Cytotoxic Potency of CD22-Ricin A Depends on Intracellular Routing Rather than on the Number of Internalized Molecules

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Cytotoxicity of immunotoxins (ITs) varies considerably depending on factors like the capability of the target antigen to internalize IT molecules, intracellular processing and routing of the IT. We studied factors that may influence cytotoxicity of CD22-ricin A IT to several B cell lines. The antigen density varied from $5.9 \times 10^3$ to $6.0 \times 10^4$ molecules/cell. The ID$_{50}$, determined by protein synthesis inhibition, varied from $2.1 \times 10^{-12}$ to $3.8 \times 10^{-11}$ M IT in absence and from $2.8 \times 10^{-14}$ M to $5.2 \times 10^{-12}$ M IT in presence of the cytotoxicity enhancer NH$_4$Cl (6 mM). In absence as well as in presence of NH$_4$Cl no correlation could be found between antigen density and ID$_{50}$. No relation was observed either with the rate of cytotoxicity. Even in cell lines with a low antigen density, such as KM3, protein synthesis was quickly inhibited. In order to investigate whether the cytotoxicity was dependent on the number of internalized molecules the kinetics of internalization and exocytosis of degraded $^{125}$I-labelled CD22 molecules were studied. After 24 h the number of internalized CD22 molecules was highest in Ramos (154,500), followed by Daudi (110,300) and KM3 (69,900). However, despite the higher internalization rate of Daudi the rate of cytotoxicity of $10^{-8}$ M IT was comparable with KM3. NH$_4$Cl did not influence the number of internalized molecules but postponed degradation of CD22. In conclusion, CD22-ricin A is a very potent and fast acting IT even for elimination of target cells that express low numbers of antigen. These results may have implication for treatment of different B cell malignancies with CD22-ricin A.

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INTRODUCTION

Ricin A chain ITs have been shown to be highly effective for the elimination of malignant B cells in vitro and in vivo [1].

Ricin A chain inhibits catalytically and irreversibly protein synthesis by cleaving the N-glycosidic bond of a single adenosine residue of 28S rRNA [2-4]. Theoretically, a single ricin A molecule that enters the cytosol has the potency to kill a cell. Since IT molecules are usually transported through cellular compartments where the ricin A may be either proteolytically cleaved or inactivated by low pH, more molecules must be endocytosed to guarantee cytotoxicity. The deliverance of the toxic compound of the IT into the cytosol depends on several factors such as the type and recognized epitope of the antigen, the number of internalized molecules [5], the intracellular routing and processing [6, 7] and the isotype of the monoclonal antibody (MoAb) [8]. This may result in a variable efficacy of the appropriate IT on different target cells by which the antigen is expressed and may complicate the treatment of different malignancies with ITs. The cytotoxic activity of ricin A ITs can be enhanced by the lysosomotropic amine NH$_4$Cl. NH$_4$Cl influences different intracellular processes such as routing and metabolism, enabling more ricin A molecules to reach their target without changing the internalization of the IT [9, 10].

We investigated the cytotoxicity of CD22-ricin A and some factors that influence this cytotoxic activity. The CD22 MoAb binds the B lymphocyte cell adhesion molecule (BLCAM) [11, 12] that is expressed on the surface of B cells in a wide range of differentiation from the early pro-B-cell stage to the immunocyte stage [13] and on their malignant counterparts.

We studied the influence of antigen density and kinetics of
internalization and intracellular degradation on the cytotoxic efficacy and the rate of activity of CD22-recombinant ricin A IT. This was investigated in absence and presence of NH4Cl in a panel of B-lineage cell lines.

**MATERIALS AND METHODS**

**Cell lines.** The B-lineage cell lines KM3 (pre-B cell), Daudi, Raji, Ramos, Ros1 [14] (Burkitt lymphomas), DoHH2, SU-DHL-6 (follicular lymphomas) and the T-lineage cell lines HPB-ALL and CEM were used. Cell lines were cultured in culture medium consisting of RPMI-1640 (Flow, Irvine, UK) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisley, UK), 2 mm glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (Flow) in a humidified incubator with 5% CO2 in air at 37°C. Cells were maintained in log phase.

