BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **222,** 58–63 (1996)
ARTICLE NO. 0697

Generation of Active Immunotoxins Containing Recombinant Restrictocin

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Received March 29, 1996

Restrictocin, a toxin produced by the fungus *Aspergillus restrictus,* is a potent inhibitor of eukaryotic protein synthesis. Recombinant restrictocin was made in *Escherichia coli* and purified to homogeneity in large amounts. The recombinant protein was found to be poorly immunogenic in mice with low toxicity, when injected intraperitoneally. Two immunotoxins were constructed by coupling the recombinant restrictocin to an antibody to the human transferrin receptor, using a cleavable and a stable linkage. The immunotoxins so generated showed specific cytotoxic activity toward receptor bearing cells in tissue culture. Immunotoxin with a cleavable linkage, however, was more active than that containing a stable linkage. Restrictocin appears to be a promising candidate to be developed as a chimeric toxin for targeted therapy.

Immunotoxins are cytotoxic molecules created by attaching protein toxins to tumor specific monoclonal antibodies. Toxins from bacterial and plant sources have been successfully used to generate active immunotoxins and chimeric toxins for targeting to cancer and virus infected cells [1, 2]. In the early clinical trials, immunotoxins and chimeric toxins containing Ricin and Pseudomonas exotoxin A look promising to be used as a modality to treat cancer either independently or in combination with the chemotherapy [3, 4]. The problems associated with these molecules, identified by the clinical trials, include dose limiting toxicity and immunogenicity which are contributed by the toxin moiety in these hybrid proteins [3]. To circumvent the immunogenicity and toxicity problems, there is a requirement to explore and characterize new toxin molecules with desirable properties, that can be used to construct potent immunotoxins. The toxin restrictocin, produced by *Aspergillus restrictus,* belongs to a class of fungal ribotoxins that are among the most potent inhibitors of translation known [5, 6] They cleave a single phosphodiester bond in the 28S rRNA, in turn inhibiting the translation [7, 8]. Restrictocin lacks a cell binding activity, and needs to be introduced into the cell to manifest its effect [5]. The single chain structure and the potent protein synthesis inhibition activity, of restrictocin makes it a potential candidate to be used in the construction of immunotoxins and recombinant toxins. We have investigated the toxic and immunogenic activities of recombinant restrictocin produced in bacteria. The recombinant restrictocin has been used to construct immunotoxins with an anti-human transferrin receptor antibody, using two different linkers, to target the toxin to cancer cells using transferrin receptor and also to elucidate the probable mode of action of these immunotoxin.

METHODS

Purification of recombinant restrictocin. E. coli cells, strain BL21 (λDE3), were transformed with a plasmid pRest that contains DNA encoding restrictocin under a phage T7 promoter. Restrictocin was isolated from the inclusion bodies and renatured as described [9]. Recombinant protein was purified to homogeneity by successive chromatography on cation exchange and gel filtration columns.

Toxicity and immunogenicity of recombinant restrictocin in mice. Different doses of recombinant protein were intraperitoneally injected, in 6–8 weeks old, Balb/c mice. Animals were observed for signs of toxicity and mortality for 96 hours. LD50 is the dose at which 50% of the animals were killed after 72 hrs of toxin administration.

For immunogenicity studies, Balb/c mice were immunized with restrictocin adsorbed on alum and antibody response against restrictocin in blood samples was evaluated by ELISA.

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Abbreviations: TFR, transferrin receptor, PE, pseudomonas exotoxin A, DT, diphteria toxin

0006-291X/96 $18.00

Construction of immunotoxins. Immunotoxins were made using the methods described [10, 11] Briefly, restrictocin was derivatized with 3-fold molar excess of 2-Iminothiolane. Purified anti-human TFR antibody, HB21, was either derivatized with N-succinimidyl 3-(2-pyridyldithio) propionate or with Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate and separately mixed with the derivatized restrictocin to respectively yield conjugates with cleavable disulfide or non-cleavable thio-ether linkages between the antibody and the toxin. Immunotoxins after conjugation were purified by gel filtration chromatography and only those fractions which did not contain any free antibody or toxin were pooled.

