

# Specific Targeting to Murine Myeloma Cells of Cyt1Aa Toxin from *Bacillus thuringiensis* Subspecies *israelensis*\*<sup>§</sup>

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Multiple myeloma is currently an incurable cancer of plasma B cells often characterized by overproduction of abnormally high quantities of a patient-specific, clonotypic immunoglobulin "M-protein." The M-protein is expressed on the cell membrane and secreted into the blood. We previously showed that ligand-toxin conjugates (LTC) incorporating the ribosome-inactivating Ricin-A toxin were very effective in specific cytotoxicity of the anti-ligand antibody-bearing target cells used as models for multiple myeloma. Here, we report on the incorporation of the membrane-disruptive Cyt1Aa toxin from *Bacillus thuringiensis* subsp. *israelensis* into LTCs targeted to murine myeloma cells. Proteolytically activated Cyt1Aa was conjugated chemically or genetically through either its amino or carboxyl termini to the major peptidic epitope VHFFKNIVTPRTP (p87–99) of the myelin basic protein. The recombinant fusion-encoding genes were cloned and expressed in acrylamidiferous *B. thuringiensis* subsp. *israelensis* through the shuttle vector pHT315. Both chemically conjugated and genetically fused LTCs were toxic to anti-myelin basic protein-expressing murine hybridoma cells, but the recombinant conjugates were more active. LTCs comprising the Cyt1Aa toxin might be useful anticancer agents. As a membrane-acting toxin, Cyt1Aa is not likely to induce development of resistant cell lines.

Chemotherapy has for many years been an important component of the cancer therapeutic arsenal, but lack of target cell specificity of most anticancer drugs leads to concentration-dependent toxicity of normal cells. The resultant use of suboptimal therapeutic doses precludes the elimination of all the cancer cells, a situation that encourages clinical recurrence of the tumor (1) as well as emergence of resistant clones (2). This scenario has stimulated development of a variety of targeted drug delivery systems, the advantages of which include, aside from target cell specificity, reduced drug dosage and increased cytotoxic efficacy (reviewed in Refs. 3, 4).

As components of the targeting system, delivery moieties with a wide range of biological functions have been employed, such as monoclonal antibodies, receptor binding peptides, liposomes, and nucleotide aptamers (4–6). Most of the cytotoxic components currently used are designed to interfere with intracellular biochemical processes (7–10). For instance, we recently demonstrated that delivery of the ribosome-inactivating protein Ricin-A efficiently destroys murine myeloma cells (11) and that targeted, chemiluminescent activation of the photodynamic process results in endomembrane destruction (12). The activity of these types of cytotoxic systems requires endocytosis, and this process often induces intracellular activation of resistance mechanisms (2). There is therefore a need to expand the portfolio of cytotoxic agents, for instance to those whose mode of action is endocytosis-independent.

Cyt1Aa is a plasma membrane-acting cytotoxin isolated from the paracrystalline  $\delta$ -endotoxin of the bacterium *Bacillus thuringiensis israelensis* (*Bti*)<sup>3</sup> (13). It is a major component (45–50%) of the mosquito-larvicidal crystal proteins of *Bti*, displaying relatively low larvicidal activity *per se* but high synergy with other *Bti* toxins (14–20). The alkali-solubilized protoxin form of Cyt1Aa (249 amino acids long; 27 kDa) is active against insect cells, erythrocytes, and mammalian cells *in vitro* without activation (21), but specific proteolysis by trypsin, proteinase K, or endogenous proteases generates a carboxyl- and amino-terminated 22–25-kDa toxin that is three times more active (22, 23). The activity of Cyt1Aa appears to depend on its ability to form a multiunit complex that binds to and then becomes embedded in the plasma membrane, leading to membrane destruction and cell lysis (24, 25). Some unsuccessful attempts have been made to exploit this activity. A Cyt1Aa-anti-Thy1.1 fusion protein, for example, lacked cytolytic specificity against a Thy 1.1-expressing mouse AKR-A/2 lymphoma cell line (26). In the same study, a Cyt1Aa-insulin conjugate displayed specificity for target cells overexpressing insulin receptors, but the activity level and response rate were lower than for the free Cyt1Aa. The reported lack of conjugated Cyt1Aa activity may be related to the site of toxin attachment to the targeting carrier that may result in interference of Cyt1Aa-membrane complex with the formation. This working hypothesis led us to compare several methods for preparing active ligand-toxin conjugates (LTCs) comprising Cyt1Aa.

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

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<sup>3</sup> The abbreviations used are: *Bti*, *B. thuringiensis israelensis*; MBPp, myelin basic protein peptide; LTC, ligand-toxin conjugate; Fmoc, *N*-(9-fluorenyl) methoxycarbonyl; FL, Friend's erythroleukemia; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

# Specific Targeting of Cyt1Aa to Myeloma Cells

**TABLE 1**

The primers used to amplify the promoter of *cyt1Aa* ( $P_{cyt1Aa}$ ), *cyt1Aa*-MBPp variants, and cleaved *cyt1Aa* fragments (*a*–*k*), and *p20*. Bold letters represent bases modified from the original sequence to encode appropriate enzyme restriction sites.

