Exploiting retrograde transport of Shiga-like toxin 1 for the delivery of exogenous antigens into the MHC class I presentation pathway

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Abstract Shiga-like toxin 1 (SLT) from *Escherichia coli* 0157:H7 enters mammalian cells by endocytosis from the cell surface to the endoplasmic reticulum before translocating into the cytosol. Here, SLT was engineered at its N- or C-terminus to carry a peptide derived from influenza virus Matrix protein for delivery to major histocompatibility complex (MHC) class I molecules. We show that SLT N-Ma was capable of sensitising cells for lysis by appropriate cytotoxic T-lymphocytes whilst no killing of SLT-resistant cells was observed. Our results demonstrate that peptide was liberated intracellularly and that retrograde transport of a disarmed cytotoxic protein can intersect the MHC class 1 presentation pathway.
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Key words: Influenza Matrix protein; Shiga-like toxin A chain; Endoplasmic reticulum

1. Introduction

Peptides, derived by proteasomal degradation of proteins in the cytosol, are normally translocated to the endoplasmic reticulum (ER) by peptide transporters associated with antigen processing (TAPs) before they associate with empty major histocompatibility complex (MHC) class 1 molecules in the ER lumen. The complexes are then transported through the secretory pathway to the plasma membrane where they can be recognised by CD8+ T cells (CTL) in a process which ultimately leads to the death of the antigen-presenting cell [1]. Such CTL responses are an important part of the immune response to viral infections and tumours [2–4] and considerable effort is now being expended in developing potential vaccine protocols that might afford protective immunity against a variety of infectious agents and other immune disorders.

A variety of approaches to generate protective CTLs are being tried (see [5] for review). Live viral vaccines, however, being potentially pathogenic, are risky to the recipient, whereas direct immunisation with a viral protein tends to lead to antigen endocytosis and a subsequent class II rather than class I response. Here we show that a disarmed (catalytically inac-

tive) protein toxin, known to be endocytosed from the cell surface to the ER lumen and then transferred to the cytosol of eukaryotic cells, is an alternative potential vehicle for the delivery of peptide epitopes in the MHC class I processing pathway.

Shiga-like toxin 1 (SLT) from *Escherichia coli* 0157:H7 is a Shiga toxin analogue comprising an enzymatic A chain (~ 32 kDa) non-covalently associated with a pentamer of B subunits (7.6 kDa/subunit) [6]. The A chain becomes cleaved during toxin uptake to generate the A1 (~ 29 kDa) and A2 (~ 3 kDa) fragments. The SLT1 holotoxin is a ribosome-inactivating enzyme that normally gains access to its substrates by initially binding to globotriose-ceramide receptors (CD77) on the surface of sensitive cells [7]. SLT1 belongs to a small group of cytotoxic proteins, which includes cholera toxin (CT) [8], whose productive cellular intoxication pathway is known to involve endocytosis from the cell surface to the ER before membrane transport to the cytosol. Intracellular trafficking of SLT1 has been studied in detail and its localisation in the ER of sensitive cells has been visualised [9,10]. We therefore constructed modified holotoxins genetically fused to an influenza virus epitope and investigated their abilities to generate a CD8+ response in vitro.

2. Materials and methods

2.1. Materials
The majority of reagents used were obtained from BDH Chemicals Ltd (UK), Fisons (USA) or Sigma (UK) and were of analytical grade if available. α-[^{35}S]dATP (1000 Ci/mmol), [^{35}S]methionine (3000 Ci/mmol), and γ-[^{32}P]ATP (5000 Ci/mmol) were from Amersham, UK, lactacystin was from Calbiochem (UK), ricin B chain from Inland Laboratories (USA), and recombinant ricin-A chain was from Zeneca Pharmaceuticals (UK). Phosphate-buffered saline (PBS) was (137 mM sodium chloride. 0.27 mM potassium chloride 10.4 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate). HeLa and Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated foetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, 300 µg/ml streptomycin. SKmel-29 and HeLa-A2 cells were grown in RPMI 1640 medium (supplemented with 10% heat inactivated foetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, 300 µg/ml streptomycin) with the latter containing 800 µg/ml (powder weight) geneticin G-418 sulphate. CTL clones were maintained in Iscove's medium (5% human serum, 100 U/ml IL-2) and restimulated every 3 weeks. CTL (5×10^5) were stimulated with matched peripheral blood lymphocytes (2×10^6) and B cells (5×10^5) in 2 ml Iscove's medium (5% human serum, 100 U/ml IL-2, 5 µg/ml pHA) and allowed to proliferate for 1 week, replacing with fresh media.

