Listeriolysin O as cytotoxic component of an immunotoxin

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Abstract: Monoclonal antibodies (mAbs) have been developed over the past years as promising anticancer therapeutics. The conjugation of tumor specific mAbs with cytotoxic molecules has been shown to improve their efficacy dramatically. These bifunctional immunotoxins, consisting of covalently linked antibodies and protein toxins, possess considerable potential in cancer therapy. Many of them are under investigation in clinical trials. As a result of general interest in new toxic components, we describe here the suitability of the bacterial protein Listeriolysin O (LLO) as cytotoxic component of an immunotoxin. Unique characteristics of LLO, such as its acidic pH optimum and the possibility to regulate the cytolytic activity by cysteine-oxidation, make LLO an interesting toxophore. Oxidized LLO shows a substantially decreased cytolytic activity when compared with the reduced protein as analyzed by hemolysis. Both oxidized and reduced LLO exhibit a cell-type-unspecific toxicity in cell culture with a significantly higher toxicity of reduced LLO. For cell-type-specific targeting of LLO to tumor cells, LLO was coupled to the dsFv fragment of the monoclonal antibody B3, which recognizes the tumor-antigen Lewis Y. The coupling of LLO to dsFv-B3 was performed via cysteine-containing polyionic fusion peptides that act as a specific heterodimerization motif. The novel immunotoxin B3-LLO could be shown to specifically eliminate antigen positive MCF7 cells with an EC_{50} value of 2.3 nM, whereas antigen negative cell lines were 80- to 250-fold less sensitive towards B3-LLO.

Keywords: cytotoxicity; hemolysis; disulfide bond; polyionic peptides; dimerization motif; B3; MCF7

Introduction

Antibody-based therapeutics have developed to become important constituents for treatment of human malignancies. The use of unconjugated monoclonal antibodies for cancer therapy relies on inherent biological activities such as induction of immune responses, blockage of highly expressed and activated growth-factor receptors on tumor cells, or inhibition of angiogenesis.¹ Tumor-specific antibodies also provide the capability for targeting therapeutic agents to tumor cells.^{1,2} Protein toxins such as the bacterial diphtheria toxin, the *Pseudomonas* exotoxin,

Abbreviations: 8H9(dsFv)-PE38, immunotoxin composed of a disulfide stabilized Fv fragment of the monoclonal antibody 8H9 and a truncated *Pseudomonas* exotoxin variant; B3-LLO, conjugated immunotoxin composed of dsFv-B3-R₈C and E₈C-LLO; B3-PE38, conjugated immunotoxin composed of dsFv-B3-R₈C and a truncated *Pseudomonas* exotoxin variant; CDC, cholesterol-dependent cytolysin; dsFv-B3, disulfide stabilized Fv fragment of the antibody B3; dsFv-B3-R₈C, dsFv-B3 extended at the C-terminus of the V_H domain by the peptide R₈CP; DTT, Dithiothreitol; E₈C-LLO, Listeriolysin O extended at the N-terminus by the peptide E₈C; E₈C-LLOox, E₈C-LLO with its singular cysteine oxidized by GSSG; E₈C-LLOred, reduced E₈C-LLO; FBS, fetal bovine serum; GST, glutathione-S-transferase; GST-E₈C-LLO, Listeriolysin O extended at the N-terminus by glutathione-S-transferase and the peptide E₈C; IPTG, Isopropyl- β -D-1-thiogalactopyranoside; LLO, Listeriolysin O; mAb, monoclonal antibody; OD, optical density; PDB, protein data bank; V_H, variable domain of the heavy chain of an antibody; V_H-R₈CP, V_H of the monoclonal antibody B3 extended at the C-terminus by the peptide R₈CP; V_L, variable domain of the light chain of an antibody; wt, wildtype.

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or the plant toxins ricin or saporin have been utilized as therapeutic agents.³ These chimaera, known as immunotoxins are described as hybrid molecules composed of an antibody or antibody fragment joined to a protein toxin.⁴ Antibody binding to the surface of cancer cells is followed by endocytosis of the antibody-toxin-conjugate, inducing cell death. The efficacy of the immunotoxin in eliminating tumor cells depends specifically on the number of cell surface receptors targeted by the antibody, the affinity of the antibody as well as the toxicity of the toxin. Here, we describe the suitability of the bacterial toxin Listeriolysin O (LLO) as cytotoxic part of an immunotoxin.