Fragment name	Primer's sequence (5'–3') (Upper forward, lower reverse)	Restriction enzyme sites
$P_{cyt1Aa}$	GGGATTTAGAGCATG <b>C</b> TTAAGTAGAATAGACG CAATGATTTAAATTTTCCATAT <b>GT</b> TAAACAACCTCC	SphI NdeI
<i>a</i> <sup>a</sup>	GGGATTTAGAGCATG <b>C</b> TTAAGTAGAATAGACG GTGTGAAGAACAAT <b>TCT</b> AGAGAAAGGGG	SphI XbaI
<i>b</i>	GGGTTATTACACATAT <b>GGT</b> TGAGGATCCAAATGAAATC AGTCTAGATTATGTACGTGGTGT <b>TACA</b> ATATTTTAAAAAAATGAACTGAGCTAACTAATGGTTGTG	NdeI XbaI
<i>c</i>	<b>AATCCACATATG</b> TTTCATTTTTTAAAAAATATTGTAACACCACGTACAGTTGAGGATCCAAATGAAATC GGTCTAGATTATGAGCTAACTAATGGTTGTGC	NdeI XbaI
<i>d</i>	GGGTTATTACACATAT <b>GGT</b> TGAGGATCCAAATGAAATC GGTCTAGATTATGAGCTAACTAATGGTTGTGC	NdeI XbaI
<i>e</i>	GTGTTGAGGATCCAAATGAAATC AGTCTAGATTATGTACGTGGTGT <b>TACA</b> ATATTTTAAAAAAATGAACTGAGCTAACTAATGGTTGTG	SphI XbaI
<i>f</i>	<b>AATCCACATATG</b> TTTCATTTTTTAAAAAATATTGTAACACCACGTACAGTTGAGGATCCAAATGAAATC GTGTGAAGAACAAT <b>TCT</b> AGAGAAAGGGG	NdeI XbaI
<i>g</i>	GGGTTATTACACATAT <b>GGT</b> TGAGGATCCAAATGAAATC GTGTGAAGAACAAT <b>TCT</b> AGAGAAAGGGG	NdeI XbaI
<i>h</i>	GGGATTTAGAGCATG <b>C</b> TTAAGTAGAATAGACG GGTCTAGATTATGAGCTAACTAATGGTTGTGC	SphI XbaI
<i>i</i>	GGGTTATTACACATAT <b>GGT</b> TGAGGATCCAAATGAAATC GCTACTAAGATTAT <b>TCTAGATTA</b> ATTAATAGCG	NdeI XbaI
<i>j</i>	GGGTTATTACACATAT <b>GGT</b> TGAGGATCCAAATGAAATC GCTAT <b>TCT</b> AGATTAGAGGGTTCC	NdeI XbaI
<i>k</i>	GGGATTTAGAGCATG <b>C</b> TTAAGTAGAATAGACG GCTAT <b>TCT</b> AGATTAGAGGGTTCC	SphI XbaI
<i>p20</i>	CCGTT <b>TCT</b> AGAGTAGAAGTCATGTTAGC CAAATTCATCTGAG <b>CTC</b> TTATATCGATTAC	XbaI SacI

<sup>a</sup> *cyt1Aa* full-length was amplified with its own promoter and with 130 bp up- and downstream.

Proteolytically activated Cyt1Aa was chemically conjugated via lysine residues to either the carboxyl or amino terminus of myelin basic protein peptide (MBPp) (amino acids 87–99). In parallel, fusion proteins were genetically engineered in which Cyt1Aa was linked in-frame to either the amino (*cyt1Aa-MBPp*) or carboxyl (*MBPp-cyt1Aa*) terminus of MBPp, and the clones were expressed via the pHT315 shuttle vector in acrySTALLIFEROUS *Bti*. Cytotoxicities of the chemically and genetically prepared LTCs were compared against mouse B cell hybridoma cells that do or do not surface express and secrete IgM clonotypic anti-MBPp antibodies. The cytotoxicity and target cell specificity of the genetically modified versions were much higher than those of the chemically linked proteins. Cyt1Aa might thus be used as a cytotoxin for targeted destruction of malignant cells.

## EXPERIMENTAL PROCEDURES

**Cells**—Friend's erythroleukemia (FL) and U136 murine hybridoma B cells, which secrete non-relevant antibodies (kindly given by Prof. Zelig Eshhar, Weizmann Institute of Science, Rehovot, Israel), were used as well as M61.13 murine hybridoma B cells, which secrete and express on the cell membrane IgM antibodies to rat MBPp (amino acids 87–99, VHFFKNIVTPRTP, molecular mass 1.56 kDa) (kindly given by Prof. Michal Schwartz, Weizmann Institute of Science) and were used as the target cells. All cells were cultured in Dulbecco's modified Eagle's medium with 10% horse serum, 30  $\mu\text{g ml}^{-1}$  of penicillin and streptomycin, and 2 mM L-glutamine (Biological Industries, Beit-Haemek, Israel), at 37 °C in a humidified incubator containing 6.5% CO<sub>2</sub>. Rabbit red blood cells were freshly prepared before each hemolytic assay.

**Bacterial Strains, Plasmids, and Growth**—Strain 4Q2–72 of *Bti* that bears pBtoxis (27) as its only plasmid was kindly supplied by D. R. Zeigler (*Bacillus* Genetics Stock Center, Columbus, OH). The acrySTALLIFEROUS plasmid-less derivative strain IPS78/11 was kindly supplied by D. J. Ellar (Cambridge, UK). *Escherichia coli* XL1-Blue MRF' (Stratagene, La Jolla, CA) was used as a host for subcloning with the *E. coli-Bti* 6.5-kb shuttle vector pHT-315 (28).

Lysogeny broth and SOC were prepared according to Sambrook *et al.* (29) and CCY sporulation medium according to Stewart *et al.* (30). Antibiotics (ampicillin to 100  $\mu\text{g ml}^{-1}$ ; erythromycin to 20  $\mu\text{g ml}^{-1}$ ) were added after cooling the autoclaved medium. Competent cells for electroporation were prepared according to Bone *et al.* (31) for IPS78/11 and according to Sambrook *et al.* (29) for XL1-Blue MRF'.

**DNA Isolation, PCR Amplification, Cloning, and Sequencing**—pBtoxis was isolated from exponentially growing *Bti* 4Q2–72 as previously described (32). The oligonucleotide primers used for PCR amplifications are listed in Table 1. All amplification processes were carried out with the high fidelity Vent DNA polymerase (New England Biolabs) in a T-gradient Thermo cycler (Biometra, Göttingen, Germany) as follows: 20–50 ng of purified pBtoxis was denatured (4 min at 95 °C) and amplified for 30 cycles, each consisting of 40 s at 95 °C, 45 s at 54 °C, 1–2 min at 72 °C, and a final extension step (72 °C for 10 min). Samples (2  $\mu\text{l}$ ) from the PCR reactions were electrophoresed (100 V, 5–30 min) on 0.7% agarose gels in 1 $\times$  TAE buffer (20 mM Tris-acetate-EDTA, pH 7.2) and DNA visualized by ethidium bromide. The amplicons were isolated by GFX PCR DNA and gel band purification kit (Amersham Biosciences) and digested by the

appropriate restriction enzyme(s), and the desired products were purified from an agarose gel with the same GFX kit.

