------|---------------------------------|--------|-------|
| CEM       | Ricin | 1                          | $10^{-8}$ | $4 \times 10^4$              | $4 \times 10^6$                  | 2      | 60    |
|           | Anti-T65 IT | 2       | $10^{-8}$ | $2 \times 10^4$              | $4 \times 10^6$                  |        |       |
|           |       | ($0.5 \times 10^{-7}$)     |          |                               |                                 |        |       |
| WEHI-7    | Ricin | 1                          | $1.5 \times 10^{-7}$ | $1.5 \times 10^6$            | $1.5 \times 10^6$                | 1.7    |       |
|           | Anti-Thy 1.2 | 3       | $10^{-8}$ | $5 \times 10^5$              | $1.5 \times 10^6$                | 20     |       |
|           | IT (IgM)   |        | ($3.3 \times 10^{-7}$)    |                               |                                 |        |       |
|           | Anti-Thy 1.2 | 2       | $3 \times 10^{-7}$        | $7.5 \times 10^5$             | $1.5 \times 10^6$                | 4      |       |

$^a$ The number of A-chain molecules/antibody was measured as already described (15).

$^b$ Dose is expressed in A-chain molarity; antibody concentration is given in parentheses.

$^c$ The number of molecules bound per cell was established by binding analysis as described under "Materials and Methods" and shown for WEHI-7 in Fig. 4.

$^d$ The $T_{1/2}$ values were obtained from kinetics curves and calculated as described under "Materials and Methods." $T_{90}$ represents the time required to reduce the rate of protein synthesis by 1 order of magnitude. In a first-order process, $T_{90}$ corresponds to the reciprocal of the first-order rate constant ($k$) multiplied by 2.303.

**Kinetics of Cytotoxicity Induced by Immunotoxins**—We have reported that ammonium chloride increased the effectiveness and the rate of action of anti-p97 immunotoxin (9). This prompted us to examine the effect of this potentator on the other immunotoxins and also to test if other agents known to affect the cellular uptake of macromolecules had the same effect. Lysosomotropic amines and carboxylic ionophores were considered and used at their highest concentration at which they did not decrease the [14C] leucine uptake in the control samples. The data shown in Table II demonstrate that the time course of protein synthesis inhibition was considerably shortened when ammonium chloride was added together with immunotoxin and kept throughout the incubation period. The $T_{10}$ values were achieved at 8.6 h with the anti-p97 immunotoxin, 5.5 h with the anti-T65 immunotoxin, and 2.2 h with the anti-Thy 1.2 immunotoxin (IgM) on their corresponding target cells. Methylamine at 10 mM or chloroquine at 100 μM was even more efficient than 10 mM ammonium chloride and generated $T_{90}$ values of 2 and 2.6 h, respectively, with anti-T65 immunotoxin. Similar acceleration was achieved with 50 nM monensin (Table II). The extent of stimulation depended on the concentration of potentiator, as shown in Fig. 5 with the anti-T65 immunotoxin on CEM cells. With from 2 to 10 mM NH$_4$Cl and from 10 to 20 mM monensin, the $T_{90}$ values diminished with increasing concentrations for both agents and then reached a saturation level with monensin. Concentrations greater than 10 mM NH$_4$Cl alone abolished protein synthesis, precluding the determination of the dose of NH$_4$Cl which produces the maximal inactivation. Although the agents could not be compared at the concentrations producing the maximal effect, on a molar basis, monensin was much more effective than ammonium chloride.

**Influence of Lysosomotropic Amines and Carboxylic Ionophores on the Cytotoxicity of Immunotoxins: Enhancement of Cytotoxicity**—Cytotoxicity was determined quantitatively using cells treated for 24 h with the relevant immunotoxin in the presence of lysosomotropic amines or ionophores. The results are presented in Table III. A considerable activation of the anti-T65 immunotoxin cytotoxicity on CEM cells was obtained with these drugs. The 50% inhibiting dose (IC$_{50}$) was reduced by a factor of 1.180 with 1 mM amantadine, 2.500 with 100 μM chloroquine, 6.700 with 10 mM NH$_4$Cl, and even 13,300 with 10 mM methylamine. However, when methyl-