**Monoclonal antibody.** The murine MoAb CD22 (CLB-B-ly/1) (IgG1) (generously provided by Dr J. Slaper, CLB, Amsterdam, The Netherlands) was purified from ascites fluid by ammonium sulphate precipitation and affinity chromatography using staphylococcal protein A-Sepharose (Pharmacia, Uppsala, Sweden).

**Radiolabelling.** The MoAb was labelled with Na125I (Amersham International plc, Buckinghamshire, UK) using the chloramin-T method [15] and purified on Sephadex G25 column (PD-10; Pharmacia). Eluted fractions containing 125I-IgG were pooled, and molecules smaller than M, 30 000 were removed by Centricon-30 concentration (Amicon, Beverly, MA, USA). The concentration was determined by Mancini assays [16] and specific binding activity was assessed by performing binding studies with limiting amounts of labelled antibody.

**Immunotoxin preparation.** CD22 MoAb was coupled to recombinant ricin A-chain (generously provided by ZENECA Pharmaceuticals, Macclesfield, UK) by means of 4-succinimidylotiocarbonyl-(2-pyridyldithio)toluene (SMPT, synthesized by the Department of Organic Chemistry, University of Nijmegen, The Netherlands) according to Thorpe et al [17]. Briefly, 10 mg CD22 (in 1 ml 25 mm sodium borate pH 9) was treated with a 10-fold molar excess of SMPT (110 μl of 1 mg/ml SMPT solved in N,N-Dimethylformamide (DMF, Merck, Darmstadt, Germany)) under gently rotating during 1 h at 20°C. Free SMPT and DMF were removed by gel filtration on a Sephadex G25 (Pharmacia) column. To introduce a free sulphhydryl group ricin A was incubated in 50 mm DTT for 30 min at 20°C. DTT was removed by gel filtration on the G25 column. The modified MoAb was incubated with a 2.5 molar excess ricin A for 72 h at 20°C resulting in a coupling ratio (ricin A: MoAb) of 0.8. Free ricin A was separated from the IT by gel filtration on a Sephacryl S200 High Resolution (Pharmacia) column. The IT-product was checked by SDS-PAGE on gradient gels of 4 to 15% using the Pharmacia LKB-Phastsystem.

**Antigen density.** The antigen density was determined by Scatchard analysis. Suspensions of 5x10^5 cells were incubated with various concentrations of 125I-labelled CD22 for 2 h at 4°C (final volume 100 μl). Before and after washing radioactivity was counted to determine the number of free and cell-associated molecules. Non-specific binding to incubation tubes was assessed by performing the experiment in the absence of targets. The number of bound molecules/cell under saturating conditions was calculated according to Scatchard [18].

**Cytotoxicity of CD22-ricin A.** Cells (10^5) in culture medium were incubated with various concentrations CD22-ricin A (10^{-14} to 10^{-8} M) in the presence or absence of 6 mM NH4Cl in a final volume of 200 μl. Incubation was performed in 96-well microtitre plates (Costar, Cambridge, MA, USA) in triplicate for 24 h at 37°C. Subsequently, 0.5 μCi [3H]leucine (Amersham) was added. After 24 h cells were harvested and the radioactivity was determined. Cytotoxicity was expressed as the percentage [3H]leucine incorporation corrected for non-specific incorporation in cells incubated in the presence of 1 mM cycloheximide (Boehringer Mannheim, Mannheim, Germany), with regard to [3H]leucine incorporation in untreated cells corrected for non-specific incorporation.

**Rate of cytotoxicity.** Cells were incubated with 10^{-8} M CD22-ricin A in culture medium for various periods of time. Thereafter cells were washed, resuspended in culture medium and incubated up to 24h after initiating the experiment. Subsequently, cells were distributed in triplicate in 96-well microtitre plates and 0.5 μCi [3H]leucine was added. Protein synthesis was determined as described.