Full-length *cyt1Aa* (Fig. 1a) was PCR-amplified with its own promoter ( $P_{cyt1Aa}$ ). The digestion product was ligated into SphI/XbaI double digested pHT315 to produce pHT-*cyA*. *p20* was ligated into the XbaI and SacI sites of pHT-*cyA* to create pHT-*cyAp20* (plasmid *a* in Fig. 1). Fragments *b*, *c*, *e*, and *f* (Fig. 1, *b*, *c*, *e*, and *f*) were amplified with the MBPp-encoding sequence fused with the cleaved *cyt1Aa* at either its 5'-end (*c* and *f*) or its 3'-end (*b* and *e*). The cleaved *cyt1Aa* (fragments *d*, *g*, *i*, and *j*) and *cyt1Aa*-MBPp variants (*c* and *f*) were amplified without  $P_{cyt1Aa}$  and separately cloned into pHT315- $P_{cyt1Aa}$ -*p20* digested by NdeI and XbaI. Fragments *b*, *e*, *h*, and *k* were amplified with  $P_{cyt1Aa}$  at the 5'-end.

All the plasmids were subcloned separately into XL1-Blue MRF', purified, and electroporated into acrycristalliferous *Bti* strain IPS78/11. Electroporation was performed in a 0.2-cm cuvette with either *E. coli* XL1-Blue MRF' or acrycristalliferous *Bti* IPS78/11 competent cells using a Bio-Rad MicroPulser electroporator apparatus setting at 2.5 kV (Ec2 program) and 1.8 kV (Ec1) for *E. coli* and *Bti*, respectively.

The plasmids containing variants of *cyt1Aa* and *cyt1Aa*-MBPp and their encoding products (Fig. 1) are listed and described in Table 2. They were isolated from XL1-Blue MRF' cells by the Wizard Plus SV Minipreps DNA purification system (Promega Madison, WI), sequenced, and electroporated into the acrycristalliferous IPS78/11 strain of *Bti*. Plasmid DNA from *Bti* was alkali-purified (33) with the following modification: addition of 4 mg ml<sup>-1</sup> of lysozyme in 37 °C for 1 h. Screening for transformants was performed on Lysogeny broth plates containing 20 μg ml<sup>-1</sup> erythromycin at 30 °C. Crystals were seen under a phase-contrast microscope (Nikon Eclipse TE2000-5 equipped with a Nikon Digital Sight DS-U1 CCD camera) at ×100 optical lens (oil). Sequence integrity of *cyt1Aa* and *p20* was verified by ABI PRISM® 3100 Genetic Analyzer.

**Purifying the Cyt1Aa-MBPp Products**—To purify the chimeric proteins, recombinant cells expressing pHT(*b-i*)*p20* were grown for a day in CCY medium. The cultures were centrifuged, washed with double distilled water, and disrupted by sonication in 50 mM Tris-HCl, pH 7.5, buffer containing 5 mM phenylmethylsulfonyl fluoride. The lysates were separated on a semi-preparative 5–20% gradient of native PAGE. The appropriate size band was excised from the gel and eluted by a Gel-Eluter (Bio-Rad) apparatus. The presence of the Cyt1Aa protein was detected by Western blot analysis using anti-Cyt1Aa and anti-MBPp.

**Crystal Protein Purification**—*Bti* strain IPS78/11 harboring *cyt1Aa*, with or without *p20*, was grown overnight in 5 ml of Lysogeny broth supplemented with 20 μg ml<sup>-1</sup> erythromycin, transferred to 500 ml of CCY medium (30) with erythromycin, and grown with aeration at 30 °C for 4 days. Cultures were centrifuged at 15,000 × *g* for 10 min, and the pellets containing crystals, spores, and cell debris were washed twice with cold water. The crystals were separated from the other components by sucrose discontinuous gradient as previously described (34).

**Solubilization, Proteolytic Activation, and Purification of Activated Cyt1Aa**—Cyt1Aa purified crystals were solubilized in 50 mM Na<sub>2</sub>CO<sub>3</sub> buffer (pH 10.5) containing 10 mM dithiothre-

itol and 1 mM EDTA at 37 °C for 1 h, followed by lowering the pH to 8.5 and proteolytic processing with proteinase K at 37 °C for 1.5 h. Activated Cyt1Aa was purified by the anion exchange chromatography system (GE Healthcare) on a DEAE-cellulose column (1 × 25 cm), pre-equilibrated with 50 mM Tris-HCl buffer, pH 8.5, and eluted with a NaCl 0–0.6 M gradient in the same buffer at a flow rate of 1.5 ml min<sup>-1</sup>. Fractions (3 ml) were collected and monitored at 280 nm. Protein concentration, homogeneity, and hemolytic activity were determined for each fraction by A<sub>280</sub> nm and the Lowry method (35) using bovine serum albumin as a standard.

**Peptide Synthesis and Chemical Conjugation of Cyt1Aa-MBPp**—Synthesis of MBPp was performed by the automated Fmoc solid phase method at BioSight Ltd., Karmiel, Israel. The peptides were synthesized using a double coupling of *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium hexafluoro-phosphate and *N*-hydroxybenzotriazole hydrate 5–10-fold excess with amino acid to resin substitution. Mix time for each coupling was 2–16 h. Wang resin was used in coupling for creation of free carboxyl at the carboxyl terminus of the peptide. Deprotection of Fmoc was performed using 20% piperidin in *N,N*-dimethylformamide. Cleavage was performed using 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% water, mixed for 2 h. The peptides were precipitated with ether and dried. The peptides were dissolved in water, purified, frozen, and lyophilized under vacuum. Purified activated Cyt1Aa was concentrated by Amicon® concentrator (MWCO 10000) and conjugated with either of two variants of MBPp by commercial cross-linkers as follows. Conjugate 1 was prepared by linking the free carboxyl terminus of the MBPp variant 1 (VHFFKNIVTPRTP) with free amines of lysine residues along the cleaved Cyt1Aa by EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) (Pierce). Conjugate 2 was prepared by linking the free -SH group of the cysteine residue in the terminus of MBPp variant 2 (CVHFFKNIVTPRTP) with free amines of lysine residues along the cleaved Cyt1Aa by the cross-linker MBS (m-maleimidobenzoyl-*N*-hydroxysuccinimide ester) (Pierce). The conjugation procedures were performed according to the Pierce manual with appropriate molar ratios that yield a minimum conjugation ratio of 1–2 MBPp:Cyt1Aa molecule. The MBPp variants and the conjugates were purified by reverse phase HPLC on LiChrosphere WP 300 RP-18 (125 × 5 mm, 5 μm; Merck) column in acetonitril/H<sub>2</sub>O.