Listeria monocytogenes is a bacterial pathogen that grows within the cytosol of infected host cells. LLO is essential to promote the phagosomal escape of the bacterium into the cytoplasm.^{5–8} LLO, first characterized by Geoffroy *et al.*,⁹ belongs to a family of cholesterol-dependent pore-forming cytolysins (CDCs).^{10–13} CDCs insert into eukaryotic cell membranes upon cholesterol-binding, leading to oligomerization of the monomers to form pores of varying sizes.^{10,13,14} This ability of LLO to form pores in eukaryotic membranes is the basis of its cytolytic activity. Members of this protein family exhibit a highly conserved sequence consisting of 11 amino acids in the C-terminal domain D4 (see Fig. 1) that is involved in membrane binding^{16–18} and is essential for the cytolytic activity.^{13,16,17} A single cysteine within this undecapeptide plays an important role, as oxidation of the thiol group leads to a loss of hemolytic activity *in vitro*, which can be reversed completely by reduction.^{9,19} LLO activity is pH-dependent,^{9,19,20} with highest activity at acidic pH,¹⁹ corresponding to acidic cell compartments such as endosomes and lysosomes. These properties make LLO a promising cytotoxic component for an immunotoxin.

We used the Fv fragment of the monoclonal antibody B3,²¹ stabilized by an engineered interchain disulfide bond^{22,23} to target LLO to tumor cells. The monoclonal antibody B3 was raised against the carbohydrate antigen Lewis Y which is highly expressed on the surface of many human tumor cells of epithelial origin like breast, lung, colon, and some epidermal cancer cells.²¹ Even though the antibody fragment B3 exhibits a comparably low affinity to its antigen with a dissociation constant in the micromolar range (SF and HL, unpublished data), it is highly selective²¹ and thus interesting as a tumor-specific targeting module of immunotoxins. Such immunotoxins have already been constructed by fusing this antibody fragment to a truncated version of the *Pseudomonas* exotoxin.^{22,24,25} The respective immunotoxins LMB-7 and LMB-9 are currently investigated in clinical trials (<http://clinicaltrials.gov>).

We utilized a highly specific heterodimerization motif for the coupling of the antibody fragment to the toxic protein LLO²⁶ consisting of oppositely charged peptides of eight arginine and glutamic acid residues,

Figure 1. Structural image of the cholesterol-dependent cytolysin Perfringolysin O (PDB file: 1PFO;¹⁵). Perfringolysin O shares a sequence identity of 42% to LLO for the whole polypeptide chain and of 64% for the C-terminal domain D4.¹³ In black, the conserved sequence consisting of 11 amino acids including the singular cysteine (spheres) is shown.

respectively, each containing an additional cysteine residue.²⁶ The polyionic interaction between these two peptides leads to a specific association; formation of a disulfide bond via the additional cysteine residues results in a covalently linked heterodimer. This heterodimerization motif has already been used in the design of immunotoxins^{27,28} and in the decoration of virus-like particles with antibody fragments successfully.^{29,30} The two proteins could be conjugated specifically due to the positively charged polyionic peptide fused to the disulfide stabilized antibody fragment B3 (dsFv-B3-R₈C)²⁹ and the LLO extended by the negatively charged peptide E₈C (E₈C-LLO). The resulting immunotoxin B3-LLO was shown to be a very efficient and cell-type-specific immunotoxin.

Results

Conjugation of the antibody fragment B3 and the toxin E₈C-LLO

We used the directed and covalent association of two proteins with a specific dimerization motif consisting

Figure 2. (A) Coomassie-stained SDS-PAGE analysis of the immunotoxin and single components under nonreducing conditions. Lane 1, molecular mass standard; lane 2, isolated E₈C-LLO; lane 3, isolated dsFv-B3-R₈C; lane 4, B3-LLO. In addition, B3-LLO was analyzed under reducing conditions, where the conjugate splits into its three parts (E₈C-LLO, V_H-R₈CP, and V_L, lane 5). (B) Analysis of the immunotoxins stability in fetal bovine serum (FBS). B3-LLO was incubated with 10% FBS for 11 h at 37°C. Subsequently, the samples were analyzed via 8% SDS-PAGE (Coomassie stained) under nonreducing conditions. Lane 1, molecular mass standard; lane 2, 10% FBS; lane 3, immunotoxin B3-LLO; lane 4, immunotoxin with 10% FBS.

of the oppositely charged cysteine containing polyionic fusion peptides R₈C and E₈C²⁶ to generate the immunotoxin B3-LLO.

The dsFv fragment of the monoclonal antibody B3 was C-terminal fused by the polyionic peptide R₈CP,²⁹ whereas the negatively charged peptide sequence E₈C was fused to the N-terminus of LLO for coupling of LLO to the antibody fragment B3. Salt conditions below 100 mM NaCl facilitated the association of the oppositely charged fusion peptides,²⁶ whereas copper ions catalyzed the formation of the disulfide bond between the reduced cysteines of E₈C and R₈CP, thus creating the covalently associated immunotoxin B3-LLO [Fig. 2(A)]. Under these conditions, the immunotoxin was formed to about 80% and could be purified from the remaining monomeric components by ion exchange chromatography.

