Themed Issue: Drug Delivery Systems for Targeted Drug Delivery
Guest Editors - Victor C. Yang, Joseph P. Balthasar, Yoon Jeong Park and Jun Feng Liang

Immunotoxins for Targeted Cancer Therapy

Submitted: January 24, 2006; Accepted: June 14, 2006; Published: August 18, 2006

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ABSTRACT

Immunotoxins are proteins that contain a toxin along with an antibody or growth factor that binds specifically to target cells. Nearly all protein toxins work by enzymatically inhibiting protein synthesis. For the immunotoxin to work, it must bind to and be internalized by the target cells, and the enzymatic fragment of the toxin must translocate to the cytosol. Once in the cytosol, 1 molecule is capable of killing a cell, making immunotoxins some of the most potent killing agents. Various plant and bacterial toxins have been genetically fused or chemically conjugated to ligands that bind to cancer cells. Among the most active clinically are those that bind to hematologic tumors. At present, only 1 agent, which contains human interleukin-2 and truncated diphtheria toxin, is approved for use in cutaneous T-cell lymphoma. Another, containing an anti-CD22 Fv and truncated *Pseudomonas* exotoxin, has induced complete remissions in a high proportion of cases of hairy-cell leukemia. Refinement of existing immunotoxins and development of new immunotoxins are underway to improve the treatment of cancer.

KEYWORDS: Monoclonal antibody, CD22, CD25, interleukin, Pseudomonas, diphtheria

INTRODUCTION

Definition of Immunotoxins

Immunotoxins are protein toxins connected to a cell binding ligand of immunologic interest. Classically, beginning 35 years ago, immunotoxins were created by chemically conjugating an antibody to a whole protein toxin, or, for more selective activity, by using a protein toxin devoid of its natural binding domain.[1,2] Immunologic proteins that are smaller than monoclonal antibodies (MAbs), like growth factors and cytokines, have also been chemically conjugated and genetically fused to protein toxins.[3] While some do not consider growth factor toxin fusions or conjugates to be immunotoxins, these newer immunotoxins, like classical immunotoxins, bind to target cells and contain a toxin that kills cells. This review will consider antibody and growth factor toxins directed to cancer cells and focus on those that have been tested clinically or developed preclinically in the past several years.

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Immunotoxins Compared With Other Surface-Targeted Therapies

One type of surface-targeted biologic therapy is unlabeled MAbs. Examples include rituximab[4] and alemtuzumab,[5] which kill cells after binding. Humanized MAbs are effective clinically in up to half of patients via mechanisms of apoptosis induction, antibody-dependent cytotoxicity, and complement-dependent cytotoxicity. Patients with malignant cells resistant to apoptosis, and patients whose immune systems will not perform antibody- or complement-dependent cytotoxicity, may be resistant. To kill cells directly without relying on these mechanisms, a second type of surface-targeted therapy is used, one in which MAbs are conjugated to radionuclides. These agents, considered radioimmunotherapy, induce responses in patients who are resistant to unlabeled MAbs.[6] However, radioimmunotherapy is limited by the potency of the radionuclide and the small number of radionuclide molecules that can be added to each MAb molecule. Patients will often incur dose-limiting toxicity to the bone marrow because of nonspecific uptake of the MAb and have an incomplete response in the tumor. A third type of surface-targeted therapy involves conjugating chemotherapy molecules to MAbs, which in many cases are more potent and cause less nonspecific damage than radionuclides. Examples include gemtuzumab ozogamicin, a conjugate of an anti-CD33 MAb and calicheamicin,[7] which is approved for acute myelogenous leukemia (AML), and the anti-CD30-monomethyl auristatin E conjugate cAC10-vcMMAE, under development for Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL).[8] In the case of gemtuzumab ozogamicin, cells that

are multidrug resistant are, as would be expected, resistant to the target chemotherapeutic.[9] Currently under development is a fourth type of surface-targeted therapy, which employs ribonucleases conjugated to MAbs.[10] Immunotoxins which are distinct from these approaches, target the surface of cancer cells with considerable potency, using protein toxins capable of killing a cell with a single molecule.[11,12] These potent proteins include plant toxins like ricin, saporin, and pokeweed antiviral protein (PAP), which inactivate ribosomes; and single-chain bacterial toxins such as diphtheria toxin (DT) and Pseudomonas exotoxin (PE), which inhibit protein synthesis by adenosine diphosphate (ADP) ribosylating elongation factor 2.[13]

Mechanism of Action of Plant Toxins

Plant holotoxins (also referred to as class II ribosome-inactivating proteins) include ricin, abrin, mistletoe lectin, and modeccin. Hemitoxins, or class I ribosome-inactivating proteins, include PAP, saporin, bryodin 1, bouganin, and gelonin.[14] As shown in Figure 1, holotoxins contain both binding and catalytic domains, whereas hemitoxins contain only catalytic domains. Plant toxins have been shown to prevent the association of elongation factor-1 and -2 (EF-1 and EF-2) with the 60s ribosomal subunit by removing the base of A^{4324} in 28s rRNA.[15] Ricin also removes the neighboring base G^{4323}.[15] Apoptosis has been shown to be involved in cell death induced by plant toxins.[16-18] Only the enzymatic domain of both holo- and hemitoxins translocates to the cytosol, so the binding domains of holotoxins must be removed by reduction of the disulfide bond prior to translocation. Exactly how plant toxins move from the cell surface to the cytosol is unknown; the process probably differs for each plant toxin. The intracellular transport of ricin is dependent on sorting receptors that cycle between the endoplasmic reticulum (ER) and the terminal compartments of the Golgi.[19] It has been shown that glycolipids that bind ricin may be transported from endosomes to the Golgi and that the Lysine-aspartic acid-glutamic acid-Leucine (KDEL) ER retention sequence, if added to ricin, enhances the delivery of this plant toxin to the cytosol.[20]

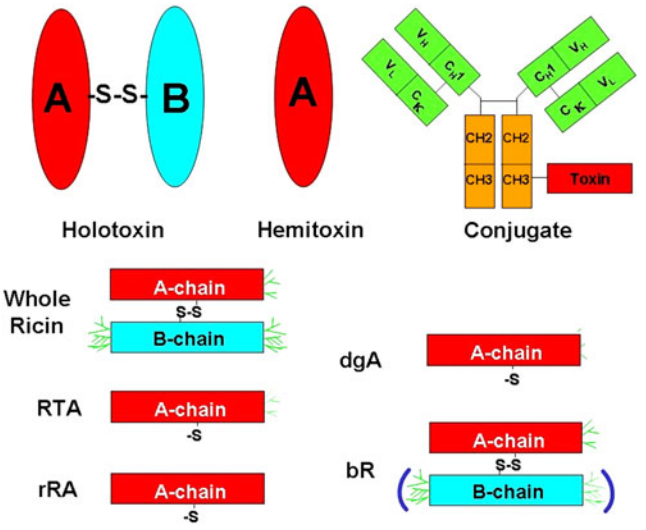

Figure 1. Plant toxins and chemical conjugation. Holotoxins such as ricin and abrin contain activity (A) and binding (B) domains disulfide-bonded together, while hemitoxins contain only activity (A) domains. Whole ricin contains carbohydrate groups and multiple residues in both A and B chains that bind to liver and other normal tissues. Reduction of the disulfide bond results in ricin A chain (RTA), which has reduced but still measurable binding to normal tissues. Options to reduce normal tissue binding further include making recombinant RTA in *Escherichia coli* (rRA), chemically deglycosylating RTA (dgA), and chemically blocking carbohydrates on whole ricin (blocked ricin, bR). An immunotoxin chemical conjugate contains a toxin chemically linked to a monoclonal antibody, optimally at a point removed from the antigen binding (V_L or V_H) domains. Generally, the toxin-ligand junction and the ratio of toxin to ligand is not constant within the conjugate mixture.