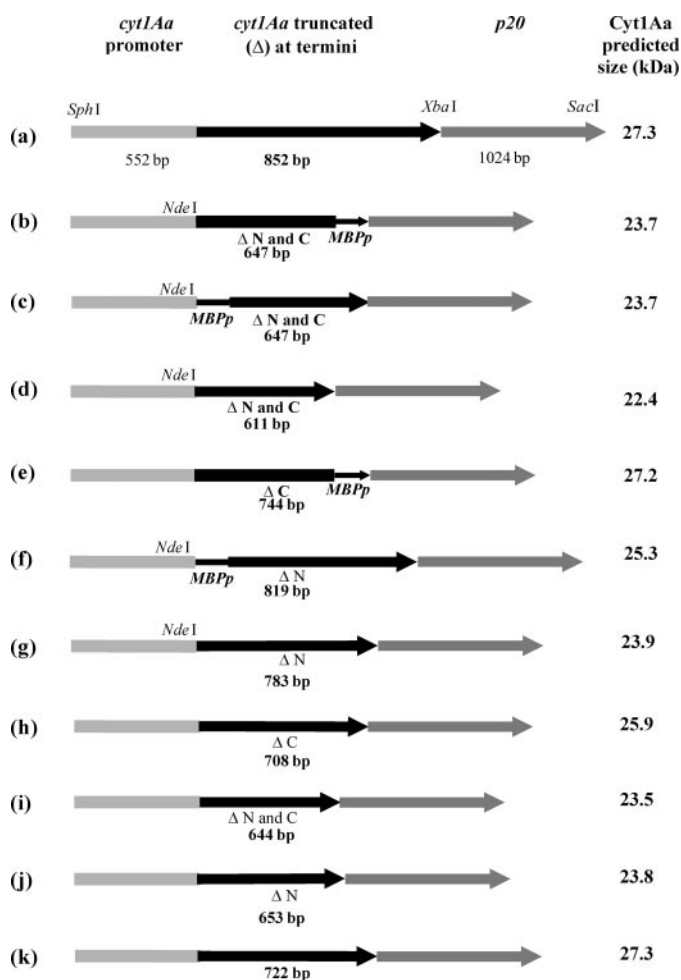
**Cytotoxicity Assays**—To establish dose-response curves for Cyt1Aa activity on hybridoma cells, log phase cells were washed twice with prewarmed Dulbecco's modified Eagle's medium and cultured in Dulbecco's modified Eagle's medium supplemented with glutamine and penicillin/streptomycin but not horse serum (as recommended in Ref. 36) at 0.5–1 × 10<sup>5</sup> cells ml<sup>-1</sup> either alone or together with various concentrations of Cyt1Aa conjugates. After 12 or 48 h (depending on the experiment), cell viability was determined by trypan blue exclusion or MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) test (37).

## RESULTS

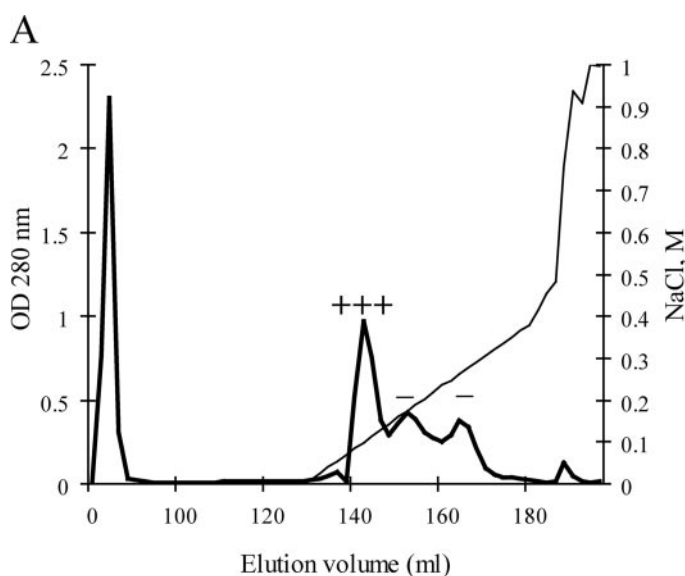
**Expression, Purification, and Activation of Cyt1Aa**—The acrycristalliferous *Bti* cells harboring pHT-*cyAp20* (Fig. 1a) were grown in CCY sporulation medium. After 36 h the cells were lysed and the accumulated Cyt1Aa was released in the form of



## Specific Targeting of Cyt1Aa to Myeloma Cells



**FIGURE 1. Variants of *cyt1Aa* with or without MBPp cloned into the SphI-SacI sites of the MCS of pHT315-p20 (6.5 kb).** a, pHT-cyAp20 (encoding full-length Cyt1Aa). b, pHTb-p20. c, pHTc-p20. d, pHTd-p20. e, pHTe-p20. f, pHTf-p20. g, pHTg-p20. h, pHTh-p20. i, pHTi-p20. j, pHTj-p20. k, pHTk-p20. The calculated molecular mass sizes are indicated at the right. Δ, terminal cleavage site.

