Construction, expression and characterization of chimaeric toxins containing the ribonucleolytic toxin restrictocin: intracellular mechanism of action

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Restrictocin is a ribonucleolytic toxin produced by the fungus *Aspergillus restrictus*. Two chimaeric toxins containing restrictocin directed at the human transferrin receptor have been constructed. Anti-TFR(scFv)–restrictocin is encoded by a gene produced by fusing the DNA encoding a single-chain antigen-combining region (scFv) of a monoclonal antibody, directed at the human transferrin receptor, at the 5′ end of that encoding restrictocin. The other chimaeric toxin, restrictocin–anti-TFR(scFv), is encoded by a gene fusion containing the DNA encoding the single-chain antigen-combining region of antibody to human transferrin receptor at the 3′ end of the DNA encoding restrictocin. These gene fusions were expressed in *Escherichia coli*, and fusion proteins purified from the inclusion bodies by simple chromatography techniques to near-homogeneity. The two chimaeric toxins were found to be equally active in inhibiting protein synthesis in a cell-free *in vitro* translation assay system. The chimaeric toxins were selectively toxic to the target cells in culture with potent cytotoxic activities. However, restrictocin–anti-TFR(scFv) was more active than anti-TFR(scFv)–restrictocin on all cell lines studied. By using protease and metabolic inhibitors, it can be shown that, to manifest their cytotoxic activity, the restrictocin-containing chimaeric toxins need to be proteolytically processed intracellularly and the free toxin or a fragment thereof thus generated is translocated to the target via a route involving the Golgi apparatus.

INTRODUCTION

Restrictocin is a ribosome-inactivating toxin produced by the fungus *Aspergillus restrictus* which is grouped in a class of protein toxins termed ribotoxins [1]. This class includes two other proteins, α-sarcin and mitogillin, produced by different strains of *Aspergillus* [2,3]. These toxins are shown to be extremely potent inhibitors of translation, and cleave a single phosphodiester bond in the 28S rRNA leading to a total collapse of the protein-synthesis machinery [4]. These toxins catalyse covalent modification of a single nucleotide out of nearly 7000 nucleotides in a mammalian ribosome, which results in the total inactivation of the ribosome and is responsible for the toxicity [5]. Ribosomes of all organisms have been found to be sensitive to inactivation by ribotoxins [6]. The ribotoxin α-sarcin has been shown to inhibit protein synthesis in certain tumour cell lines, albeit when present at very high concentrations [7]. Ribotoxins do not bind to any cell surface receptor and, when introduced inside the cell by artificial means, they manifest extremely potent cytotoxicity [8]. α-Sarcin has been shown to be a powerful inhibitor of protein synthesis in picornavirus-infected cells and also in *Xenopus* oocytes on microinjection [9,10]. Gasset and co-workers [11,12] have demonstrated interaction of α-sarcin with phospholipid vesicles in model systems, which could be an indication of their ability to translocate intracellularly to reach their target in the cytosol.

Protein toxins from bacterial, plant and fungal sources have been successfully employed to make immunotoxins and chimaeric toxins by chemical means and gene-fusion technology respectively to selectively kill cells bearing specific receptors or antigens [13–15]. In the preclinical and clinical evaluation these chimaeric toxins appear to be quite promising for the treatment of a variety of malignancies [13–19]. In addition to their practical application as therapeutic agents, chimaeric toxins are also proving to be useful tools for the study of the mechanisms of toxin action, intracellular translocation and sorting of proteins [20–23]. The mechanism of cell intoxication by protein toxins involves cell binding, intracellular translocation and target modification. Since ribotoxins do not have any intrinsic cell binding activity and have potent toxic activity along with a probable intrinsic translocating activity, they appear to be potential candidates for development as chimaeric toxins.

Earlier ribotoxins have been used in the construction of immunotoxins by chemical means [24–28]. We have found recombinant restrictocin to have poor immunogenic activity, a desirable property for components of immunotoxins/chimaeric toxins, and therefore we made active immunotoxins targeted at the human transferrin receptor [28]. Although chimaeric molecules have been made using the bacterial toxins *Pseudomonas* exotoxin A (PE) and diphtheria toxin (DT), fungal ribonucleolytic toxins, although being equally potent *in vitro*, have not so far been used. Here we report on the construction and characterization of restrictocin-based chimaeric toxins. Single-chain antigen-combining region (scFv) of an anti-(human transferrin receptor) antibody [anti-TFR(scFv)] was used as a model ligand and genetically fused separately at the N- and C-termini of restrictocin. The proteins were expressed in *Escherichia coli* and purified to homogeneity. The chimaeric toxins were tested on a variety of human cancer cell lines for their cytotoxic activity. Also, their intracellular mode of action was investigated with respect to proteolytic processing and translocation.