Mutant Plant Toxins for Connecting to Ligands

Originally, antibodies were chemically conjugated through a disulfide bond to the catalytic subunits of holotoxins such as ricin or abrin, each of which had been removed from its binding domain by reduction.[2] Even without its binding domain, however, ricin A chain (RTA) was taken up nonspecifically by macrophages and hepatic nonparenchymal Kupffer cells.[21] This uptake was due to glycosylated side residues of RTA (Figure 1) binding to mannose receptors on the liver.[22] The most successful technique for reducing nonspecific uptake of RTA was through chemical deglycosylation. Deglycosylated ricin A chain (dgA) immunotoxins had significantly prolonged lifetimes in mice, leading to an improved therapeutic index.[21,23] Half-lives improved further when the disulfide bond between the MAb and the toxin was formed in a hindered fashion using the derivatizing agent 4-succinimidyloxycarbonyl-a-methyl-a(2-pyridyldithio)toluene (SMPT).[24] Because the ricin B chain facilitates the cytotoxicity of RTA-containing immunotoxins,[25] whole ricin has been targeted after blocking its oligosaccharide binding sites to prevent normal cell binding (Figure 1). These sites on ricin were blocked with ligands prepared by chemical modification of glycopeptides containing triantennary N-linked oligosaccharides.[26] The resulting blocked ricin (bR) was then chemically conjugated to antibodies to make immunotoxins.

Attempts to Construct Fusion Toxins Using Plant Toxins

The cytotoxicity of both plant and bacterial toxins is optimal when the catalytic domain alone translocates to the cytosol.[27] A binding domain can be translocated to the cytosol if

placed within the catalytic domain, but cytotoxic activity is significantly reduced.[28] In an attempt to construct a fusion toxin containing RTA from which free A-chain could be generated, interleukin-2 (IL-2) was fused to recombinant RTA through a linker that contained a proteolytic cleavage site for DT or clotting factor Xa.[29] Although the recombinant toxin could be cleaved extracellularly, it could not selectively target cells since the ligand and toxin were no longer connected. Later, IL-2 was fused to a mutant of PAP, but the fusion toxin was not purified and was not very cytotoxic.[30] Ligands fused to plant toxins have produced recombinant toxins with significant cytotoxic activity, including 1 containing a CD40 single-chain antibody and bryodin 1,[31] 1 containing urokinase binding domain and saporin,[32] and 1 containing human fibroblast growth factor and saporin.[33] For these molecules, it is not known whether (1) the recombinant toxin entered the cytosol of target cells intact, or (2) the ligand was unstable after internalization, permitting the catalytic domain alone to translocate to the cytosol. The ability of even stable ligands to predictably separate from the catalytic domain is an important feature of recombinant toxins[34] and a unique feature among all toxins provided by the bacterial toxins PE and DT.

Mechanism of Action of Bacterial Toxins

Both PE and DT enzymatically ADP-ribosylate EF-2 in the cytosol.[13] They each catalyze the ADP-ribosylation of histidine-699 of EF-2, which is posttranslationally modified to a diphthimide residue.[35] Despite their similar action, PE and DT differ greatly in their amino acid sequence, and in fact PE's enzymatic domain is near the carboxyl terminus, while DT's is near the amino terminus. Conversely, PE's binding domain is near its amino terminus, and DT's is near its carboxyl terminus.

Mechanism of Intoxication of PE

Full-length 613-amino-acid PE, as shown in Figure 2, is a single-chain protein containing 3 functional domains.[36,37] Domain Ia (amino acids 1-252) is the binding domain, domain II (amino acids 253-364) is responsible for translocating the toxin to the cytosol, and domain III (amino acids 400-613) contains the ADP-ribosylating enzyme that inactivates EF-2 in the cytosol. The catalytic process of ADP ribosylation has been shown to involve residues His440 and Glu553.[42] His440 binds nicotinamide adenine dinucleotide (NAD) via Adenosine monophosphate (AMP) ribose. The carboxyl group of the Glu553 side chain, through a water-mediated hydrogen bond with Tyr481 and Glu546, allows Tyr481 to bind NAD through a ring-stacking mechanism. The function of domain Ib (amino acids 365-399) is unknown. Thus, a current model of how PE kills cells contains the following steps: (1) The C-terminal residue

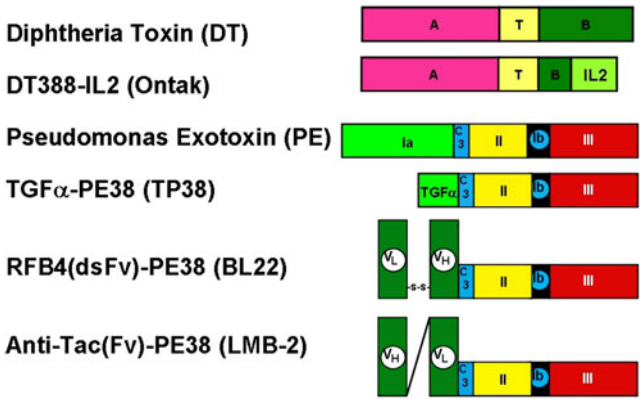

Figure 2. Schematic structure of bacterial toxins and recombinant toxins. PE is a single-chain 613-amino-acid protein containing 3 functional domains.[36,37] Domain Ia (amino acids 1-252) is the binding domain, domain II (amino acids 253-364) is the translocating domain, and domain III (amino acids 400-613) contains the adenosine diphosphate ribosylating enzyme that inactivates elongation factor 2 in the cytosol, resulting in cell death.[38] Domain Ib separates domains II and III and contains amino acids 365 to 399. DT is 535 amino acids in length and is composed of the enzymatic A domain (amino acids 1-193)[39] and the binding B domain (amino acids 482-535).[40] The translocation or transmembrane (T) domain is located in between.[41] PE38 is a 38 kDa truncated form of PE containing amino acids 253 to 364 and 381 to 613. The truncated form of DT used in recombinant toxins is DT388 in DT388-GM-CSF (DTGM) or DAB_{389} in denileukin diftitox (shown), each of which contain methionine followed by the first 388 amino acids of DT. The single-chain recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) contains the variable heavy domain (V_H) of the anti-Tac monoclonal antibody (MAb) fused via the peptide linker $(G_4S)_3$ to the variable light domain (V_L), which in turn is fused to PE38. The recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) is composed of the V_L from the MAb RFB4 disulfide bonded to a fusion of V_H with PE38. The disulfide bond connecting V_H and V_L is formed between 2 cysteine residues replacing Arg44 of V_H and Gly100 of V_L. For single-chain recombinant toxins containing DT, the ligand is at the carboxyl rather than at the amino terminus of the toxin.