2.2. Expression and purification of Shiga-like toxin-peptide chimeras
Recombinant SLT fusions were expressed in a 1-l culture of *Escherichia coli* TG2 cells harbouring pUC19 containing the SLT1 bicistronic operon [11] during a 3-h induction with 1 mM isopropyl 1-thio-

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β-D-galactoside. Cells were harvested, resuspended in 20% (w/v) sucrose, 300 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM MgCl$_2$, repelleted and resuspended in 10 ml 1 mM ice-cold Tris-HCl pH 7.5 to osmotically lyse the cells' periplasm and release assembled SLT1 oligomers. The resulting high-speed supernatant was filter-sterilised and applied to a 1 ml synthetic globotriose-Sepharose affinity chromatography column equilibrated in PBS. SLT1 was subsequently eluted with 6 M guanidine hydrochloride, immediately dialysed against PBS overnight and selected samples were quantified and analysed by reducing SDS-PAGE.

2.3. Protein inhibition assays

HeLa-A2 cells were plated in flat-bottomed 96-well plates (1.5×10^4/well) in RPMI+5% foetal calf serum, in a total volume of 150 µl per well. Cells were then incubated with various concentrations of SLT proteins in quadruplicate, for 16 h at 37°C before adding [^{35}S]methionine (1 µCi/well) and incubating for a further 2 h. Cell proteins were precipitated by washing 3 times with 5% trichloroacetic acid, and radioactivity measured by liquid scintillation counting. Protein synthesis was measured by the incorporation of [^{35}S]methionine into protein and a percentage calculated in relation to toxin-free control cells.

2.4. CTL assays

Matrix-specific CTL were generated from peripheral blood lymphocytes purified from healthy volunteers. A CTL clone was isolated by single cell sorting using HLA-A2/Matrix 58-66 tetramers [12] and used as effectors. Targets were HeLa cells stably transfected with HLA-A2 DNA (kindly provided by Dr H. Stauss, Hammersmith Hospital, London). 1×10^6 HeLa-A2 cells were incubated with D167SLT-Ma chimeras (20 ng/ml) overnight. Cells were then washed and labelled with ^{51}Cr (50 µCi) for 90 min at 37°C, washed and incubated for a further 3 h at 37°C. Cells were then plated with CTLs at 5:1 and 2:1 effector/target ratios and incubated for 4 h at 37°C before ^{51}Cr release counting. HeLa A2 cells were treated with IFN-γ (100 U/ml) for 48 h before the incubation with the toxin. As controls, HeLa-A2 cells were infected for 90 min with 5 plaque-forming units (pfu) of vaccinia virus encoding influenza Matrix (M1). Cells were then washed and allowed to express influenza Matrix protein overnight prior to ^{51}Cr labelling. SKmel-29 cells were incubated with SLT N-Ma toxin as described above and the ability of these cells to be sensitised for cytotoxicity was determined using the A2 MAGE-3 peptide 271–279 (10 µM for 1 h). Target cells were then incubated with HLA-A2 MAGE-3 specific CTL (effector/target ratios were 27:1 and 9:1). In some experiments cells were incubated with 5 µg/ml brefeldin A (BFA) for 1 h before addition of toxin or M1, and BFA was maintained at this concentration throughout the assay.

