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Recombinant Immunotoxins Containing Truncated Bacterial Toxins for the Treatment of Hematologic Malignancies

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Abstract

Immunotoxins are molecules that contain a protein toxin and a ligand that is either an antibody or a growth factor. The ligand binds to a target cell antigen, and the target cell internalizes the immunotoxin, allowing the toxin to migrate to the cytoplasm where it can kill the cell. In the case of recombinant immunotoxins, the ligand and toxin are encoded in DNA that is then expressed in bacteria, and the purified immunotoxin contains the ligand and toxin fused together. Among the most active recombinant immunotoxins clinically tested are those that are targeted to hematologic malignancies. One agent, containing human interleukin-2 and truncated diphtheria toxin (denileukin diftitox), has been approved for use in cutaneous T-cell lymphoma, and has shown activity in other hematologic malignancies, including leukemias and lymphomas. Diphtheria toxin has also been targeted by other ligands, including granulocyte-macrophage colony-stimulating factor and interleukin-3, to target myelogenous leukemia cells. Single-chain antibodies containing variable heavy and light antibody domains have been fused to truncated *Pseudomonas* exotoxin to target lymphomas and lymphocytic leukemias. Recombinant immunotoxins anti-Tac(Fv)-PE38 (LMB-2), targeting CD25, and RFB4(dsFv)-PE38 (BL22, CAT-3888), targeting CD22, have each been tested in patients. Major responses have been observed after failure of standard chemotherapy. The most successful application of recombinant immunotoxins today is in hairy cell leukemia, where BL22 has induced complete remissions in most patients who were previously treated with optimal chemotherapy.

1. Immunotoxins Defined

Immunotoxins are chimeric proteins containing a protein toxin and a targeting ligand derived from the immune system. Although the classic targeting ligand from the immune system is the antibody, growth factors are also proteins of immunologic interest, and growth factors connected to toxins are also considered a type of immunotoxin.[1-4] Originally, beginning 35 years ago, immunotoxins were made by chemically conjugating an antibody, either polyclonal or monoclonal, to a whole protein toxin. For more selective activity, a protein toxin devoid of its natural binding domain was connected to the antibody.[5-10]

1.1 Recombinant Immunotoxins, the Modern Immunotoxins

Immunotoxins containing chemical conjugates of ligand and toxin were often difficult to produce because the ligand and toxin had to be purified separately, the conjugation procedure was difficult and often of low yield, and the junction between the toxin and ligand could be at many different sites (for example, at any lysine residue). A percentage of the conjugate product

would have a toxin : ligand ratio much greater or less than unity, and consequently would suffer from inactivity. To facilitate the commercial development of immunotoxins, toxins and ligands were produced as single chains that were fused together genetically. Thus, in recombinant immunotoxins, the toxin-ligand junction could be defined, and the immunotoxin could be produced in and purified from *Escherichia coli* as a single-chain fusion. Recombinant immunotoxins have been produced to target cancer, infectious agents, and activated lymphocytes. This review focuses on recombinant immunotoxins used clinically in patients with hematologic malignancies, including leukemias, lymphomas, and Hodgkin disease.

1.2 Protein Toxins

The most potent protein toxins kill cells by inhibiting protein synthesis enzymatically, and may be derived from bacteria or plants. Plant holotoxins, also referred to as class II ribosome inactivating proteins, contain both binding and catalytic domains, and include ricin, abrin, mistletoe lectin, and modeccin. Hemitoxins, also called class I ribosome inactivating proteins, contain only catalytic domains and include pokeweed antiviral protein (PAP), saporin, Bryodin 1, bouganin, and gelonin.[11] Plant toxins have been shown to prevent the association of elongation factor (EF)1 and EF2 with the 60S ribosomal subunit by removing the base of A⁴³²⁴ in 28S ribosomal RNA (rRNA).[12,13] Ricin also removes the neighboring base G⁴³²³. [12] Toxin cytotoxicity is optimal when the catalytic domain alone translocates to the cytosol.[14,15] A binding domain can be translocated to the cytosol if placed within the catalytic domain, but at significant expense in terms of cytotoxic activity.[16] Attempts to fuse plant toxin fragments to ligands to make recombinant toxins have not resulted in molecules suitable for drug development. The difficulty has been either in premature separation of the toxin and ligand before cell binding, or, after internalization, lack of separation of toxin and ligand before translocation to the cytosol.[17-19] The ability of ligands to separate predictably from the catalytic domain only after internalization is an important feature of recombinant toxins,[20] and is a unique feature provided by the bacterial toxins *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT).