For any potential applications as a therapeutic agent, B3-LLO must be stable against proteases present in serum. To analyze this, the immunotoxin was incubated with 10% fetal bovine serum (FBS, not heat inactivated) for 11 h at 37°C and afterwards analyzed via SDS-PAGE. As shown in Figure 2(B), there was no significant dissociation or degradation of B3-LLO upon incubation with FBS. Thus, the immunotoxin is sufficiently stable for analysis of its cytotoxic potential in cell culture experiments.

Functional analysis of dsFv-B3-R₈C, B3-LLO and E₈C-LLO

The immunotoxin B3-LLO as well as its single components were analyzed for functionality. The antibody fragment B3 and its conjugate were analyzed by antigen-dependent cell binding monitored by fluorescence

Figure 3. Fluorescence images of MCF7 and HT-29 cells. The cells were treated with 0.1 nM of dsFv-B3-R₈C, E₈C-LLO, and B3-LLO, respectively. All proteins were labeled with fluorescein. (A) MCF7 cells, positive for the antigen Lewis Y, exhibited green fluorescence after binding of the antibody fragment dsFv-B3-R₈C. (B) HT-29 cells, negative for Lewis Y, did not fluoresce after incubation with dsFv-B3-R₈C. (C) MCF7 cells also showed green fluorescence due to the binding of the immunotoxin B3-LLO, whereas HT-29 did not (D). The isolated cytolysin E₈C-LLO interacted with both MCF7 cells (E) and HT-29 cells (F). On the left side there are fluorescence images, the right side shows the corresponding images from transmitted light. The length of the bar is equivalent to 20 μ m.

microscopy and fluorescence activated cell sorting (data not shown) with fluorescein labeled proteins. The fluorescence images in Figure 3 show the binding of dsFv-B3-R₈C to the Lewis Y positive MCF7 cells [Fig. 3(A)], whereas the Lewis Y negative cell line HT-29 exhibited no antibody binding [Fig. 3(B)], indicating a functional antibody fragment B3 with cell-type-specific binding activity. The polyionic fusion peptide did not influence the antigen binding.^{29,30} It was further investigated whether the coupling of E₈C-LLO to dsFv-B3-R₈C affects the Lewis Y binding of the antibody fragment. As demonstrated in Figure 3(C), the GSSG-oxidized immunotoxin B3-LLO bound to MCF7 cells but not to HT-29 cells [Fig. 3(D)], showing that the coupling of E₈C-LLO to the antibody fragment B3 does not affect antigen binding. Surprisingly, the immunotoxin B3-LLO does not interact with HT-29 cells, even though the pore-forming cytolysin LLO is able to insert into eukaryotic membranes. This was not due to modification of the protein with the polyionic fusion peptide, as oxidized E₈C-LLO could insert

into the membranes of both MCF7 [Fig. 3(E)] and HT-29 cells [Fig. 3(F)]; coupling of the antibody fragment B3 to E₈C-LLO completely abolished this membrane interaction for HT-29 cells [Fig. 3(D)]. Thus coupling of dsFv-B3-R₈C to E₈C-LLO generates an immunotoxin which binds specifically to the antigen Lewis Y on target cells and exhibits no unspecific cell binding mediated by E₈C-LLO.

Hemolysis

The interaction of LLO with cholesterol containing membranes depends initially on a highly conserved sequence consisting of 11 amino acids in its C-terminal region.^{16–18} This sequence contains a single cysteine whose oxidation status plays an important role for the activity of LLO as shown by hemolysis studies.^{9,19} Furthermore, LLO activity is pH-dependent,⁹ with the hemolytic activity decreased to about 30% at neutral pH in comparison to pH 5.5.¹⁹

Both E₈C-LLO and B3-LLO were assayed for hemolysis. The disruption of erythrocytes was monitored

Figure 4. Hemolytic activity of different LLO variants. Sheep erythrocytes were treated with different concentrations of the respective LLO species and immunotoxin, respectively. After 30 min the light scattering signal at 350 nm was determined. The signal decreases due to the lysis of the erythrocytes with increasing toxin concentrations. Titrations are shown for reduced wtLLO (∇), B3-LLO ($\bullet$) and oxidized wtLLO ($\triangle$) at pH 7.4. The measurements were performed in triplicate, the error bars represent the SEM of the mean value. The lines represent the fit of the experimental data to the EC_{50} values listed in Table I.