3. Results

A minigene encoding the influenza Matrix 58–66 (M58–66-peptide GILGFVFTL, using the single-letter code) was genetically fused with the 5′- or 3′-terminus of genomic DNA encoding the catalytic A chain of the toxin. The bicistronic operon encoding SLT B chain and the modified A chain permits expression of holotoxins designated SLT N-Ma or SLT C-Ma, depending whether M58–66 is located at the N- or C-terminus of the toxic A chain. The structures of the SLT-peptide chimeras are shown schematically in Fig. 1. To significantly reduce the native cytotoxicity of the toxin portion, an active site mutation (glutamate-167 to aspartate; E167D), previously reported to cause a 1000-fold reduction in protein synthesis-inactivating activity [13], was simultaneously created. Recombinant holotoxins were purified to homogeneity from *E. coli* by receptor-ligand based chromatography using globotriose (α-D-galactose-(1→4)-β-D-galactose-(1→4)-β-D-glucose-(1→)) coupled to a Sepharose matrix (an example is shown in Fig. 2A). By denaturing SDS-PAGE, the peptide-containing A chains were visually larger than native SLT A chain (Fig. 2B), with molecular masses of 33148.47 ± 0.31 and
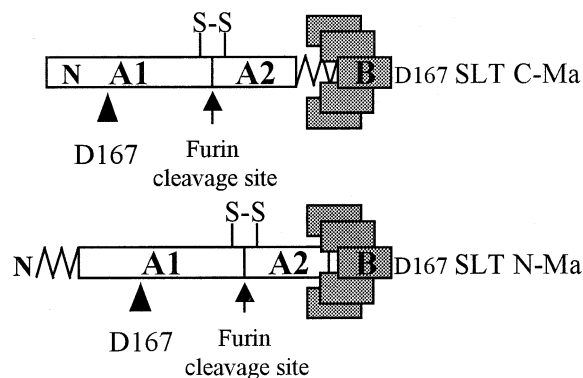
Fig. 1. Schematic representation of SLT-peptide chimeras. SLT holotoxin comprises a single A chain non-covalently associated with a pentamer of B chains. Upon cellular uptake the A chain becomes proteolytically cleaved at a furin recognition site (arrow) into an A1 fragment with catalytic activity and a C-terminal A2 fragment which remains associated with the five B chains (for clarity here the A2 fragment and the B chains are drawn apart) and disulphide bonded with A1. D167 refers to a catalytic site mutation. Influenza Matrix peptide, M58–66 (GILGFVFTL, in single letter code) is denoted by the zig-zag at either the N- or C-terminus of the SLT A chain.

33148.08 Da ± 2.83 for SLT N-Ma and C-Ma respectively, as determined by electrospray ionisation mass spectrometry. The predicted molecular mass is 33148.58. Thus, the influenza Matrix peptide (Ma) remained intact at the termini of the SLT A chains during expression and subsequent purification.

To ensure that the presence of the peptide did not interfere with normal trafficking of the toxin moiety, M58–66 was conjugated to the wild type toxin. When applied to HeLa cells, the peptide/wild type SLT was as potent as SLT without the M58–66 (Fig. 2C) with an IC$_{50}$ value (i.e. concentration of toxin required to inhibit protein synthesis by 50%) of 0.01 ng/ml. Furthermore, when cells were pre-treated with 5 µg/ml BFA to collapse the Golgi stack, the toxicity of wild type SLT-carrying peptide was completely blocked (Fig. 2C). This indicates that the uptake pathway of toxin chimeras was identical to that of native toxin without antigenic peptide. Peptide chimeras engineered using E167D SLT were, as expected, approximately 100-fold less active than native toxin in direct assays on mammalian ribosomes in vitro [14] (data not shown), and 1000-fold less toxic in vivo (IC$_{50}$ 10 ng/ml) (Fig. 2C), supporting the view that SLT can be significantly disarmed by the incorporation of a single mutation that is unlikely to cause major structural perturbation.