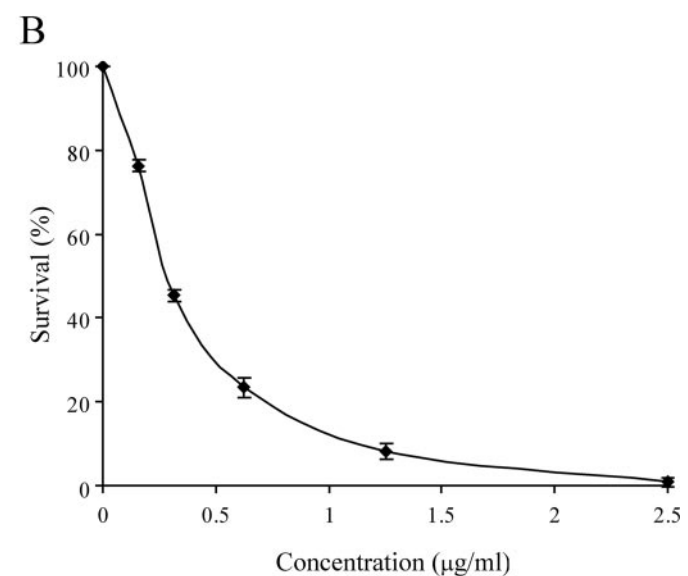


large parasporal bipyramidal crystals ( $\sim 1.5 \mu\text{m}$ ) together with the spores (phase and electron micrographs are shown in the supplemental data). The Cyt1Aa crystals were purified by a sucrose gradient, solubilized, and the native protein proteolytically activated by 1% proteinase K. A major peak was eluted in the prewash fraction, but SDS-PAGE analysis showed that this material was not a protein. Four additional peaks were obtained, but only the first of these, that which eluted at  $\sim 0.1 \text{ M NaCl}$ , displayed high activity against red blood cells (Fig. 2A, + + +). This fraction was also tested against M61.13 cells and showed  $\text{LC}_{50}$  of  $0.3 \mu\text{g ml}^{-1}$  (Fig. 2B). This second peak contains only activated Cyt1Aa ( $\sim 22 \text{ kDa}$ , Fig. 3A, lane 1) and was used for chemical conjugation to MBPp.

**Toxicity of the Chemical Conjugates**—M61.13, FL, and U136 cells were treated for 48 h with different concentrations of conjugates 1 or 2 (Cyt1Aa cleaved at both termini and chemically cross-linked to the carboxyl or amino terminus of MBPp, respectively). Both conjugates caused decreased viability (MTT test) at doses above  $3 \mu\text{g ml}^{-1}$ . Values of  $\text{LC}_{50}$  were 6 and  $5.5 \mu\text{g ml}^{-1}$  for conjugate 2 and conjugate 1, respectively (Fig. 4). The control cells were not affected under the same conditions.

The kinetics of cytotoxicity induced by both conjugates was determined during incubation of  $12.5 \mu\text{g ml}^{-1}$  for 1, 3, 24, and 48 h (Fig. 5). As expected, activated free Cyt1Aa was not specific, killing all three cell types with similar intensities. Conjugates 1 and 2 were not toxic for FL and U136 cells, but the target M61.13 cells were killed by either conjugate in a time-dependent manner. The percentage of killing was, however, lower than by free Cyt1Aa. Conjugate 2 appeared to be more effective than conjugate 1, but the differences were not significant. In the kinetic assay both conjugates were specifically toxic to M61.13 cells even after 1 h of incubation, and more than 65% of these cells were lysed after 24 h. The killing rate ( $\sim 90\%$  of the cells) even after 48 h was less than that of free Cyt1Aa (Fig. 5).

**Genetic Fusion of Both Terminally Cleaved Cyt1Aa to MBPp**—In addition to the chemically conjugated peptides, two variants



**FIGURE 2. A**, purification of the active Cyt1Aa fragment by chromatography on a DEAE cellulose column. The eluate was monitored at 280 nm, and the fractions corresponding to each absorbance peak were collected manually. The hemolytic activity of each peak was measured; + + + denotes high hemolysis activity of  $85 \pm 5\%$ ; -, 0% activity. **B**, cytotoxicity against M61.13 cells as examined by MTT test.

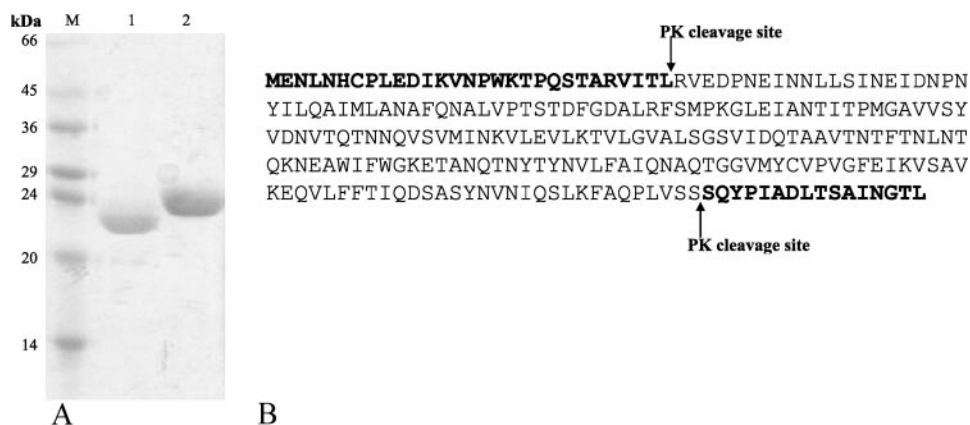


FIGURE 3. A, SDS-PAGE of the full-length and the proteolytic cleaved forms (~22.5 kDa) of Cyt1Aa purified on a DEAE cellulose column. B, the protein sequence of cleaved Cyt1Aa (residues Arg-30-Ser-231) is bordered by two arrows depicting the proteinase K cleavage sites (22).

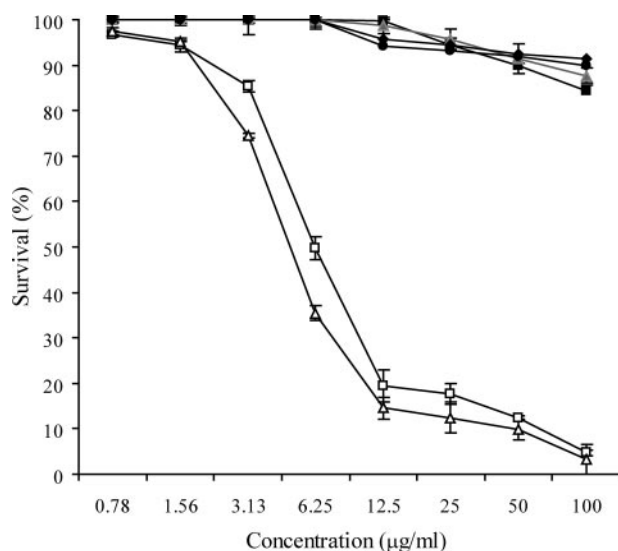


FIGURE 4. Dose-survival curve of M61.13 target cells and U136 and FL control cells following treatment with elevated concentrations of Cyt1Aa-MBPp conjugates 1 and 2. ◆, FL cells with conjugate 1; ▲, U136 cells with conjugate 1; ●, FL cells with conjugate 2; ■, U136 cells with conjugate 2; □, M61.13 with conjugate 2; △, M61.13 with conjugate 1. The results are relative to survival kinetics in the non-treated cells and represent mean  $\pm$  S.D. for triplicate in three different assays.