| Kinetics of cytotoxicity induced by immunotoxins (IT) and ricin at comparable amount of A-chain molecule bound per cell |
|---------------------------------------------------------------|
| Cell line          | Toxin     | A-chain/Toxin molecule$^a$ | Dose$^b$ | Toxin molecules bound per cell | A-chain molecules bound per cell | $T_{1/2}$ | $T_{90}$ |
|--------------------|------------|----------------------------|---------|-------------------------------|---------------------------------|--------|-------|
| CEM                | Ricin      | 1                          | $10^{-8}$ | $4 \times 10^4$              | $4 \times 10^6$                  | 2      | 60    |
|                    | Anti-T65 IT | 2       | $10^{-8}$ | $2 \times 10^4$              | $4 \times 10^6$                  |        |       |
|                    |            | ($0.5 \times 10^{-7}$)     |          |                               |                                 |        |       |
| WEHI-7             | Ricin      | 1                          | $1.5 \times 10^{-7}$ | $1.5 \times 10^6$            | $1.5 \times 10^6$                | 1.7    |       |
|                    | Anti-Thy 1.2 | 3       | $10^{-8}$ | $5 \times 10^5$              | $1.5 \times 10^6$                | 20     |       |
|                    | IT (IgM)   |        | ($3.3 \times 10^{-7}$)    |                               |                                 |        |       |
|                    | Anti-Thy 1.2 | 2       | $3 \times 10^{-7}$        | $7.5 \times 10^5$             | $1.5 \times 10^6$                | 4      |       |

$^a$ The number of A-chain molecules/antibody was measured as already described (15).

$^b$ Dose is expressed in A-chain molarity; antibody concentration is given in parentheses.

$^c$ The number of molecules bound per cell was established by binding analysis as described under "Materials and Methods" and shown for WEHI-7 in Fig. 4.

$^d$ The $T_{1/2}$ values were obtained from kinetics curves and calculated as described under "Materials and Methods." $T_{90}$ represents the time required to reduce the rate of protein synthesis by 1 order of magnitude. In a first-order process, $T_{90}$ corresponds to the reciprocal of the first-order rate constant ($k$) multiplied by 2.303.
The kinetics of immunotoxin intoxication of target cells was examined by measuring the rate of protein-synthesis inhibition induced by immunotoxins of various specificities and was compared to that obtained with ricin. From this study, it appeared that the intoxication induced by both immunotoxins and ricin was characterized by 1) a lag period preceding the start of protein-synthesis inhibition; 2) a monoeponential decrease in protein-synthesis rate, indicating a first-order process; and 3) a rate of inactivation which is a function of the toxin concentration and which was maximal when all the receptors were occupied (Figs. 1 and 2).

The presence of a lag period for both ricin and the immunotoxins is consistent with the finding that they must first be processed by endocytosis to gain access to the cytosol compartment, as recently demonstrated for ricin by Sandvig and Olsnes (20) and confirmed for immunotoxins (data not shown).

Slower intoxication of cells was obtained when the amount of toxin molecules bound per cell decreased (Fig. 1), demonstrating that the inactivation rate was a function of the number of toxin molecules bound. However, in the case of ricin, maximal inactivation rate was achieved before all ricin receptors were fully saturated. The fact that ricin was much more efficient than immunotoxins could be explained by the presence of the B-chain which could facilitate the transmembrane passage of the A-chain into the cytosol compartment and/or select a special receptor mediating rapid entry.

The nature of the binding moiety also seems to be important for the activity of immunotoxin. Thus, immunotoxin containing IgM and IgG molecules directed against the same antigen was very different in toxicity (Table I). The reason for the difference between immunotoxins containing the two Ig classes is not known, but it could be due to differences in capacity to induce capping or to the smaller size of IgG which may bring the A-chain into closer contact with the cell membrane than does IgM. This could be much more favorable for the passage of the A-chain.

We have recently observed that the rate of cell killing by immunotoxins of different specificities differed greatly, suggesting that the target antigen chosen may account for the dissimilarities (comparisons were made on a basis of an identical number of molecules bound per cell). This also could explain in part the variation obtained with the immunotoxins examined here.

