Internalization and Action of an Immunotoxin Containing Mistletoe Lectin A-Chain

Antoni Więtęocha, Kirsten Sandvig, Hermann Walzel, Czeslaw Radzikowski, and Sjur Olsnes

Institute for Cancer Research at The Norwegian Radium Hospital, Montebello, 0310 Oslo 3, Norway [A. W., K. S., S. O.]; Institute of Biochemistry, Rostock University, Rostock, Germany [H. W.]; and Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Czerska 12, Wroclaw, Poland [A. W., C. R.]

ABSTRACT

An immunotoxin consisting of the enzymatically active A-chain of mistletoe lectin I and a monoclonal antibody against a surface protein on mouse leukemia L1210V cells was found to inhibit protein synthesis in these cells as efficiently as the native mistletoe toxin. The immunotoxin was somewhat more slowly endocytosed than the native toxin, but in both cases the endocytic uptake continued under conditions in which uptake from clathrin-coated pits was inhibited by mild acidification of the cytosol. This indicates that the toxin and the immunotoxin were at least partially internalized by a non-clathrin-dependent uptake mechanism and that uptake by this pathway is responsible for most of the toxic effect on the cells. The results indicate that efficient immunotoxins can be made with antibodies against cell surface epitopes that are endocytosed by a mechanism not involving clathrin-coated pits.

INTRODUCTION

In recent years a large number of monoclonal antibodies have been used to direct bacterial and plant toxins to malignant cells in an attempt to achieve selective cell killing (1, 2). A major problem in this work is that most conjugates are much less toxic than desired, and therefore great effort is being made to increase the toxic effect of the conjugates. It appears that after binding of the conjugates to their respective cell surface antigens, endocytic uptake is required for toxicity (3, 4). The possibility therefore existed that the antibody part of the molecule must be directed against epitopes that are rapidly endocytosed from clathrin-coated pits. This endocytic pathway has been studied in a variety of cell lines, and it has been found to be involved in the uptake of a number of physiologically important ligands as well as toxins and viruses (5). A large number of immunotoxins has been constructed with antibodies that are likely to be internalized by endocytosis from coated pits. Little is known about the further mechanism of translocation of the toxic moiety to the cytosol, but transport to the trans Golgi network appears to be required (6, 7).

There is now evidence that both fluid-phase markers and certain membrane-bound markers are internalized, at least to some extent, by invaginations of non-clathrin-coated areas of the cell surface. Thus, endocytosis of these molecules continues when the coated pit/coated vesicle pathway is blocked by hypertonic medium (8), by low cytosolic pH (9), or by removal of clathrin-coated pits by hypertonic shock and incubation in the absence of potassium (10). There is evidence that material endocytosed by this pathway may be delivered to endosomes, but it is not clear whether ligands taken up by each of the two endocytic pathways follow the same intracellular route.

In the present paper we have studied uptake and toxicity in L1210V cells of an immunotoxin of the A-chain of ML13 and a monoclonal antibody against a cell surface protein. The results indicate that the immunotoxin, as well as the parent toxin ML1, are endocytosed from non-clathrin-coated areas of the cell surface and that uptake by this pathway is responsible for most of the toxic effect. The results indicate that efficient immunotoxins can be made by conjugation of a toxic moiety to an antibody that is internalized by clathrin-independent endocytosis.

MATERIALS AND METHODS

Materials. [3H]Leucine and Na251 were from the Radiochemical Centre, Amersham, United Kingdom. Transferrin, horseradish peroxidase, Hapes, Pronase, SPDP, NaN3, lactose, and 2-deoxy-d-glucose were obtained from Sigma Chemical Co., St. Louis, MO. Transferrin was saturated with iron as described (11), and 125I-labeled ligands were prepared by the Iodo-Gen method (12).

Monoclonal Antibody. Purified MoAb-16 monoclonal antibody was prepared as described (13, 14). MoAb-16 is of mouse IgM isotype and binds specifically to leukemias L1210V, L1210, RLô1 and to 13-day-old mouse embryo cells. For the production of this antibody, immune spleen cells from BALB/c mice immunized with L1210V cells were used (13). The antibody recognizes a Mr, 30,000 cell surface protein.4 Transplantable Tumor Line. Mouse leukemia L1210V was selected from L1210 leukemia cells. All passages of the transplantable tumor line were made in syngeneic recipients (DBA/2 mice). Appropriate suspensions of L1210V cells were obtained from ascites fluid by dilution in ice-cold PBS on the third day after inoculation. For in vitro experiments the cells were propagated in Hepes medium, pH 7, supplemented with 10% fetal calf serum in an atmosphere containing 5% CO2.

