Immunotoxins: magic bullets or misguided missiles?

Ellen S. Vitetta, Philip E. Thorpe and Jonathan W. Uhr

Thirteen years have passed since specific in vitro *and* in vivo *killing of tumor cells by immunotoxins was first described. Why, then, has it taken so long to determine whether these pharmaceuticals will have a major impact on the treatment of cancer, AIDS and autoimmune disease? The answer is that the transfer of basic discoveries to the clinic is a slow, multistep, interdisciplinary process. Thus, immunotoxin molecules must be designed and redesigned by the basic scientist depending on the efficacy and toxicity shown* in vitro *and in relevant experimental models. Next, each version must be evaluated by clinicians in humans through a lengthy process (1–3 years) in which the dose regimen is optimized and in which new problems and issues frequently emerge. These problems must again be modeled and studied in animals before additional clinical trials are initiated. In this article, Ellen Vitetta and colleagues discuss both basic and clinical aspects of the development of immunotoxin therapy.*

Immunotoxins are chimeric molecules in which cell-binding ligands are coupled to toxins or their subunits. If the ligand moiety is tumor cell-specific, the immunotoxin should kill tumor cells selectively, unlike conventional chemotherapy and radiotherapy, which kill rapidly dividing or metabolizing cells, whether malignant or normal.

Components of an immunotoxin

The toxins used for different types of immunotoxins are depicted in Table 1, and their components, ligand, toxin and crosslinker, are discussed below[1].

Ligand

Monoclonal antibodies The ligand most frequently used is a cell-reactive monoclonal antibody 'mAb)[1]. Although tumor-reactive mAbs often react with some normal tissue, crossreactivity does not necessarily prohibit their use. Thus, low antigen density, anatomical barriers or poor endocytosis could prevent the killing of a cell that has a crossreacting antigen[1,2]. Conversely, some cross-reactions not detectable by conventional techniques can damage life-sustaining tissues[3]. Hence, a primate model in which the mAb reacts with the primate antigen is desirable to test the safety of an immunotoxin to be used in humans.

Only a proportion of mAbs make potent immunotoxins[4]. Depending on their specificity, they may not be internalized or, if they are, they may not be routed to the appropriate intracellular compartment for translocation of their attached toxin into the cytosol. Hence, mAbs must also be screened for effectiveness as carriers of toxin.

mAbs can be used as intact molecules or as fragments[1]. While fragments are less immunogenic, they have a shorter half-life *in vivo* and are often partially inactivated by their coupling to toxins. These problems should be circumvented by generating fusion proteins containing portions of the constant regions of the heavy chain, which confer a long half-life in the circulation.

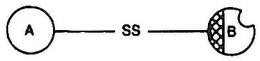
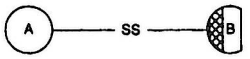
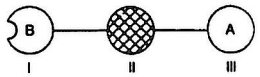
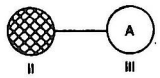
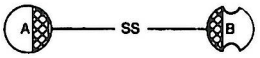
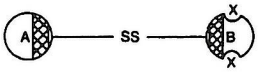
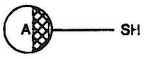
Growth factors Other ligands for preparing immunotoxins are growth factors[5,6]. Although these bind to normal cells, tumor cells frequently express elevated levels of growth factor receptors. Advantages of using growth factors as ligands include their relative lack of immunogenicity, high affinity for their receptors, and the availability of cloned genes for generating fusion proteins. Problems include rapid *in vivo* clearance, stimulation of target cells by small amounts of bound immunotoxin insufficient to kill the cells, and the presence of circulating ligands or soluble receptors that compete for the immunotoxin.