(Lys613) is removed by a carboxypeptidase in the plasma or culture medium.[43] (2) Domain Ia binds to the α2 macroglobulin receptor that is present on animal cells and is internalized via endosomes to the transreticular Golgi.[44] (3) After internalization, the protease furin cleaves domain II between amino acids 279 and 280.[45] (4) The disulfide bond between cysteines 265 and 287, which joins the 2 fragments generated by proteolysis, is reduced.[46] (5) Amino acids 609 to 612 Arginine-glutamic acid-aspartic acid-leucine (REDL) bind to an intracellular sorting receptor that transports the 37 kDa carboxy terminal fragment from the transreticular Golgi apparatus to the ER.[47,48] (6) Amino acids 280 to 313 mediate translocation of the toxin to the cytosol.[49,50] (7) The ADP-ribosylating enzyme within amino acids 400 to 602

inactivates EF-2.[13] (8) While inhibition of protein synthesis is sufficient to induce cell death eventually, recent experiments indicate that cell death from toxins is facilitated by apoptosis.[51,52]

Mechanism of Intoxication of DT

DT is a single-chain protein 535 amino acids in length. It is composed of an enzymatic A domain (amino acids 1-193) and a binding B domain (amino acids 482-535).[40] A third domain, which is the translocation or transmembrane (T) domain, is located in the center of the molecule.[53] Based on DT's 3-dimensional structure in the presence and absence of NAD,[54] DT is thought to undergo these steps to kill cells: (1) DT is proteolytically cleaved outside the cell between Arg193 and Ser194,[55] which is within a disulfide loop formed by Cys186 and Cys201. (2) DT binds on the cell surface via residues 482 to 535 to a complex of heparin-binding Epidermal growth factor (EGF)-like growth factor precursor and CD9.[40] (3) DT internalizes into an endosome and unfolds at low pH,[56] and the disulfide bond linking amino acids 186 and 201 is reduced. (4) The TH8 (amino acids 326-347) and TH9 (amino acids 358-376) domains form a hairpin, which inserts into the membrane of the endosome and forms a channel through which the enzymatic fragment translocates to the cytosol,[41] probably from early endosomes.[57] (5) In the cytosol, NAD binds to the active-site cleft of DT (amino acids 34-52), and the ADP ribose of NAD is transferred to EF-2.[58,59] (6) As with PE, cell death is facilitated by apoptosis.[52]

Mutated Bacterial Toxins for Fusing to Ligands

The structures of mutated and truncated forms of DT and PE are shown in Figure 2. To improve specificity, toxins for labeling MAbs are mutated to prevent their binding to normal cells. DT is mutated by converting Leu390 and Ser525 each to phenylalanine, resulting in CRM107.[60] Truncated forms of PE and DT include PE40, containing amino acids 253 to 613 of PE, and Diptheria toxin A and B domains (DAB_{486}), containing the first 485 amino acids of DT.[36,61,62] Shorter versions more recently used include PE38, composed of amino acids 253 to 364 and 381 to 613 of PE, and DT388 or DAB_{389}, containing the first 388 amino acids of DT.[63-66] To allow the ADP-ribosylating domain to translocate to the cytosol without the ligand, the ligand is placed at the amino terminus of PE and at the carboxyl terminus of DT. Another form of PE has an altered carboxyl terminus from the Arginine-glutamic acid-aspartic acid-leucine-lysine (REDLK) to the KDEL sequence, which binds with higher affinity to the KDEL receptor and results in increased cytotoxicity.[48] Immunotoxins containing mutants of PE ending in KDEL (ie, PE38KDEL or PE40KDEL) are more cytotoxic than comparable immunotoxins where the PE mutant ends in the native sequence REDLK.[48,64,67] The translocated fragment of PE38 is 35 kDa in length beginning with Gly280, and since methionine in this position does not alter activity, the new mutant PE35 is produced; it begins with a methionine at position 280 and contains amino acids 281 to 364 and 381 to 613.[68] This molecule would not be appropriate for fusing to ligands, but since it contains a single disulfide bond, it is ideal for chemically conjugating to ligands.

Production of Immunotoxins

Chemical conjugates of growth factor and toxin generally involve either reducible disulfide (S-S) or nonreducible thioether (S-C) bonds.[69] A thioether bond is appropriate if the ligand is conjugated to a bacterial toxin in the part that does not translocate to the cytosol, such as the binding domain.[70] Otherwise, a disulfide bond is commonly used. Derivatization of the toxin requires only reduction in the case of RTA and its mutants, and also in the case of PE35, since both contain only 1 cysteine each. Derivatization of the ligand requires care to produce sulfhydryls without harming the molecule, unless the ligand also has a single cysteine. Once the ligand and toxin are derivatized, they must be purified and conjugated, and then the conjugate of correct toxin-ligand ratio must be repurified. The difficulty and cost of these multiple steps have pushed development of recombinant toxins, which may be produced in Escherichia coli transformed with a plasmid encoding the recombinant toxin. A common method of producing material for clinical trials is harvesting recombinant protein from insoluble bacterial inclusion bodies.[71-73] The insoluble protein can be washed extensively with detergent to remove endotoxin, solublized, denatured, and reduced in guanidine-dithioerythritol solution. The recombinant protein is then renatured by rapid dilution into refolding redox buffer containing arginine and glutathione, and the dialyzed renatured protein purified by anion exchange and sizing chromatography. Other published methods of producing recombinant toxins from E coli involve harvesting the protein from cytoplasm or cell lysate[74] and then using an affinity column to capture the dilute protein. Reverse-phase chromatography followed by sizing chromatography has also been used. Eukaryotic expression systems normally fail with recombinant toxins since eukaryotic EF-2 is highly susceptible to the toxin. However, insect and plant cells have been produced that are resistant to toxin and can produce active toxin.[75,76]

Testing Immunotoxins in Preclinical Models

Immunotoxins are typically first tested on a cell line that contains the receptor or antigen that attracts the binding domain. To determine whether the immunotoxin might be effective in vivo, murine models are produced in which mice contain human xenografts of tumor cell lines. In this

regard, solid tumors have been found more difficult to treat than disseminated leukemia of the same cell line.[77] Once antitumor activity is found in vivo, it is still not clear that the agent would result in responses in patients. One reason for this is that cell lines may grossly overestimate the number of antigen-binding sites/cell in patients. Thus, primary tumor cells freshly isolated from patients are often tested ex vivo to determine sensitivity to the immunotoxin. Another problem with murine models is that patients may have much more unwanted toxicity than mice since the murine receptor or antigen may not even bind the immunotoxin as the human antigen would. For this reason, nonhuman primates that display the antigen on their normal cells are used for toxicity experiments. Even so, expensive experiments of this type are often not predictive of human toxicity. The remainder of this review will focus on immunotoxins tested in patients or being developed for clinical testing.

IMMUNOTOXINS TARGETING HEMATOLOGIC TUMOR ANTIGENS

Antigens Targeted by Immunotoxins in Hematologic Tumor Clinical Trials

Hematologic malignancies are optimal for treating with immunotoxins, since malignant cells are often intravascular and accessible to intravenously administered drug, and since patients often lack sufficient immunity to make antibodies against the toxin. That said, only a handful of antigens have been used to target immunotoxins to hematologic malignancies in patients. These are summarized in Table 1.