Toxins and Immunotoxins. ML1 toxin was prepared as described (15, 16). The A-chain was prepared by affinity chromatography on a d-galactose-Sepharose 6B column. After binding of the whole lectin to the column via the A-chain, the A-chain was obtained by reductive elution with PBS containing 5% (v/v) 2-mercaptoethanol. The A-chain was linked to MoAb-16 by the SPDP method in the following way. To 16 mg IgM dissolved in 2 ml PBS were added 80 µl 20 mm SPDP in ethanol. After 2 h the obtained PDP-IgM was separated from the low-molecular-weight reaction products on a Sephadex G-25 column and reacted with 3 mg of ML1 A-chain for 4 h at room temperature. Finally, the conjugate was purified on a Sephacryl S300 column. Quantitation of the amount of ML1 A-chain in the immunoonjugate was performed by enzyme-linked immunosorbent assay as described (16). Ricin was extracted and purified as earlier described (17).

Measurement of Protein Synthesis. After incubation with or without toxin and immunotoxin as described in the legends to the figures, the cells were transferred to medium without inhibitors and the ability of the cells to incorporate [3H]leucine during 15 min was measured as described earlier (18).

3 The abbreviations used are: ML1, mistletoe lectin I (viscum); SPDP, 3-[2-pyridyldithio]propionioic acid- N-hydroxy succinimide ester; PBS, phosphate-buffered saline (140 mm NaCl-10 mm sodium phosphate, pH 7.4); MoAb, monoclonal antibody; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
4 Unpublished data.
Assay of Cytotoxicity and Kinetics of Intoxication. The cytotoxic effects of ML I toxin and the immunotoxin were assessed by measuring the inhibition of cellular protein synthesis relative to untreated controls, as described in the figure legends.

Measurement of Endocytosis. To measure endocytosis of I25I-labeled MoAb-16 L1210V cells were incubated with the conjugate for 15 min at 37°C. The cells were then incubated for 60 min at 0°C in Hepes medium containing 0.1 M mercaptoethanol and 2 mg/ml Pronase. Then the cells and the medium were transferred to Eppendorf tubes and centrifuged for 2 min, and the radioactivity in the pellet and supernatant was measured.

The amount of surface bound and internalized transferrin was measured as described by Ciechanover et al. (11). Briefly, cells were incubated with I25I-transferrin for the indicated periods of time at 37°C, washed three times with ice-cold PBS, and then treated for 1 h at 0°C with 0.25 ml serum-free medium containing 2 mg/ml Pronase. Subsequently, the cells and the medium were transferred to Eppendorf tubes and centrifuged for 2 min, and the radioactivity in the pellet and in the supernatant was measured.

Endocytosis of I25I-labeled ricin was measured as the amount of toxin that could not be removed from the cells with lactose, as described previously (19).

RESULTS

Toxic Effect of ML I and MoAb-16-ML I-A-chain Immunotoxin. To measure the toxic effect of ML I and the corresponding immunotoxin, L1210V cells were incubated with increasing concentrations of the proteins for 3 h at 37°C, and then the rate of protein synthesis was measured. As shown in Fig. 1, the immunotoxin was essentially as efficient as the native ML I in intoxicating the cells. In the case of the native toxin, the presence of 0.1 M lactose strongly inhibited the toxic effect, whereas it had no inhibitory effect on the immunotoxin (not demonstrated). On the other hand, unconjugated antibody strongly prevented the effect of the immunotoxin, but not that of unconjugated ML I. This demonstrates that the functional binding of the immunotoxin to the cells was by the monoclonal antibody.

Rate of Entry of ML I and Immunotoxin. To measure the kinetics of the functional entry of the toxin from the cell surface, cells were incubated with toxin and immunotoxin at 0°C to allow binding to occur. Unbound ligand was then removed and the cells were incubated at 37°C. After increasing periods of time a neutralizing amount of antibodies against the ML I was added to inactivate toxin remaining at the cell surface. The cells were further incubated overnight to allow the endocytosed toxin time to exert its effect. As shown in Fig. 2, the immunotoxin became shielded from the antibody somewhat more slowly than the native toxin. Thus, with the native toxin antibodies added after 2 h had little protective effect, whereas in the case of the immunotoxin ~90% could still be inactivated by the antibody at this time point. It therefore appears that the immunotoxin is endocytosed more slowly than the native toxin.

