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*Review*

# Immunotoxins: The Role of the Toxin †

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† This review is dedicated to the memory of Phil Thorpe, an immunotoxin pioneer and esteemed colleague. He is sorely missed.

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**Abstract:** Immunotoxins are antibody-toxin bifunctional molecules that rely on intracellular toxin action to kill target cells. Target specificity is determined via the binding attributes of the chosen antibody. Mostly, but not exclusively, immunotoxins are purpose-built to kill cancer cells as part of novel treatment approaches. Other applications for immunotoxins include immune regulation and the treatment of viral or parasitic diseases. Here we discuss the utility of protein toxins, of both bacterial and plant origin, joined to antibodies for targeting cancer cells. Finally, while clinical goals are focused on the development of novel cancer treatments, much has been learned about toxin action and intracellular pathways. Thus toxins are considered both medicines for treating human disease and probes of cellular function.

**Keywords:** immunotoxin; antibody; toxin; cancer; immunotherapy; apoptosis; translocation; ricin; diphtheria; *Pseudomonas*

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## 1. Introduction

In the late 1970s three seminal papers set the stage for future immunotoxin development. One by Yamaizumi *et al.* confirmed the potency of diphtheria toxin for mammalian cells [1] and coined the now famous phrase “one molecule of diphtheria toxin (DT) can kill a cell”. Thus the potency of DT

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and similar protein toxins was established. Potency apparently resides in both the turnover rate and intracellular stability of the toxin's enzyme domain. The second paper, by Thorpe *et al.*, introduced the concept of using antibodies to redirect toxin killing activity in a purposeful way [2]. Specifically, the report described the use of anti-lymphocyte antibodies to kill lymphoblastoid tumor cells. The strategy involved the use of chemical linking agents to attach DT to these antibodies and so "early" immunotoxins were born. And, finally, the "antibody world" itself changed as monoclonal antibodies emerged onto the scene [3] allowing for the construction of bimolecular agents with toxins chemically attached to antibodies of a single defined specificity [4]. Then for a while "favorite" monoclonal antibodies were attached chemically to "favorite" toxins and new agents were produced on a regular basis, mostly for cancer therapy [5–9]. The next leap forward involved the application of molecular cloning techniques. This allowed for the production of fusion proteins composed of antibody fragments joined to enzymatically active toxin domains [10,11]. Mostly these fusion proteins were expressed in *E. coli*, which allowed for efficient production of a homogeneous product.

Over 30 years of development, progress with immunotoxins as cancer treatment agents followed a predictable path: promising results in tissue culture systems led to experimentation in animal tumor models which progressed to large animal toxicology/pharmacology studies and then to the planning and implementation of clinical trials. Various immunotoxins derived either from the plant toxin ricin or the bacterial toxins DT or *Pseudomonas* exotoxin (PE) entered clinical trials. Many of these trials are now published with some describing very encouraging results [12–24]. However, also described are dose-limiting toxicities: including vascular leak syndrome, hemolytic uremic syndrome and pluritis [21,25,26]. Improved immunotoxin design should minimize these side effects. To date only one targeted toxin, DT-IL2 (termed denileukin diftitox—trade name Ontak), directed to the IL2 receptor, has been approved for human use [27,28]. The approval of other immunotoxins awaits favorable results from Phase III trials. Despite having a reputation for potency, immunotoxins have been co-administered with "enhancing agents" even from the earliest days—in the hopes of making a good reagent even better [29–31]. Because cancer therapies usually require combination treatments this is not an unreasonable approach: and, in the future, successful immunotoxin development will likely depend on discovering the best agents for co-administration.

While immunotoxins are most frequently studied as cancer therapy agents other uses have been suggested and evaluated—for a recent comprehensive immunotoxin review see Shapira and Benhar [32]. These include modulating immune responses: such as preventing graft versus host disease [33,34], removing T-cells from grafts [35,36] or the elimination T-regulatory cells [37–40]. Some progress has been made also in producing immunotoxins with anti-viral [41–43] or anti-parasitic activity [44]. *Ex-vivo* uses are also anticipated whereby unwanted cells are killed before infusing bone marrow or other stem cell like preparations [45,46].