EXPERIMENTAL

Materials

Restriction and modifying enzymes were purchased from Gibco–BRL or Boehringer. [^{3}H]Leucine and ^{125}I were obtained from Amersham. Reagents for *in vitro* translation assay were from Promega and Pharmacia. All cell culture reagents were

Abbreviations used: Anti-TFR(scFv), single-chain Fv region of anti-(transferrin receptor) antibody; DT, diphtheria toxin; PE, *Pseudomonas* exotoxin A; DMEM, Dulbecco's modified Eagle's medium; Tos-Lys-CH_2Cl, tosyl-lysylchloromethane ('TLCK').

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from Gibco–BRL. All cancer cell lines and 5E9C11, a hybridoma producing the anti-(transferrin receptor) monoclonal antibody HB21, were obtained from the ATCC.

Construction of plasmids

pAnti-TFR(scFv)–restrictocin

pAnti-TFR(scFv)–restrictocin contains DNA coding for anti-TFR(scFv) at the 5′ end of restrictocin DNA under the control of a phage T7 promoter. Plasmid pJB anti-TFR(Fv)-1108 [29], containing anti-TFR(scFv) insert, was used as template to amplify the anti-TFR(scFv) fragment by PCR such that it contained recognition sites for *Xba*I and *Nde*I at the 5′ and 3′ ends respectively. The 760 bp fragment obtained was digested with *Xba*I and *Nde*I and ligated into pRest, which contains restrictocin in a T7-promoter-based bacterial expression vector, which was also digested with the same enzymes. *E. coli* strain DH5α was used for DNA manipulation. The correct clones were identified by restriction analysis and protein expression.

pRestrictocin–anti-TFR(scFv)

pRestrictocin–anti-TFR(scFv) contains DNA coding for anti-TFR(scFv) at the 3′ end of restrictocin DNA under the control of a phage T7 promoter. Restrictocin DNA was amplified by PCR using pRest as template, and *Nde*I recognition sites were created at both the 5′ and 3′ ends of the fragment. DNA coding for anti-TFR(scFv) was amplified by PCR using pJB anti-TFR(Fv)-1108 as template such that the fragment contained *Nde*I and *Eco*RI sites respectively at the 5′ and 3′ ends. A three-fragment ligation was set up with restrictocin fragment digested with *Nde*I, scFv fragment digested with *Nde*I and *Eco*RI and the expression vector, pVex11, digested with *Nde*I and *Eco*RI. The correct clones were identified by restriction analysis and protein expression.

Expression and purification of chimaeric toxins

E. coli strain BL21 (λDE3) was used for expression. Cells were separately transformed with pAnti-TFR(scFv)–restrictocin or pRestrictocin–anti-TFR(scFv) and grown in super broth at 37 °C containing 100 μg/ml ampicillin. The cultures were induced at an A_{600} of 2.0, with 1 mM isopropyl β-D-thiogalactopyranoside, for 2 h. Inclusion bodies were isolated from the total cell pellet and processed using the protocol described [30]. Briefly, inclusion bodies were denatured in guanidinium chloride and reduced by dithioerythritol, followed by renaturation in refolding buffer containing arginine and oxidized glutathione. Renatured material after dialysis was loaded on an S-Sepharose column (Pharmacia), equilibrated with 50 mM sodium phosphate buffer, pH 6.3. Fusion protein was eluted with a linear gradient from 0 to 1 M NaCl in 50 mM sodium phosphate, pH 6.3, using an FPLC system (Pharmacia), and purified to homogeneity by gel-filtration chromatography on a TSK 3000 column (LKB).

Ribonucleolytic activity of chimaeric toxins

The ribonucleolytic activity of fusion proteins and recombinant restrictocin was assayed by measuring the inhibition of protein synthesis in the presence of toxins in a rabbit-reticulocyte-lysate-based *in vitro* translation assay system. Rabbit reticulocyte lysate was prepared and the assay performed as described [31]. Serial dilutions of chimaeric toxins were tested over the range 0.5–500 ng/ml. Incorporation of [^{3}H]leucine was measured as a function of toxin concentration. ID_{50} was calculated by comparing with uninhibited protein samples.

Cytotoxicity and specificity of chimaeric toxins

The activity of fusion proteins was tested on a variety of human cancer cell lines. Protein synthesis in these cells was assayed in the absence and presence of various concentrations of toxins by measuring [^{3}H]leucine incorporation. Adherent cells were plated at a density of 5×10^3/well in 96-well plates, 16 h before the addition of toxin. Cells growing in suspension were seeded at a density of 5×10^3/well in 96-well plates in 80% leucine-free Dulbecco's modified Eagle's medium (DMEM) containing 18% RPMI 1640 and 2% serum, and used immediately. All dilutions of the toxin were made in PBS containing 0.2% human serum albumin. After 48 h, adherent cells were washed twice with leucine-free DMEM and labelled for 2 h with 0.25 μCi of [^{3}H]leucine. Suspensed cells were directly labelled with 0.5 μCi of [^{3}H]leucine for 2 h. The cells were harvested and counted on filtermats using an LKB β-plate counter. The results are expressed as percentage of control, to which no toxin was added. For competition experiments, 10 μg of anti-(transferrin receptor) antibody (HB21) was added per well before the addition of the fusion protein.