Targeting DT to the IL-2 Receptor

The IL-2 receptor (IL2R) binds IL-2 with high affinity ($K_d \sim 10^{-11}$M) and is composed of a complex of alpha (CD25), beta (CD122), and gamma (CD132) subunits.[117] The complex of CD122 and CD132 bind IL-2 with intermediate affinity ($K_d \sim 10^{-9}$M), and CD25 alone binds IL-2 with low affinity ($K_d \sim 10^{-8}$M). IL2Rs of some type are present on a wide variety of hematologic malignancies, including cutaneous T-cell lymphoma (CTCL), adult T-cell leukemia (ATL), HD, and other B- and T-cell leukemias and lymphomas.[118-121] IL2Rs are also displayed by normal T cells and these T-cells can mediate graft rejection and graft vs host disease (GVHD). Only a small percentage of T cells are ordinarily IL2R+.[122] To target the IL2R, human IL-2 was fused to truncated DT, originally a fragment of DT containing methionine plus the first 485 amino acids of DT.[62,123] Clinical trials showed some efficacy with DAB_{486}IL-2 in hematologic malignancies with dose-limiting transaminase elevations.[124-126] A new fusion toxin was created by removing amino acids 389 to 485, and it was found that DAB_{389}IL-2, also called denileukin diftitox or Ontak (Figure 2), had improved half-life, cytotoxicity, and tolerance in animals.[65] In phase I testing there were 5 complete remissions (CRs) and 8 partial responses (PRs) in 35 patients with CTCL, and 1 CR and 2 PRs out of 17 patients with NHL.[127] The maximum tolerated dose (MTD) was 27 μg/kg daily (QD) × 5, and the dose-limiting toxicity was asthenia (fatigue). Common toxicities included transaminase elevations (62%), hypoalbuminemia (86%), rashes (32%), and hypotension (55%).[65] In the pivotal phase III CTCL trial, 7 CRs and 14 PRs were achieved in 71 patients, but most patients had objective skin improvement.[128,129] Two dose levels were tested (9 μg/kg and 18 μg/kg QD × 5), and patients with more advanced disease benefited from the higher dose. Vascular leak syndrome (VLS), attributed to cytokine release after the killing of perivascular T cells in the dermis, was usually without pulmonary edema and could be prevented with steroid prophylaxis.[95,128,130] Immunogenicity toward anti-DAB_{389}IL-2 increased from 32% baseline to nearly 100% after 1 cycle, but retreatment was sometimes effective, indicating that antitoxin antibodies were not always neutralizing.

Postapproval Testing of Denileukin Diftitox

Denileukin diftitox was approved by the Food and Drug Administration for the treatment of advanced CTCL. Other treatments like bexarotene are indicated for early CTCL.[131] Denileukin diftitox has also shown activity in clinical trials of other tumors and in autoimmune disease, including peripheral T-cell lymphoma,[132] panniculitic lymphoma,[133] B-Chronic lymphocytic leukemia (CLL),[96] B-NHL,[97] and psoriasis.[134] Out of 18 patients with B-CLL with an average of 4.5 prior treatments/patient, 12 received at least 3 cycles at 9 or 18 μg/kg QD × 5, and 6 (50%) of 12 had 95% to 99% reductions of circulating malignant cells. Four (33%) out of 12 patients had 29% to 80% reductions in lymph nodes, with 2 qualifying for PR, lasting 14 and >19 months. Out of 45 evaluable patients with NHL treated in a phase II trial, there were 3 (7%) CRs and 8 (18%) PRs. Durability of response was somewhat limited, with a 7-month median time to treatment failure in responding patients.[97] Thus, denileukin diftitox, the only targeted protein toxin so far approved for use, is effective in several hematologic malignancies. One limitation for CTCL, CLL, and NHL is the lack of high-affinity IL2Rs in a large percentage of cases, usually because of lack of CD122. Several types of agents, including rexinoids[135,136] and arginine butyrate,[136] can upregulate CD25 and/or CD122 and could potentially expand the clinical utility of this recombinant toxin.

Targeting PE to CD25, Preclinical Studies

To target IL2R+ disorders expressing CD25 regardless of the presence of other subunits of the IL2R, the anti-CD25 MAb anti-Tac was used as a ligand instead of IL-2. The

Table 1. Immunotoxins Tested Clinically in Recent Years*

Chemical Conjugates

Agent	Antigen	Ligand	Truncated Toxin	Basic Toxin	Diseases	References
RFT5-dgA	CD25	MAb	dgA	Ricin	HD	78,79
RFB4-dgA	CD22	MAb	dgA	Ricin	B-NHL, CLL	80,81
RFB4-Fab'-dgA	CD22	Fab'	dgA	Ricin	B-NHL	82
HD37-dgA	CD19	MAb	dgA	Ricin	B-NHL	83
Anti-CD7-dgA	CD7	MAb	dgA	Ricin	T-NHL	84
K_i-4.dgA	CD30	MAb	dgA	Ricin	HD	85
LMB-1	Le^y	MAb	Lys-PE38	PE	Carcinoma	70
TF-CRM107	TFR	Tf	CRM107	DT	Glioma	86
B43-PAP	CD19	MAb	PAP	PAP	ALL	87
Anti-B4-bRicin	CD19	MAb	bR	Ricin	B-NHL	88-90
Ber-H2-Sap6	CD30	MAb	Sap6	Saporin	HD	91
Anti-My9-bRicin	CD33	MAb	bR	Ricin	AML	90
454A12-rRA	TFR	MAb	rRA	Ricin	CSF cancer	92
N901-bR	CD56	MAb	bR	Ricin	SCLC	90,93,94

Recombinant toxins

Agent	Antigen	Ligand	Truncated Toxin	Basic Toxin	Diseases	References
Ontak	IL2R	IL-2	DAB_{389}	DT	CTCL, CLL, NHL	95-97
BL22	CD22	dsFv	PE38	PE	HCL, CLL, NHL	98
LMB-2	CD25	scFv	PE38	PE	NHL, leukemias	99,100
DT388-GM-CSF	GM-CSF	GM-CSF	DT388	DT	AML	101
B3(Fv)-PE38	Le^y	scFv	PE38	PE	Carcinoma	102
B3(dsFv)-PE38	Le^y	dsFv	PE38	PE	Carcinoma	103
TP40	EGFR	TGFα	$PE40^{4a}$	PE	Bladder cancer, CIS	104
TP38	EGFR	TGFα	PE38	PE	Glioblastoma	105
BR96(scFv)-PE40	Le^y	scFv	PE40	PE	Carcinoma	106-108
erb38	erbB2	dsFv	PE38	PE	Breast cancer	109
NBI-3001	IL4R	IL-4(38-37)	PE38KDEL	PE	Glioma	110,111
IL13-PE38QQR	IL13R	IL-13	PE38QQR	PE	Renal cell	112-114
SS1(dsFv)-PE38	Mesothelin	dsFv	PE38	PE	Mesothelioma	115
DAB_{389}EGF	EGFR	EGF	DAB_{389}	DT	Carcinoma	116