2. Mechanism of Action of Bacterial Toxins

Both PE and DT enzymatically modify EF2 in the cytosol.[21-23] Both catalyze the adenosine diphosphate (ADP) ribosylation of residue His699 of EF2 which is post-translationally modified to a diphthimide residue.[24-26] Both toxins are produced as single-chain proteins, containing a catalytic domain that is fused to the binding domain through a central translocation domain which facilitates transfer of the catalytic domain into the cytosol. In each case, the toxin is proteolytically cleaved within the translocation domain, and a disulfide bond holds the two fragments together until it is reduced. While they act similarly, PE and DT have very different amino acid sequences, and, as shown in figure 1, the enzymatic domain of PE is near the carboxyl terminus while that of DT is near the amino terminus. Conversely, the binding domain of PE is near its amino terminus and that of DT is near its carboxyl terminus.

2.1 Mechanism of Intoxication of *Pseudomonas* Exotoxin (PE)

Full-length PE, as shown in figure 2, is a single-chain 613 amino-acid protein containing three functional domains.[27,28] 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 function that inactivates EF2 in the cytosol. The function of domain Ib (amino acids 365–399) is unknown, but it contains a disulfide bond and separates domains II and III.

A current model of how PE kills cells, as shown in figure 2, contains the following steps:

1. The C-terminal residue (Lys613) is removed by a carboxypeptidase in the plasma or culture medium,[29] leaving the terminus REDL (Arg-Glu-Asp-Leu).
2. Domain Ia (amino acids 1–252) binds to the $\alpha 2$ macroglobulin receptor, present on animal cells, and is internalized via endosomes to the transreticular Golgi.[30]
3. The protease furin cleaves domain II (amino acids 253–364) between amino acids 279 and 280.[31–33]
4. The disulfide bond between cysteines 265 and 287, which joins the two proteolytic fragments, is reduced.[34]
5. Amino acids 609–612 (REDL) bind to the KDEL (Lys-Asp-Glu-Leu) intracellular sorting receptor, which transports the 37 kDa carboxy terminal fragment from the transreticular Golgi apparatus to the endoplasmic reticulum (ER).[35,36] Alternatively, lipid sorting to the ER may occur.[37]
6. Amino acids 280–313 mediate translocation of the toxin to the cytosol.[38,39]
7. The ADP-ribosylating enzyme function within domain III (amino acids 400–602) inactivates EF2.[21] ADP-ribosylation involves residues His440 and Glu553.[21, 40–42] His440 binds nicotinamide adenine dinucleotide (NAD) via adenosine 5'-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.

While inhibition of protein synthesis can induce eventual cell death, cell death from toxins occurs through apoptosis.[43,44]

2.2 Mechanism of Intoxication of Diphtheria Toxin (DT)

DT is a single-chain protein 535 amino acids in length, composed of an enzymatic A domain (amino acids 1–193) and a binding B domain (amino acids 482–535),[22,23,45] which are separated by an internal translocation or transmembrane (T) domain.[46] Based on structural analysis,[47,48] as shown in figure 2, DT is thought to undergo the following steps to kill cells:

1. DT is proteolytically cleaved outside the cell between amino-acid residues Arg193 and Ser194,[49] which is within a disulfide loop formed by Cys186 and Cys201.
2. DT binds on the cell surface via residues 482–535 to a complex of heparin-binding epidermal growth factor (EGF)-like growth factor precursor and CD9.[45,50]
3. DT internalizes into an endosome and unfolds at low pH,[51] and the disulfide bond linking amino acids 186 and 201 is reduced.
4. The T-domain helices TH8 (amino acids 326–347) and TH9 (amino acids 358–376) form a hairpin which inserts into the membrane of the endosome and forms a channel through which the enzymatic fragment translocates to the cytosol,[52–55] probably from early endosomes.[56]
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 EF2.[48,57]
6. As with PE, cell death occurs through apoptosis.[44]

3. Truncated Bacterial Toxins for Making Recombinant Toxins

To improve specificity, toxins for attachment to ligands have deletions in their binding domains to prevent their binding to normal cells. The most common truncated form of DT is DT388 or DAB389, containing the first 388 amino acids of DT.[58–61] The most common truncated

form of PE is PE38, composed of amino acids 253–364 and 381–613 of PE. As shown in figure 3, 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.

4. Production and Clinical Testing of Immunotoxins

Conventional immunotoxins, containing a toxin chemically conjugated to a ligand, required derivatization of the toxin and ligand, purification, conjugation, and re-purification of the conjugate with the correct toxin-ligand ratio. The difficulty and cost entailed in these multiple steps have pushed the development of recombinant toxins, which may be produced in *E. coli* transformed with a plasmid encoding the recombinant toxin. A common method of production of material for clinical trials is to harvest recombinant protein from insoluble bacterial inclusion bodies.[62–64] The insoluble protein can be washed extensively with detergent to remove endotoxin, solubilized, denatured, and reduced in a guanidine-dithioerythritol solution. The recombinant protein is then renatured by rapid dilution into a redox refolding buffer containing L-arginine and glutathione. The dialyzed renatured protein is then purified by anion exchange and gel-filtration chromatography. Recombinant toxins from *E. coli* may also be produced by harvesting the protein from *E. coli* cytoplasm or cell lysate[65,66] and then using an affinity column to capture the dilute protein. Reverse-phase chromatography followed by gel-filtration chromatography has also been used.[67] Insect, plant, and yeast cells have been produced that are resistant to toxin and can therefore produce active toxin-containing protein.[68,69] The remainder of this review will focus on the immunotoxins listed in table I, which have been tested in patients with hematologic malignancies.

4.1 The Interleukin (IL)2 Receptor as a Target for DT

The interleukin (IL)2 receptor (IL2R), which binds IL2 with high affinity ($K_d \approx 10^{-11}$ mol / L), is composed of a complex of α (CD25 or p55 or Tac), β (CD122 or p75) and γ (CD132 or p64) subunits.[70] The complex of CD122 and CD132 binds IL2 with intermediate affinity ($K_d \approx 10^{-9}$ mol / L), and CD25 alone binds IL2 with low affinity ($K_d \approx 10^{-8}$ mol / L). IL2Rs containing some or all subunits are present in cutaneous T-cell lymphoma, adult T-cell leukemia, Hodgkin disease, and other B- and T-cell leukemias and lymphomas.[71–76] IL2Rs are also found on normal activated T cells which mediate graft rejection and graft versus host disease (GVHD), as well as on normal T-regulatory cells mediating tolerance and inhibiting cancer vaccination.[77] Only a small percentage of T cells are ordinarily IL2R+.[74] To target the IL2R, human IL2 was fused to truncated DT, originally a fragment of DT containing methionine plus the first 485 amino acids of DT.[4,78,79] Clinical trials showed efficacy with DAB486-IL2 in hematologic malignancies and dose-limiting transaminase elevations.[80–82] DAB486-IL2 was improved by removal of part of the toxin containing a disulfide bond, resulting in improved half-life and tolerance in animals. DAB389-IL2, also called denileukin diftitox (figure 3), contains methionine followed by the first 388 amino acids of DT fused to IL2.[60] In a phase I trial, five complete remissions (CRs) and eight partial responses (PRs) were observed in 35 patients with cutaneous T-cell lymphoma (CTCL). One CR and two PRs were observed out of 17 patients with non-Hodgkin lymphoma (NHL).[67] The maximum tolerated dose was 27 μ g/kg every day for 5 days, limited by asthenia (fatigue). Common toxicities included transaminase elevations (62% of patients), hypoalbuminemia (86%), rashes (32%), and hypotension (55%). In the pivotal phase III CTCL trial, denileukin diftitox induced seven CRs and 14 PRs in 71 patients, and most patients had objective skin improvement.[83,84] Two dose levels were tested, 9 and 18 μ g/kg every day for 5 days, 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, usually occurred without pulmonary edema and could be prevented with corticosteroid prophylaxis.[83,85,86] Immunogenicity towards anti-DAB389-IL2 increased from 32% at

