**Pseudomonas exotoxin: recombinant conjugates as therapeutic agents**

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**Pseudomonas exotoxin: structure and function**

*Pseudomonas* exotoxin (PE) is a bacterial protein produced by *P. aeruginosa* that is toxic for most mammalian cells [1, 2]. The mature protein is a proenzyme and, after activation, exhibits both NAD hydrolase activity and ADP-ribosyltransferase activity. When added to mammalian cells the toxin binds to the alpha 2-macroglobulin receptor which mediates internalization to the endosomal compartment [3]. In the endosome the toxin is cleaved ('nicked') by a cellular protease. Disulphide bond reduction then results in the release of an enzymatically active toxin fragment which translocates to the cytosol where it inhibits protein synthesis. In the cytosol, PE hydrolyses NAD and transfers the ADP-ribose moiety of NAD to elongation factor 2 (EF-2). When EF-2 is modified in this way it can no longer participate in the synthesis of new protein. The inactivation of EF-2 is lethal for the cell.

Structural analysis of PE crystals by McKay and colleagues [4] revealed three prominent domains. These were termed domains I, II, and III: domain I was further divided into domains Ia and Ib. This study was followed first by a deletion analysis of the structural gene [5] and then by many individual studies focused on determining the location of important sequences within each domain. The results from all these studies has revealed that domain Ia (amino acids 1-252) is responsible for the toxin binding to its surface receptor; domain II (amino acids 253-364) is the substrate for the cellular protease and, after cleavage, has translocating activity; domain Ib (amino acids 365-399) has no known function and most of it can be deleted without loss of toxin activity; and domain III (amino acids 400-613) has the ADP-ribosylating activity and contains an endoplasmic retention sequence.

A closer look at the interactions of PE with cells has revealed that a lysine at position 57 in domain I is needed for cell binding [6]. Changing this basic residue to an acidic residue such as glutamic acid or inserting a dipeptide reduces binding by 100-fold. After binding the toxin is internalized and cleaved within the endosomal compartment. Cleavage occurs in an arginine-rich loop. Specifically, the peptide bond between arginine 279 and glycine 280 is broken. The toxin is then held together by the disulphide bond that joins cystine 265 with cystine 287. While it is clear that the reduction of this bond is needed to produce an enzymatically active C-terminal fragment of 37 kDa, neither the intracellular location of the reduction step nor the mechanism of reduction are known. After reduction the 37 kDa fragment translocates to the cytosol [7]. Translocation is only successful if PE has an endoplasmic retention-like sequence at its C-terminus [8]. The sequence at the C-terminus of native PE is REDLK. When this is changed to KDEL, a mutant toxin is generated with slightly enhanced (3-fold) toxicity. However, if this sequence is jumbled, extended with other amino acids or removed, there is no toxicity for cells, indicating that an interaction with the endoplasmic reticulum is an important step for toxin translocation to the cytosol. In the cytosol, the C-terminal fragment ADP-ribosylates EF-2. To do this it binds NAD, hydrolyses it, and then transfers the ADP-ribose portion. Glutamic acid 553 has been shown to be important for NAD binding [9] (see Fig. 1 for summary of PE pathway).

**Targeting PE to diseased cells**

Because of its potency, PE has been joined to various targeting ligands and directed to kill specific populations of cells [2, 10]. In early experiments native PE was conjugated chemically to epidermal growth factor [11] or various monoclonal antibodies [12, 13]. These conjugates were active against receptor-positive or antigen-positive cells but retained some of the toxicities of native PE since no major structural changes had been imposed on the toxin. It was not until recombinant versions of the toxin that lacked the toxin's binding domain, termed PE40, were available, that it was possible to make conjugates with high specificity for target cells and low toxicity for normal tissue [2, 14]. Because PE40 lacked binding activity but retained the portions of PE responsible for translocation and enzyme activity, it was considered a suitable candidate for making toxin conjugates. Experiments in this regard involved attaching PE40

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Abbreviations used: PE, *Pseudomonas* exotoxin; EF-2, elongation factor-2; Ab, antibody.
Pathway of PE within mammalian cells

PE binds and is internalized from coated pit structures. When PE is delivered to the endosomal compartment processing begins. The toxin is proteolytically cleaved near Arg 279 to produce major fragments: the N-terminal fragment is 28 kDa and the C-terminal fragment is 37 kDa. The fragments are separated by reduction of the disulphide bond that joins cystines 265 and 287. The C-terminal fragment, containing the ADP-ribosylating activity then translocates to the cytosol and inhibits protein synthesis. PE is shown with its three major domains. For simplicity, domain IIb is shown as part of domain I.

ER = endoplasmic reticulum.

Antibody–PE conjugates and chimerics

Currently antibody–PE (Ab–PE) immunotoxins are made in two different ways. PE40 is chemically attached to a native antibody molecule, or a recombinant Ab–PE40 chimeric is constructed by combining the cDNA for an Ab single chain Fv fragment with the DNA for PE40. Each type of immunotoxin has distinct properties (see Fig. 2). By analysing the results of experimentation with these immunotoxins, insights into toxin-related issues concerning cell biology, animal physiology, pathology and pharmacology can be gained.

In early experiments, conjugating native PE to

Either chemically or genetically to binding ligands and testing for cell killing activity. Results showed that conjugates with potent cytotoxic activities could be produced when PE40 was attached chemically to monoclonal antibodies such as HB21, which recognizes the human transferrin receptor [14], anti-interleukin 2 receptor (anti-Tac) which binds the p55 subunit of the interleukin 2 receptor [15], or B3 [16] which recognizes a Lewis Y carbohydrate on the surface of various carcinomas; or when it was fused genetically to cDNAs for transforming growth factor alpha [17, 18], cytokines or various single chain monoclonal antibodies [15, 19, 20].