**Kinetics of binding and internalization of 125I-CD22.** The number of cell bound, intracellular, broken down and subsequently excytosed 125I-labelled MoAb molecules was determined during continuous incubation using a modification of a previously described procedure [19]. Briefly, a suspension of 5x10^5 cells was incubated with 10^{-8} M MoAb for various times at either 37°C or 4°C in a final volume of 100 μl. Cells were washed and radioactivity was calculated to determine the total amount of cell associated radioactivity. Subsequently, cells were incubated with isotonic glycine buffer (pH2) at 4°C for 30 min to remove surface-bound molecules, washed, and radioactivity was again determined. The absolute number of intracellular molecules was calculated from the glycine buffer-treated cells initially incubated at 37°C after correcting for radioactivity in cells treated similarly at 4°C.

The number of molecules bound to the cell surface was determined by subtracting the amount of intracellular radioactivity from the total radioactivity associated with cells after incubation at 37°C.

**Degradation of 125I-CD22.** After incubation with labelled MoAb at 37°C or 4°C, cells were pelleted by centrifugation. Seventy-five microlitres of supernatant were treated with 1 ml of 15% trichloroacetic acid (TCA) and 100 μl 1% bovine serum albumine at 4°C for at least 15 min and then centrifugated at 1500 g for 15 min. The intracellularly degraded and subsequently excytosed amount of MoAb was determined by measuring radioactivity of the TCA-soluble fraction of culture supernatant. The radioactivity of the soluble fraction from cells incubated at 4°C was subtracted from that obtained at 37°C to remove any background radioactivity.

**Internalization of 125I-CD22.** Internalization was defined as the total number of molecules transported across the cell membrane and was calculated by summation of the amount of intracellular MoAb and the amount of broken down and excytosed MoAb.

**RESULTS**

**Antigen density**

The antigen density for 125I-labelled CD22 was determined by Scatchard analysis and varied between the different B cell lines (Table 1). The pre-B cell line KM3 appeared to bind less than the more mature Burkitt (Daudi, Raji and Ramos) and follicular lymphoma (DoHH2 and SU-DHL-6) cell lines. 

Table 1. CD22 antigen density in comparison with the cytotoxicity of CD22-ricin A in absence and in presence of 6 mM NH₄Cl

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Antigen density⁴</th>
<th>IT - NH₄Cl⁵</th>
<th>IT + NH₄Cl⁵</th>
<th>Factor of enhancement⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>KM3</td>
<td>5.9±0.7</td>
<td>1.9x10⁻¹¹</td>
<td>3.4x10⁻¹³</td>
<td>56</td>
</tr>
<tr>
<td>Daudi</td>
<td>42.3±8.6</td>
<td>4.2x10⁻¹²</td>
<td>5.1x10⁻¹⁰</td>
<td>8</td>
</tr>
<tr>
<td>Raji</td>
<td>60.4±11.1</td>
<td>3.4x10⁻¹¹</td>
<td>5.2x10⁻¹²</td>
<td>7</td>
</tr>
<tr>
<td>Ramos</td>
<td>52.4±11.8</td>
<td>3.5x10⁻¹²</td>
<td>3.8x10⁻¹³</td>
<td>9</td>
</tr>
<tr>
<td>Rosl</td>
<td>21.0±1.2</td>
<td>3.8x10⁻¹¹</td>
<td>2.2x10⁻¹²</td>
<td>17</td>
</tr>
<tr>
<td>DoHH2</td>
<td>52.2±8.5</td>
<td>5.6x10⁻¹²</td>
<td>6.3x10⁻¹⁴</td>
<td>93</td>
</tr>
<tr>
<td>SU-DHL-6</td>
<td>51.1±1.6</td>
<td>2.1x10⁻¹²</td>
<td>2.8x10⁻¹⁴</td>
<td>75</td>
</tr>
</tbody>
</table>

⁴ The antigen density was determined in Scatchard analysis and expressed as molecules per cell. Experiments were performed in duplicate and mean of three experiments is shown.