Toxin

The toxins used for immunotoxins are derived from bacteria and plants and all inhibit protein synthesis (as described below; and see Table 1). Unlike chemotherapeutic agents, these toxins kill both resting and dividing cells. Hence, as immunotoxins, they have the potential to kill tumor cells that are not in cycle at the time of treatment (dormant tumor cells) and that may be spared by conventional chemotherapy. These toxins share common features[7]:

1 They are all synthesized as single chain proteins and are processed either post translationally or in the

Table 1. Structure and function of toxins and RIPs used for immunotoxins

Toxin	Structure of mature form	Toxin receptor	A-chain action	LD_{50} of immunotoxins (mice, mg kg^{-1} total protein)
Diphtheria toxin (DT)		heparin-binding epidermal growth factor-like precursor	ADP-ribosylation of elongation factor 2	0.3
Truncated diphtheria toxin (DAB486)		none	ADP-ribosylation of elongation factor 2	>1.0
Pseudomonas exotoxin (PE)		α_2-macroglobulin receptor-like molecule	ADP-ribosylation of elongation factor 2	0.1
Truncated *Pseudomonas* exotoxin (PE40)		none	ADP-ribosylation of elongation factor 2	2.0
Ricin/abrin		galactose	*N*-glycosidase for 28S ribosomal RNA	0.1–0.2
Blocked ricin/abrin		none	*N*-glycosidase for 28S ribosomal RNA	0.4–0.8
Ricin toxin A-chain (RTA)		none	*N*-glycosidase for 28S ribosomal RNA	20
Ribosome inactivating protein (RIP)		none	*N*-glycosidase for 28S ribosomal RNA	5–20

A, B: different polypeptide chains; ⊠: hydrophobic region in the polypeptide; indentations: cell-binding sites; X: partial or complete blockade of lectin activity at the binding site.

target cell to which they are delivered into two-chain molecules with interchain disulfide bonds.

2 The disulfide bond linking the two chains is critical for cytotoxicity.

3 All toxins have subunits or domains devoted to binding to cells, translocation across membranes, and the destruction of protein synthesis in the cell. These domains can be separated or genetically manipulated to delete those that are unwanted.

Plant toxins The most widely used plant toxins, ricin and abrin, consist of two disulfide-linked polypeptides, A and B (Ref. 8). The toxin binds via the B-chain to galactose-containing glycoproteins and glycolipids that are present on the surface of all cell types. The toxin is then endocytosed and routed to the trans-Golgi network which is believed to be the site where the A-chain translocates to the cytosol. The A-chain then kills the cell by enzymatically removing a crucial adenine residue from the 60S ribosomal subunit which is needed for the binding of elongation factor 2 (EF-2) during protein synthesis[9]. Ribosome inactivating proteins (RIPs) are single-chain proteins found in many plants[10], and have the same enzymatic properties as the A-chain of ricin[11].

Bacterial toxins The active form of diphtheria toxin (DT) is a disulfide-bonded two-chain molecule[12]. The toxin binds via the B-chain to an epidermal growth factor-like receptor that is present on most cell types in DT-sensitive species[13]. The toxin is then endocytosed and, within an acidic intracellular compartment, the B-chain undergoes a conformational change to expose hydrophobic regions, which are thought to be important in enabling the A-chain to translocate across the membrane to the cytosol[14]. The A-chain then kills the cell by catalysing a modification of EF-2 that prevents its participation in protein synthesis[15].

Pseudomonas exotoxin (PE) is produced by the bacterium as a single-chain protein[16]. It binds via its N-terminal region (domain I) to an α_2-macroglobulin receptor-like molecule present on the surface of most cell types[17]. The toxin is then endocytosed and becomes converted through the action of proteolytic enzymes into a disulfide-bonded two-chain form[18]. The C-terminus of domain III (the equivalent of the A-chain) has an endoplasmic reticulum retention sequence, REDLK, which causes the toxin to concentrate in the endoplasmic reticulum – probably the site where domain III enters the cytosol. Once in the cytosol, the toxin kills the cell in the same manner as DT.

Crosslinker

The crosslinker used to join the ligand and the toxin must remain stable extracellularly but be labile intracellularly so that the toxic fragment can enter the cytosol. The choice of crosslinker depends on whether intact toxins, A-chains or RIPs are used. A-chains and RIPs are coupled to ligands using linkers that introduce a disulfide bond between the ligand and the A-chain[1,20]. Bonds that cannot be reduced render these immunotoxins much less toxic or nontoxic probably because the A-chain must be released from the ligand by reduction to be cytotoxic[21]. Such immunotoxins tend to be labile *in vivo* unless hindered crosslinkers are used. These crosslinkers place bulky groups around the disulfide bond to protect it from attack by thiols in the blood and tissues[1]. Intact toxins are usually linked to ligands using nonreducible (e.g. thioether) linkages to prevent release of active free toxin *in vivo*. Recombinant immunotoxins have been prepared by splicing the genes encoding truncated DT (e.g. DAB486) or *Pseudomonas* exotoxin (e.g. PE40) to the gene encoding the ligand and expressing the entire immunotoxin as a fusion protein[22]. Recombinant immunotoxins are highly stable *in vivo* because they contain nonreducible peptide bonds.