baseline to nearly 100% after one treatment cycle, and immunogenicity assessment by ELISA correlated with assessment by neutralizing antibody assay. Some responding patients continued to respond after re-treatment, despite the development of neutralizing antibodies.

4.2 Post-Approval Testing of Denileukin Diftitox

After the approval of denileukin diftitox for advanced CTCL, efficacy was shown in clinical trials of other tumors and in autoimmune disease, including peripheral T-cell lymphoma,[87] panniculitic lymphoma,[88] B-cell chronic lymphocytic leukemia (B-CLL),[89] B-cell NHL [90] and psoriasis.[91] Out of 18 patients with B-CLL, 12 received at least three cycles at 9 or 18 µg/kg every day for 5 days, and six (50%) of the 12 had 95–99% reductions in circulating malignant cells. Four (33%) of the 12 patients had 29–80% reductions in lymph nodes with two patients qualifying as PRs, lasting 14 and >19 months. Of 28 CLL patients in a phase II trial, 22 received at least two cycles and resulted in one CR (4%) and five PRs (23%).[92] Out of 45 evaluable patients with NHL treated in a phase II trial, there were three CRs (7%) and eight PRs (18%). The median time to treatment failure in responding patients was 7 months. [90] Another phase II trial reported a response rate of 10% in 29 patients with indolent NHL, and no difference in response rate between CD25+ and CD25– cases.[93] In combination with rituximab, a 32% response rate was observed in 38 evaluable patients.[94] In CTCL, response rates of 49–63% were reported in early and advanced disease.[95] Thus, denileukin diftitox, the only targeted protein toxin so far approved for use, is effective in several hematologic malignancies. Efficacy in CTCL is related to CD25 expression[96] and was improved by combination with bexarotene.[97] One limitation for CTCL, CLL or NHL is the lack of high affinity IL2Rs in a large percentage of cases, usually due to lack of CD122.

4.3 Targeting PE to CD25

To target IL2R+ disorders expressing CD25 with or without other IL2R subunits, the anti-CD25 monoclonal antibody (mAb) anti-Tac was used as a ligand instead of IL2. CD25 alone binds with higher affinity to anti-Tac ($K_d \approx 10^{-10}$ mol/L) than to IL2 ($K_d = 10^{-8}$ mol/L),[98] and CD25 greatly outnumbers CD122 and CD132 on most malignant cell types.[71,72] Early studies indicated that CD25 alone would not internalize anti-Tac,[99] but CD25 alone does internalize bound recombinant toxin.[59,100,101] A recombinant single-chain Fv[102,103] was constructed in which the variable heavy domain (V_H) was fused to the variable light domain (V_L) via the peptide linker (G_4S)₃, and V_L was fused to truncated PE.[104] The resulting recombinant immunotoxin anti-Tac(Fv)-PE40 and the slightly shorter derivative anti-Tac(Fv)-PE38 (called LMB-2, figure 3) were selectively cytotoxic towards CD25+ malignant cell lines and towards leukemic cells freshly obtained from patients.[59,101,105–108] Studies on fresh leukemic cells were important because malignant cells freshly obtained from patients typically display far fewer CD22 sites/cell compared with cell lines. Antitumor studies in mice with CD25+ human xenografts showed complete regressions, and biodistribution studies showed concentration of LMB-2 into CD25+ tumors.[101,106] Fresh malignant cells from patients with adult T-cell leukemia (ATL) and hairy cell leukemia (HCL) were much more sensitive than were CLL cells from patients, probably due to lower CD25 expression in the latter. Phosphorothioate oligodeoxynucleotides can up-regulate CD25 expression on CLL cells.[109] Cyclosporin has been reported to increase the sensitivity of ATL toward anti-Tac (Fv) toxin.[110]