⁵ Cytotoxicity of CD22-ricin A was determined in a protein synthesis inhibition assay in absence and in presence of 6 mM NH₄Cl and expressed as ID₅₀. Assays were performed in triplicate; SDs were less than 10%.

⁶ The factor of enhancement is the product of ID₅₀ in absence of NH₄Cl divided by ID₅₀ in presence of NH₄Cl.

The Burkitt lymphoma cell line Rosl bound 2.1x10³ CD22 molecules per cell, two to three times less than the other cell lines except KM3. The affinity of the MoAb on the different cell lines was comparable (Kₐ = 1-3x10⁻⁷ M).

*Cytotoxicity of CD22-ricin A IT*

CD22-ricin A strongly inhibited protein synthesis in the different B cell lines which was determined by [³H]leucine incorporation. Strikingly strong cytotoxicity was found even for cells that express low numbers of target antigen. This cytotoxicity was independent on the maturation stage of the target cell. Cytotoxic activity, expressed by the 50% inhibition dose (ID₅₀), varied between 3.8x10⁻¹¹ M and 2.1x10⁻¹² M (Table 1). Addition of 6mM NH₄Cl enhanced significantly the cytotoxicity of the IT. Enhancement varied considerably from a factor 7 in Raji to 93 in DoHH2 suggesting a different handling of the IT. In presence of NH₄Cl the most mature follicular lymphoma cell lines DoHH2 and SU-DHL-6 were most sensitive for the IT.

In absence as well as in presence of NH₄Cl no correlation could be detected between antigen density and cytotoxicity of the IT (in both P = 0.76, Spearman’s coefficient) (Table 1, Fig. 1). Although KM3, Raji and Rosl varied strongly in antigen expression the ID₅₀ for these cell lines in absence of NH₄Cl were comparable (1.9-3.8x10⁻¹¹ M). In contrast, in Daudi, Ramos, DoHH2 and SU-DHL-6 the antigen expressions and ID₅₀ were comparable. Nevertheless ID₅₀ in the presence of NH₄Cl varied strongly.

IT concentrations of 10⁻⁸ M inhibited protein synthesis only completely in Ramos, DoHH2 and SU-DHL-6. However, in presence of NH₄Cl 10⁻⁸ M IT could inhibit protein synthesis completely in all cell lines except in Rosl (data not shown). Concentrations up to 10⁻⁷ M CD22-ricin A did not reduce protein synthesis in the antigen negative control T cell lines HPB-ALL and CEM. No differences could be detected with respect to the sensitivity for unconjugated ricin A. The ID₅₀ appeared to be 4x10⁻⁷ M for all cell lines in absence as well as in presence of NH₄Cl (data not shown).

Fig. 1. Relationship between cytotoxicity of CD22-ricin A and antigen density. Cytotoxicity of CD22-ricin A was determined in a protein synthesis inhibition assay. Cells were incubated for 24h at 37°C with various concentrations of IT (10⁻¹⁴-10⁻⁸ M) in absence (A) or in presence (B) of 6mM NH₄Cl. [³H]leucine was added for another 24 h whereafter the cells were harvested. Protein synthesis is expressed as a percentage of [³H]leucine incorporation of untreated cells. Cytotoxicity is expressed as ID₅₀. Mean of three experiments which were performed in triplicate is shown; SDs were less than 10%. Antigen density was determined in a Scatchard plot analysis. Cells were incubated with different concentrations of [¹²⁵I]-labelled CD22 for 2h. Afterwards cells were washed twice and radioactivity was measured. Mean of three experiments which were performed in duplicate is shown; SDs were less than 10%. The cell lines are depicted by numbers: KM3 [1]; Daudi [2]; Raji [3]; Ramos [4]; Rosl [5]; DoHH2 [6] and SU-DHL-6 [7].
Table 2. CD22-ricin A protein synthesis inhibiting activity in comparison with the number of internalized molecules