Cytotoxicity assay. Cells were incubated with various dilutions of the toxin made in phosphate buffer saline containing 0.2% human serum albumin. After 48 hours, cells were labeled with [^{3}H] leucine, harvested and counted on filtermats using a LKB β-plate counter. For competition experiments, 10 μg of antibody was added per well prior to the addition of the immunotoxin.

For competition binding analysis, cells were incubated for two hours at room temperature with various concentrations of cleavable and non-cleavable anti-TFR-restrictocin containing ^{125}I-HB21. Cells were washed and counted directly in a gamma counter (LKB). HB21 was iodinated using the lactoperoxidase method [12].

RESULTS AND DISCUSSION

An ideal toxin molecule for use in immunotoxin construction will be one with no non-specific toxicity and poor immunogenicity. Since restrictocin by itself is unable to bind eukaryotic cells because it lacks a cell binding activity, it appears to be a promising molecule for constructing immunotoxins. Earlier, we have cloned the gene for restrictocin and refolded the active protein from the bacterial inclusion bodies. In this study the recombinant toxin was tested *in-vivo,* in mice for its toxicity and immunogenicity. No mortality was observed in mice injected with upto 400μg (i.e. 20mg/kg of body wt.) of restrictocin, while all the animals in the group injected with 800μg of protein died after 72 hours. Thus the LD50 of restrictocin was found to be >20 mg/kg body weight. Restrictocin was found to be poorly immunogenic, as administration of 10μg of restrictocin adsorbed on alum, produced a maximum of 25ng/ml of anti restrictocin antibody, after three weeks, which gradually declined and no detectable antibody levels were present after the fifth week (Figure 1). As compared to other toxins toxicity and immunogenicity of restrictocin in mice appears to be significantly lower [13, 14].

To evaluate the usefulness of recombinant restrictocin in the construction of immunotoxins, it was chemically coupled to a murine monoclonal antibody, HB21, directed against the human transferrin receptor. The transferrin receptor has been shown to be overexpressed in a number of cancers and has been used earlier to evaluate the efficacy of immunotoxins [10, 15]. To generate an immunotoxin, the monoclonal antibody could be coupled to the toxin either by a cleavable

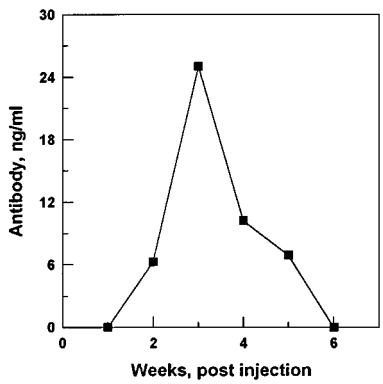

FIG. 1. Immunogenicity of recombinant restrictocin in mice. Recombinant restrictocin, at a concentration of 1mg/ml, was mixed with equal volume of alum and incubated overnight at 4°C. 6–8 week old inbred Balb/c mice were intraperitoneally immunized, with 10μg of restrictocin adsorbed on alum. Blood samples were collected at weekly intervals, and antibody response against restrictocin was evaluated by ELISA using restrictocin as the coating antigen. A sandwich ELISA, using anti-mouse antibody as the coating antigen followed by known amount of mouse IgG and anti-mouse IgG-HRP, was used to plot the standard curve and quantitate the antibody generated against restrictocin.

linkage e.g. disulfide or a stable non-cleavable linkage e.g. thio-ether. Although immunotoxins with a non-cleavable linkage are more stable *in vivo*, not all toxins could be coupled through this linkage. It has been shown that ricin-based immunotoxins are active only when a cleavable linkage is used to couple the toxin with the antibody [1], whereas, PE and DT could be coupled to antibodies with both cleavable and non-cleavable linkages to make active immunotoxins [1]. In this study, the recombinant restrictocin was coupled to the anti-TFR antibody using both a disulfide and a thio-ether linkage to produce a cleavable and a non-cleavable anti-TFR-restrictocin respectively.