4.4 Clinical Development of LMB-2 in CD25+ Hematologic Malignancies

LMB-2 was tested in a phase I trial in 35 patients with chemotherapy-resistant leukemia, lymphoma and Hodgkin lymphoma. Seven PRs and one CR were achieved, all in the 20 patients who received a total dose of >60 µg/kg/cycle. All of four patients with HCL responded, with one CR and three PRs.[111] CR was associated with the resolution of pancytopenia and

eradication of circulating malignant cells. Patients with CLL, ATL, CTCL and Hodgkin lymphoma achieved PR.[112] The most common toxicities included transaminase elevations, which were associated with fever and thus appeared to be mediated by cytokines.[113,114] Only six of the 35 patients were prevented from receiving re-treatment due to neutralizing antibodies. The eight CLL patients did not develop neutralizing antibodies after a total of 16 treatment cycles. In 11 patients with Hodgkin lymphoma, high levels of neutralizing antibodies were observed in three patients after one cycle and in two additional patients after two to three cycles. Phase II trials are currently underway in CD25+ CLL, CTCL, and HCL. A phase I/II trial is underway in ATL following fludarabine and cyclophosphamide to prevent immunogenicity to LMB-2 and rapid progression in ATL after response to LMB-2.[128] Prior to LMB-2, HCL patients are now being treated in clinical trials with the anti-CD22 recombinant immunotoxin BL22 (figure 3).

4.5 Targeting CD22 with Immunotoxins

Chemical conjugates were previously constructed to target CD22 on B-cell malignancies, including the mAb RFB4 conjugated to deglycosylated ricin A-chain.[115-119] The first anti-CD22 immunotoxin containing PE contained the mAb LL2, and induced complete regression in human xenograft models.[120,121] However, LL2 as a single-chain Fv was unstable and an active recombinant immunotoxin could not be made. Fortunately, a stable recombinant immunotoxin, RFB4(Fv)-PE38, could be made from the variable domains of the RFB4 mAb, and was shown to be cytotoxic toward CD22+ cell lines.[122]

4.6 BL22

4.6.1 Preclinical Development—The stability of RFB4(Fv)-PE38 was increased by mutation of residues Arg44 in the V_H domain and Gly100 in the V_L domain to cysteines, leading to disulfide bond formation between the variable domains. V_H was fused to PE38, resulting in BL22 (figure 3).[123] BL22 is considered fully recombinant, since the disulfide bond between V_L and V_H-PE38 forms naturally during *in vitro* renaturation of the two fragments (in the redox buffer with L-arginine) and chemical conjugation is not needed. BL22 has resulted in complete regressions in human CD22+ B-cell lymphoma xenografts in mice at plasma levels that were safely tolerated in Cynomolgus monkeys.[124] Malignant cells obtained directly from patients with CLL or NHL were sensitive to BL22.[125] Several-fold and up to >10-fold greater activity towards CLL cells was observed with the mutant HA22, which has higher affinity (lower off-rate) for CD22 due to the amino acids Thr, His, Trp replacing Ser, Ser, Tyr at positions 100, 100a and 100b of the V_H domain.[126,127]

