IMMUNOTOXINS: A MAGIC BULLET IN CANCER THERAPY

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Abstract:
Immunotoxins are composed of a protein toxin connected to a binding ligand such as an antibody or growth factor. These molecules bind to surface antigens and kill cells by catalytic inhibition of protein synthesis within the cell cytosol. Immunotoxins have evolved with time and technology and recently, third generation immunotoxins are made by recombinant DNA techniques. The majority of these immunotoxins targeted antigens selectively expressed on cancer cells. It has been hoped that these agents could cause regression of malignant disease in patients. The studies carried over the plasma clearance of antibody-ricin-A-chain immunotoxins have been shown that after intravenous injection in animals of different species, immunotoxins are rapidly eliminated from the bloodstream. It is due to the mannose residues on the rich A-chain moiety which are specifically recognized by liver cells. The coadministration of yeast mannan with immunotoxin enhances the level of active immunotoxin in circulation by inhibition of liver uptake, which drastically improves the anti-cancer efficacy of immunotoxin in vivo. Immunotoxins can be given in combination with other anticancer therapies to manage tumour penetration. Immunotoxins also firmly increase the viral mortality when used along with effective anti-retroviral drugs. Besides many advantages, the development of many immunotoxins for cancer therapy has still many challenges like immunogenicity, unwanted toxicity, and difficulty in production, limited half-life, and resistance.

Key words: *Immunotoxins, antitumor, rDNA technique, ricin-A-chain immunotoxins, antiretroviral.*

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INTRODUCTION:

Since last many years researchers have been searching for novel therapeutic agents of high potency and high selectivity. Recently the search for specific as well as effective anti-tumour chemotherapeutic agents has been the main aim of such research activities. There is no doubt that drug combinations today can produce long-term remissions and even cures for several, formerly fatal tumours, particularly those of the lymphatic and circulatory systems. Nevertheless the side effects of these agents are formidable and many of the commonest tumours do not respond well. Tumours such as the small cell ("oat cell") carcinoma of the lung respond well to conventional chemotherapy, with many patients achieving long term remission. However, despite these patients having no measurable tumour burden, nearly all patients relapse. Clearly some cells have evaded treatment. For these reasons several groups have attempted to combine the toxicity of cytotoxic agents or radioisotopes with the specificity of polyclonal or monoclonal antibodies, raised against tumour specific antigens or key cell differentiation markers. In practice, an antigen absolutely specific to a given tumour cell has not been found, and some of the most effective immunotoxins have been directed against sub-populations of cells bearing specific differentiation markers [1].

An immunotoxin is a human-made protein that consists of a targeting portion linked to a toxin. When the protein binds to that cell, it is taken in through endocytosis, and the toxin kills the cell. They are used for the treatment of some kinds of cancer and a few viral infections. These chimeric proteins are usually made of a modified antibody or antibody fragment, attached to a fragment of a toxin. The targeting portion is composed of the Fv portion of an antibody that targets a specific cell type. The toxin is usually a cytotoxic protein derived from a bacterial or plant protein, from which the natural binding domain has been removed so that the Fv directs the toxin to the antigen on the target cell [2].

Immunotoxins have recently been tested clinically in hematologic malignancies and solid tumors and have demonstrated potent clinical efficacy in patients with malignant diseases that are refractory to surgery, radiation therapy and chemotherapy - the traditional modalities of cancer treatment. This therapy is thus evolving into a separate modality of cancer treatment, capable of rationally targeting cells on the basis of surface markers. Efforts are underway to obviate impediments to clinical efficacy, including immunogenicity and toxicity to normal tissues. Immunotoxins are now being developed to new antigens for the treatment of cancer [3].

HISTORICAL DEVELOPMENT:

i. Chemical conjugates of full-length and truncated PE: The first PE-based immunotoxins were composed of full-length PE protein attached to whole monoclonal antibodies (MAbs). Unfortunately, these immunotoxins retained some ability to bind to normal noncancerous cells, resulting in high toxicity in animals. Evolving data about the structure and function of PE during the 1980s enabled us to identify and delete regions of the toxin that were not needed for cell killing. The most important of these regions was the cell binding domain of PE. Eventually, we produced PE38, a smaller, truncated toxin, which by itself did not bind to or kill cells, but could be directed to cells by attaching it to an antibody. These early immunotoxins containing either full-length or truncated forms of PE were attached to MAbs via conventional chemical coupling methods. Their chemical heterogeneity, large molecular size that limited their entry into bulky tumors, and high production costs were among the tribulations associated with these antibody-toxin conjugates.

ii. Recombinant immunotoxins: In the 1990s, advances in recombinant DNA techniques enabled the development of single chain recombinant immunotoxins. DNA sequences encoding only the antigen-binding site of the antibody (the Fv portion) were fused to DNA sequences encoding PE38, the truncated form of PE. These single-chain recombinant proteins were homogeneous in composition and had a smaller molecular size than their chemical conjugate predecessors, and they could be produced in Escherichia coli, resulting in lower production costs.