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inhibition of protein synthesis (%)(^a)</th>
<th>Internalized mol./cell(^b)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>–NH(_4)Cl + NH(_4)Cl</td>
<td>–NH(_4)Cl + NH(_4)Cl</td>
</tr>
<tr>
<td>KM3</td>
<td>1 h 64 97</td>
<td>20 300 17 800</td>
</tr>
<tr>
<td></td>
<td>24 h 81 100</td>
<td>69 900 60 700</td>
</tr>
<tr>
<td>Daudi</td>
<td>1 h 74 93</td>
<td>33 000 38 700</td>
</tr>
<tr>
<td></td>
<td>24 h 87 100</td>
<td>110 300 106 400</td>
</tr>
<tr>
<td>Ramos</td>
<td>1 h 100 100</td>
<td>52 000 46 900</td>
</tr>
<tr>
<td></td>
<td>24 h 100 100</td>
<td>154 500 159 300</td>
</tr>
</tbody>
</table>

\(^a\) Inhibition of protein synthesis was determined as described in Materials and Methods in rate of cytotoxicity. Experiments were performed in triplicate and mean of three experiments is shown. SDs were less than 5%.

\(^b\) The numbers of internalized molecules/cell were determined as described in Materials and Methods in kinetics of binding and internalization of \(^{125}\)I-CD22. Mean of three experiments which were performed in duplicate is shown; SDs were less than 10%.

Rate of cytotoxicity

To study the influence of the incubation time on the cytotoxicity of CD22-ricin A in relation to the antigen density, the rate of protein synthesis inhibition was determined in the lines Daudi, KM3 and Ramos, which varied in antigen density and susceptibility to cytotoxicity. Cells were incubated with \(10^{-8}\) M IT during various periods of time followed by an IT-free incubation up to 24 h after initiating the experiment. This procedure was used to exclude differences in cytotoxicity due to the delay in action of the IT by binding to and transport into the cell. The kinetics in Daudi and KM3 were comparable. Both cell lines showed a fast inhibition of protein synthesis within 1 h of incubation (respectively 74% and 64%). Thereafter inhibition rate slowed down for up to 24 h (Table 2). In contrast, protein synthesis in Ramos was blocked completely within 1 h of incubation even in absence of NH\(_4\)Cl (Table 2). In presence of NH\(_4\)Cl the incubation time to block protein synthesis completely in Daudi and KM3 was reduced from more than 24 h to 8 and 2 h, respectively (data not shown).

Kinetics of internalization and intracellular degradation of \(^{125}\)I-CD22

To investigate the relation between cytotoxicity of CD22-ricin A and the number of internalized molecules, the kinetics of internalization and intracellular degradation were studied by means of \(10^{-8}\) M \(^{125}\)I-labelled CD22 in Daudi, KM3 and Ramos. After an initial binding phase the number of molecules bound to the cell membrane at 4°C did not change in KM3 and Ramos up to 24 h. In Daudi a slight decrease of bound molecules was detected (Fig. 2). In contrast to Daudi and KM3 in Ramos a discrepancy was found between the number of bound molecules (40 000) and the antigen density determined by Scatchard analysis (52 000). This was due to the not completely saturating conditions of incubation with \(10^{-8}\) M IT for Ramos as determined in the Scatchard analysis. This, however, did not count for Daudi and KM3. The number of bound molecules per cell at 37°C was four to five times less for KM3 in comparison with Daudi and Ramos. This disagreed with the difference in antigen density of seven to eight times fewer antigen molecules when compared with Daudi and Ramos. This may be explained partly by the non-saturation conditions for Ramos and by

Fig. 2. Binding of \(^{125}\)I-CD22 during continuous incubation and internalization. Cells were incubated at 37°C (●) and 4°C (○) for various periods of time with \(10^{-8}\) M CD22 at 37°C and at 4°C as described in Materials and Methods. The number of surface-bound molecules was measured by counting cell-bound radioactivity that was removable by glycine buffer pH 2. Mean of three experiments which were performed in duplicate is shown; SDs were less than 10%.