4.6.2 BL22 Phase I Trial in Patients with B-Cell Malignancies—BL22 was administered to 46 patients with B-cell lymphomas and leukemias.[128,129] A total of 265 cycles of BL22 were administered, with up to 33 cycles/patient. Responses were best in the 31 patients with HCL, all of whom had received one to six separate prior courses of cladribine. A total of 19 (61%) of the 31 HCL patients had CRs and six (19%) had PRs. CR was achieved in all of three patients with the poor prognosis variant HCLv.[130-133] BL22 induced CR after cycle 1 in 11 patients, and after cycles 2–14 in eight patients. At the time of CR, only one of the 19 patients with CRs had minimal residual disease in bone marrow biopsy by immunohistochemistry, which is reported to be a risk factor for early relapse.[134] Cytopenias improved in all responders. CRs have lasted for 5–67 (median 36) months, with seven (37%) of 19 still in CR at 35–67 (median 51) months of follow-up. Of the first three HCL patients to relapse, all three achieved a second CR when re-treated with BL22. There was one PR among 11 CLL patients and no responses in four patients with NHL. High levels of neutralizing antibodies were observed in 11 (24%) of the total 46 patients after cycles 1–8. Plasma levels in patients with high disease burden were much greater on subsequent cycles after patients responded, compared with cycle 1. Dose-limiting toxicity included a cytokine release

syndrome associated with immunogenicity in one patient, and hemolytic uremic syndrome (HUS) in five. Of the five patients with HUS, four had HCL, and in all four HCL patients HUS presented clinically with hematuria and hemoglobinuria by day 8 of cycle 2. These patients required 6–10 days of plasmapheresis but not dialysis for complete resolution of renal function and correction of thrombocytopenia and anemia. In all cases, pre-existing cytopenias of HCL resolved, as well as those related to HUS.

BL22 was 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. A phase II trial of BL22 in 36 patients with cladribine-resistant HCL has been completed, which has confirmed the phase I experience. Rates of HUS and immunogenicity were reduced by re-treating only those patients who did not achieve blood counts consistent with a CR after 1 cycle. The CR and overall response rates were significantly higher in patients with spleens present and <200 mm, compared with patients with larger spleens or post-splenectomy. This argues against delay or splenectomy prior to BL22 in HCL patients resistant to cladribine. Finally, HA22 (the high-affinity mutant of BL22) is currently undergoing phase I testing in HCL.[135]

4.7 Targeting GM-CSFR with DT388-GM-CSF

To target the granulocyte-macrophage colony stimulating factor receptor (GM-CSFR), which is expressed in acute myelogenous leukemia (AML) cells from most patients, human GM-CSF was fused to truncated bacterial toxins, and DT388-GM-CSF (DTGM) was found to be more cytotoxic than GM-CSF-PE38KDEL.[136] DTGM was tested in 31 patients with relapsed or refractory AML, all of whom were resistant to chemotherapy.[137] One CR and two PRs were observed, and the major toxicity was cytokine release syndrome. Pre-existing antibodies to DT were observed in 28 of 31 patients.[137,138] Cytotoxic plasma levels of DTGM could be detected in 14 of 20 patients with anti-DT antibody concentrations <2.2 µg/mL, and in 2 of 11 patients with anti-DT antibody concentrations >2.2 µg/mL.[137] To avoid cytokine release syndrome, IL3, which does not bind to monocytes and macrophages as does GM-CSF, was fused to truncated diphtheria toxin.