Effect on Endocytosis of Acidification of the Cytosol. To study by which mechanism the immunotoxin is internalized, we took advantage of our previous observation that mild acidification of the cytosol inhibits the coated pit pathway of endocytosis in a number of different cell lines (9). To test if this was also the case in L1210V cells, we measured the effect of acidification on endocytosis of transferrin, a ligand known to be endocytosed by the coated pit pathway (11).

To acidify the cytosol we either preincubated the cells with ammonium chloride which was then removed (Fig. 3, A, B), or we added sodium acetate to the cells (Fig. 3, C, D). In the first case ammonia, which is membrane permeable, will leave the cells, whereas the protons are left behind. As a result, an immediate acidification of the cytosol occurs. The extent of acidification increases with increasing concentration of ammonium chloride during the preincubation (9). The cells were kept in Na+-free and bicarbonate-free buffer to prevent normalization of the intracellular pH by Na+/H+ exchange and Na+-linked HCO3/Cl- exchange (9). As shown in Fig. 3A, there was a strong inhibition of transferrin endocytosis at the highest ammonium chloride concentrations used.

Cells with acidified cytosol were found to bind more transferrin than nonacidified cells (Fig. 3B). This is in accordance with earlier findings with Vero cells (9). Also when the acidification was carried out by addition of acetate to the medium, transferrin endocytosis was strongly reduced, and the amount of transferrin receptors at the cell surface was increased (Fig. 3, C, D).

Earlier results have shown that acidification of the cytosol does not strongly inhibit endocytosis of ricin in Vero cells, Hep2 cells, MCF 7 cells, and A431 cells, indicating that this toxin is largely endocytosed by an alternative mechanism (9). The data in Fig. 4 and in Table 1 show that this was also the case in L1210V cells. On the other hand, when both kinds of endocytosis were inhibited by pretreatment of the cells with NaN3 and 2-deoxyglucose to strongly reduce the ATP level, the endocytosis of ricin was also strongly reduced (Table 1).

To test if the manipulations used were toxic to the cells, we tested the ability of the cells to incorporate [3H]leucine. As shown in Table 2, acidification of the cytosol reduced the
incorporation 30 min after the manipulations, whereas the cells had largely recovered 12 h later.

We then measured the endocytic uptake of the MoAb-16 antibody. As shown in Fig. 5, the uptake was only moderately reduced at the highest concentrations of ammonium chloride and acetate (Table 3). The results are similar to those obtained with ricin and different from those obtained with transferrin. This indicates that, like ricin, MoAb 16 is internalized to a large extent by a pathway not involving coated pits.

Effect of Acidification on the Toxic Effect of ML I and Immunotoxin. To test whether immunotoxin internalized by the alternative endocytic pathway was able to intoxicate cells, we tested whether immunotoxin endocytosed by acidified cells was also able to inhibit cellular protein synthesis. The cells were preincubated in the absence and presence of 50 mM NH_4Cl, then NH_4Cl and Na^+ were removed, and the cells were exposed to increasing concentrations of ML I or immunotoxin for 15 min in a buffer containing 0.14 M KCl instead of NaCl. Subsequently, antitoxin was added to inactivate toxin present at the cell surface, and the cells were transferred to normal medium. They were incubated for 18 h to allow internalized toxin time to exert its toxic effect, and finally the [3H]leucine incorporation 30 min after the manipulations, whereas the cells had largely recovered 12 h later.

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MISTLETOE IMMUNOTOXIN

Fig. 5. Effect of acidification of the cytosol by NH₄Cl prepulsing (A, B) and acetic acid (HAc) (C, D) on binding and endocytosis of MoAb-16 antibody. In A and B, L1210V cells in 96-well plates (2.5 x 10⁵ cell/well) were incubated for 30 min at 37°C in Hepes medium, pH 7.0, with the indicated concentrations of NH₄Cl. The medium was then removed, and 0.2 ml 0.14 M KCl, containing 2 mM CaCl₂ and 20 mM Hepes, pH 7.0, was added. After 5 min further incubation ¹²⁵I-labeled MoAb-16 (80 ng/ml, 26,400 cpm/ng) was added and cell-bound and endocytosed antibody was measured after 15 min as described in "Materials and Methods." In C and D, the cells were incubated for 5 min at 37°C in Hepes medium (0.2 ml, pH 5.0) with and without acetic acid in the concentrations indicated. ¹²⁵I-labeled MoAb-16 (80 ng/ml, 26,400 cpm/ng) was then added and, after 10-min incubation, endocytosed and surface bound antibody was measured as described in "Materials and Methods."