Immunotoxin experimentation with eukaryotic cells has led directly to the identification of novel toxin features and functional domains. Similarly, the concept of toxins-as-probes of eukaryotic biology has been exploited to uncover previously unknown pathways or properties of cells. A very early example of the latter stemmed from the observation in the 1960s by Kim and Groman that ammonium chloride protected cells from DT [47]. This led to the understanding that endocytic vesicles are maintained at acidic pH. And as we now know, acidic pH is required for DT transport to the cytosol [48,49].

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## 2. Toxin Candidates

Protein toxins first came to prominence as pathogenic factors released by bacteria or poisons ingested from toxic plants and were noteworthy because, as "single agents", they caused such severe morbidity and mortality. Many decades later, it is intriguing to note that several of these toxins share a common biochemical mechanism *i.e.*, they inhibit protein synthesis. DT and PE have similar mechanisms: they ADP-ribosylate elongation factor 2 and halt protein synthesis at the elongation step [50]. Ricin A chain is an *N*-glycosidase and is toxic because it depurinates a critical adenine in 28S rRNA [51]. And it is from these toxins that investigators have turned most often to construct immunotoxins. Originally there were three toxin candidates: the plant toxin ricin (and similar toxins expressed from other plants [52,53]) and the bacterial toxins DT and PE. Because of toxin complexity and reagent loyalty, rarely did individual researchers use more than one toxin, so direct toxin-to-toxin comparisons were seldom undertaken. Even to this day, these three toxins remain among the top choices for immunotoxin development; although, other plant toxins and fungal toxins are also used to make immunotoxins [53,54]. So what were the features that characterize toxin utility?

Toxin structure, orientation of domains, expression and purification yields, ease of cloning, sugar binding, immunogenicity, and non-specific toxicity have each contributed to researchers choosing to work with one toxin over another. Many of these issues have been discussed in a recent review [32] and won't be discussed at length here but a few key points should be mentioned. Each toxin has an active enzyme domain that must reach the cell cytosol to kill cells. Each toxin also has a cell-binding domain that has to be eliminated or nullified before attachment to an antibody. Finally there is the "translocation" function, which may or may not be encompassed in a single functional domain. The "job" of the translocation domain is the transport of the toxin's enzyme domain across an intracellular membrane into the cell cytosol. And, even today, understanding the mechanism or mechanisms of toxin translocation remains a challenge. In broad terms, DT translocates from acidic endosomes with the aid of its T-domain [55], while ricin and PE associate with the ER prior to translocation; although the case for the ER pathway is stronger for PE [56] (with a known KDEL-like sequence at the *C*-terminus) than it is for ricin [57,58]. For each toxin, translocation apparently involves unfolding prior to reaching the cytosol [59,60] and refolding once in the cytosol, leading the speculation that chaperones may be needed for the most efficient translocation [61]. DT and PE have distinct binding and enzymatic domains-at each termini and an alpha helical domain in the middle (Figure 1). The role of the helical domain is more clearly defined for DT than for PE but it is intriguing to note that multi-helical domains of protein toxins may be involved in membrane insertion and possible pore formation [62]. In fact, the membrane insertion of the T domain of DT has been used to model the molecular behavior of Bax and Bak, the proapoptosis Bcl2 proteins that cause pores in mitochondria, leading to the release of cytochrome C and the initiation of apoptosis [62]. In the case of ricin distinct binding (the B chain) and enzyme domains (the A chain) are also defined (Figure 1) while translocation activity is harder to locate precisely. However, it is noteworthy to point out the presence of a 5-helix structure in the middle of the A chain. Ricin A (RTA) is clearly able to translocate to the cytosol when coupled to some monoclonal antibodies [63]. And several trials are on-going evaluating the utility of this form of the toxin [19,63]. However, when RTA is coupled to other antibodies, there is poor cell killing and researchers are "forced" to include the B chain as well [22,64] suggesting that in