Lyosomal amines such as ammonium chloride, chloroquine and methylamine, which have been described as inhibiting the action of diphtheria toxin (21), strongly accelerate the inhibition of protein synthesis by all the immunotoxins tested and reduced the $T_{90}$ values to 2.2-9.6 h (Table II). Similarly, treatment with the carboxylic ionophore monensin, which also inhibits the action of diphtheria toxin (20, 22), reduced the $T_{10}$ to a similar extent. The effects of NH$_4$Cl and monensin were found to be dose-dependent processes. On a concentration basis, monensin was approximately 10$^5$ times more potent than NH$_4$Cl. It should be noticed that the concentration of monensin which generated the maximal effect on immunotoxins was 20-fold lower than that required to protect cells against diphtheria toxin (22). The fact that the activity of ricin and A-chain was affected by monensin but not by NH$_4$Cl (Fig. 6) suggests that these two activators act in different ways, as already proposed by Ray and Wu (23). The reason for the increase in activity of immunotoxins in the presence of these potentiators could be: (a) an increase in

![Figure 5. Dose-response curves of ammonium chloride and monensin effect on the inactivation rate of protein synthesis induced by immunotoxins.](image)

DISCUSSION

ammonium, dimethylamine, and trimethylamine were compared, the primary amine was a much better potentiatior than the secondary amine, which was again better than the tertiary amine.

With the carboxylic ionophore nigericin at 10 nM, an IC$_{50}$ similar to that observed with 10 mM NH$_4$Cl was measured. More dramatic results were obtained with 50 nM grisoroxin (the IC$_{50}$ was reduced by a factor of 25,000), 1 mM lasalocid (33,000), and with 50 mM monensin (50,000). Ionophores such as nonactin (10 nM), valinomycin (1 nM), and calcimycin (10 nM) produced no activation of anti-T65 immunotoxin, nor did the protease inhibitors leupeptin and pepstatin used at 1 mM.

When 10 mM NH$_4$Cl or 50 mM monensin was added, the IC$_{50}$ of anti-p97 immunotoxin on SK-MEL 28 cells was decreased by a factor of 42- and 420-fold, respectively. The smallest potentiation effect was seen with the anti-Thy 1.2 immunotoxin (IgM) acting on WEHI-7 cells. In this case, the cytotoxicity increased only 5.7-fold with NH$_4$Cl and 4.4-fold with monensin.

When CEM cells were exposed to NH$_4$Cl for 4 h at 37 °C and then washed prior to anti-T65 immunotoxin treatment, no stimulation was observed, demonstrating that the simultaneous presence of immunotoxin and NH$_4$Cl is required for the effect. With monensin, a similar procedure led to a 20-fold reduction in the sensitizing effect; possibly monensin cannot be completely removed by washing.

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P. Casellas, unpublished experiments.
the binding of the immunotoxin to the cell surface, (b) an increase in the efficiency of the transport system, or (c) an increase in the intrinsic toxicity of the A-chain. On the basis of binding studies, we ruled out the possibility that these agents played any direct role in the initial binding step (data not shown). It could also be demonstrated that these agents had no influence on the effect of the A-chain of ricin on ribosomes as evaluated in a cell-free protein-synthesizing system prepared from rat liver (data not shown). Taken together, these facts suggest that NH4Cl and monensin might act on the immunotoxin internalization process. In the presence of these compounds, this process may be extremely potent. Thus, IC50 was reached with as little as 10 or 100 anti- 

Although the in vivo utilization of potentiating drugs remains to be explored, there is an immediate clinical application for immunotoxins used in combination with one of these compounds to eliminate metastatic tumor cells from bone-marrow samples in patients undergoing autologous transplantation in order to avoid recurrence from the graft. Promising preclinical results have already been obtained, showing that a treatment of a mixture of clonogenic CEM cells and normal human bone marrow with anti-T65 immunotoxin plus NH4Cl led to the reduction by more than 6 orders of magnitude of tumor cells, whereas bone-marrow progenitor cells were unaffected by such a treatment.4

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Methylamine. were purchased from (Clemint-Ferrand France). The human intestinal cell line CEM carrying the Thy-1 antigen was obtained from Dr. Carrel (Ludwig Institute, Basel, Switzerland). The T cell line SK-MEL 23 carrying the p97 antigen, was obtained from Dr. E.D. Melcher (Fred Hutchinson Cancer Research Center, Seattle, U.S.A.). The human T-cell line HSB2 lacking the Thy-1 antigen and non-T cells in monolayer culture were maintained in a blood-serum medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Tests and IC50

Ricin, ricin A-chain, and ITs were prepared as previously described(9,13). Four monoclonal antibodies were used: an IgG mouse antibody specific for the mouse differentiation antigen Thy-1.2 purchased from Dako (England), an IgG rabbit antibody, AT4M, specific for the human T-cell differentiation antigen Thy-1 (14), an IgG mouse antibody, TIO, specific for the human T-cell differentiation antigen Thy-1 (15), and an IgG mouse antibody specific for the mouse-associated antigen Thy-2 (16). The corresponding FITC was labelled with anti-FITC IgG2a and anti-FITC IgG1, respectively. The IC50 contained an average of 1.5-3 dpm/molecule per antibody molecule.