by two different methods, the decrease in light scattering and the release of hemoglobin from the cells, respectively. Both methods showed identical results. Titration curves for reduced wtLLO (wtLLOred), oxidized wtLLO (wtLLOox), and the oxidized immunotoxin B3-LLO at pH 7.4 are shown in Figure 4 and EC_{50} values of all titrations are listed in Table I. As expected, reduced wtLLO is significantly more active than the oxidized protein. The calculated EC_{50} value of 38 ± 7 pM for reduced E_8C -LLO at pH 7.4 is in good agreement with that for wtLLOred (21.9 ± 7 pM). Oxidized E_8C -LLO exhibits an EC_{50} of 39.3 ± 11.7 nM, comparable with that for oxidized wtLLO (EC_{50} 10.9 ± 1.7 nM). Thus, the polyionic fusion peptide does not change the characteristics of LLO. Under acidic conditions (pH 5.5), the hemolytic activities of reduced E_8C -LLO and wtLLO are 3–7 times higher when compared with the activity at pH 7.4. Most importantly, the immunotoxin B3-LLO, with its single cysteine of E_8C -LLO oxidized by GSSG, shows a very low activity with an EC_{50} value of 6.5 ± 1.2 nM, comparable to the activity of both oxidized wtLLO and E_8C -LLO. Hemolytic activity of B3-LLO under reducing conditions could not be assayed because this leads to the dissociation of the heterobifunctional construct.

These hemolysis studies show that the high cytolytic activity of LLO can be controlled by oxidation of the single cysteine of LLO in its C-terminal domain and that this reduction in activity is maintained in the immunotoxin B3-LLO.

Cytotoxicity of the immunotoxin

The conjugated immunotoxin B3-LLO was analyzed for its ability to specifically and efficiently kill tumor cells expressing the B3 antigen Lewis Y on the surface. To assay cytotoxicity, Lewis Y positive MCF7 and SKBR-3 cells and Lewis Y negative HT-29 and HeLa cells were titrated with the various protein constructs and the number of viable cells analyzed by fluorescence activated cell sorting (FACS). The immunotoxin B3-LLO was very efficient in eliminating the Lewis Y positive cell lines, with an EC_{50} value of 2.3 ± 0.5 nM for MCF7 [Fig. 5(A) and Table II] and of 12.7 ± 1.94 nM for SKBR-3, respectively. The slightly lower cytotoxicity of the construct for SKBR-3 when compared with MCF 7 cells is expected because SKBr-3 cells are known to possess less antigen on the cell surface.

In contrast, only a cell-type-unspecific cytotoxic activity (EC_{50} value of 168.9 ± 38.9 nM) could be determined for HT-29 cells [Fig. 5(B) and Table II] and for HeLa cells (EC_{50} value of 503.78 ± 164.11 nM). This low cytotoxicity is in the same range as that of the isolated oxidized E_8C -LLO (E_8C -LLOox) (Table II). Nevertheless, the immunotoxin B3-LLO discriminates between antigen positive and antigen negative cells, whereas E_8C -LLOox is unspecific. Reduction of E_8C -LLO results in a dramatic increase in cytotoxicity; E_8C -LLOred exhibits the same activity as the immunotoxin B3-LLO on MCF7 and SKBR-3 cells, but is again cell-type-unspecific (Table II). It should be emphasized that the oxidized B3-LLO possesses an equivalent decreased activity as E_8C -LLOox. We suggest that following antibody-mediated binding and uptake of the immunotoxin into the cell, B3-LLO is reduced intracellularly and the former inhibited activity of E_8C -LLO is reversed. Following uptake of B3-LLO into antigen positive cells, the immunotoxin confers nearly the same cytotoxic activity as observed for the maximal active species E_8C -LLOred.

Discussion

The treatment of cancer is characterized by a major conflict in which treatment should be as aggressive as possible to completely destroy tumor cells but with minimal side effects. To circumvent this problem, cancer therapies based on tumor-specific monoclonal

Table I. Hemolytic Activity of LLO

Toxin	EC_{50} (pM)		
	Reduced (pH 7.4)	Reduced (pH 5.5)	Oxidised (pH 7.4)
wtLLO	21.9 ± 7	7 ± 0.3	$10.9 \times 10^3 \pm 1.7 \times 10^3$
E_8C -LLO	38 ± 7	5.6 ± 0.7	$39.3 \times 10^3 \pm 11.7 \times 10^3$
B3-LLO	—	—	$6.5 \times 10^3 \pm 1.2 \times 10^3$

EC_{50} values of the immunotoxin and isolated toxins in reduced and oxidized form in hemolysis under physiological (PBS, pH 7.4) and acidic (citrate, pH 5.5) conditions.

Figure 5. Cytotoxicity of the immunotoxin B3-LLO. MCF7 as Lewis Y positive and HT-29 as Lewis Y negative cell lines were incubated for 48 h with different concentrations of B3-LLO or the isolated toxins in reduced (E_8C -LLOred) or oxidized (E_8C -LLOox) form, respectively. Afterwards the cells were analyzed by FACS and the amount of viable cells was determined. All measurements were performed in triplicate, the error bars represent the SEM of the mean value. The effect of B3-LLO (●), E_8C -LLOred (▲), and E_8C -LLOox (□) on MCF7 cells (A) and on HT-29 cells (B) is shown. The EC_{50} values are given in Table II.