Since HeLa cells do not express the required HLA-A2 haplotype, we used HeLa cells stably transfected with HLA-A2 DNA and confirmed the A2 expression by FACS staining (data not shown). HeLa-A2 cells treated with the SLT N-Ma were sensitised for lysis by Matrix 58–66-specific CTL (Fig. 3). In contrast, no lysis was observed after addition of the SLT C-Ma, even though it could be internalised by HeLa-A2 cells (Fig. 2C). To demonstrate that HeLa-A2 cells were capable of processing and presenting intracellular antigens, we showed that infection with a vaccinia virus encoding influenza Matrix protein sensitises them for lysis by influenza-specific CTL. Since a CTL clone was used rather than a CTL line, the background killing against a wild type, non-recombinant vaccinia was negligible [15]. It is of interest that presentation of the M58–66 epitope contained within the full length Matrix
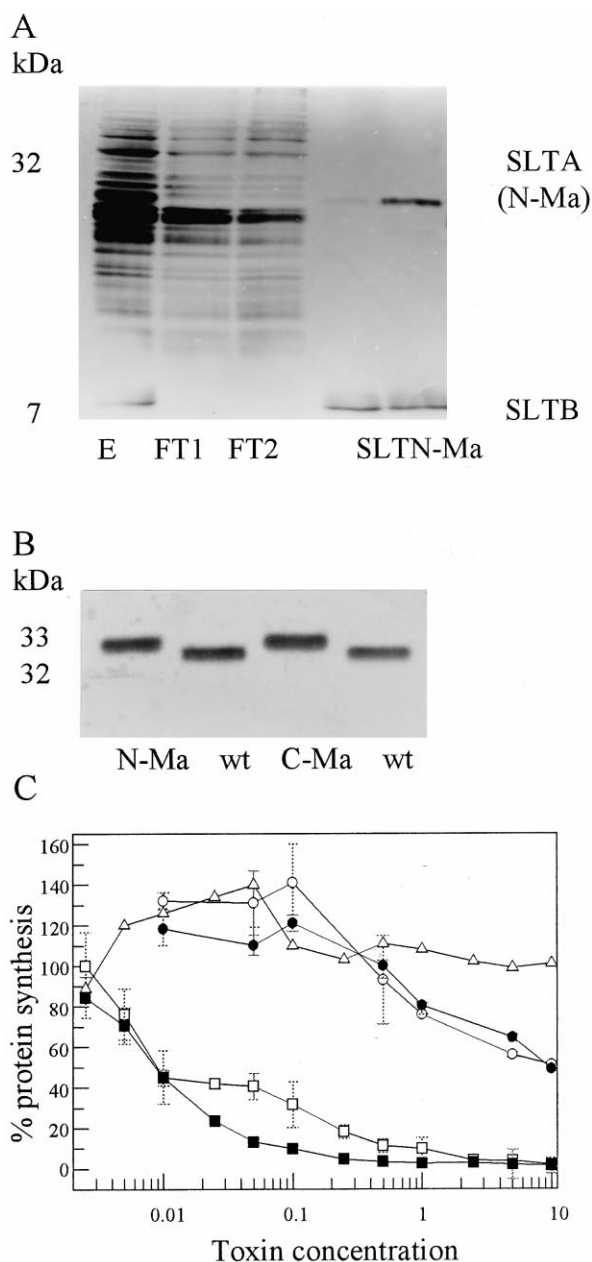
Fig. 2. Purification, sizing and cytotoxicity of SLT-peptide chimeras. A: Expression of recombinant SLT fusions. Samples were subjected to reducing SDS-PAGE and silver staining. E refers to a sample of total *E. coli* extract; FT refers to a sample of column flow through. The positions of SLT A and B chain are indicated. B: Reducing SDS-PAGE comparing the apparent molecular weights of SLT N-Ma and SLT C-Ma A chains with native SLT A chain. C: Toxicity of wild type and D167 SLT-peptide chimeras towards HeLa-A2 cells, and wild type SLT towards SKmel-29 cells. ■, wild type SLT N-Ma; ●, E167D SLT N-Ma; □, wild type SLT C-Ma; ○, E167D SLT C-Ma; ◆, SK-mel; △, HeLa-A2 cells pre-treated with BFA for 1 h prior to addition of wild type SLT N-Ma. BFA was maintained throughout the toxin incubation and pulse labelling period.

Fig. 3. HeLa-A2 cells incubated with SLT N-Ma are sensitised for lysis by Matrix 58–66 specific CTL. A: HeLa-A2 cells were incubated overnight with 20 ng/ml of the D167SLT-Ma chimeras. Cells were then loaded with ^{51}Cr and plated with CTLs at effector/target ratios of 5:1 (black bars) and 2:1 (white bars) as described in Section 2. Where indicated HeLa A2 cells were pre-treated with IFN-γ for 48 h before the addition of toxin. Controls include HeLa-A2 cells infected for 90 min with vaccinia virus encoding influenza Matrix (M1). B: SKmel-29 cells were incubated with SLT N-Ma and the ability of these cells to be sensitised for cytotoxicity was determined by using the A2 MAGE-3 peptide 271–279 and HLA-A2 MAGE-3-specific CTLs at effector/target ratios of 27:1 (black bars) and 9:1 (white bars). C: HeLa-A2 cells were pre-treated with IFN-γ as described in Section 2 and, where indicated, with BFA for 1 h before addition of toxin or M1. BFA was maintained at this concentration throughout the assay.

protein and the SLT N-Ma was enhanced by IFN-γ treatment (Fig. 3A,C). These results, which were observed in seven independent experiments, were consistent with a IFN-γ-dependent upregulation of the class I processing and presentation pathway [16]. Expression of the proteasome MHC-encoded subunits, LMP2 and LMP7, in HeLa cells was significantly

enhanced after incubation with IFN-γ (data not shown), indicating that the class I presentation pathway was indeed upregulated upon incubation with IFN-γ. To confirm that the M58–66 epitope was generated by intracellular proteases rather than serum proteases, we used as target cells the melanoma line SKmel-29, which is naturally resistant to SLT (data not shown). The results of these experiments showed that SKmel-29, which were killed after pulsing with synthetic peptides, were not sensitised for lysis after adding the SLT N-Ma (Fig. 3B).