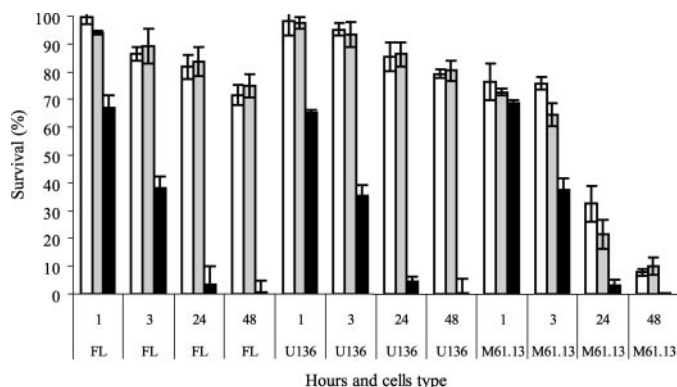


FIGURE 5. The cytotoxicity rate of chemical conjugates 1 (white column) and 2 (gray column) in concentrations of  $12.5 \mu\text{g ml}^{-1}$  and  $0.3 \mu\text{g ml}^{-1}$ -free Cyt1Aa (■) to M61.13, U136, and FL cells as measured by MTT analysis after 1, 3, 24, and 48 h. The results are relative to survival kinetics in the non-treated cells and represent mean  $\pm$  S.D. for triplicate in three different assays.

were genetically constructed encoding Cyt1Aa fused to MBPp at the carboxyl or amino terminus (Fig. 1, *b* and *c*, respectively) and introduced to acrySTALLIFEROUS *Bti*. All recombinant bacteria sporulated and lysed after 2 days in CCY medium, but no crystals were observed by phase microscopy. Bands with anticipated sizes were, however, detected by a Western blot analysis (Fig. 6) with anti-Cyt1Aa serum in lysates of 1-day cultures of sporulated recombinant cells expressing *b*, *c*, and *d* LTCs (Fig. 1). The lysate of cells expressing pHTb-*p20* (NCΔCyt1Aa-MBPp; Fig. 1*b*,

Table 2) exhibited two bands on native and denatured PAGEs, corresponding to monomer and dimer (Fig. 7, *A* and *B*).

**Toxicity of the Genetic Fusion Conjugates**—Toxicity of the recombinant conjugates against M61.13, U136, and FL cells was quantified over a wide range of concentrations (up to  $1.25 \mu\text{g ml}^{-1}$ ). The cells were cultured without serum together with toxin fragments *b* (Cyt1Aa-MBPp), *c* (MBPp-Cyt1Aa), or *d* (free Cyt1Aa) (Table 2 and Fig. 1, *b–d*), and cell viability was determined by MTT test after 16 h. Fragment *b* was the most cytotoxic to M61.13 target cells (Fig. 7*A*),  $LC_{50} \cong 180 \text{ ng ml}^{-1}$  compared with 260 and  $360 \text{ ng ml}^{-1}$  of fragments *c* and *d*, respectively. Conversely, fragment *b* was not toxic to U136 and FL cell lines (Fig. 7, *A* and *B*, respectively). Fragment *d* (free Cyt1Aa) showed nonspecific cytotoxicity and had a similar toxicity ( $LC_{50}$  range between 340 and  $360 \text{ ng ml}^{-1}$ ) against all the cell types tested.

**The Toxicity of Partially Cleaved Cyt1Aa**—To assess whether cleavages at both the amino and carboxyl termini are necessary for optimal activity of Cyt1Aa, seven additional constructs designated *e–k* were prepared (Table 2 and Fig. 1, *e–k*) that express (under *cyt1Aa* promoter) *p20* and *cyt1Aa* or *cyt1Aa-MBP* variants, cleaved at one terminus only. When introduced into acrySTALLIFEROUS *Bti* cells, none of these variants produced crystals, but their lysates contained soluble fragments as detected by anti-Cyt1Aa antibodies (Fig. 6). The polypeptides *e–h* were eluted from native gel and their toxicities tested against the three cell lines; none was toxic to any of the cells, even at high ( $100 \mu\text{g ml}^{-1}$ ) concentrations (data not shown). The hemolytic capability of fragments *g* and *h* cut at either end was four times less than that of fragment *d* ( $22 \pm 3$  versus  $83 \pm 2\%$ ), which was cut at both ends. Fragments cut at either end and fused to MBPp at the cleavage site (*e*, *f*) were non-hemolytic and not cytotoxic.

## DISCUSSION

Almost all of the cytotoxic agents currently used in cancer chemotherapy require cellular internalization and repeated applications that most likely induce resistance. Utilization of the membrane-active capacity of Cyt proteins, which rapidly disrupt the phospholipid bilayer of the cell membrane (23, 24), provides an alternative source of toxins that may bypass these

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limitations. Of the numerous Cyt proteins isolated so far from several *Bt* subspecies, Cyt1Aa is the most active against cells of various origins. Hence, we tested the cytotoxicity of the active fragment of this protein toward M61.13 hybridoma cells used as a clonotypic model of multiple myeloma (11).

Linking Cyt1Aa via Cys-190 to the insulin B-chain elevated its specificity to NIH 3T3 NIR3.5 cells overexpressing the insu-

lin receptor (26), but the cytotoxicity of the conjugate was lower than that of the free Cyt1Aa. The structure of Cyt1Aa has still not been solved, but comparing its amino acid sequence to that of Cyt2Aa (38) one can see that Cys-190 is located in the middle of  $\beta$ 6 strand that is thought to be important for channel formation (38, 39). Therefore, linkage of the target insulin moiety through the Cys-190 residue may explain the decreased activity of the conjugate. Hence, we adopted other linking and recombinant DNA strategies to improve the efficacy of Cyt1Aa-based LTCs.