Preclinical evaluation

Cytotoxic potency and specificity

Immunotoxins prepared from holotoxins (i.e. complete toxins containing both A- and B-chains or analogous domains) are usually more potent than those containing A-chains (or RIPs) because the toxin moiety can interact with toxin receptors in or on the target cell[23,24]. This enables the immunotoxin to enter and kill the cell by the highly efficient entry pathway used by the native toxin. Predictably, however, holotoxin-containing immunotoxins are highly toxic to animals because they can bind to toxin receptors that are present on non-target cells. The problem of nonspecific toxicity can be reduced with ricin-containing immunotoxins by reversibly blocking the galactose-binding sites of the toxin either sterically by the ligand itself or with galactose-based affinity labels[1,25]. Such 'blocked' ricin immunotoxins appear to act by being degraded inside the cell to release unblocked ricin or ricin fragments.

Immunotoxins prepared from ricin A-chain or RIPs are highly specific for their designated target cells but vary in potency depending on the affinity of the ligand, the cell surface molecule and epitope that it recognizes and the capacity of that molecule to enter an intracellular compartment that is favorable for toxin translocation[1,26,27]. Immunotoxins prepared from DAB486 or PE40, which lack cell-binding domains, are also highly specific in their cytotoxic action on target cells[22,28]. It is unclear whether immunotoxins containing DAB486 or PE40 are more uniformly cytotoxic than their A-chain counterparts, as might be expected if the truncated toxins have, for example, hydrophobic regions that assist the entry of the enzymatic fragment or subunit into the cytosol. Thus far, the evidence with PE40-containing immunotoxins is that they have variability in potency similar to that of A-chain immunotoxins.

Immunotoxins prepared with A-chains can often be made more potent by lysosomotropic amines and carboxylic ionophores, which inhibit the fusion of endosomes with lysosomes (where the A-chains are destroyed) or retard the transit of the immunotoxins through a compartment favorable for A-chain translocation[29,30].

Toxicity

Many immunotoxins can cause hepatotoxicity[1] (Tables 1 and 2). In the case of ricin A-chain (RTA), mannose- and fucose-containing oligosaccharides bind to liver cells leading to rapid clearance and hepatic damage[1]. This problem has been successfully circumvented either by deglycosylating RTA (chemically or enzymatically)[1] or using recombinant RTA (expressed in a non-glycosylating cell)[31]. In the case of blocked ricin immunotoxins, the oligosaccharides on the A- and B-chains and the affinity labels used to block the B-chain's lectin sites result in liver homing and liver damage[32]. Bacterial toxins and RIPs produce hepatotoxicity by binding to molecules other than carbohydrate receptors on liver cells or by binding to serum proteins that have receptors in the liver[1,33].

RTA-based immunotoxins cause vascular leak in humans, which is manifested by extravasation of fluids and proteins from the vasculature into the periphery causing edema and weight gain, and, occasionally, life-threatening pulmonary edema[1,34]. The mechanisms underlying vascular leak are not known, although recent evidence suggests that they may be related to the binding of the RTA to vascular endothelial cells[35]. In addition, these immunotoxins cause myalgias (rarely, rhabdomyolysis) via unknown mechanisms.

Pharmacokinetics

An effective immunotoxin must have a serum half-life of sufficient duration for a cytotoxic quantity of it to access the target cells. When the target cells are intravascular (for example, circulating tumor cells or normal lymphocytes), access is not a problem and the immunotoxins are highly effective, but when the target cells reside in large solid tumor masses with a poor blood supply and high interstitial pressure[36], the need for a long serum half-life becomes critical. The half-life of immunotoxins prepared with mAbs is longest when the mAbs are intact, the crosslinker is stable and the toxin moiety does not bind to normal tissues. In contrast, when the ligand is an antibody fragment or growth factor, the crosslinker is not stable, or the toxin displays some nonspecific binding, the half-life is short[37]. The problem of a rapid half-life can be partially solved by continuous intravenous infusion of the immunotoxin, although increasing the half-life may also increase the likelihood that these immunotoxins will gain access to other tissues and cause unwanted toxicities.