Kinetics of protein-synthesis inhibition

The time course of inhibition of protein synthesis was investigated by incubating K562 cells at a density of 2×10^4/well in a 96-well plate with different concentrations of chimaeric toxin at 37 °C for various time periods. At the end of each incubation period, protein synthesis was measured. Results are expressed as described above.

Binding studies

Anti-(transferrin receptor) antibody (HB21) was iodinated by the lactoperoxidase method [32]. ^{125}I-labelled antibody was added as tracer at a concentration of 1.5 ng per assay. HUT102 and A431 cells, at a density of 4×10^5/well, were used for the assay. Cells were washed twice with binding buffer (DMEM containing 0.1% BSA), before the addition of various concentrations of fusion proteins in 0.2 ml of binding buffer. Cells were incubated with shaking for 2 h at room temperature. At the end of the incubation, cells were washed three times with binding buffer, and counted directly in a γ-counter (LKB).

Effect of metabolic inhibitors on the cytotoxicity of chimaeric toxins

K562, A431 and HUT102 cells were used to study the effect of NH_4Cl and brefeldin A. Cells were incubated for 2 h with 5 mM NH_4Cl and then for a further 36 hours after the addition of fusion protein. The effect of brefeldin A was evaluated by adding it at a concentration of 0.05 μg/ml, 18 h after the addition of toxin. Cells were incubated for a further 18 h and then [^{3}H]leucine was added as described above.

Effect of protease inhibitors on the cytotoxicity of chimaeric toxin

A549 cells were used to study the effect of pepstatin A, leupeptin and tosyl-lysylchloromethane (Tos-Lys-CH_2Cl) on the cytotoxicity of chimaeric toxins. Cells were incubated for 48 h with chimaeric toxins in the presence of pepstatin A (0.15 mM), leupeptin (0.40 mM) or Tos-Lys-CH_2Cl (0.27 mM). Cytotoxicity was measured as described.

RESULTS

We have previously shown that immunotoxins constructed with anti-(transferrin receptor) monoclonal antibody and

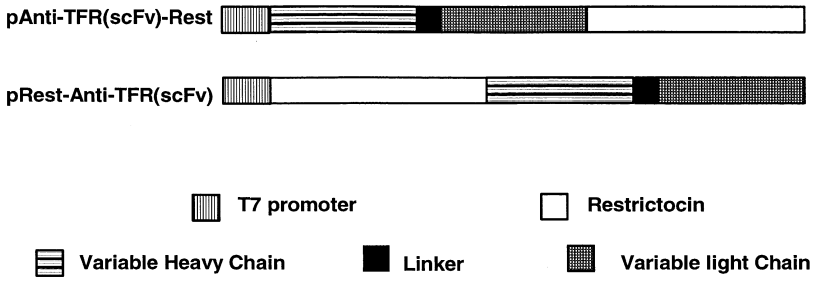

Figure 1 Schematic representation of chimaeric toxins

In anti-TFR(scFv)–restrictocin, anti-TFR(scFv) was fused at the N-terminus of restrictocin, whereas in the case of restrictocin–anti-TFR(scFv) it was fused at the C-terminus of restrictocin. The scFv region contains the first 110 amino acids of the heavy and light chains of the anti-(transferrin receptor) antibody held together by a 15-amino-acid peptide linker composed of $(Gly_4Ser)_3$.

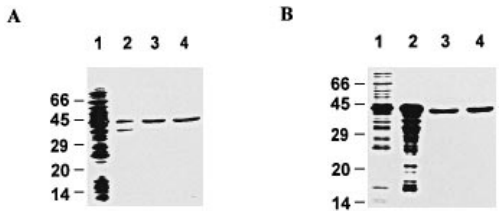

Figure 2 Purification of chimaeric toxins

(**A**) and (**B**) represent anti-TFR(scFv)–restrictocin and restrictocin–anti-TFR(scFv) respectively. An SDS/12% polyacrylamide gel was run and stained with Coomassie Blue. Lane 1, total cell pellet; lane 2, inclusion bodies; lane 3, protein after S-Sepharose column; lane 4, protein after gel filtration. The molecular masses of the markers (in kDa) are shown on the left.