Fig. 3. Internalization of $^{125}$I-CD22. The intracellular number of molecules was measured by counting cell-bound radioactivity that was not removable by glycine buffer pH 2 (■). The number of internalized and subsequently degraded and exocytosed molecules was measured by counting TCA-soluble radioactivity of the supernatant of incubation medium (□). The absolute amount of internalized molecules was determined as the sum of intracellular molecules and intracellular molecules that were degraded and subsequently exocytosed (○). Mean of three experiments which were performed in duplicate is shown; SDs were less than 10%. Internalization of $^{125}$I-CD22 was determined in absence (A) and in presence (B) of 6 mM NH$_4$Cl.

Differences in internalization and/or re-expression of antigen.

In absence of NH$_4$Cl the course of the kinetics of internalization of CD22 (defined as the sum of the intracellular amount of CD22 and the amount of intracellularly degraded and subsequently exocytosed CD22) in Daudi, KM3 and Ramos were comparable (Fig. 3A). After 2 h the intracellular amount of CD22 was maximal. Thereafter the intracellular amount of CD22 remained high in KM3 and Ramos, whereas in Daudi a reduction of CD22 was found. This suggest a difference in intracellular handling of CD22 or a difference in internalization of antigen-antibody complexes.

For all cell lines degradation and exocytosis of CD22 could be determined after 1 h of incubation. However, the degradation rate in Daudi and Ramos was at least twice as high as in KM3 notwithstanding the intracellularly present amount of CD22, also suggesting a different intracellular handling.

All these cell lines showed a very fast internalization of CD22 during the first hour whereafter the internalization rate slowed down but the total number of internalized CD22 increased up to at least 24 h. Although Ramos internalized the highest number of CD22 within 24 h, KM3 internalized 11.8 times more CD22 than the available antigen (70,000 molecules CD22 per 5,900 molecules antigen) whereas Daudi and Ramos internalized respectively 2.6 and 3.0 times more CD22 than the available antigen. This suggests a difference in the mean internalization activity of the CD22 antigens on the varying B cell lines.

The number of internalized molecules seemed to be related to cytotoxicity. However, this was rejected by close analysis of the data (Table 2) since 52,000 molecules internalized by Ramos inhibited protein synthesis more (100%) than 69,000 and 110,300 molecules internalized by respectively KM3 (81%) and Daudi (87%).

Addition of NH$_4$Cl did not influence the total amount of internalized CD22 (Fig. 3B). Intracellular degradation,
however, was delayed from within 2 to approximately 8 h. A higher cytotoxic potency of CD22-ricin A due to NH₄Cl could not be explained by a higher amount of internalized molecules, but by a reduced degradation rate during the first hours of treatment.

**DISCUSSION**

Three processes are underlying the cytotoxic activity of ricin A ITs. The MoAb moiety must bind to the specific target cell antigen. This binding has to be followed by an internalization of the IT. After splicing from the MoAb the ricin A must be translocated to the cytosol where it will affect the ribosomal activity resulting in an irreversible inhibition of protein synthesis [5, 20, 21]. In different B cell lines we investigated which factors contribute most to the cytotoxic activity of CD22-ricin A IT. For the production of the IT we used aglycosyl recombinant ricin A that already has been used for in vivo studies in mice [22].

In absence and presence of the lysosomotropic amine NH₄Cl, that enhances cytotoxicity of ricin A ITs [19, 23], we found no correlation between antigen density and cytotoxicity of CD22-ricin A. The antigen density on the cell lines, determined by Scatchard analysis, varied from 5,900 to 60,400 molecules per cell. CD22-ricin A appeared to be highly cytotoxic even to cells expressing low numbers of antigen such as KM3. Cytotoxicity expressed by ID₅₀ values varied from 2.1x10⁻¹² M to 3.8x10⁻¹¹ M in absence of NH₄Cl and from 2.8x10⁻¹₁ to 5.2x10⁻¹² M in presence of 6 mM NH₄Cl.