Two types of Cyt1Aa-peptide conjugates were prepared. In the first group, the lysine residues of enzymatically activated Cyt1Aa were chemically cross-linked to either the carboxyl or amino terminus of MBPp (conjugates 1 and 2, respectively). This synthetic strategy was chosen as there are eight lysine residues in the activated Cyt1Aa sequence; two of them (Lys-154 and Lys-163) are important for providing hydrophilic balance to the exposed edge of the  $\beta$ -sheets, whereas another one (Lys-225) is located toward the carboxyl-terminal region of  $\beta$ 7 strand and may have relevance to the toxin's attachment site to the cell membrane (38). Replacement of Lys-225 with alanine reduced Cyt1Aa toxicity toward mosquito larvae and various other cells (40). We found that the cytotoxic effect of free Cyt1Aa toward M61.13 (Fig. 3) was maintained in both conjugates 1 and 2 (Fig. 4); they were target-specific, not affecting either non-relevant antibody-bearing cells (U136) or control erythroleukemic cells (FL). However, comparing the  $LC_{50}$  parameter showed that conjugation reduced toxin activity against the target M61.13 cells by more than an order of magnitude (0.3 versus  $5.5 \mu\text{g ml}^{-1}$  of free Cyt1Aa). One explanation for this result is that the conjugation procedure may have also coupled the peptide to Cyt1Aa through its lysine Lys-225 that interfered with the interaction of this strand and the cell membrane, hence reducing toxicity and rate of killing. Although it would be preferable to employ only the non-critical lysines in the coupling procedure, this is impossible to achieve chemically. In addition, chemical conjugation would produce a heterogeneous mixture of conjugates that are difficult to separate and study. We therefore engineered a series of recombinant *MBPp-cyt1Aa* coding for fusion proteins, cloned them into *acrySTALLIFEROUS Bti*, and isolated the expressed products. This bacterial strain was used because it was hoped at the outset that the products would assemble to crystals, thus assisting in their rapid isolation and purification (34). The chimeric proteins

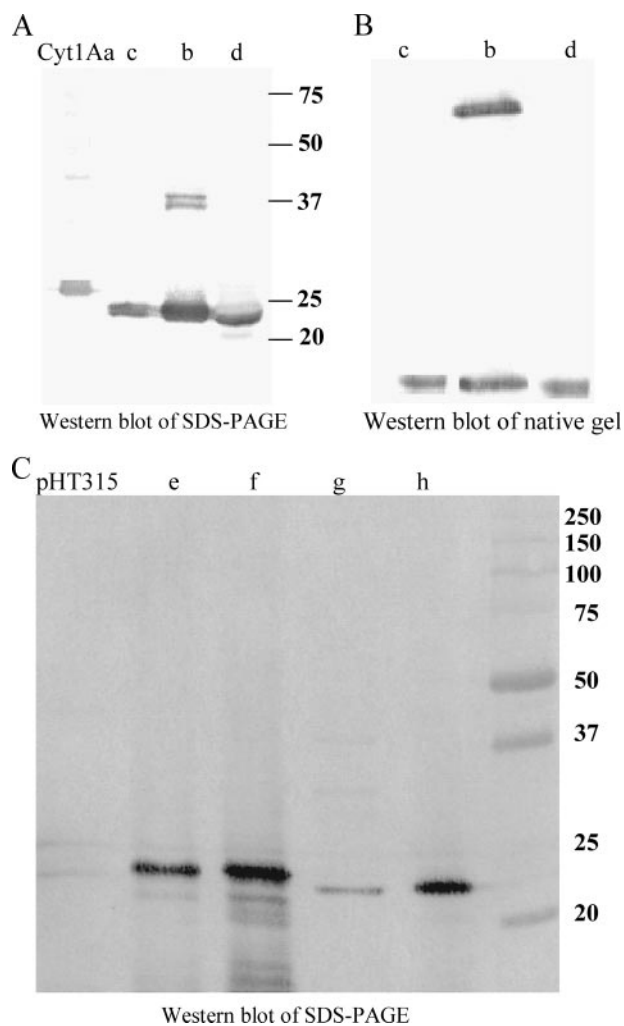


FIGURE 6. Western blot analyses of SDS-PAGE (A) and 5–20% gradient native gel (B) of bacterial lysate of *b*, *c*, and *d* fragment-expressing cells. C, SDS-PAGE of bacterial lysate of cells expressing fragments *e*, *f*, *g*, and *h* as detected by an anti-Cyt1Aa serum.

TABLE 2

### Cyt plasmids containing inserts with variants of *cyt1Aa*, *MBPp-cyt1Aa*, or *cyt1Aa-MBPp*, their encoding products termed *a–k*

The proteinase K cleavage site (22) at the amino terminus of Cyt1Aa is before Arg-30, and the carboxyl cleavage site is before Ser-233. The MBPp is fused in-frame in the indicated fragments.  $\Delta$ , cleaved terminus (amino and/or carboxyl). All engineered genes were cloned downstream the *cyt1Aa* promoter and upstream *p20* as delineated in Fig. 1. Plasmids *a*, *f*, and *g* contain 130 bp downstream the stop codon.

Plasmid	Encoded fragment	Cyt1Aa fragment cleaved at
pHT- <i>cyAp20</i>	Full-length Cyt1Aa ( <i>a</i> )	
pHT <i>b-p20</i>	NC $\Delta$ Cyt1Aa-MBPp ( <i>b</i> )	Amino and carboxyl termini and fused from the carboxy terminus onward with MBPp
pHT <i>c-p20</i>	MBPp-NC $\Delta$ Cyt1Aa ( <i>c</i> )	Amino and carboxyl termini and fused to MBPp at the amino terminus
pHT <i>d-p20</i>	NC $\Delta$ Cyt1Aa ( <i>d</i> )	Amino and carboxyl termini, without appendage of MBPp
pHT <i>e-p20</i>	MBPp-C $\Delta$ Cyt1Aa ( <i>e</i> )	Carboxyl terminus. MBPp fused from this site onward
pHT <i>f-p20</i>	N $\Delta$ Cyt1Aa-MBPp ( <i>f</i> )	Amino terminus and fused to MBPp at this site
pHT <i>g-p20</i>	N $\Delta$ Cyt1Aa ( <i>g</i> )	Amino terminus without appendage of MBPp
pHT <i>h-p20</i>	C $\Delta$ Cyt1Aa ( <i>h</i> )	Carboxyl terminus without appendage of MBPp
pHT <i>i-p20</i>	NC $\Delta$ Cyt1Aa ( <i>i</i> )	Amino terminus and after Asn-246 (3 amino acids before the carboxyl terminus) without appendage of MBPp
pHT <i>j-p20</i>	N $\Delta$ Cyt1Aa ( <i>j</i> )	Amino terminus without appendage of MBPp. The sequence downstream to the stop codon is omitted
pHT <i>k-p20</i>	Full-length Cyt1Aa ( <i>k</i> )	The sequence downstream to the stop codon is omitted