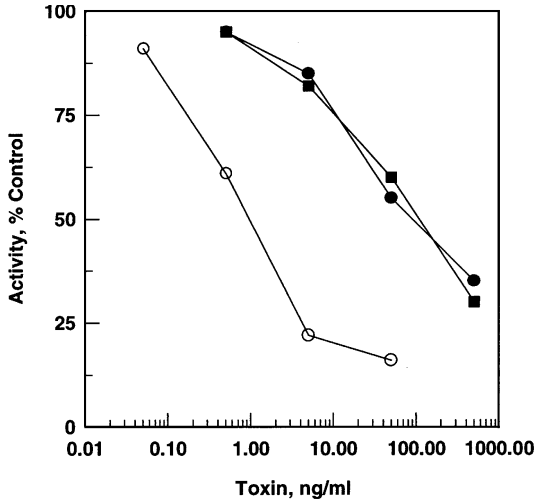

Figure 3 Effect of chimaeric toxins on translation in cell-free assay system

Rabbit reticulocyte lysate was incubated with different concentrations of anti-TFR(scFv)–restrictocin (●), restrictocin–anti-TFR(scFv) (■) and restrictocin (○) for 1 h at 30 °C, and proteins were precipitated with 20% trichloroacetic acid. Precipitated material was collected on a glass-fibre filter and counted on a β-counter for [^{3}H]leucine incorporation.

recombinant restrictocin possess considerable cytotoxicity *in vitro* against a wide variety of target cells [28]. In the present study we constructed chimaeric toxins in which cDNA encoding anti-TFR(scFv) was fused to that for restrictocin. We produced two molecules that differed in the site of attachment of the ligand with respect to the toxin. Human transferrin receptor has been frequently used as a model target for investigating the efficacy of immunotoxins, since it is identified as a marker of rapid cell proliferation and is expressed at higher densities on tumour cells than on most normal cells [17,28,29,33,34]. Transferrin receptor has been successfully used for targeting ricin-A-chain- and DT-based immunotoxins to treat central nervous system malignancies [17].

Construction of chimaeric toxins

DNA encoding anti-TFR(scFv) was cloned separately at the 5′ and 3′ ends of restrictocin DNA in a T7-promoter-based bacterial expression vector to respectively generate gene fusions coding for anti-TFR(scFv)–restrictocin (referred to from here on as Fv–restrictocin) and restrictocin–anti-TFR(scFv) (referred to from here on as restrictocin–Fv). The structures of these constructs are illustrated in Figure 1. The scFv region consists of variable heavy and light chains of the anti-(human transferrin receptor) antibody held together by a 15-amino-acid linker containing $(Gly_4Ser)_3$ and has been shown to contain full antigen-binding activity of the antibody [29]. In the construction of a chimaeric toxin, the site of attachment of the ligand on the toxin can be critical. As chimaeric toxins were being made for the first time with restrictocin, we constructed Fv–restrictocin and restrictocin–Fv, containing the Fv portion of the antibody respectively at the N- and C-termini of restrictocin (Figure 1). In both cases, protein of the expected molecular mass (44 kDa) was overexpressed in *E. coli*, where it accumulated in spheroplasts in the form of insoluble inclusion bodies (Figure 2). Inclusion bodies were purified from the total cell pellet, and solubilized in guanidinium chloride. The reduced and denatured proteins were refolded in an arginine-rich buffer containing oxidized glutathione. After renaturation, proteins were purified by successive chromatography on cation-exchange and gel-filtration columns (Figure 2). Both proteins were more than 90% pure after the gel-filtration step. The yield of restrictocin–Fv was considerably higher than that of the Fv–restrictocin, typical yields being 6–8 mg/l and 1–2 mg/l respectively.

In vitro activity of chimaeric toxins

Protein-synthesis-inhibitory activity of the chimaeric toxins containing restrictocin was evaluated in a cell-free translation assay system containing rabbit reticulocyte lysate to investigate whether extending the N- or C-terminus of restrictocin by fusing proteins alters the activity of the toxin. As shown in Figure 3,