*Toxins, several of which are shown schematically in Figure 1, include recombinant ricin A chain (rRA), blocked ricin (bR), deglycosylated ricin A chain (dgA), pokeweed antiviral protein (PAP), truncated diphtheria toxin (DT388 or DAB_{389}), truncated *Pseudomonas* exotoxin (PE38 or PE40), and mutated diphtheria toxin (CRM107). Non-monoclonal antibody (MAb) ligands include interleukin-2, -4, and -13 (IL-2, IL-4, and IL-13); granulocyte-macrophage colony stimulating factor (GM-CSF); epidermal growth factor (EGF); transforming growth factor (TGFα); and transferrin (Tf). $PE40^{4A}$ is PE40 with alanine substituted for cysteine at positions 265, 287, 372, and 379. PE38QQR is PE38 with 2 glutamine residues and 1 arginine replacing the 3 lysine residues of PE38 at positions 590, 606, and 613. Diseases include non-Hodgkin's lymphoma (NHL, B- or T-cell), cutaneous T-cell lymphoma (CTCL), Hodgkin's disease (HD), chronic lymphocytic leukemia (CLL), carcinoma in situ (CIS), acute myelogenous leukemia (AML), metastatic tumor involving the cerebrospinal fluid (CSF cancer), renal cell carcinoma (renal cell), small cell lung cancer (SCLC), Acute lymphoblastic leukemia (ALL) and hairy cell leukemia (HCL).

rationale is based on the higher binding of CD25 alone to anti-Tac ($K_d \sim 10^{-10}$M) than to IL-2 ($K_d = 10^{-8}$M).[137] CD25 greatly outnumbers CD122 and CD132 on most malignant cell types.[118,119] Although early studies indicated CD25 alone would not internalize anti-Tac,[138] CD25 alone does internalize bound recombinant toxin.[64,139,140] A recombinant single-chain Fv[141,142] was constructed containing the variable heavy domain (V_H) fused to the variable light domain (V_L) via the peptide linker $(G_4S)_3$, and V_L was fused to truncated PE.[143] The resulting recombinant immunotoxin anti-Tac(Fv)-PE40 and its slightly shorter derivative anti-Tac(Fv)-PE38 (called LMB-2) were selectively cytotoxic toward CD25+ malignant cell lines and toward leukemic cells freshly obtained from patients.[64,140,144-147] Antitumor

studies in mice bearing CD25+ xenografts showed complete regressions, and biodistribution studies showed a concentration of LMB-2 in such tumors in vivo.[140,145] Primary ATL and hairy cell leukemia (HCL) cells were much more sensitive than primary CLL cells, probably because of lower CD25 expression in the latter, and CLL cells have been shown to upregulate CD25 by phosphorothioate oligodeoxynucleotides.[148] Cyclosporine has been reported to increase the sensitivity of ATL cells toward anti-Tac(Fv) toxin, but such cells were already very sensitive.[149]

Clinical Development of LMB-2 in CD25+ Hematologic Malignancies

LMB-2 was administered to 35 patients with chemotherapy-resistant leukemia, lymphoma, and HD. There were 7 PRs and 1 CR, all in the 20 patients receiving a total dose of >60 μg/kg/cycle. All 4 patients with HCL responded, with 1 CR and 3 PRs.[99] CR was associated with resolution of severe pancytopenia and eradication of circulating malignant cells. Patients with CLL, ATL, CTCL, and HD achieved PR.[100] The most common toxicities included transaminase elevations that were associated with fever and thus appeared to be mediated by cytokines.[150,151] Immunogenicity resulted in 6 out of 35 patients being excluded from further treatment after the first cycle. The 8 CLL patients did not make neutralizing antibodies after a total of 16 cycles. In HD, high levels of neutralizing antibodies were observed in 3 out of 11 patients after 1 cycle and after 2 to 3 cycles in 2 additional patients. Phase II trials are currently underway in CD25+ CLL and CTCL. HCL patients are being treated now with the anti-CD22 recombinant immunotoxin BL22 (see below) instead of LMB-2.

Development of RFT5-dgA for CD25+ Malignancies

The anti-CD25 MAb RFT5 was isolated and chemically conjugated to dgA, and the resulting immunotoxin RFT5-dgA induced 2 PRs out of 18 patients with HD treated at the optimal dose level.[78,79,152,153] RFT5-dgA was also tested in the prevention of GVHD in patients undergoing allotransplantation.[154] It was reported that patients receiving RFT5-dgA had a higher incidence of grade III/IV GVHD than historical controls, suggesting that CD4+/CD25+ T-regulatory cells were targeted and that activated T cells may have been spared because of reduction of CD25 expression by cyclosporine.[155]

Preclinical Development of Other Recombinant Toxins Targeting CD25

Production of a more stable form of LMB-2 was accomplished by replacing the peptide linker between the variable domains with a disulfide bond via cysteine residues engineered into the framework region; the resulting disulfide-stabilized recombinant immunotoxin had improved stability, but binding, cytotoxicity, and antitumor activity were not compromised.[156,157] Mik-β1(Fv)-PE40, a recombinant immunotoxin targeting CD122, was produced and shown to cointernalize with LMB-2 into cells expressing both CD25 and CD122 subunits of the IL2R.[158] This agent was cytotoxic toward natural killer leukemia cells, which express more CD122 than CD25, but has not been developed further. The MAb RFT5 was converted to the recombinant immunotoxin RFT5(scFv)-ETA'. This recombinant immunotoxin showed antitumor activity in Severe combined immunodeficiency (SCID) mice bearing disseminated human HD.[159-161]

Targeting CD22 With Immunotoxins

Chemical conjugates were previously constructed to target CD22 on B-cell malignancies, including the MAbs H6 or RFB4 conjugated to dgA,[162,163] and the MAbs HD6 and HD39 linked to saporin.[164] RFB4-dgA resulted in 2 CRs and 10 PRs out of 41 patients with B-cell lymphoma/leukemia (combining both bolus and continuous infusion trials), and patients had dose-limiting VLS.[80,81,165] To avoid VLS, a derivative of recombinant RTA was prepared containing an N87A mutation. RFB4-N87A led to significantly less VLS in mice, suggesting that this new immunotoxin may be useful in patients.[166,167] The first anti-CD22 immunotoxin with PE contained the MAb LL2 and induced complete regression in human xenograft models.[68,168] However, LL2 as a single-chain Fv was unstable, and an active recombinant immunotoxin could not be made. Therefore, the variable domains from RFB4 were cloned and a stable recombinant immunotoxin RFB4(Fv)-PE38 was made and shown to be cytotoxic toward CD22+ cell lines.[169]

Preclinical Development of BL22

To improve the stability of RFB4(Fv)-PE38, the variable domains were connected by a disulfide bond instead of a peptide linker, and V_H was fused to PE38, resulting in BL22.[170] The disulfide bond is between cysteine residues replacing framework residues Arg44 of V_H and Gly100 of V_L. This technology had been used for stabilizing Fvs of a variety of different MAbs, including anti-Tac.[156] The double-chain immunotoxin, termed RFB4(dsFv)-PE38 or BL22, is considered fully recombinant since the disulfide bond between V_L and V_H-PE38 forms naturally during in vitro renaturation of the 2 fragments and chemical conjugation is not needed. BL22 induced complete regressions in mice of human CD22+ B-cell lymphoma xenografts at plasma levels that could be tolerated in cynomolgus monkeys.[171] Leukemic cells freshly obtained from patients with CLL and NHL were found to be sensitive to BL22.[172] This study, which showed specific killing of such cells, was important

for preclinical development because malignant cells freshly obtained from patients typically display far fewer CD22 sites/cell than do cell lines. Much greater activity toward the CLL cell was observed with the mutant HA22, which has higher affinity for CD22 because of THW replacing amino acids SSY at positions 100, 100a, and 100b of V_H.[173]