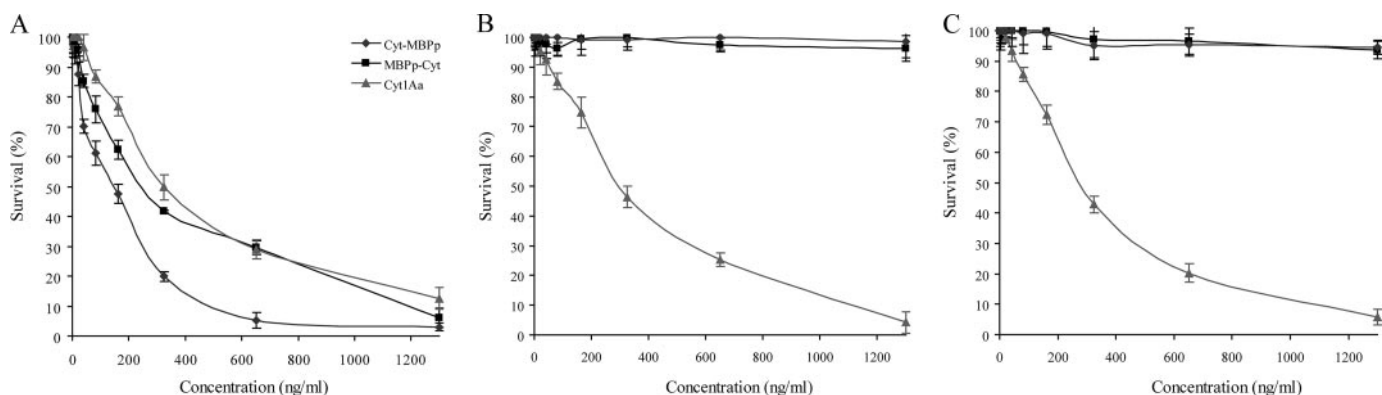


FIGURE 7. Dose-survival curve of M61.13 target cells (A), U136 cells (B), and FL control cells (C) following treatment with elevated concentrations of recombinant fragments. *b*, Cyt1Aa-MBPp,  $\blacklozenge$ ; *c*, MBPp-Cyt1Aa,  $\blacksquare$ ; *d*, Cyt1Aa,  $\blacktriangle$ . The results were calculated in relation to survival rate in the non-treated cells and represent mean  $\pm$  S.D. for triplicate in three different assays.

were, however, only expressed in soluble form in the cytosol. This may be explained by the lack of sequences upstream or downstream the coding regions that are required for *in vivo* crystallization, which were removed as designed. Several lines of evidence strengthen this proposal. (i) Mutation of residues located at the amino and carboxyl termini that are normally excised by proteolytic cleavage, *e.g.* replacement of Arg-25, Arg-30 (upstream), and Asp-240 (downstream) to alanines, led to prevention and reduction, respectively, of *in vivo* crystallization of Cyt1Aa in *Bacillus subtilis* (40). It seems that the termini (especially the amino) are important for inter-molecular interactions such as dimerization. Proteolytic removal of Cyt termini prevents association between  $\beta 1$ - $\beta 1'$  strands at the amino terminus and formation of disulfide bonds between Cys-7 and other cysteine residues that assist in attachment of the toxin molecules to the membrane (38). (ii) In this study, removal of only one terminus or the downstream sequence following the open reading frame (Fig. 1, fragments *b*–*k*) resulted in production of only soluble product. (iii) We have found that cleavage of Cyt1Aa and Cyt2Ba by proteinase K and *Bti* endogenous proteases enhanced solubility of the proteins (data not shown). Their aggregation in solution is connected directly to the capability of the monomers to interact with each other.

Recombinant Cyt1Aa lacking both amino and carboxyl termini (fragment *d*) was cytotoxic for the cell types (Fig. 7, A–C), reinforcing a previous assumption that removal of both termini is necessary for proper toxin activity (22). Fusion of this truncated Cyt1Aa to MBPp at either the carboxyl or amino termini (fragments *b* and *c*, respectively) maintained and even enhanced cytotoxicity (Fig. 7). Furthermore, conjugation to MBPp imbued the conjugates with target specificity toward M61.13 cells (Fig. 7A). According to the three-dimensional model of Cyt2Aa (38), its membrane-perforating region is near the carboxyl terminus. Our finding that enhanced cytotoxicity was particularly pronounced with fragment *b* might therefore suggest that binding of MBPp to its cell surface antibody facilitates interaction between the membrane and the active regions of the protein close to the carboxyl terminus, as previously suggested (39). Although it was recently reported that Cyt1Aa fused to green fluorescent protein at the carboxyl terminus lost its toxicity (41), there is a possibility that green fluorescent protein (27 kDa) that is larger than the active Cyt1Aa (22 kDa),

disturbs the toxin activity, whereas MBPp is a small peptide (1.56 kDa) and not a disrupting factor.

For the treatment of B cell leukemias, circulating anti-ligand antibodies such as the M-protein in multiple myeloma may theoretically reduce the efficacy of LTC-based therapy by blocking conjugate activity. We considered this possibility and in our previous study (11) demonstrated that at least a 7-M excess of soluble anti-ligand did not interfere the LTC destruction of target myeloma cells. Furthermore, LTC therapy would likely be most effective as second-line treatment following initial chemotherapy to reduce tumor burden and also to eliminate residual disease, at which time the level of circulating anti-ligand antibodies would be minimal.

**Concluding Remarks**—It was demonstrated that conjugation of activated Cyt1Aa to a peptide can confer two characters to the activated toxin: (i) abolishment of the non-target-specific activity of Cyt1Aa against mammalian cells, and (ii) maintenance of membrane cytotoxicity, particularly when the amino termini of the protein are not conjugated to a carrier molecule. This strategy can be further developed for treatment of tumor cells bearing unique surface receptors, such as chronic lymphocytic leukemia, wherein cells express surface immunoglobulins with clonotypic variable (V) domains that might be targeted with LTCs composed of a V domain binding peptide and a cytotoxin. In this regard, Cyt1Aa represents an attractive cytotoxin because it is a membrane-acting toxin, and its exploitation would broaden the spectrum of cellular mechanisms that may be targeted by anti-cancer drugs.

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