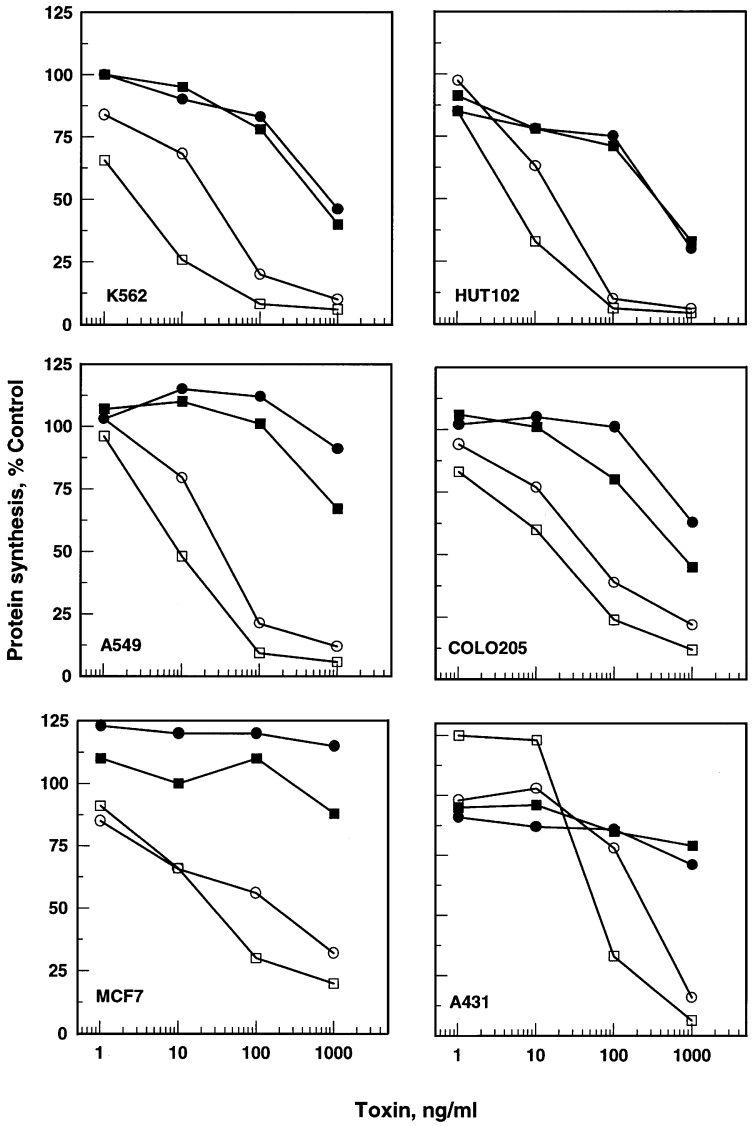

Figure 4 Effect of chimaeric toxins on various cell lines

Anti-TFR(scFv)–restrictocin (○, ●) or restrictocin–anti-TFR(scFv) (□, ■) was added to the cells in the absence (○, □) or presence (●, ■) of excess (50 μg/ml) anti-(transferrin receptor) antibody for 48 h at 37 °C. [^{3}H]Leucine incorporation was measured as described.

Fv–restrictocin and restrictocin–Fv inhibited translation of endogenous globin mRNA in a dose-dependent manner with an ID_{50} of 2.2 nM. Chimaeric toxins were about 30-fold less active than recombinant restrictocin, which had an ID_{50} of 70 pM in the same assay (Figure 3).

Cytotoxicity and specificity of chimaeric toxins

Activity of both the fusion toxins was tested on a variety of human cell lines by a quantitative assay in which their ability to inhibit protein synthesis was measured on target and non-target cell lines. The fusion proteins inhibited protein synthesis in target cells in a dose-dependent manner (Figure 4). Both restrictocin–Fv and Fv–restrictocin showed maximum activity on K562 cells, which express transferrin receptor in excess, with ID_{50} values of 0.05 and 0.56 nM respectively. The chimaeric toxins exhibited similar activities on HUT102 cells, which have fewer transferrin receptors than K562 cells, with ID_{50} values of 0.1 and 0.4 nM for restrictocin–Fv and Fv–restrictocin respectively (Table 1, Figure 4). Invariably, restrictocin–Fv was found to be more active than Fv–restrictocin, the cytotoxicity being 3–12-fold higher depending on the cell line (Table 1). The cytotoxic activity of chimaeric toxins was specific, as addition of an excess of anti-(transferrin receptor) antibody prevented the cytotoxic effect of the chimaeric toxins (Figure 4). In addition, both proteins had no cytotoxic activity on a murine cell line L929, and no inhibition of protein synthesis was observed even at a concentration of 115 nM, indicating the specific binding of the proteins to the human transferrin receptor (Table 1). Restrictocin alone also did not show any activity up to 115 nM (results not shown).

Binding of chimaeric toxins to the transferrin receptor

The cytotoxicity of restrictocin–Fv, depending on the cell line, was 3–12-fold higher than that of Fv–restrictocin. In no case

Table 1 Cytotoxic activity of fusion proteins on various cell lines

Human cancer cell lines K562 (erythroleukaemia), HUT102 (T-cell leukaemia), MCF7 (breast adenocarcinoma), COLO205 (colon adenocarcinoma), A431 (epidermoid carcinoma), A549 (lung carcinoma), HeLa (cervical carcinoma) and L929 (a mouse fibroblast cell line) were used to test the cytotoxic activity of the chimaeric toxins. All assays were carried out at least three times, and variations in the ID_{50} values were within 10%. Relative cytotoxicity is the ratio of activity of restrictocin–anti-TFR(scFv) to anti-TFR(scFv)–restrictocin on the same cell line.