antibodies (mAbs) have been developed, especially because these antibodies show only minor effects. A couple of mAbs have already been approved as therapeutics and many others are currently in clinical tri-

als.^{1,31} Despite this initial success, however, there is still an urgent need to enhance the efficacy of antibodies as anticancer therapeutics. One solution to this problem is to combine the targeting specificity of mAbs with the tumor-killing potency of cytotoxic effector molecules to produce immunoconjugates. The cytotoxic drugs used in these immunoconjugates could be chemotherapeutic agents such as, for example, antifolates,³² anthracyclines,³³ radionuclides,^{34,35} small toxic molecules like calicheamicin,³⁶ or protein toxins.^{37,38} The antibody fragment B3, used in this study, has already been evaluated in the context of immunotoxins^{22,24,25} and applied in clinical trials (immunotoxins LMB-7 and LMB-9; details at: <http://clinicaltrials.gov>). The cytotoxic potency of immunotoxins depends on several properties, such as the number of antigens on the cell-surface, the antigen-binding affinity, their internalization rate and their intracellular processing.³⁹ To use toxins as therapeutics, they often have to be modified. For example, their binding sites for targets expressed in normal tissue have to be removed⁴⁰ or they have to be deglycosylated to circumvent rapid clearance by liver cells expressing mannose receptors.⁴¹ Currently, there is no single protein toxin that has all of these ideal features. The currently used toxic parts of immunotoxins that are investigated in clinical trials are truncated variants of *Pseudomonas* exotoxin, gelonin, diphtheria toxin, and RNase.¹ However, the search for new toxins is still in progress.

Here, we describe Listeriolysin O as a new toxic component of an immunotoxin. Because it does not require modification and shows unique characteristics, that is, the cytolytic activity that can be regulated by oxidation of the singular cysteine and the acidic pH-optimum,^{9,19,20} it represents an excellent candidate for novel immunotoxin generation. The cysteine in LLO serves not only as an ideal site for chemical modification and conjugation⁴² but also as an appropriate regulator of the hemolytic activity. Consequently, we first coupled LLO to the tumor-specific antibody fragment B3 through disulfide formation between the single cysteine of LLO and the cysteine within the R₈C fusion peptide of dsFv-B3-R₈C. This conjugation led to a biologically active and cell-type-specific immunotoxin; however, the yield of heterodimer formation was very low (data not shown). Coupling of the antibody

Table II. Cytotoxic Activity of the Immunotoxin B3-LLO

Toxin	EC_{50} (nM)			
	MCF7 (Lewis Y ⁺)	SKBR-3 (Lewis Y ⁺)	HeLa (Lewis Y ⁻)	HT-29 (Lewis Y ⁻)
E_8C -LLOred	1.9 ± 0.4	2.49 ± 0.15	10.34 ± 2.23	1.9 ± 0.25
B3-LLO	2.3 ± 0.5	12.7 ± 1.94	503.78 ± 164.11	168.9 ± 38.9
E_8C -LLOox	153.8 ± 41	314.8 ± 40.67	910.63 ± 164.07	231.5 ± 58.1

EC_{50} values of the immunotoxin and isolated toxins in reduced (E_8C -LLOred) and oxidized form (E_8C -LLOox) in cell culture experiments using different antigen positive (Lewis Y⁺) and antigen negative (Lewis Y⁻) cell lines.

fragment B3 to the protein toxin LLO via oppositely charged polyionic fusion peptides, which act as a specific heterodimerization motif²⁶ increased the yield of conjugation product. This coupling procedure has already been exploited in the design of other immunotoxins^{27,28} and the modification of virus-like particles.^{29,30}

Fusion of the polyionic fusion peptide to the N-terminus of LLO did not affect the biological activity of the protein as monitored by hemolysis. Although Geoffroy *et al.*⁹ reported that they did not observe any hemolytic activity of LLO at neutral pH it did have a high activity under acidic conditions. This pH-dependent difference in hemolytic activity was, however, not as pronounced in a study of Giammarini *et al.*^{19,43} We could also demonstrate that LLO had hemolytic activity at neutral pH but this activity was much lower than under acidic conditions. The pH optimum of LLO activity is around pH 5.5. The oxidation of the singular cysteine of LLO leads to a dramatic loss of hemolytic activity.^{9,19} These characteristics were maintained in the case of the polyionic variant E₈C-LLO and the immunotoxin B3-LLO. The ability to regulate its activity by cysteine-oxidation and pH makes LLO an attractive cytotoxic component.