Immunogenicity

Individuals with a functional immune system make antitoxin antibodies even when humanized antibodies or human growth factors are used as carriers[34,38]. Strategies to decrease such immunogenicity, such as

concomitant administration of immunosuppressive drugs, have not yet been successful in humans[34]. In contrast, multiple courses of immunotoxin can be given to highly immunosuppressed individuals, such as B-cell lymphoma patients, without a resultant immune response[39]. Even in these patients, when earlier disease is treated, immunogenicity will become a problem. Circulating antibodies can inhibit the efficacy of immunotoxins by increasing their rate of clearance, and/or by blocking the binding site on the antibody or the enzymatic site on the toxin. Despite these considerations, immunotoxins have been administered in the face of serum antibody and, in some cases, have been effective[38]. With immunotoxins of a very short half-life (e.g. IL-2-DAB486; Ref. 38), the binding of non-neutralizing antibody may, in fact, increase the half-life. Nevertheless, immunogenicity will remain a problem until the entire immunotoxin is humanized. This may be possible by using human 'toxins', such as ribonuclease, attached to human antibody[40]. However, even this strategy may not avoid the formation of new immunogenic epitopes created by linking autologous proteins.

Immunotoxin-resistant mutants

In several rodent tumor models, immunotoxins have produced excellent tumor regressions but have failed to cure the animals because immunotoxin-resistant tumor cells emerge[41]. These are usually antigen-deficient mutants whose outgrowth can be prevented by administering immunotoxin cocktails directed against alternative tumor-associated antigens[42,43]. However, mutants have also been observed that have defects in intracellular transport of the endocytosed immunotoxin[41]. Importantly, mutants with toxin-resistant ribosomes have not been observed, suggesting that such mutations may be lethal.

Difficulties in evaluating immunotoxins

From experimental studies and theoretical considerations, the optimal efficacy of immunotoxin should be obtained by administration of a single short course in patients with minimal[1], dormant[44], or premalignant[45] disease. The latter is a particularly attractive state for intervention since the development of full-blown malignancy appears to require an additional rare, stochastically determined genetic event. Hence, killing of 100–1000 premalignant cells would probably prevent development of malignancy.

However, the design of clinical trials does not allow this strategy to be tested readily. The initial trials (Phase I) require treatment of patients with intractable disease. Dose escalations of the drug are performed in small cohorts of patients until the maximally tolerated dose (MTD) is established. Side-effects, pharmacokinetics, and immunogenicity are analysed. As in most Phase I clinical trials, clinical benefit is unlikely to occur because the patients have far-advanced, bulky tumors and organ damage from previous therapy. Alterations in the drug or the protocol are usually not acceptable until completion of the trial. Therefore, lack of efficacy in a Phase I trial should not preclude further clinical testing of the drug.

The drug is then administered at a safe dose to patients with less advanced disease to determine efficacy (Phase II trial). Generally, a response rate of 20–40% (partial or complete remissions) must be observed at Phase II, or drug development is halted. This may be too stringent a criterion for an agent that is likely to be most effective when used in the treatment of minimal disease and in combination with one or two other immunotoxins and chemotherapy. An additional problem is that an MTD established in patients with bulky tumor may be very different from that in patients with minimal disease.

Phase III trials require several hundred patients to be treated in multiple clinical centers along with controls (who usually receive placebos or the current 'best' therapy) and, therefore, requires support by a pharmaceutical company. The result of the above considerations is that few immunotoxins have proceeded beyond the stage of Phase I or II trials. Therefore it might be wiser to test immunotoxins by an alternative strategy, for example to establish MTD (Phase I) in patients with less bulky disease and then use a safe dose in combinatorial therapy (Phase II) before proceeding to randomized Phase III trials in which immunotoxins plus or minus additional therapies are compared for efficacy.