The activity of both immunotoxins was tested on a variety of human cancer cell lines, expressing varying numbers of transferrin receptor, by a quantitative assay in which their ability to inhibit protein synthesis was measured. Anti-TFR-restrictocin, with a cleavable linkage, inhibited protein synthesis in a dose dependent manner in most of the cell lines studied, and was most active on HUT 102 cells with an ID_{50} of 30 pM (Figure 2, Table 1). Anti-TFR-restrictocin containing a non-cleavable linkage also was most active on HUT 102 cells with an ID_{50} of 135 pM (Figure 2, Table 1). However, on other cell lines studied, the conjugate with the non-cleavable linkage exhibited very little cytotoxic activity (Table 1). The cytotoxicity of both the conjugates was specific, because in the presence of excess unconjugated antibody there was no inhibition of protein synthesis even at the highest immunotoxin concentration studied (Figure 2). Also, neither conjugates showed cytotoxic activity on a murine cell line L929 thereby establishing their specific binding to the human transferrin receptor (Table 1). Recombinant restrictocin alone did not inhibit protein synthesis in any of the cell lines studied, up to a concentration of 62.5 nM.

To investigate if the differential activities of two immunotoxins were due to differences in their binding affinity for the human transferrin receptor, a competition binding analysis was performed on HUT 102 and A431 cells. Both immunotoxins were found to equally and efficiently compete for binding to the human transferrin receptor and the competition was the same as that with unlabeled native antibody (Figure 3). Toxin alone did not compete for the binding of labeled antibody to its receptor.

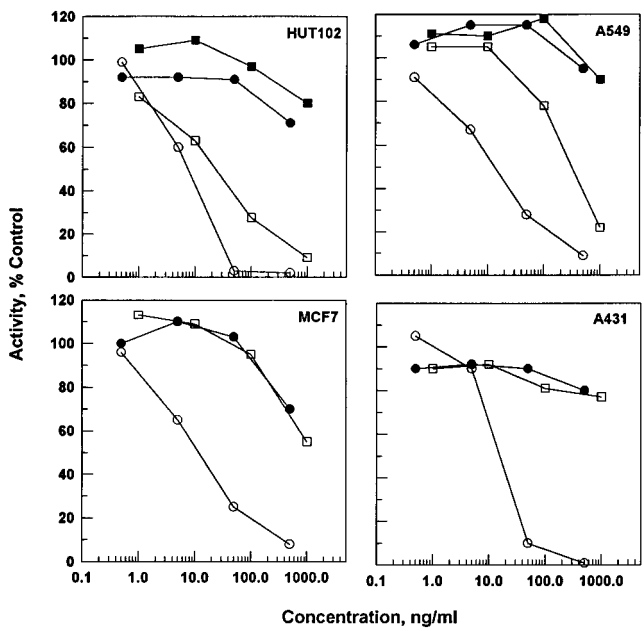

FIG. 2. Activity of anti-TFR-restrictocin on various cell lines. Circles and squares respectively represent cleavable and cleavable anti-TFR-restrictocin. Immunotoxins were added to the cells in the absence (○, □) and presence (●, ■) of excess of anti-TFR antibody (50μg/ml) for 48 hrs at 37°C. [^{3}H] incorporation was measured as described.

TABLE 1
Cytotoxic Activity of Restrictocin Containing Immunotoxins

	ID_{50} (pM)	
Cell line	Cleavable	Non-cleavable
HUT102	30	140
A549	85	1600
U937	170	2000
MCF7	66	>5000
A431	85	>5000
COLO205	80	>5000
DU145	950	>5000
KB	1100	>5000
SK-BR-3	2500	>5000
L929	>25,000	>25,000

Adherent cells were plated at a density of 5×10^3 cells per well in 96 well plates, 16 hours before addition of toxin. Suspension cells were seeded in 80% leucine free DMEM containing 18% RPMI 1640 and 2% serum and used immediately. Cells were incubated with various dilutions of immunoconjugates at 37°C. After 48 hours, adherent cells were washed twice with leucine free DMEM and labeled for two hours with 0.25 μCi of [^{3}H] leucine. Suspension cells were directly labeled with 0.5 μCi of [^{3}H] for two hours. The cells were harvested and counted on filtermats using a LKB β-plate counter. ID50 is the amount of conjugate required to inhibit [^{3}H] leucine incorporation by 50% to that of control where no toxin was added.