Chimera– PE conjugates with PE and its derivatives

The removal of domain Ia eliminates toxin binding but also produces a molecule which can be redirected simply by adding binding ligands of known specificity. As mentioned this can be done in one of two different ways. The PE40 protein can be produced in large amounts in *Escherichia coli* and then purified. It is then conjugated to various monoclonal antibodies. When PE40 was conjugated to the following antibodies, active immunotoxins were produced: antitransferrin receptor (HB21) [14, 21], anti-Tac [14] anti-adenocarcinoma antibody (B3) [16]. These immunotoxins were prepared by using heterobifunctional cross linking agents that formed thioether linkages. When these immunotoxins were targeted to a variety of carcinomas or leukaemic cell lines, they had potent cytotoxic activity.

The removal of the DNA encoding domain Ia and the replacement of it with a specific cDNA also allowed for the construction of gene fusions. The cDNA insert then targets the toxicity of the fusion protein. This strategy has been used to construct a variety of growth factor–toxin, cytokine–toxin and antibody–toxin fusions proteins.

The cDNA for the growth factor, cytokine or antibody is placed at the 5' end of the fusion. This is in contrast to the situation with the gene fusions involving diphtheria toxin where the growth factor is positioned at the 3' end [22]. Fusion proteins are expressed at high levels in *E. coli*. Once purified, the chimeric proteins are directed to receptors on target cells and killing is often directly related, with a small number of exceptions, to the number of receptors on the cell surface [23]. Since the growth factors retain their agonist activity, failure to achieve complete cell killing could result in undesired cell growth [24]. When cells with high levels of the receptor are being targeted, killing is often more efficient with the chimeric toxin than with native PE [18]. For example, on A431 cells transforming growth factor alpha–PE40 is 50 times more potent than native PE.

Several growth factor–PE40 chimeric proteins have demonstrated cell killing activity in tumor models [25–28] and other models of human disease [29, 30]. This has generated a lot of enthusiasm for this approach as a new class of therapeutic agent.
Fig. 2  
Properties of PE40 immunotoxins

When PE40 is attached chemically to a monoclonal antibody the following general properties can be expected: the size of the 1:1 conjugate will be 190,000 Da; the half-life after IV injection will be approximately 2-5 h; the antibody will be bivalent, and unless attachment of PE40 is close to the combining site, there will be no loss of binding affinity; and conjugates can be made with relatively high efficiency. 20% of starting material is typically recovered, and unless attachment of PE40 is close to the combining site, there will be a 3-5-fold decline in binding affinity due to the monovalent nature of the construction; and recovery of correctly folded material is only 5-10%.

When the PEN gene is fused with a single chain (Fv) antibody the following properties can be expected: the size will be 65,000 Da; the half-life after injection will be approximately 15-20 min; unlike the chemical conjugates, there will be a precise attachment of antibody sequences with the toxin; there is usually a 3-5-fold decline in binding affinity due to the monovalent nature of the construction; and recovery of correctly folded material is only 5-10%.

**Properties of PE40 immunotoxins**


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*Endocytosis, Toxins, Immunotoxins and Viruses*
Cell surface and intracellular functions for galactose binding in ricin cytotoxicity

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Introduction

Ricin is a heterodimeric plant protein which is potently cytotoxic to mammalian cells. Both polypeptide subunits of ricin have distinct roles in the intoxication process [1]. The A subunit (RTA) is a ribosomal RNA N-glycosidase which, having entered the target cell cytosol, specifically removes an adenine residue from 28S rRNA (A4324 in the case of rat liver 28S rRNA) [2, 3]. This particular adenine residue, which is present in a highly conserved, surface-exposed loop in 28S rRNA, has a crucial role in the binding of elongation factors during translation. Ribosomes containing depurinated 28S RNA are no longer capable of protein synthesis. The ricin B subunit (RTB) is a lectin which has two galactose binding sites. Interaction between these sites and galactosides present on plasma membrane glycoproteins or glycolipids bind the holotoxin to the surface of target cells [1]. After endocytic uptake of surface-bound toxin, the B subunit is believed to have a further function in that it facilitates, either directly or indirectly, delivery of the toxic A subunit into the target cell cytosol.

Role of RTB in cytotoxicity

The notion that RTB has two functional roles in the intoxication process, binding to cell surface galactose residues and facilitating RTA translocation into the cell cytosol (hereinafter referred to as the binding and translocation functions) arose from studies with ricin-containing immunotoxins. Initially RTA and RTB were separated by reducing the intrachain disulphide bond, and RTA was purified to homogeneity. The RTA was then conjugated via a disulphide bond to a monoclonal antibody raised against a cell type-specific surface antigen. In spite of binding readily to cells bearing the relevant antigen, such RTA-containing immunotoxins frequently displayed disappointingly low cytotoxicity [4]. In contrast, corresponding immunotoxins in which whole ricin was conjugated to the antibody were potently cytotoxic, even when surface binding via the RTB galactose binding sites was prevented by the addition of free galactose or lactose [5, 6]. The greater cytotoxicity of whole ricin containing

Abbreviations used: RTA, ricin A subunit; RTB, ricin B subunit; TGN, trans Golgi network; BFA, brefeldin A; ER, endoplasmic reticulum.

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