4. Discussion

In this paper we describe how nanomolar amounts of a disarmed SLT1 A chain carrying, at its N-terminus, a well defined peptide epitope for influenza virus Matrix protein can be used to deliver antigen for intracellular processing and interaction with MHC class I molecules in vitro. This leads to subsequent surface recognition by CTLs in association with MHC class I molecules.

Precisely where in the cell the SLT-Ma chimeras are being processed (ER or cytosol) remains unclear. It is known that SLT holotoxin must reach the ER [9,10] where it is then believed to exploit the ER export machinery normally associated with the ejection of aberrantly folded ER proteins to the cytosol for degradation [17,18]. If processing occurs in the cytosol, this may explain the lack of presentation when using SLT C-Ma. SLT C-Ma has M58–66 located at the C-terminal end of the B chain-binding A2 fragment (Fig. 1), a region that appears not to translocate from the ER to the cytosol. From studies of CT [8], it appears far more likely that only the N-terminal A1 fragment of SLT1 (generated by proteolytic cleavage of holotoxin during endocytosis [19]) is translocationally competent after its reduction from the A2 fragment in the ER lumen. Since only SLT N-Ma contains the antigenic peptide on the toxin A1 fragment, this could explain why SLT N-Ma rather than SLT C-Ma is capable of being properly processed. Surprisingly, the proteasomal inhibitor lactacystin did not block the processing and presentation of Ma from SLT N-Ma (data not shown), as has also been shown for the full length influenza Matrix protein. This observation suggests that non-proteasomal cytosolic proteases may be involved in the generation of the M58–66 epitope.

Treatment of the cells with the Golgi stack-disrupting agent BFA successfully blocked the presentation of the M58–66 epitope by HLA-A2 molecules at the cell surface (Fig. 3C). Toxin uptake is blocked by BFA (Fig. 2C) leading to toxin accumulation within endosomes where it could conceivably interact with MHC class I molecules recycling from the cell surface [20]. The data here however, clearly suggest that the M58–66 epitope from SLT fusions follows the classical class I processing pathway (ER to cell surface via the Golgi) and that its binding to class I molecules does not take place in endosomes. Furthermore, the finding that BFA blocks the cytotoxicities of wild type SLT N-Ma (Fig. 2C) reveals that, upon endocytosis, the toxin fusions follow the same pathway as native SLT, a route that involves passage through the Golgi to the ER for membrane transport, rather than translocation across endosomal membranes.

Other toxins (diphtheria toxin (DT), anthrax toxin and *Pseudomonas* exotoxin A (PE)) have been used to deliver peptide epitopes [21–25] but with one exception [23], the intra-

cellular routing pathway or the location of processing was not known or investigated. Where DT and anthrax toxin lethal factor (LF) were used, translocation of the antigenic peptide presumably occurred from acidified endosomes, the site of LF entry into the cytosol, and processing involved cytosolic proteasomes. PE, like SLT-A, is believed to translocate from the ER, but, unlike SLT, has never been visualised there. Indeed, where PE was used to deliver peptides, BFA was without effect on antigen presentation suggesting that a different internalisation/processing pathway was being used [22]. Recently, the B chain of a relative of SLT, namely the *Shigella dysenteriae* Shiga toxin B chain, has been used to successfully deliver a peptide antigen from the Mage 1 tumour antigen to MHC class 1 proteins [26]. As here, processing and presentation appeared to involve trafficking of toxin chimeras to the ER of target cells. The B fragment of Shiga toxin is believed not to translocate to the cytosol from the ER (our unpublished observations) suggesting that processing of the Mage peptide from the toxin-peptide chimera could occur within this organelle. SLT holotoxin with its membrane translocating A chain component may be of greater utility when whole antigens rather than short peptides are to be delivered. In these instances, it seems reasonable to anticipate a requirement for conventional proteasomal degradation. What remains clear from the present study and that of Lee et al. [26] is that both subunits of the Shiga toxin class of proteins can be used to effectively carry very different antigenic peptides. This is important if such proteins are to fulfill their potential as future T cell vaccines. Moreover, since the ST/SLT family of toxins are known to reach the ER by endocytosis in amounts that permit visualisation by conventional methods, it should now be possible to use these proteins to further probe the MHC class I restricted antigen processing pathway.

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