Cell line	ID_{50} (nM)		Relative cytotoxicity
	Restrictocin–Anti-TFR(scFv)	Anti-TFR(scFv)–Restrictocin	
K562	0.05	0.56	11.2
HUT102	0.10	0.40	4.0
A549	0.22	0.68	3.1
COLO205	0.45	1.30	2.9
MCF7	0.63	2.50	4.0
HeLa	0.90	11.30	12.5
A431	1.50	6.80	4.5
L929	> 115.00	> 115.00	

were the cytotoxicities of the two proteins similar or Fv–restrictocin more active than restrictocin–Fv. As the *in vitro* ribonucleolytic activity of the two proteins is similar, the differences in the cytotoxic activities could be due to differences in (i) their affinities for the transferrin receptor or (ii) their intracellular translocation or processing. To check whether the differential cytotoxic activity was due to a difference in the binding of the two proteins to transferrin receptor, competition binding analyses were performed on HUT102 and A431 cells by measuring the ability of the two chimaeric toxins to compete for the binding of ^{125}I-anti-(transferrin receptor) antibody. The two proteins were found to be equally potent in binding to the transferrin receptor (Figure 5). Also, their binding activities were found to be similar to that of the native antibody, indicating that the folding of the ligand was correct (Figure 5). Since the fusion proteins bind the cell surface receptor equally well and have similar activities in the *in vitro* translation assay system, the differences in their cytotoxic activity appear to be due to intracellular factors or events.

Table 2 Kinetics of intoxication of chimaeric toxins

K562 cells at a density of 2×10^4/0.2 ml of medium were incubated with the fusion toxins for various lengths of time. [^{3}H]Leucine incorporation was measured as described.

Time (h)	ID_{50} (ng/ml)	
	Restrictocin–anti-TFR(scFv)	Anti-TFR(scFv)–restrictocin
12	> 1000	> 1000
24	300	1000
36	20	60
48	10	35
60	20	42
72	30	50

Kinetics of protein-synthesis inhibition

To determine the minimum amount of time required to induce maximum inhibition of protein synthesis, K562 cells were incubated with the fusion proteins for various time intervals ranging from 12 to 72 h at 37 °C. Protein synthesis was measured as described. After an initial lag of 12 h, Fv–restrictocin and restrictocin–Fv started to show protein-synthesis-inhibitory activity, and by 24 h had respective ID_{50} values of 1000 and 300 ng/ml. Activities reached a peak by 48 h when the ID_{50} values were 35 and 10 ng/ml respectively (Table 2). The values remained relatively stable for the rest of the experiment (Table 2). Intoxication kinetics of restrictocin-based chimaeric toxins are slow compared with those of PE- and DT-containing toxins, where peak cytotoxicity is observed in less than 24 h [29]. The slow kinetics of intoxication for the restrictocin-based immunotoxins further indicates involvement of an intracellular rate-limiting step subsequent to the internalization of the immunotoxins.

Effect of metabolic inhibitors on the toxicity of fusion protein

On internalization, the endocytosed protein toxins need to be translocated intracellularly to meet the cytosolic target, which may involve different pathways for different toxins [20,35,36]. To trace the intracellular translocation pathway followed by restrictocin-based chimaeric toxins, the effect of NH_4Cl and

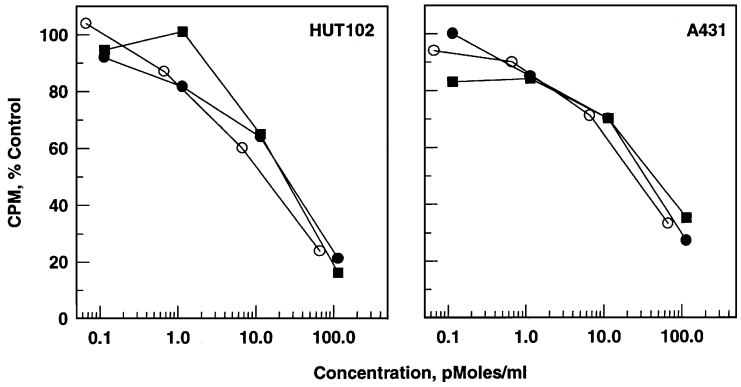

Figure 5 Binding activity of chimaeric toxins

Iodinated anti-(transferrin receptor) antibody was added as tracer with increasing amounts of anti-TFR(scFv)–restrictocin (●), restrictocin–anti-TFR(scFv) (■) or native anti-(transferrin receptor) antibody (○) on HUT102 and A431 cells. Cells were incubated at room temperature for 2 h; bound label was then assessed using a γ-counter.

Table 3 Effect of metabolic inhibitors on the cytotoxicity of chimaeric toxins

K562 cells at a density of 2×10^4/0.2 ml of medium were incubated with chimaeric toxins in the absence or presence of the indicated inhibitors. [^{3}H]Leucine incorporation was measured as described.