The cell-type-specific targeting of the immunotoxin B3-LLO was mediated by the tumor-specific antibody fragment dsFv-B3-R₈C. LLO exhibited only very low activity within B3-LLO because it was oxidized. Thus, the cell-type unspecific toxicity of B3-LLO was low, as demonstrated for the Lewis Y negative cell lines HT-29 and HeLa. For Lewis Y positive cells, the situation was very different due to the cell-type-specific targeting and internalization of B3-LLO. After the internalization of the immunotoxin by endocytosis into the Lewis Y positive cells, it was located in acidified endosomes/lysosomes. It is likely that the activity of E₈C-LLO could be restored by intracellular reduction of B3-LLO. The reduction of disulfide bonds in endosomes and the *trans*-Golgi network is a well-known mechanism for activation of bacterial toxins, such as diphtheria and cholera toxin. Here, we used this concept for activation of the artificially designed immunotoxin B3-LLO. This activation results in a substantial decrease of the EC₅₀ value for Lewis Y positive cells when compared with the control cell lines. A cell-type-specific toxicity of the immunotoxin with an EC₅₀ value of 2.3 nM could be observed for MCF7 and of 12.7 nM for SKBR-3, respectively.

In contrast to B3-LLO, reduced E₈C-LLO could eliminate all cell lines in equal measure, resulting in nearly identical EC₅₀ values when compared with the cell-type specific activity of B3-LLO on MCF7 and SKBR-3 cells. This was surprising, because B3-LLO and E₈C-LLO probably act by two different mechanisms. It is conceivable that reduced LLO binds to the cell membrane and forms pores leading to cell lysis. In contrast, the immunotoxin is internalized into cells

and LLO develops its cytotoxic effect intracellularly. It is possible that after activation LLO destroys the structural integrity of the endosomes/lysosomes. Because lysosomal cathepsins have been implicated in the activation of caspases, and therefore apoptosis,^{44,45} the release of lysosomal proteins and enzymes into the cytoplasm could lead to cell death.⁴⁵ Several studies have demonstrated that infection of cells with *Listeria*⁴⁶ or the treatment of cells with sublytical concentrations of LLO⁴⁷ leads to apoptosis. Alternatively, it is conceivable that LLO, after its release from endosomes/lysosomes, uniformly distributes throughout all cellular membranes, which could lead to cell death.

Surprisingly the EC₅₀ values of cytotoxicity assays were approximately three orders of magnitude higher than those for hemolysis. This could be due to differences in the membrane constitution of erythrocytes and cancer cells, especially in terms of cholesterol content. Because LLO binds to cholesterol-containing membranes, a difference in the susceptibility of erythrocytes towards the cytotoxic LLO in comparison with cancer cells is possible. Moreover, it could be shown that living cells are able to repair a limited number of pores,⁴⁸ which also might be a reason for the differences in hemolytic and cytotoxic activity of LLO. Nevertheless, the toxicity of the immunotoxin B3-LLO with an EC₅₀ value of 2.3 nM for MCF7 and 12.7 nM for SKBR-3 is comparable with that of other immunotoxins based on the antibody B3.^{24,27}

Materials and Methods

Cloning, expression, and purification of toxin variant E₈C-LLO

The DNA for the E₈C-LLO variant was amplified from wtLLO DNA and two restriction sites (5'BspEI and 3'SalI) were introduced during PCR. The fragment was then cloned into pGEX-4T-1-vector with N-terminal polyionic extension coding for the amino acid residues E₈C.²⁷ We created the E₈C-LLO variant using standard polymerase chain reaction and cloning techniques. The cloned plasmid sequence was verified by sequencing analysis. The resulting expression clone pE₈C-LLO encoded a fusion protein consisting of the N-terminal glutathione-S-transferase (GST), a thrombin cleavage site between GST and the toxin, and the toxin with the N-terminal E₈C extension and a C-terminal histidine₆-tag. The processed N-terminal amino acid sequence of the mature toxin variant is GSPEFE₈C-LLO.

The gene was expressed in the *Escherichia coli* strain BL21 (DE3), cultured in LB medium supplemented with 100 µg/mL ampicillin. At OD_{600nm} = 0.8, the recombinant protein expression was induced by adding 1 mM IPTG to the medium. After induction the cultivation temperature was decreased from 37°C

to 24°C. The cells were harvested 4 h after induction by centrifugation (8980g, 20 min).