Phase I Testing of BL22 in Patients with B-Cell Malignancies

In one study, BL22 was administered to 46 patients with HCL B-cell lymphomas and leukemias.[98,174] A total of 265 cycles of BL22 were administered to 16 patients, with up to 33 cycles/patient. All patients were pretreated with 1 to 6 separate courses of cladribine. A total of 19 out of 31 patients (61%) had CR, and 6 patients had PR (19%). Seven patients had marginal responses, with up to 99.5% reductions in circulating HCL counts but less than 50% decreases in lymph node masses. CR was achieved in all 3 patients with the poor-prognosis variant HCLv.[175] Eleven had CR after cycle 1, and 8 had CR after cycles 2 to 9. Only 1 out of 19 CRs had minimal residual disease in the bone marrow biopsy by immunohistochemistry, which is reported to be a risk factor for early relapse.[176] Cytopenias resolved in all responders. Within the follow-up time of 5 to 67 (median 36) months, 7 patients were still in CR. High levels of neutralizing antibodies were observed in 11 patients after cycles 1 to 5. Plasma levels in patients with high disease burden were much greater on subsequent cycles after patients responded, compared with cycle 1. In the HCL patients, dose-limiting toxicity included a cytokine release syndrome in 1 patient with fever, hypotension, bone pain, and weight gain (VLS) without pulmonary edema; this resolved within 3 days. Also, 4 patients with HCL had completely reversible hemolytic uremic syndrome (HUS), confirmed by renal biopsy. HUS presented clinically with hematuria and hemoglobinuria by day 8 of cycle 2 in each case. These patients required 6 to 10 days of plasmapheresis but not dialysis for complete resolution of renal function and correction of thrombocytopenia and anemia. Three of these 4 HCL patients achieved CR, and in all 4, there was resolution of preexisting cytopenias as well as those related to HUS. BL22 is the first agent since purine analogs reported to induce CR in the majority of patients with HCL. Its success in chemoresistant patients is clearly related to the fact that CD22 is highly conserved at high density on HCL cells despite purine analog resistance.

Targeting the Granulocyte-Macrophage Colony Stimulating Factor Receptor With DT388-GM-CSF

To target the granulocyte-macrophage colony stimulating factor receptor (GM-CSFR), which is expressed in AML cells from most patients, human GM-CSF was fused to truncated bacterial toxins. DT388-GM-CSF (DTGM) was found to be more cytotoxic than GM-CSF-PE38KDEL.[177] DTGM was tested in 31 patients with relapsed or refractory AML, all of whom were resistant to chemotherapy.[101] One CR and 2 PRs were observed, and the major toxicity was cytokine release syndrome. Preexisting antibodies to DT were observed in 28 of 31 patients.[101,178] Cytotoxic plasma levels of DTGM could be detected in 14/20 patients with anti-DT antibody concentrations <2.2 μg/mL and in 2/11 patients with anti-DT antibody concentrations >2.2 μg/mL.[101]

Targeting CD19 or Both CD19 and CD22 With Immunotoxins

The anti-CD19 immunotoxin anti-B4-blocked ricin (anti-B4-bR) had previously been tested in phase I trials, which showed responses, including CRs, but later trials showed more limited activity, possibly because of limited tumor penetration.[88] Subsequent trials used anti-B4-bR in the setting of minimal residual disease or in combination with chemotherapy. In these nonrandomized trials, the most recent of which was in patients with acute lymphoblastic leukemia in first CR, no obvious activity was observed.[89,179-183] The combination of anti-CD19 HD37-dgA and anti-CD22 RFB4-dgA was tested in an animal model[184] and had some efficacy in patients with B-cell malignancies,[185] but safety could be established in only patients with circulating tumor cells.

Targeting CD30 With Immunotoxins

The anti-CD30 MAb K_i-4 conjugated to dgA was tested in 15 patients with NHL and HD, achieving 1 PR.[186] Preclinical work with a related immunotoxin, K_i-3-dgA, showed that inhibition of metalloproteinases enhanced internalization and cytotoxicity.[187] K_i-4 was converted to a recombinant immunotoxin, termed K_i-4(scFv)-ETA', which displayed antitumor activity in mice with disseminated human HD.[188,189] Without a hybridoma being obtained, anti-CD30 single-chain Fvs were obtained by immunizing mice with DNA encoding human CD30, harvesting the spleens, and constructing an scFv phage display library. Several anti-CD30 recombinant immunotoxins were obtained with selective cytotoxicity toward cell lines, and anti-CD30-CL2(Fv)-PE38KDEL had antitumor activity in a CD30+ human solid tumor mouse xenograft model.[190] For higher-affinity recombinant immunotoxins targeting CD30, mice were DNA-immunized and the hybridomas isolated prior to scFv construction. Two molecules, T25(dsFv)-PE38 and T6(dsFv)-PE38, were found to have potent cytotoxicity toward CD30+ cell lines.[191]

Preclinical Studies With Other Immunotoxins Targeted to Hematologic Tumors

To target AML cells with a molecule that would not cross-react with monocytes and macrophages and hence would

not cause cytokine release syndrome, a DT containing human IL-3 was produced. DT388-IL3 was found to be cytotoxic toward AML cell lines[192] and primary AML or Chronic myelogenous leukemia (CML) cells[193,194] but not normal hematopoietic progenitors.[195] DT388-IL3 prolonged survival in tumor-bearing mice[196] and has been produced for phase I clinical testing.[197] To target multiple myeloma and T-cell leukemias, CD38 was targeted with anti-CD38 saporin and improved cytotoxicity was observed in combination with anti-CD7 saporin.[198] Retinoic acid was found to induce CD38 expression and enhance cytotoxicity to anti-CD38 gelonin.[199] A recombinant anti-CD7 immunotoxin was found to kill T-cell acute lymphoblastic leukemia (T-ALL) cells by apoptosis.[200] To target CD20+ B cells, rituximab was conjugated to saporin-S6 and synergy was found with fludarabine.[201] CD64 was targeted using an RTA MAb conjugate,[202] and a recombinant anti-CD64 immunotoxin was produced and found to be cytotoxic toward AML cells.[203] Anti-CD80 and anti-CD86 were used as conjugates with gelonin to target HD cells, and safety was established in monkeys.[204] The antigen JL1 on leukemias was targeted using an MAb-gelonin conjugate.[205] Finally, CTLA4 was targeted using 2 different Fvs, each conjugated to saporin-S6, and activity against both lymphoid and myeloid leukemias was observed.[206]

Immunotoxins Targeting Solid Tumor Antigens

Targeting solid tumors with immunotoxins is much more difficult than targeting hematologic tumors. Not only are the cellular junctions tighter and the tumor cells more tightly packed, but the patients are less immunosuppressed and more likely to make neutralizing antibodies to the toxin. Below, recent published information regarding solid tumor immunotoxin trials, along with recent preclinical development, is discussed. The findings are summarized in Table 1.