We have earlier used the same anti-TFR antibody to make chemical conjugates with a truncated version of PE and the activities reported are similar to those seen with cleavable anti-TFR-restrictocin in the present investigation [10, 15]. The cytotoxic activity of anti-TFR-restrictocin is also comparable to that shown for DT388-Anti-TFR(Fv), a chimeric toxin containing DT and Fv portion of the monoclonal antibody HB21 [15]. Previously, restrictocin and α-sarcin purified from the fungal culture have been used to make immunoconjugates which were found to have poor cytotoxic activity on target cells, ID_{50}'s being in μM range [16–18]. Recombinant mitogillin has been used to produce an immunotoxin which was active on a target cell line [19]. The two

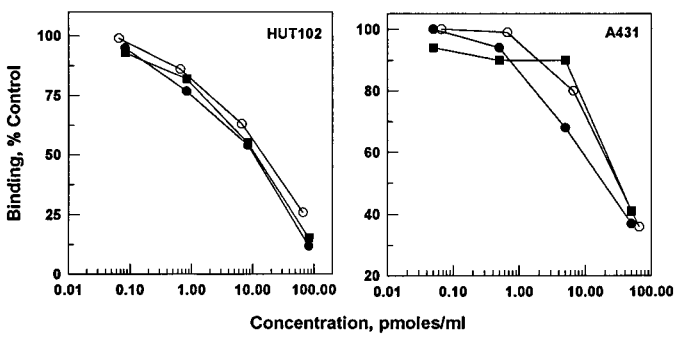

FIG. 3. Binding activity of anti-TFR-restrictocin. ^{125}I-labeled HB21 as tracer was incubated with cleavable anti-TFR-restrictocin (●), non-cleavable anti-TFR-restrictocin (■), and native HB21 (○) on HUT102 and A431 cells. 4×10^5 cells were washed twice with binding buffer (DMEM containing 0.1% BSA) and incubated for two hours at room temperature with various concentrations of cleavable and non-cleavable anti-TFR-restrictocin containing 1.5 ng of ^{125}I-HB21 in 200 μl of binding buffer. Cells were washed thrice with binding buffer and counted directly in a gamma counter (LKB).

conjugates made in this study differ in the nature of linkage between the antibody and recombinant restrictocin. The non-cleavable conjugate was inactive on most of the cell lines studied which implies that for ribotoxin-based immunotoxins to be maximally active, the linkage has to be cleavable. However, the same conjugate does show activity on HUT102 cell line which is also most sensitive to the cleavable conjugate. Since HUT 102 cells have lower number of transferrin receptors as compared to A431, A549, MCF7 and KB, it appears that factor(s) other than receptor mediated endocytosis are also involved in the cytotoxicity of restrictocin-based immunotoxins. The mechanism by which immunotoxins kill cells involves binding to the receptor, internalization and subsequent translocation of the toxin to the target. There may be a processing step involved before translocation, as shown earlier for DT and PE [1], and Ricin A chain containing chimeras [20]. In conjugates containing DT and PE, furin, a protease has been shown to be responsible for the release of the active fragment. In our study, the two restrictocin-based immunotoxins do not differ in receptor binding activity, thus the differential cytotoxic activity may be due to differences in processing and/or translocation. It appears that for cytotoxic activity to be manifested by the restrictocin containing immunotoxins, active toxin or a fragment thereof has to be released from the conjugate. The release of active restrictocin from the cleavable conjugate could be achieved by the hydrolysis of disulfide linkage between the antibody and the toxin, which would not happen with the conjugate having a stable linkage. Since both conjugates in the current study are most active on HUT 102 cells, it appears that these cells produce a protease that might be involved in releasing the active moiety from the toxin molecule. The other cell lines, on which the cleavable conjugate shows potent cytotoxic activity but the stable conjugate is poorly active, may be producing the protease in lower quantities which may not be optimum for the processing of the stable conjugate. Also, since the cleavable conjugate is active on a variety of cell lines, it appears that though the toxin lacks a cell binding activity it does have translocation activity within the molecule.

In conclusion, we have shown that recombinant restrictocin can be used to make potent immunotoxins using appropriate antibodies. The intracellular processing of restrictocin-based immunotoxins may not be ubiquitous and could be a property of selective cells. This could be explored further to generate restrictocin based recombinant chimeric toxins for selective elimination of particular cell types.

ACKNOWLEDGMENTS

The work was supported by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India. Technical assistance of Mr. Kevlanand is acknowledged. Dharmendar Rathore is a Senior Research Fellow of the Council of Scientific and Industrial Research (CSIR).

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