Assay of protein synthesis

Cells suspended in fresh culture medium (8 x 10⁶ cells/ml) for leucine cells or 2 x 10⁶ cells/ml for melanoma cells were dispensed in 30 ml aliquots into wells of microplate tubes. Tests and IC50 were added in a volume of 10 ml and incubated with cells for various periods of time, as indicated in the figure. Protein synthesis was determined by measuring [35S]methionine incorporation into cell protein. For the uptake was determined for 45 min in the presence of 2 μCi of [35S]methionine. Cells were then immediately chilled on ice and harvested from the well with extraction buffer. Surface radioactivity was measured by counting 100 tryps, in 300 μl buffer, before harvesting. Incorporation of radioactivity into cell proteins was determined by the cold trypsinization method. Each point corresponds to the mean of quadruplicate assays.

IC50 determination: cells were exposed to IT for 24 h. For each IT, the concentration based on the amount of compound that caused a 50% inhibition of incorporation was determined. This concentration was termed as the IC50 value.

TIO determination: kinetics of cytotoxicity were obtained by exposing the target cell line in order of magnitude, treated TIO was used to compare the products. With ricin, TIO was 1.4-1.6 h irrespective of the cell line used. 80 h with anti-Ag IT on the CEM cells and 60 h with anti-Thy IT on the SK-MEL 23 cells. IC50 inhibition rates which varied in a large extent (3 fold) and significantly were higher than those for the ricin on the same cell line.

The possibility that the slow response to IT was due to a slow binding was ruled out by kinetic studies of the time needed for the 8-chain to be transported to the cytoplasmic site of action.

Kinetics of leucocyte binding

The cell binding kinetics of the designated ITs were measured using a cell marker (FITC 14 = Rosenthal, Basle, Switzerland). Cells (10⁶/ml) were incubated with IC50 (C, 100 μg/ml) or A-chain (D, 100 μg/ml) labeled with 125I (specific activity 10⁶ cpm/mg protein) for 1 h at 37°C in culture medium containing 10% fetal calf serum and an on-cell assay to the final concentration of time point at 4°C. The concentration required to have a desired number of high-affinity binding sites per cell was defined from the binding curves. The results were expressed as specific radioactivity in arbitrary units after calibration by fluorescent microspheres.

Determination of TIO and IT bound at the cell surface

Measurement of TIO and IT bound at the surface of intact cells was done by using binding assays with the radiolabeled products. Antibodies were labeled with 125I in the presence of chloramine-T as described by Kroll et al. (19). Binding assay were performed under the same conditions as for the assay of protein synthesis. IC50 in the culture medium containing 10% fetal calf serum and an on-cell assay to the final concentration of time point at 4°C. The concentration required to have a desired number of high-affinity binding sites per cell was defined from the binding curves. The results were expressed as specific radioactivity in arbitrary units after calibration by fluorescent microspheres.

Determination of ricin and IT bound at the cell surface

Measurement of ricin and IT bound at the surface of intact cells was done by using binding assays with the radiolabeled products. Antibodies were labeled with 125I in the presence of chloramine-T as described by Kroll et al. (19). Binding assay were performed under the same conditions as for the assay of protein synthesis. IC50 in the culture medium containing 10% fetal calf serum and an on-cell assay to the final concentration of time point at 4°C. The concentration required to have a desired number of high-affinity binding sites per cell was defined from the binding curves. The results were expressed as specific radioactivity in arbitrary units after calibration by fluorescent microspheres.

Kinetics of cytotoxicity induced by ricin and ITs

Effect of IC50 inhibition of protein synthesis induced by ricin (2 x 10⁶ cpm) and TIO (10⁵ cpm) were measured at defined time intervals over 2 days and plotted on a semilogarithmic scale in figure 2. Points were selected to provide a 0-100% range for each IT for the IC50. Protein synthesis decreased linearly, demonstrating the existence of an apparent first order process for cytotoxicity induced by both ricin and ITs. The time required to reduce the rate of protein synthesis by 10% from the maximum, treated TIO, was used to compare the products. With ricin, TIO was 1.4-1.6 h irrespective of the cell line used. 80 h with anti-Ag IT on the CEM cells and 60 h with anti-Thy IT on the SK-MEL 23 cells. IC50 inhibition rates which varied in a large extent (3 fold) and significantly were higher than those for the ricin on the same cell line.