	ID_{50} (ng/ml)	
Inhibitors	Restrictocin–anti-TFR(scFv)	Anti-TFR(scFv)–restrictocin
None	8	62
NH_4Cl	10	90
Brefeldin A	200	1200

brefeldin A (which affect the normal functioning of different subcellular organelles) was checked on the cytotoxicity of Fv–restrictocin and restrictocin–Fv. NH_4Cl diffuses readily into the cells in the uncharged form, and, in intracellular acidic compartments, tends to become protonated and unable to diffuse out, leading to an increase in the pH of the endosomal compartment [37]. Brefeldin A causes dissolution of *cis*-, medial and *trans*-Golgi lamellae and blocks the transport of proteins into post-Golgi organelles [38]. NH_4Cl did not affect the activities of Fv–restrictocin and restrictocin–Fv in any of the cell lines tested; results for K562 cells are shown in Table 3. In the same experiment, NH_4Cl protected the cells from the toxicity of DT–anti-TFR(Fv), a chimaeric toxin containing DT fused to the anti-TFR(scFv) (results not shown). An acidic endosomal pH is required for DT activity, and an increase in pH therefore protected the cells from DT toxicity. NH_4Cl alone inhibited protein synthesis by 25–30%. The results have been corrected for this inhibition. Brefeldin A protected the cells from restrictocin–Fv and Fv–restrictocin toxicity, their cytotoxicities decreasing by about 20-fold on K562 cells in the presence of brefeldin A (Table 3). A similar result was obtained for HUT102 and A431 cells (results not shown). A long exposure of cells to brefeldin A was found to be lethal, therefore experiments were carried out using low concentrations of brefeldin A and exposing the cells for only 18 h.

Effect of protease inhibitors on the cytotoxicity of fusion proteins

Chimaeric toxins, subsequent to internalization, may require proteolytic processing to generate an active fragment of the toxin capable of interacting with the intracellular target [39–41]. To investigate the probable involvement of an intracellular proteolytic processing step, the effect of the protease inhibitors pepstatin A, leupeptin and Tos-Lys-CH_2Cl on the cytotoxicity of restrictocin-based chimaeric toxins was investigated. The aspartic protease inhibitor, pepstatin A, strongly inhibits acid proteases such as pepsin [42], whereas leupeptin and Tos-Lys-CH_2Cl prevent the activity of serine and thiol proteases such as trypsin, plasmin, kallikrein, papain and ficin [43,44]. Pepstatin A has previously been shown to inhibit intracellular proteolysis of the ricin A chain [45], and leupeptin has been shown to inhibit intracellular proteolytic processing of DT [41], resulting in reduction in the respective cytotoxicities of these toxins. In the present study, under similar conditions, pepstatin A and leupeptin did not affect the cytotoxic activities of either restrictocin–Fv or Fv–restrictocin (Table 4). Tos-Lys-CH_2Cl, however, protected the cells from the cytotoxicity of both restrictocin–Fv and Fv–restrictocin, the ID_{50} values increasing 26- and 33-fold respectively (Table 4). This indicates that proteolytic processing, perhaps carried out by serine or cysteine protease, is involved in the cytotoxicity of chimaeric toxins containing restrictocin.

Table 4 Effect of protease inhibitors on the cytotoxicity of chimaeric toxins

A549 cells seeded at a density of 5×10^3/well in 96-well plates 16 h before the experiment were incubated with the fusion proteins in the absence or presence of various protease inhibitors for 48 h. [^{3}H]Leucine incorporation was measured as described.

	ID_{50} (ng/ml)	
Protease inhibitor	Restrictocin–anti-TFR(scFv)	Anti-TFR(scFv)–restrictocin
None	4.5	30.0
Pepstatin	6.0	30.0
Leupeptin	6.0	28.0
Tos-Lys-CH_2Cl	115.0	1000.0

DISCUSSION

Recombinant chimaeric toxins are rationally designed fusion proteins in which novel cell-binding specificities are generated by fusing appropriate ligands to toxins such that they are specifically toxic to the target cell types. A variety of toxins have been used to construct chimaeric toxins, but the approach cannot be used universally with every potential toxin to generate a new chimaeric toxin. Ribotoxins, which lack cell-binding activity and contain potent toxic activity with low immunogenicity, are excellent candidates for chimaeric toxin development. In this study we designed and developed active chimaeric toxins using the ribotoxin restrictocin, and studied their probable intracellular mode of action. It has previously been established that the site of attachment of a ligand to a toxin is crucial. For instance, in the case of PE, a ligand can only be attached at the N-terminus, and a free C-terminus is essential for the cytotoxic activity of the molecule, whereas, in contrast, the ligand must be fused to the C-terminus in the case of DT-based chimaeric molecules [46,47]. Thus we have made two chimaeric toxins which have the ligand, anti-TFR(scFv), separately fused at the N- or C-terminus of restrictocin. We have previously shown that the addition of a few amino acids at the N-terminus of restrictocin does not affect its ribonucleolytic activity [48], but the addition of a bulky ligand at either end of restrictocin resulted in an appreciable loss of its ribonucleolytic activity in the present study. The decreased ribonucleolytic activity observed could be due to a change in the folding pattern of restrictocin, and/or steric hindrance of the active site by the bulky ligand. The cytotoxic activities of fusion proteins did not correlate fully with cell surface antigen density. Both proteins were found to be much more active on K562 than A431 cells, even though the two cell types express similar numbers of transferrin receptors. This indicates that, in addition to receptor-mediated endocytosis, intracellular factor(s) also contribute to the cytotoxicity of restrictocin-based chimaeric toxins. This is in agreement with the results obtained previously with chemical conjugates containing restrictocin targeted at the human transferrin receptor using the monoclonal anti-(transferrin receptor) antibody HB21 [28].