Compared with CD22-ITs used by other investigators our CD22-recombinant ricin A IT appears to be more cytotoxic to Daudi (ID₅₀ 4.2x10⁻¹² M) than HD-6-native ricin A (3.7x10⁻¹¹ M [24] and 1.3x10⁻¹¹ M [25]), HD-6-deglycosylated ricin A (1.4x10⁻¹¹ M [24]), UV22-1-native ricin A (2.1x10⁻¹¹ M [25]) and UV22-2-native ricin A (3.8x10⁻¹¹ M [25]), but less toxic than RFB4-native ricin A (1.2x10⁻¹² M [25]) and RFB4-deglycosylated ricin A (1.2x10⁻¹² M [25, 26]). These differences may be due to recognition of different epitopes by the CD22-MoAbs, to the ricin A variants, to variations in Daudi lines in the different laboratories and to different experimental procedures.

We investigated also the relationship between antigen density and cytotoxicity after shorter incubation periods. For this reason we studied the rate of cytotoxicity of 10⁻⁸ M IT in cell lines which vary in antigen density and susceptibility to CD22-ricin A. We did not find a correlation. The rate of cytotoxicity in KM3 was comparable with those in Daudi, which bound 7-times more CD22. Protein synthesis in Ramos, which bound CD22 at the same level as Daudi, was completely inhibited after 1 h whereas in Daudi only 87% was inhibited after 24 h. NH₄Cl reduced the time required for complete inhibition by 10⁻⁴ M IT in Daudi and KM3 from longer than 24 h to 8 and 2 h, respectively. These results suggest that the antigen density is not the most important factor for the cytotoxic efficacy of CD22-ricin A. This implies that patients with B cell malignancies expressing low numbers of CD22 antigens should also be considered for CD22-ricin A treatment.

Subsequently, we investigated whether the cytotoxicity of CD22-ricin A was related more to the amount of molecules internalized into the target cells. The internalization kinetics were related to the antigen density although variations in internalization are less pronounced than those in antigen density. The number of internalized CD22 molecules per antigen molecule within 24 h was higher in KM3 (11.8 molecules) than in Daudi and Ramos (2.6 and 3.0 molecules, respectively). This suggests differences in the mean internalization activity of the antigen on the varying cell lines. The total amount of internalized CD22 exceeded the number of molecules that could be maximally bound implying that the antigen for CD22 was re-expressed or newly synthesized after binding.

The number of internalized molecules did not correlate with the cytotoxic activity of the IT. Within 24 h Daudi internalized twice as many molecules as KM3 (Table 2) whereas inhibition of protein synthesis was comparable (87% and 81%, respectively; Table 2). Within 1 h of incubation Ramos internalized 52,000 molecules resulting in a complete blocking of protein synthesis (Table 2). In contrast Daudi internalized 110,300 molecules during 24 h which resulted in only 87% inhibition (Table 2). These results indicate differences in intracellular processing of CD22-ricin A which influences strongly the cytotoxic activity.

Treatment of cells with NH₄Cl increases the endosomal pH [27], resulting in reduction of endosomal proteolysis of IT [23]. This induces a higher chance on translocation of ricin A over the endosomal/lysosomal membrane resulting in an improvement of IT activity. We showed that NH₄Cl induced a delay of intracellular degradation of IT which increased cytotoxicity of CD22-ricin A. These data show that intracellular processing is essential for the cytotoxicity of ricin A ITs.

The importance of intracellular routing for the potency of ricin A ITs is supported by findings of other authors. It is found that cytotoxicity of ricin A ITs also can be increased by addition of the endoplasmatic reticulum retention signal, KDEL, to the carboxyl terminus of the ricin A chain [28, 29]. The KDEL facilitates ricin A entry into the cytosol by interaction with the intracellular KDEL receptor which promotes transport of the toxin to the endoplasmatic reticulum, where translocation into the cytosol occurs.

We conclude that the CD22-ricin A IT has a high cytotoxic potency even for cells expressing low numbers of antigen molecules. The intracellular routing of the IT is a major factor for the cytotoxicity of CD22-ricin A. Investigations elucidating the involvement of the activation state of the antigen are in progress. Patients suffering from B cell malignancies expressing low numbers of CD22 antigens should also be considered for treatment with CD22-ricin A.

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