Clinical trials

The completed or ongoing clinical trials involving systemic therapy with immunotoxins are summarized in Table 2. The major findings to emerge are:

1 The side-effects of immunotoxin therapy are different from those of conventional therapy, in that there is no damage to rapidly dividing normal tissues. Blocked immunotoxins consisting of ricin, DT and *Pseudomonas* exotoxin routinely cause hepatotoxicity. All the ricin-based immunotoxins cause reversible vascular leak and myalgias. The MTD appears inversely related to the half life and the stability of the immunotoxin are directly related to the size of the antigenic sink. Multiple courses of immunotoxin therapy have been well tolerated, indicating that toxicity is not cumulative.

2 Severe neurotoxicity was observed in two trials and was due to cross-reactivities of the antibody portion of the immunotoxins with neural cells[3,46]. This emphasizes the importance of carefully screening antibodies for unexpected cross-reactivities with life-sustaining tissues and, when possible, selecting mAbs which cross-react with their homologs in non-human primates. Conversely, administration of an anti-CD19 immunotoxin that was known to cross-react with astrocytes[45] did not cause CNS lesions, presumably because the astrocytes were inaccessible to the immunotoxin, or were insensitive to it.

3 Optimal regimens for administration of the immunotoxins have not yet been devised. The half-lifes in trials to date have generally been shorter than would be predicted to induce an optimal therapeutic index.

4 A general problem is that techniques for isolating and immunophenotyping the malignant progenitor cells have not been developed for the majority of tumors. The assumption usually has to be made that

Table 2. Summary of clinical trials of immunotoxins

Disease	Trial phase	Immunotoxin	Maximum tolerated dose (total mg kg^{-1})	Toxicity
Metastatic melanoma	I	Xomazyme-Mel	>3	vascular leak syndrome, myalgia
	II	Xomazyme-Mel	n.d.	vascular leak syndrome
	II	Xomazyme-Mel plus cyclophosphamide	n.d.	vascular leak syndrome
Colorectal carcinoma	I	Anti-gp72–ricin toxin A-chain	>1	vascular leak syndrome, aphasia
Metastatic breast carcinoma	I	260F9–ricin toxin A-chain (bolus)	0.05	vascular leak syndrome, myalgia, paresthesia
	I	260F9–ricin toxin A-chain (continuous infusion)	0.4	vascular leak syndrome, myalgia, neuropathies
Ovarian carcinoma	I	Anti-OVB3–*Pseudomonas* exotoxin	n.d.	SGOT/SGPT elevations, abdominal pain, encephalopathy
Non-Hodgkin's lymphoma	I/II	Anti-CD19–blocked ricin (bolus)	0.25	SGOT/SGPT elevations, thrombocytopenia
	I	Anti-CD19–blocked ricin (continuous infusion)	0.35	SGOT/SGPT elevations, thrombocytopenia, edema
	I	Fab' anti-CD22–deglycosylated ricin A-chain	1.8	Vascular leak syndrome, myalgia
	I	IgG anti-CD22–deglycosylated ricin A-chain	0.7	vascular leak syndrome, myalgia
Hodgkin's disease; non-Hodgkin's lymphoma	I	IL-2–truncated diphtheria toxin (DAB 486)	1.5	hepatic transaminase elevations, hypoalbuminemia, hypersensitivity, creatinine elevations, thrombocytopenia, renal insufficiency
Hodgkin's disease	I	Anti-CD30–saporin	n.d.	thrombocytopenia, SGOT/SGPT elevations, proteinuria
B-cell chronic lymphocytic leukemia	I	Anti-CD5 (T1-01)–ricin toxin A-chain	n.d.	fever
T-cell lymphoma	I	Anti-CD5 (H65)–ricin toxin A-chain	3.3	vascular leak syndrome, dyspnea
B-cell acute lymphoblastic leukemia	I	Anti-CD19 (B43)–PAP	Not yet reached	hypoalbuminemia
Steroid-resistant graft-versus-host disease	II	Anti-CD5 (H65)–ricin toxin A-chain	1.3	vascular leak syndrome, myalgia, hematuria, tremors

*Human antibodies made by the patient against the two components of the immunotoxin, measured by radio- or enzyme-linked-immunoassay.
AR: anti-ricin A-chain antibody; AM: anti-mouse Ig antibody; ADT: anti-diphtheria toxin antibody; AIL-2: anti-IL-2 antibody; AS: anti-saporin antibody.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.