After binding to its receptor, a chimaeric toxin is internalized by receptor-mediated endocytosis, followed by proteolytic processing in some cases; subsequently the active fragment of the toxin is translocated to the cytosol [35,36,39–41]. In PE and DT fusion proteins the translocation-competent toxin fragment is generated by a specific proteolytic processing step that occurs

within the target cell [39–41]. It has been shown, using protease inhibitors, that the cytotoxicity of the ricin A chain is dependent on the action of specific proteases, and endosomal proteolysis precedes ricin A chain toxicity in macrophages [45,49]. However, in chimaeric toxins containing ricin A chain, processing is inefficient, and introduction of a proteolytically cleavable spacer sequence dramatically improves the cytotoxicity of these chimaeric toxins [50]. A proteolytic processing step also appears to be involved in the cytotoxic action of restrictocin-based chimaeric toxins. The protease responsible for this processing does not appear to be an acid protease, since pepstatin A did not inhibit the cytotoxic activities of the chimaeric toxins. Although both leupeptin and Tos-Lys-CH_2Cl are strong inhibitors of serine proteases, the cytotoxicity of restrictocin-based chimaeric toxins was prevented only by the latter. This variation in susceptibility to the inhibitors is not surprising, since enzymes using similar catalytic mechanisms could be members of different evolutionary families and thus structurally quite dissimilar [51].

However, the two restrictocin-based chimaeric toxins, which differ in the position of the Fv portion on the toxin, do not differ in their *in vitro* ribonucleolytic activity, receptor-binding activity and intracellular routing, and yet restrictocin–Fv was found to be more cytotoxic than Fv–restrictocin. The precise reason for the differential activity remains to be discovered, but it appears that intracellular proteolytic processing of restrictocin–Fv and Fv–restrictocin might result in restrictocin fragments that differ in translocation efficiency and/or interaction with the target RNA. The activity of both restrictocin–Fv and Fv–restrictocin remained unaffected in the presence of NH_4Cl, indicating that an acidic endosomal environment is not a prerequisite for processing/translocation of the fusion protein, which is in further agreement with the observation that inhibition of acid proteases by pepstatin A did not affect the cytotoxicities of these proteins. Brefeldin A was able to protect cells from the toxicity of both restrictocin–Fv and Fv–restrictocin, indicating that the toxin is translocated intracellularly via a route involving the Golgi apparatus. Brefeldin A has been shown to prevent intoxication by ricin, abrin and modeccin [36,52,53]. A similar effect is seen with PE-based chimaeric toxins [54]. Brefeldin A does not affect DT intoxication, as processing and translocation of the active moiety take place in the endosomal compartment [36]. It is probable that the Golgi is the site of proteolytic processing of restrictocin–based chimaeric toxins. In comparison with other protein toxins, intracellular processing/translocation of the fusion proteins containing restrictocin is apparently less efficient, which could be the reason for the slow kinetics of intoxication.

Targeted therapy employing chimaeric toxins and immunotoxins has great promise, and a number of molecules are at different stages of preclinical and clinical evaluation [15–19]. There are some associated limitations that are now recognized, and attempts are being made to resolve them [15–19]. Two major problems are immunogenicity and dose-limiting toxicity [13,15,17,19]. It has also been realized that smaller chimaeric toxins will have better tumour penetration than the bulky conventional immunotoxins [13]. A small toxin with poor immunogenicity and low non-specific toxic activity would thus be ideal for constructing immunotoxins. Furthermore, once an anti-toxin response begins, a chimaeric toxin directed at the same target but containing a different toxin could be employed to circumvent the neutralization. In this context there is a need to explore novel toxins with desirable properties that could be used as components of chimaeric toxins, and the present study is a step in that direction.

In conclusion, we have shown that it is possible to develop active restrictocin-based chimaeric toxins by placing the binding ligand at the C-terminus of the toxin. After internalization the chimaeric toxins appear to be proteolytically processed, and translocated to the intracellular target via the Golgi apparatus. The first generation of chimaeric toxins developed with the ribotoxin restrictocin are quite potent even though they do not contain the full ribonucleolytic activity of the toxin and it should now be possible to improve the activity of these chimaeric toxins by further engineering.

The work was supported by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India. We thank Professor Sandip K. Basu for a critical review of the manuscript and helpful discussions. The technical assistance of Mr. Kevlanand is acknowledged. D.R. is a Senior Research Fellow of the Council of Scientific and Industrial Research (CSIR).

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Received 16 December 1996/3 February 1997; accepted 12 February 1997