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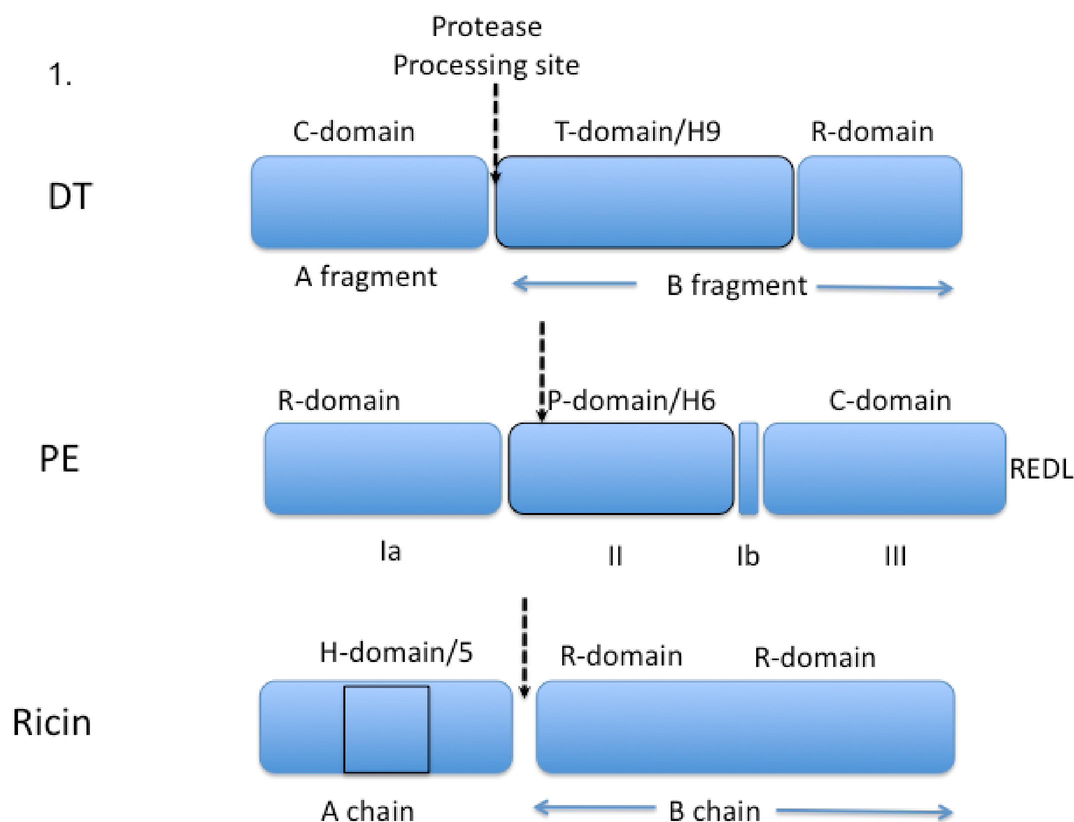
some instances the B chain is needed to direct the routing of the A chain. To nullify normal B chain binding to surface galactose residues, immunotoxins were developed using "blocked" ricin. Blocked ricin retains sugar-binding residues but their active sites are blocked via chemical modification. Retaining the entire B-chain, albeit with reduced binding activity, has also been reported for DT immunotoxins constructed with CRM9 [35,36].