4.8 Targeting IL3R with DT388-IL3

The recombinant toxin DT388-IL3 was found to be cytotoxic towards AML cell lines,[139] primary AML or CML cells,[140,141] but not to normal hematopoietic progenitors.[142] DT388-IL3 prolonged survival in tumor-bearing mice[143] and was produced for phase I clinical testing.[144] In phase I testing, one CR and one PR were observed among 40 evaluable AML patients, and one PR was observed among five patients with myelodysplastic syndrome.[145]

5. Obstacles and Opportunities in the Development of Immunotoxins for Hematologic Malignancies

5.1 Immunogenicity

While immunogenicity is much less frequent in hematologic malignancies compared with solid tumors, it still remains a considerable obstacle to effective treatment. The incidence of immunogenicity after a single cycle of immunotoxin is 0–40% for hematologic tumors, being very low in CLL and higher in T-cell malignancies. Patients have been reported to respond to some fusion toxins after immunogenicity is detected.[83] Antibodies that are neutralizing can be detected by determining whether serum neutralizes the cytotoxicity of the immunotoxin towards target cells. The presence of neutralizing antibodies results in a decrease in biologically

active immunotoxin. This compromises efficacy unless clinical benefit is based on a different mechanism. Several approaches have been tested to prevent immunogenicity of recombinant immunotoxins. Polyethylene glycol (PEG) modification (PEGylation), which is effective in blocking immunogenicity of interferon[146] and L-asparaginase,[147] resulted in only modest reduction in immunogenicity of LMB-2 preclinically.[148,149] PEGylation of a toxin is more challenging than PEGylation of smaller molecules, since few sites on toxins can be disturbed without significantly reducing toxin activity. A large number of B-cell and T-cell epitopes have been identified on *Pseudomonas* exotoxin,[150-152] and mutations of these epitopes might lead to at least partial 'humanization' of the molecule. Rituximab, which depletes B lymphocytes, was not effective in avoiding immunogenicity in patients receiving LMB-1 for solid tumors.[153] Patients with CLL have very low immunogenicity to recombinant immunotoxins, due either to prior treatment or to disease. To determine whether chemotherapy can reduce immunogenicity, a phase I/II trial of fludarabine and cyclophosphamide is planned at the National Institute of Health to determine whether these agents in ATL can block subsequent immunogenicity to LMB-2.

5.2 Normal Tissue Toxicity

Immunotoxins commonly cause VLS, which is expected because cytotoxic protein must pass through endothelial cells to exit the blood vessels. However, VLS is rarely dose limiting for recombinant immunotoxins, which have a relatively short half-life in plasma. Hepatotoxicity is commonly dose limiting in mice, but only rarely in patients. HUS appears to be a dose-limiting toxicity restricted to BL22, since hundreds of cycles of other recombinant immunotoxins containing the same toxin do not cause HUS. The lack of requirement for plasmapheresis and lack of abnormality of the metalloprotease ADAMTS-13 in patients with HUS from BL22 make this syndrome distinct from other causes of HUS, and further investigation will be needed to elucidate its mechanism.

5.3 Potential for Combination with Chemotherapy

For many types of disease, immunotoxins are unlikely to work alone. Their half-lives may be too limited for diffusion into solid lymphomatous masses. Clinical trials of immunotoxins administered by continuous infusion have thus far not suggested significant improvements in efficacy over the bolus infusion route.[118,154,155] It is possible that combination with other therapeutic agents having non-overlapping toxicities will result in better responses. One mechanism of synergy could be the reduction of high concentrations of soluble receptor in tumors by chemotherapy, allowing better distribution of the immunotoxin to target cells.[156] Similarly, treatment of microscopic disease might be useful, after cytoreduction by surgery, chemotherapy, or radiotherapy. Finally, the antigen and the disease targeted remain major determinants of immunotoxin efficacy. As combinations of diseases and antigen targets are chosen, we can anticipate exciting successes in the future development of immunotoxins.

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Fig. 1.

Schematic structure of bacterial toxins. *Pseudomonas* exotoxin is a single-chain 613 amino-acid protein containing three functional domains.[27-28] 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 ADP ribosylating enzyme which inactivates elongation factor 2 in the cytosol, resulting in cell death.[33] Domain Ib separates domains II and III and contains amino acids 365–399. Diphtheria toxin is 535 amino acids in length and is composed of the enzymatic (A) domain (amino acids 1–193)[22] and the binding (B) domain (amino acids 482–535).[45] The translocation or transmembrane (T) domain is located in between. [55]

Fig. 2.