For protein preparation, 25 g of wet cell paste were suspended in 50-mL buffer A (50 mM NaH₂PO₄/Na₂HPO₄ (pH 7.3), 150 mM NaCl, 5 mM DTT) with one tablet of protease inhibitor, EDTA-free (Roche). The cells were then disrupted by high pressure dispersion. The DNA in the crude extract was digested with 20 µL Benzonase (Merck) for 30 min at 20°C in the presence of 3 mM MgCl₂. The extract was centrifuged for 30 min at 75,600g at 4°C in a JA30.50 rotor (Beckman Instruments). The supernatant was loaded onto a GSH-Sepharose column (GE Healthcare), equilibrated in buffer A. The column was first washed with buffer B (50 mM NaH₂PO₄/Na₂HPO₄ (pH 7.0), 300 mM NaCl, 0.1% (v/v) Tween 20, 10% (v/v) glycerol, 5 mM DTT) and then with buffer C (buffer B without Tween 20 or glycerol). The protein was eluted from the column in 50 mM NaH₂PO₄/Na₂HPO₄ (pH 8.0), 300 mM NaCl, 20 mM GSH. GST-E₈C-LLO fractions were pooled and dialyzed against thrombin-cleavage buffer (20 mM Tris-HCl (pH 7.3), 200 mM NaCl, 1.5 mM KH₂PO₄, 80 mM MgCl₂, 2.7 mM KCl). 10 U/mL thrombin (Merck) were added to the protein solution and incubated for 11 h at 20°C. Subsequently the protein was applied on a Ni-NTA HisTrapTM crude FF column (GE Healthcare), equilibrated in 50 mM NaH₂PO₄/Na₂HPO₄ (pH 7.3), 700 mM NaCl, 10 mM mercaptoethanol. GST-E₈C-LLO and E₈C-LLO were bound to the column, whereas GST and thrombin was found in the flow-through. After washing the column with 50 mM NaH₂PO₄/Na₂HPO₄ (pH 7.3) including 20 mM imidazole and 10 mM mercaptoethanol, the protein was eluted using a linear gradient of imidazole from 20 to 600 mM. The eluted protein was dialyzed against buffer A before the final purification step and loaded onto GSH-Sepharose column, previously equilibrated with buffer A. The flow-through contained the cleaved toxin, whereas noncleaved GST-E₈C-LLO remained bound to the column. Finally, the toxin was dialyzed against storage buffer (25 mM NaH₂PO₄/Na₂HPO₄ (pH 7.3), 300 mM NaCl, 1 mM EDTA, 2 mM DTT). The concentration of the purified E₈C-LLO was determined spectroscopically using an extinction coefficient of ϵ (280 nm) = 71,850 M⁻¹ cm⁻¹.

Recombinant wtLLO was purified as described²⁰ using a Ni-NTA HisTrapTM FF column (GE Healthcare). As an additional purification step a Poros HS column, equilibrated in 50 mM NaH₂PO₄/Na₂HPO₄ (pH 6.0) including 100 mM NaCl, 1 mM EDTA, and 5 mM DTT, was used. After washing the column with the same buffer, the bound wtLLO could be eluted using a linear gradient of 0.1–1M NaCl. The protein eluted at 300 mM salt. Finally, wtLLO was extensively dialyzed against storage buffer (50 mM NaH₂PO₄/Na₂HPO₄ (pH 6.0), 1M NaCl, 1 mM EDTA, 5 mM DTT).

Preparation of recombinant antibody fragment dsFv-B3-R₈C

For targeting of tumor cells, we chose the Fv fragment of the tumor-specific antibody B3, which is directed against Lewis Y antigen.²¹ This Fv fragment is stabilized by an engineered interchain disulfide bond.^{22,23} The V_H-domain with its C-terminal polyionic extension encoding the amino acid sequence R₈CP and the V_L-domain were expressed separately in *E. coli* BL21 (DE3) and isolated as inclusion bodies.⁴⁹ The inclusion bodies were refolded to dsFv-B3-R₈C and purified as described.^{27,29} The protein concentration was determined spectroscopically using an extinction coefficient of ϵ (280 nm) = 52,060 M⁻¹ cm⁻¹.

Conjugation of E₈C-LLO and dsFv-B3-R₈C

Before the conjugation of both proteins, E₈C-LLO and dsFv-B3-R₈C were reduced with 5 mM glutathione (GSH) for 1 h at 20°C and with 2 mM GSH for 40 min at 20°C, respectively. Excess GSH was removed by gel filtration (PD-10 column, GE Healthcare). The conjugation was performed in 25 mM NaH₂PO₄/Na₂HPO₄ (pH 8.5) including 100 mM NaCl and 1 µM CuCl₂ with each protein at a concentration of 3 µM. After 20 h at 22°C, the reaction mixture was supplemented with 1 mM oxidized glutathione (GSSG) for 2 h at 22°C to oxidize the thiol group of the single cysteine of the E₈C-LLO. Subsequently, the reaction mixture was shifted to a pH of 6.5 with H₃PO₄ and diluted 1:20 with 50 mM NaH₂PO₄/Na₂HPO₄ (pH 6.5) containing 1 mM EDTA. Subsequently, the sample was loaded onto an ion exchange column (HiTrap SP HPTM, GE Healthcare), equilibrated in the same buffer. The conjugated product eluted at 30 mM NaCl in a linear gradient of NaCl from 0–1M and thus could be readily separated from the nonconjugated proteins. The protein concentration of the purified immunotoxin B3-LLO was determined spectroscopically using an extinction coefficient of ϵ (280 nm) = 123,910 M⁻¹ cm⁻¹.