![Graphs showing effect of NH₄Cl and HAc on MoAb-16 binding and endocytosis.](image)

**Table 3** Effect of acidification of the cytosol on the ability of L1210V cells to endocytose ¹²⁵I-MoAb-16 antibody

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Endocytic uptake of ¹²⁵I-MoAb-16</th>
</tr>
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<tbody>
<tr>
<td>Hepes medium, pH 7.0</td>
<td>16 ± 7</td>
</tr>
<tr>
<td>Hepes medium, pH 5.0</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>5 mM acetic acid in Hepes medium, pH 5.0</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>10 mM NaCl + 50 mM 2-deoxy-D-glucose in Hepes medium, pH 7.0</td>
<td>4 ± 2</td>
</tr>
</tbody>
</table>

*Cells growing in 96-well plates (5.0 x 10⁵ cells/well) were incubated in Hepes medium, pH 5.0, with or without acetic acid for 5 min at 37°C and in Hepes medium, pH 7.0, with or without metabolic inhibitors for 20 min at 37°C. The cells were then incubated with ¹²⁵I-MoAb-16 (80 ng/ml, 26,400 cpm/ng) for 15 min at 37°C. Finally, endocytosed antibody was determined as described in "Materials and Methods."

**DISCUSSION**

The data here presented indicate that the MoAb-16 antibody and its conjugate with ML I toxin are to a large extent internalized by endocytosis from non-clathrin-coated areas of the plasma membrane and that toxin taken up by this pathway is able to enter the cytosol and intoxicate the cells. This finding suggests that antibodies raised against molecules that are excluded from coated pits can be equally useful in the construction of immunotoxins as antibodies internalized by the coated pit/coated vesicle mechanism.

It should be noted that in the present work NH₄Cl was used only during the preincubation and was removed from the cells before toxin or immunotoxin were added. Endosomes and lysosomes would therefore recover acidity before exposure to toxins. The effects here observed therefore appear to be unrelated to the enhancing effect of NH₄Cl found with many immunotoxins (2).

As earlier described for ricin (5, 19), which is at least partly endocytosed by a clathrin-independent pathway, the uptake of ML I and the corresponding immunotoxin also occurs comparatively slowly. This is in contrast to the rapid endocytic uptake of ligands endocytosed by the coated vesicle pathway (5). However, factors other than rapid internalization may be more important for efficient intoxication. There is some evidence that ligands internalized by the two different pathways eventually become, at least partially, colocalized in endosomes (20). However, it is not known whether ligands internalized by the two pathways have the same probability of being transported to the Golgi apparatus.

Endocytic uptake from coated pits is involved in efficient uptake of a large number of ligands and it has been studied in considerable detail (5, 11). Most of the cell surface components known to be internalized from clathrin-coated pits are glycoproteins, and it has been suggested that certain amino acids in these proteins are responsible for the aggregation in coated pits (21-24). Tetanus and cholera toxin bind to surface glycolipids and appear to be internalized from non-clathrin-coated areas of the plasma membrane (20, 25). On the other hand, it was recently shown that also a lipid-binding ligand, Shiga toxin, can aggregate in coated pits (26).

Although there is an increasing amount of evidence for the existence of an alternative, non-clathrin-dependent endocytic pathway, thus far no morphological correlate has been identified. Material that is not clathrin has been observed at the cytosolic side of the membrane in some cell types (27, 28), but
it is not clear whether these structures are involved in endocytosis or whether they have another function.

It was recently shown that it is possible to modulate the non-clathrin-coated endocytosis, at least in some cell types (29). Thus, cytochalasins selectively inhibited non-clathrin-coated endocytosis in Vero cells. On the other hand, non-clathrin-coated endocytosis in A431 cells was stimulated by epidermal growth factor and by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. A similar stimulation of endocytosis by growth factors has been observed also in other cell lines (30–33). The role of the different endocytic processes may vary from one cell line to another, and it is still not clear whether there is a systematic difference between normal and malignant cells in this respect. In any event, the results here described strongly suggest that immunotoxins taken up by non-clathrin-coated endocytosis may be highly toxic.

REFERENCES

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