**Figure 1.** Graphic representations of three toxins, diphtheria toxin (DT), *Pseudomonas* exotoxin (PE) and the plant toxin, ricin. Above each "domain" is a functional label. Below each domain is a common name that was used in early publications. DT has an *N*-terminus catalytic domain (C-domain) also known and the A fragment followed by a protease processing site, then a nine helix domain (commonly known as the "T" or translocation-domain) followed by a receptor binding domain (R-domain). The B-fragment includes both the T-domain and the R-domain. PE has an *N*-terminal receptor-binding domain followed by a processing domain. Then at the *C*-terminus there is a catalytic domain followed by a KDEL-like sequence. Ricin has a catalytic domain at the *N*-terminus, followed by a processing site and then a duplicated receptor-binding domain with a preference for binding galactose residues. Each toxin has a helical domain where several helices follow in close sequence. For DT there are nine helices while PE has 6; and these helices are arranged in what appears to be a separate domain between C and R-domains. Ricin also has a cluster of helices but these are located in the middle of its catalytic domain. A simple view of these helical domains is that they function in the translocation of each toxin's C-domain. However, this has only been established for the T-domain of DT. The site of proteolytic processing is shown for each toxin.

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Finally, toxins that interact with mammalian cells invariably need "processing" steps to convert a precursor molecule to an active one [65]. In addition, toxins act in the cell cytosol and must reach their destination via a collaboration between the toxin and the target cell [57]. By tracking the fate of toxin molecules one can learn about cellular functions and thus toxins are probes for the cells they attack. For DT and PE minimum processing includes a protease cleavage step [66] (Figure 1) and a reduction of a key disulfide bond [67]. Other features include a transient unfolding step-followed by refolding [61] For PE, there is a KDEL-like ER retention sequence at the *C*-terminus that is essential for cell killing activity [56]. So for PE and PE-derived immunotoxins there are four known steps prior to reaching the cytosol: (1) receptor binding, (2) furin cleavage, (3) disulfide reduction and (4) interaction with KDEL receptor 2. For DT in addition to protease "nicking" there is a cytosolic chaperone and reductase that have been identified as being important for toxin action [61]. Proteolytic processing of ricin occurs in the germinating castor bean, producing the A and B chains (Figure 1). Because ricin interacts with terminal galactose residues displayed on many different surface receptors, tracking its fate can be challenging [68,69]. Ricin also requires an intracellular reduction step. Recent studies using RNAi highlighted important genes in the ricin pathway and compared these with genes involved in PE intoxication [70,71]. These genetic screens along with chemical screens to identify anti-ricin compounds should provide new insights into ricin's intracellular trafficking pathway [72].

## 3. Early Immunotoxin Development

Thorpe *et al.* set the stage for immunotoxin development by confirming that protein toxins could be redirected to kill selected cell types over bystander cells [2]. However, their result was achieved with a poorly defined antibody preparation. Using the same concept but with the benefit of Kohler and Milstein's monoclonal antibody technology [3,73], well defined immunotoxins of a single specificity were produced. These included, ricin-, DT- and PE-derived immunotoxins. Besides antibody and toxin selection, other steps in the manufacture of immunotoxins included the use of different chemical "glues" (called cross linkers) to join the two molecules in a manner that kept both parts functional [74,75]. Early on it was appreciated that antibodies alone were rarely cytotoxic. This fueled research into making antibodies more potent by attaching protein toxins to them. Potency depended not only on internalization but also on the "correct" internal conditions within the cell. For instance, in the case of early immunotoxins to CD5 made with the T101 antibody, neutralization of acidic pH was deemed important for optimal killing [76]. In other immunotoxins, disulfide linkers allowed for cytotoxic activity while thioether linkers did not, confirming the need for the appropriate reducing environment to allow separation of toxin from antibody [75,77].

For PE the first immunotoxins were made via thioether linkage from an intact monoclonal antibody to the native intact toxin (Figure 2B). When the functions of the toxin's structural domain were discovered, it made sense to delete the receptor binding domain, producing a molecule termed PE40-based on its molecular weight. However, the deletion of the *N*-terminal domain (harboring many lysine residues for chemical conjugation) created a problem of how to attach PE40 to antibodies. This was solved by the introduction of a novel lysine residue near the terminus of PE40, producing Lys-PE40 (Figure 2C). Together, these chemical conjugates made up first and second generations of immunotoxins.

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