Immunotoxins Targeting the Epidermal Growth Factor Receptor

Some of the earliest chemical conjugates and recombinant fusion toxins contained either epidermal growth factor (EGF) or transforming growth factor α (TGFα), both ligands for the EGF receptor (EGFR), and Pseudomonas exotoxin.[28,207-210] Antitumor activity in tumor-bearing mice was demonstrated,[211] but tolerated doses were low because of expression of EGFR by the liver. Thus, TGFα toxins were tested nonsystemically, either by intravesical treatment of bladder cancer[104] or by intracerebral injection of patients with glioblastoma multiforme.[105] In the latter trial, several patients responded, including 1 with long-term CR. EGF fused to diphtheria was also developed for targeting EGFR-bearing tumors.[212] $DAB_{389}EGF$ was administered systemically in phase I trials to patients with prostate, gastrointestinal, head and neck, renal, lung, and breast cancer,[116] and the dose-response rate was limited by renal tubular acidosis and immunogenicity. More recently, $DAB_{389}EGF$ and anti-EGFR immunotoxins have been developed for local treatment of brain and pancreatic tumors.[213-215] One method for improving specificity for EGFR+ malignant cells is to target mutant versions of the EGFR with recombinant immunotoxins.[216,217] Another is to target the heparin binding form of the EGFR, which can be modulated by heparin.[218-220]

Targeting the Le^y Antigen on Solid Tumors

To target the carbohydrate antigen Le^y,[221] a chemical conjugate of B3 with PE38, termed LMB-1, was produced, developed preclinically,[222-224] and tested in 38 patients with Le^y-expressing carcinomas of breast, ovarian, and gastrointestinal origin.[70] One CR and 1 PR were achieved, the first major responses to immunotoxins for metastatic breast and colon cancer, respectively. The dose-limiting toxicity was due to VLS. Experiments with human umbilical vein endothelial cells indicated that the MAb B3 rather than PE38 was binding to the Le^y antigen on endothelial cells.[225] To target Le^y-expressing tumors with a smaller immunotoxin that would leave the vasculature quickly before causing VLS, the Fv of B3 was cloned and fused to PE38.[226] B3(Fv)-PE38 (LMB-7) and B3(dsFv)-PE38 (LMB-9) are 2 recombinant immunotoxins that have recently undergone clinical testing, the former having a single-chain structure like LMB-2 and the latter having a disulfide-stabilized structure like BL22. LMB-9 is more appropriate for administration by continuous infusion, because of its extreme stability at 37°C.[103] The recently published clinical results of the anti-Le^y recombinant immunotoxin BR96(sFv)-PE40 indicated that the molecule was reasonably stable and the dose was limited by gastrointestinal toxicity rather than by VLS.[106]

Recombinant Toxins Targeting erbB2

To target the erbB2 antigen expressed in poor-prognosis breast cancer and other carcinomas,[227] several MAbs that vary in affinity have been cloned to produce recombinant single-chain or disulfide-stabilized immunotoxins.[228-230] One of these, erb-38, containing a disulfide-stabilized Fv fused to PE38, was tested in patients with carcinomas, mostly breast cancer.[109] Erb-38 bound to normal liver tissue, resulting in dose-limiting toxicity at a low dose level. This supports the important principle that targeting any antigen expressed even at very low levels on an organ that is already sensitive to a toxin's effects may prevent selective tumor targeting. Thus, antitumor activity in animals that do not express the targeted antigen on normal cells may not translate into response in humans.[231] Further preclinical development of such immunotoxins is proceeding using the

intratumoral injection route.[232] In a clinical trial of ScFv(FRP5)-ETA, 6 of 10 patients achieved tumor regression of cutaneous metastases of colon and breast cancers.[233]

Recombinant Toxins Targeting the IL-4 Receptor

The IL-4 receptor (IL4R) is widely expressed by solid tumors and hematologic malignancies.[234,235] Early IL4-PE fusions had limited binding because the toxin interfered with the IL4-IL4R binding site. To optimize binding, the circularly permuted mutant IL-4 toxins were made, containing IL-4 amino acids 38 to 129 connected through the peptide linker GGNGG to IL-4 amino acids 1 to 37, which were in turn fused to the toxin.[236-238] This resulted in enhanced cytotoxicity and antitumor activity.[238-240] Because IL4(38-37)-PE38KDEL was highly toxic to the liver at low doses, it was developed for intratumoral therapy of glioblastoma multiforme.[240,241] Of the first 9 patients treated in this fashion, 1 multiply relapsed patient had extensive tumor necrosis followed by a long-term (>18-month) CR.[110] Toxicity was usually related to the edema associated with high infusion volumes and rates. In some patients requiring reoperation, toxicity to normal brain tissue caused by the toxin was excluded histologically. In a phase I/II trial of 31 patients, tumor necrosis was observed in 71%, and 1 patient experienced long-term survival.[111]

Targeting the IL-13 Receptor on Solid Tumors

The IL13 receptor, which is related to the IL4R, is also expressed in a variety of solid tumors.[242,243] The recombinant fusion toxin IL13-PE38QQR is cytotoxic and showed antitumor efficacy toward a variety of tumor cell lines.[112,113] This molecule is currently undergoing phase I clinical testing in patients with metastatic renal cell carcinoma. In an interim report, out of 46 patients across 3 trials, histopathologic tumor effect was seen at drug concentrations of 0.5 to 2 μg/mL.[114]

Targeting the Transferrin Receptor by Compartmental Administration

Transferrin receptors are present on all normal cells that are actively taking up iron, particularly in the liver. Using a chemical conjugate containing human transferrin and a mutant form of DT,[60] Tf-CRM107 was infused directly into the tumors of 18 patients using catheters placed stereotactically. Two CRs and 7 PRs were documented in 15 evaluable patients.[86] In 6 of 9 patients who responded and in some of the nonresponders, tumors exhibited early central necrosis. There was evidence that the chimeric toxin escaped from the central nervous system, resulting in transient transaminase elevations, hypoalbuminemia, and an increase in anti-DT titer. At doses at or above 1 μg/mL, peritumoral brain toxicity was observed, consisting of thrombosed cortical vessels, attributed to the presence of Transferrin receptor (TFR) on endothelial cells. In a phase II trial, Tf-CRM107 resulted in a 35% response rate[244] at the maximum tolerated dose (MTD), 0.66 μg/mL. One strategy for improving the safety of Tf-CRM107 is to coadminister chloroquine intravenously, which blocks the toxicity of DT toward endothelial cells that express TFR.[245] The use of this strategy showed promising results in an animal model.

Targeting the Mesothelin Antigen on Solid Tumors

Phage display technology was used to generate new Fvs binding to mesothelin, an antigen on mesotheliomas, ovarian and pancreatic carcinomas, and other tumors.[115,246,247] A recombinant immunotoxin was obtained that underwent affinity improvement, and the recombinant immunotoxin generated, SS1(dsFv)-PE38 (SS1P), was developed for systemic therapy of patients.[248-252] SS1P is now undergoing clinical testing.