The possibility that the slow response to IT was due to a slow binding was ruled out by kinetic studies of the time needed for the 8-chain to be transported to the cytoplasmic site of action.
Kinetics of Cytotoxicity Induced by Immunotoxins

FIG. 2: Binding of ITs to the target cells.

10 20 30 40 50 60 Time (min)

[Graph showing binding of ITs to target cells over time]

Immunoassay binding (% of maximum binding)

[Table showing binding data]

Table II: Effect of monoclonal antibodies and recombinant proteins on the cytotoxicity of ITs

| Cell Type | Antibody | IC50 Value for IT | Effect on Specificity |
|-----------|-----------|-------------------|----------------------|
| CEM       | anti-Thy 1.2 IgG | 5.9 x 10^-9 M | 6.4 x 10^-9 M |
| CEM       | anti-Thy 1.2 IgG | 6.2 x 10^-9 M | 6.5 x 10^-9 M |
| CEM       | anti-p97 IT | 5.7 x 10^-9 M | 6.0 x 10^-9 M |

**TABLE III:** Effect of monoclonal antibodies and recombinant proteins on the cytotoxicity of ITs

| Antibody | Specific activity [% of control] |
|----------|---------------------------------|
| None     | 100                             |
| Anti-Thy 1.2 IgG | 80                             |
| Anti-p97 IT     | 90                             |

**TABLE IV:** Effect of monoclonal antibodies and recombinant proteins on the cytotoxicity of ITs

| Antibody | Specific activity [% of control] |
|----------|---------------------------------|
| None     | 100                             |
| Anti-Thy 1.2 IgG | 80                             |
| Anti-p97 IT | 90                             |

Fig. 3: Binding curves of anti-Thy 1.2 antibody (IgG and IgM) and ricin on WEHI-7 cells.

[Graph showing binding curves]

Ricin or antibody concentration [M]

[Graph showing concentration of ricin and antibody]

*The results were obtained from kinetic curves and calculated as described in Materials and Methods.

**TABLE V:** Percentage of maximum amount of IT bound to target cells.

| ITs | Percentage of maximum amount bound |
|-----|-----------------------------------|
| Anti-Thy 1.2 IgG | 75% |
| Anti-p97 IT | 80% |

*All drugs were tested at serial, non-damaging concentrations established in prior experiments.

**Fig. 4:** The specific activity of ITs based on specific activity required to reduce protein synthesis of target cells by 50% after a 24-hour exposure.

**TABLE VI:** Specific activity factors of ITs.

| ITs          | Specific activity |
|--------------|-------------------|
| Anti-Thy 1.2 IgG | 5.5 x 10^-9 M |
| Anti-p97 IT | 6.0 x 10^-9 M |

**Fig. 5:** Effect of treatment with monoclonal antibodies and recombinant proteins on the activity of ITs.

[Graph showing effect of treatment]

**TABLE VII:** Specificity factors of ITs.

| ITs          | Specificity Factor |
|--------------|--------------------|
| Anti-Thy 1.2 IgG | 6.5 x 10^-9 |
| Anti-p97 IT | 7.0 x 10^-9 |

*Specificity factors were calculated from the data shown in Table VI.

**Fig. 6:** The specificity factor of ITs in the presence of monoclonal antibodies and recombinant proteins.

[Graph showing specificity factors]

**TABLE VIII:** Specificity factors of ITs.

| ITs          | Specificity Factor |
|--------------|--------------------|
| Anti-Thy 1.2 IgG | 6.5 x 10^-9 |
| Anti-p97 IT | 7.0 x 10^-9 |

*Specificity factors were calculated from the data shown in Table VI.

**Fig. 7:** The effect of NH4Cl and monensin on the activity of ITs.

[Graph showing effect of NH4Cl and monensin]

*All drugs were tested at serial, non-damaging concentrations established in prior experiments.
Kinetics of cytotoxicity induced by immunotoxins. Enhancement by lysosomotropic amines and carboxylic ionophores.

P Casellas, B J Bourrie, P Gros and F K Jansen

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