Modification of proteins with oxidized glutathione (GSSG)

E₈C-LLO and wtLLO were reduced with 5 mM DTT for 30 min at 20°C. Subsequently, excess DTT was removed by gel filtration (PD-10 column, GE Healthcare) and 50 mM GSSG was added to the protein solution. After 4 h at 22°C, the GSSG was removed by dialysis against 25 mM NaH₂PO₄/Na₂HPO₄ (pH 7.4) containing 200 mM NaCl. Quantitative oxidation of LLO with GSSG was confirmed by mass spectrometry.

Cell culture

The MCF7 (breast carcinoma) cell line was grown in RPMI 1640 Dutch Modification (PAA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 1% (v/v) GlutaMAX I (Gibco), 0.5% (v/v) Gentamicin

(Gibco). The HT-29 (colon carcinoma) cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose, with sodium pyruvate and pyridoxine (Gibco), supplemented with 10% (v/v) FBS, 1% (v/v) GlutaMAX I, and 0.5% (v/v) Gentamicin. The SKBR-3 (breast carcinoma) and HeLa (cervix carcinoma) cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose, with sodium pyruvate (PAA), supplemented with 10% (v/v) FBS, 1% (v/v) GlutaMAX I, and 0.5% (v/v) Gentamicin.

Fluorescence labeling of proteins and imaging techniques

Proteins (dsFv-B3-R₈C, E₈C-LLO, immunotoxin) were labeled with 5-(and 6)-carboxyfluorescein succinimidyl ester (FITC; Molecular Probes) with a spectroscopically determined degree of labeling of 2–3 molecules fluorescein per protein molecule. The labeling procedure was performed according to the manufacturer's specifications. For fluorescence images, cells of MCF7 and HT29 were trypsinized, resuspended in fresh medium, and counted. Approximately 20,000 cells of each cell line were seeded in an 8-well chamber slide (Nunc) and grown overnight. Next day, cells were washed with PBS (PAA) and incubated in incubation buffer (PBS with 3% (v/v) FBS) for 1 h at 4°C with immunotoxin-fluorescein and dsFv-B3-R₈C-fluorescein, respectively, and for 30–60 min at 20°C with E₈C-LLO-fluorescein. In each case, a protein concentration of 0.1 nM was used. Cells were then washed with incubation buffer and images were taken on a fluorescence microscope Axiovert 200M (Carl Zeiss), whereas FITC fluorescence was detected with the filter set 09 (excitation BP 450–490 nm, emission LP 515 nm; Carl Zeiss).

Hemolysis assay

Hemolysis describes the disruption of native erythrocytes and the release of hemoglobin from the cells. Hemolysis data were determined by measuring the differences in light-scattering of intact and lysed erythrocytes. For the hemolysis assay, sheep blood (Dade Behring) was centrifuged for 10 min at 400g (20°C). The erythrocytes were washed three times with 20 mM sodium citrate (pH 5.5) containing 150 mM NaCl for acidic conditions or PBS (PAA) at pH 7.4, respectively, and diluted to a light-scattering signal of 0.8–0.9 at 350 nm. Erythrocyte suspension (900 µL) was added to 100 µL of protein solution, which had varying protein concentrations. After an incubation time of 30 min at 37°C the light-scattering at 350 nm was determined. Alternatively, the release of hemoglobin from disrupted erythrocytes was monitored. Therefore, the cell suspension was centrifuged (5 min at 1700g, 20°C) and the absorbance of the supernatant was measured at 541 nm. The measurements were performed in 20 mM sodium citrate buffer (pH 5.5) con-

taining 150 mM NaCl under acidic conditions and in PBS (PAA) at pH 7.4, respectively. The EC₅₀ value of hemolysis was calculated from the titration curves according to the following equation: $y = y_{\min} + y_{\max} / (1 + 10^{(\log(x) - \log(EC_{50}))})$, where $y_{\min}$ = light scattering signal at the highest toxin concentration; $y_{\max}$ = light scattering signal at the lowest toxin concentration; x = toxin concentration.

Cytotoxicity of the immunotoxin

For the cytotoxicity assay, cells were seeded in 24-well plates with ~25,000 cells per well and grown overnight. Next day, the cells were washed with PBS. Subsequently, medium supplemented with immunotoxin or control proteins, prediluted in PBS, was added at different concentrations. After an incubation time of 48 h (37°C, 5% CO₂, humidified atmosphere), the cells were harvested by trypsinization, washed with PBS, resuspended in 10 mM HEPES/NaOH (pH 7.4) containing 140 mM NaCl and 5 mM CaCl₂, and stained with 1 µg/mL propidium iodide for 15 min at 4°C. The percentage of viable cells was determined by FACS analysis. The data were fitted according to the equation $y = y_{\min} + y_{\max} / (1 + 10^{(\log(x) - \log(IC_{50}))})$, where $y_{\min}$ = % of surviving cells at the highest toxin concentration; $y_{\max}$ = % of surviving cells at the lowest toxin concentration; x = toxin concentration.

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