Intoxication of target cells by toxins. Current models of intoxication are shown for the bacterial toxins *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT). After internalization, PE and DT undergo proteolysis and disulfide-bond reduction to separate the catalytic (domain III for PE, A-chain for DT) from the binding (domain Ia for PE, B-chain for DT) domains.[31-33, 49] PE undergoes both removal of the carboxy terminal lysine (K) residue[29] and processing between residues 279 and 280, resulting in a 37 kDa carboxy terminal toxin fragment ending in the residues REDL (Arg-Glu-Asp-Leu). This fragment is believed to be transported intracellularly via the KDEL (Lys-Asp-Glu-Leu) receptor from the Golgi to the endoplasmic reticulum (ER),[36] where it translocates to the cytosol. DT undergoes processing between residues 193 and 194.[49] The catalytic A-chain (amino acids 1–193) then translocates to the cytosol through the endosome with the help of translocation (T) domain residues 326–347 and 358–376, which form an ion channel.[52-55] Cell death caused by bacterial toxins has been shown to occur through apoptosis.[44,157] **EF2** = elongation factor 2.

DAB389-IL2 (denileukin diftiox)

DT388-GMCSF (DTGM)

DT₃₈₈IL3

Anti-Tac(Fv)-PE38 (LMB-2)

RFB4(dsFv)-PE38 (BL22)

Fig. 3.

Schematic structure of recombinant immunotoxins. PE38 is a 38 kDa truncated form of *Pseudomonas* exotoxin (PE) containing amino acids 253–364 and 381–613. The truncated form of diphtheria toxin (DT) used in recombinant toxins is DT388 in DTGM or DAB389 in denileukin diftiox (shown), each of which contains 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 (G₄S)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 domain from the mAb RFB4 disulfide bonded to a fusion of the V_H domain with PE38. The disulfide bond connecting V_H and V_L is formed between two 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 the amino terminus of the toxin. **A** = enzymatic domain of the diphtheria toxin; **B** = binding domain of the diphtheria toxin; **C3** = ASGGPE (Ala-Ser-Gly-Gly-Pro-Glu) connector; **DTGM** = DT388-GM-CSF; **GM-CSF** = granulocyte macrophage colony-stimulating factor; **IL2** = interleukin-2; **IL3** = interleukin-3; **T** = transmembrane or translocation domain of the diphtheria toxin.

Table I
Recombinant immunotoxins tested clinically in recent years against hematologic malignancies

Agent	Antigen	Ligand	Truncated toxin	Basic toxin	Diseases	References
Demileukin diftitox	IL2R	IL2	DAB389	DT	CTCL, CLL, NHL	86,89-90
BL22	CD22	dsFv	PE38	PE	HCL, CLL, NHL	128
LMB-2	CD25	scFv	PE38	PE	NHL, leukemias	111-112
DT388-GM-CSF	GM-CSFR	GM-CSF	DT388	DT	AML	137
DT388-IL3	IL3R	IL3	DT388	DT	AML, MDS	145

AML = acute myelogenous leukemia; **CLL** = chronic lymphocytic leukemia; **CSF** = colony-stimulating factor; **CSFR** = CSF receptor; **CTCL** = cutaneous T-cell lymphoma; **ds** = double strand; **DT** = diphtheria toxin; **GM** = granulocyte macrophage; **HCL** = hairy cell leukemia; **IL2** = interleukin-2; **IL2R** = IL2 receptor; **IL3** = interleukin-3; **IL3R** = IL3 receptor; **MDS** = myelodysplastic syndrome; **NHL** = non-Hodgkin lymphoma; **PE** = *Pseudomonas* exotoxin; **sc** = single strand.