Targeting the N901 Antigen on Solid Tumors

Over 10 years ago, N901-bR, targeting the small cell lung cancer (SCLC) antigen NCAM (also named CD56) was tested in patients with SCLC and found to induce 1 PR out of 19 patients and dose-limiting VLS.[93,253] The complete phase I report included 1 PR in 21 patients.[94] In a phase II trial of N901-bR after CR or near-CR from chemotherapy, 9 patients were treated, 1 with a long-term (>6-year) survival, but the trial was closed because of toxicity (VLS).[254]

Recent Preclinical Development of Other Immunotoxins for Solid Tumors

To target the urokinase receptor (also called uPAR or CD87), present on many types of hematologic[255,256] and solid[257,258] tumors, the amino terminal fragment (ATF) of urokinase was fused to PE38 and PE38KDEL.[259] The recombinant toxins ATF-PE38 and ATF-PE38KDEL were very cytotoxic toward leukemia cells and extremely cytotoxic toward glioblastoma multiforme cells. The recombinant toxin DT388-ATF (DTAT) was also produced and found to be cytotoxic.[260-263] The bispecific immunotoxin DTAT13 was produced and found to be toxic to both CD87 and IL13R+ cells.[264] Gelonin-containing immunotoxins have been developed to target gp240 on melanoma.[265,266] Immunotoxins targeting the high-molecular-weight melanoma antigen have been developed for both melanoma and glioblastoma multiforme.[267,268] To combat prostate cancer, toxins were targeted by antibodies to prostate-specific membrane antigen.[269,270] To target childhood sarcomas and neuroblastoma, recombinant immunotoxins were directed to a glycoprotein using MAb 8H9 and observed to cause antitumor activity in

SCID mice at doses that were tolerated in monkeys.[271] Activity in neuroblastoma was also reported, targeting GD_2 using the recombinant immunotoxin DT5F11.[272] Antitumor activity was observed with the recombinant anti-Ep-CAM immunotoxin 4D5MOCB-ETA', which is undergoing phase I testing in patients with squamous cell carcinoma of the head and neck.[273] Finally, in an elaborate use of immunotoxins, the gene for vascular endothelial growth factor fused to truncated DT or PE was introduced into T15 T cells that were raised to recognize leukemia cells.[274]

Problems and Opportunities in Immunotoxin Development

There are challenges associated with the development of many immunotoxins for cancer therapy. Several of these problems, including immunogenicity, unwanted toxicity, difficulty in production, limited half-life, and resistance, will be considered below, along with potential opportunities for improved development of immunotoxins.

Immunogenicity

Based on a wide range of clinical trials, the incidence of immunnogenicity after a single cycle of immunotoxin ranges from 50% to 100% for solid tumors, and from 0% to 40% for hematologic tumors. Patients have been reported to respond to some fusion toxins after immunogenicity is detected,[128] but in these cases antitoxin antibodies are detected by enzyme-linked immunosorbent assay and are probably not neutralizing. Antibodies that are neutralizing can be detected by determining whether serum containing them can block the cytotoxicity of the immunotoxin toward cultured cells. The presence of neutralizing antibodies lowers the levels of biologically active immunotoxin and compromises efficacy. Several approaches can be used to prevent immunogenicity. The method most useful for other biologic agents, such as interferon[275] and L-asparaginase,[276] is PEGylation, which not only blocks immunogenicity but also prolongs half-life. Limited success has been achieved in preclinical studies of a PEGylated form of LMB-2.[277,278] PEGylating a toxin appears much more challenging than PEGylating simpler molecules, since disturbing sites on a toxin reduces toxin activity. Immunologic studies have found a large number of B-cell and T-cell epitopes on *Pseudomonas* exotoxin,[279-281] suggesting that "humanization" of the molecule would be extremely difficult. Agents to nonspecifically suppress the immune response, such as deoxyspergualin[282] and CTLA4Ig,[283,284] have shown efficacy in preclinical models but have not been tested clinically. Rituximab has proven ineffective in preventing immunogenicity in patients receiving LMB-1.[285] Nevertheless, it is noteworthy that no patients with CLL have ever produced neutralizing antibodies to LMB-2 or BL22. This suggests that artificial replication of the humoral immune deficiency in CLL, from either treatment or disease, might prevent the immunogenicity of immunotoxins.

Unwanted Toxicity

A variety of toxicities have been observed with immunotoxins that have limited the dose and hence the efficacy. The most common toxicity is VLS, which is not surprising, given that a cytotoxic protein must traverse endothelial cells to exit the blood vessels. Studies have shown that RTA binds directly to endothelial cells, while truncated PE requires a ligand that cross-reacts with the endothelium.[225] Other studies have suggested that specific residues on RTA and also truncated PE and IL-2 can bind to endothelial cells and can elicit VLS by a mechanism independent of the normal toxin-induced cell death.[286,287] Such studies led to a mutant form of RTA that shows less VLS in an animal model.[168] Hepatotoxicity, a typical side effect of recombinant immunotoxins, is attributed to the binding of basic residues on the Fv to negatively charged hepatic cells.[152,288] Hepatoxicity appears to be related to cytokine production, possibly by the Kupffer cells of the liver.[153] Although recombinant immunotoxins that specifically bind to antigens expressed on the liver are not well tolerated systemically,[109] recombinant immunotoxins like LMB-2 and BL22 that cause transaminase elevations are not associated with decreased hepatic function.[98,100] Renal toxicity due to immunotoxins is less well defined and could be nonspecific at least in part because the kidneys are the dominant route of excretion of recombinant immunotoxin.[142]

Difficulty in Production

Originally, chemical conjugates were made for clinical trials since manufacturers of recombinant toxins faced problems of endotoxin contamination and low yield. Advances in the production of other recombinant proteins for clinical use have solved many of these problems and have allowed large-scale production of recombinant toxins with high purity and reasonable cost. It is anticipated that corporate development will further improve yield and cut costs.

Potential for Future Development

For many types of disease, immunotoxins are unlikely to work by themselves. Their half-lives may be too limited for diffusion to occur into solid tumor masses. Clinical trials of immunotoxins administered by continuous infusion have thus far not found significant improvements in efficacy over the bolus infusion route.[81,289,290] It is possible that combination with other therapeutic agents having nonoverlapping toxicities will result in better responses. Similarly, treatment of microscopic disease may be useful after cytoreduction by surgery, chemotherapy, or radiotherapy. Finally, the antigen

and disease targeted remain major determinants of immunotoxin efficacy and resistance. As combinations of diseases and antigen targets are chosen, we can anticipate exciting successes in the future development of immunotoxins.

CONCLUSIONS

In the past 3 to 4 decades, a wide variety of immunotoxins have been tested against a wide variety of malignancies in cell culture, in animal models, and in patients. The most useful of these agents appear to be the relatively small recombinant fusion toxins that contain either growth factor or Fv fragments as ligands. The most sensitive diseases appear to be hematologic malignancies. Future development will need to address combinations of immunotoxins with other anticancer therapies in order to overcome problems of tumor penetration, toxicity, and immunogenicity.

ACKNOWLEDGMENTS

This work was supported by the intramural program of the National Cancer Institute.

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