The first recombinant immunotoxin that was developed at NCI and that showed evidence of clinical activity in hematologic malignancies was LMB2, an immunotoxin targeting the CD25 antigen.

iii. PE-based anti-CD22 immunotoxins for B-cell malignancies: CD22, an antigen expressed on the surface of normal B cells and in the vast majority of B-cell leukemias and lymphomas, subsequently became a new target for immunotoxin development. In 1997, using a murine anti-CD22 antibody developed by Peter Amlot (Royal Free Hospital, London, United Kingdom), we (FitzGerald and Pastan) synthesized a single-chain immunotoxin, RFB4(scFv)-PE38, that was cytotoxic to CD22-expressing cells *in vitro*.

iv. BL22[RFB4(dsFV)PE38, CAT-3888]: Further improvements in anti-CD22-PE38 immunotoxins occurred with the introduction of a disulfide bond as the link between the heavy and light chain domains of the Fv antibody fragment to replace the peptide linker. This led to RFB4 (dsFv)-PE38, an immuno toxin with significant improvement in stability compared with its predecessor, RFB4 (scFv)-PE38. This new immunotoxin was named BL22 which exhibited high activity both *in vitro* and *in vivo* against B-cell malignancies, supporting clinical evaluation. BL22 was then clinically evaluated in patients with refractory/resistant hairy cell leukemia (HCL), non-Hodgkin lymphoma (NHL), and chronic lymphocytic leukemia (CLL) in a phase 1 study and in HCL patients in a phase 2 study. In the phase 1 study (ClinicalTrials.gov identifier: NCT00021983), the highest activity was obtained among HCL patients—81% overall response rate (ORR), 61% complete response (CR), and 19% partial response (PR). Dose-limiting toxicities were reversible hemolytic uremic syndrome (HUS) and vascular leak syndrome. Reversible HUS was observed in 5 of 46 patients (11%) enrolled in the study. In the phase 2 study (ClinicalTrials.gov identifier: NCT00074048), the ORR was 72% (CR, 47%; PR, 25%). Best responses to BL22 after cladribine failure were achieved before the patients developed massive splenomegaly or underwent splenectomy. Grade 3 reversible HUS developed in 2 patients (6%) and grade 1 HUS in 1 patient (3%). Other common adverse events observed in patients treated with BL22 were grade 1 or 2 hypoalbuminemia, elevated alanine and aspartate aminotransferases, edema, myalgia, proteinuria, fatigue, nausea, and fever. The mechanism underlying HUS is not completely understood; patients develop thrombocytopenia, hyperbilirubinemia, hemolysis, creatinine elevations, and proteinuria; and in most instances, HUS is completely reversible. BL22 has also been studied in pediatric acute lymphoblastic leukemia (ALL) and NHL. BL22 exhibited modest activity during the phase 1 study (ClinicalTrials.gov identifier: NCT00077493) in 23 children and adolescents with ALL and NHL. No ORRs were obtained and a modest hematologic response was seen among ALL patients. BL22 was patented and initially produced at the NCI. In 2005, Cambridge Antibody Technology (CAT) licensed BL22 and named it CAT-3888.

v. Moxetumomab pasudotox (HA22, CAT-8015): To further optimize the anti-CD22-PE38 immunotoxin, three point mutations were introduced to the heavy chain domain of BL22 using phage display. This resulted in an immunotoxin with a higher binding affinity to CD22 and a 10-fold higher cytotoxicity against malignant B cells in vitro. This new immunotoxin was named HA22. In 2005, HA22 was licensed to CAT, which named the drug CAT-8015. In 2007, AstraZeneca acquired CAT, and currently MedImmune, a subsidiary of AstraZeneca, is developing the immunotoxin in collaboration with the NCI. Its generic name, moxetumomab pasudotox, was recently approved [4].

On the 16 May 2013, AstraZeneca announced that CAT-8015 had started Phase III clinical trials [5].

GENERAL CHARACTERISTICS:

Immunotoxins are chimeric proteins composed of an antibody or antibody fragment derived from the immune system (conferring target specificity) that is fused or conjugated to a toxic protein. Immunotoxins have evolved with time and technology, and can be generally divided into three generations. The first generation immunotoxins were produced by chemically coupling native toxins to antibodies using cross linking reagents that form disulfide bonds connecting the toxin to the antibody. Second generation immunotoxins, like the first generation, were made by chemical coupling methods. The first immunotoxins contained full length toxins, including their targeting domains that are not cancer cell-specific. As research progressed, toxin autonomic cell-binding domains were recognized and removed. The resulting toxin fragment, that could no longer bind normal cells, was coupled to an antibody. Third generation immunotoxins are made by recombinant DNA techniques and combine variable fragments of an antibody (Fv) and toxins without their cell binding domains on the same protein.

More than 1,000 third generation immunotoxins have been developed since the first report of Fv production in 1988, in which the variable domains of the heavy chain and light chain of the antibody were connected by a peptide linker. The majority of these immunotoxins targeted antigens selectively expressed on cancer cells. It has been hoped that these agents could cause regression of malignant disease in patients. Being of bacterial or plant origin, toxins and toxin domains are highly immunogenic to humans and the immune reaction to the immunotoxin restricts the treatment of each patient to a few doses. Today, efforts are being made to eliminate immunogenic epitopes from toxin surfaces to reduce the immunogenicity of the native toxins [6].

DESIGN OF IMMUNOTOXINS:

i. The Antibody Moiety:

Antibodies used are generated using monoclonal antibody technology. The target protein (termed an antigen), usually a surface antigen of malformed cell is injected into a mouse, and when the mouse has developed a sufficient immune response to the antigen (including many protein-specific, antibody-producing B cells), its spleen cells (containing B cells) are harvested and fused to myeloma cells. Myeloma cells are an immortal cell line that will allow the fused cells to grow indefinitely at a fast rate. These myeloma-B cell hybrid cells are called hybridoma cells, and can be selected for and tested to verify that they produce the desired antibody. The hybridoma clones that produce the antibody demonstrating the required specific binding activity can be grown in large quantities, and the monoclonal antibodies can be harvested for use in an immunotoxin.

Since antibodies have high molecular mass, they have a hard time penetrating solid cancer tumors where the blood supply is fairly restricted. As a solution, immunotoxins are created that utilize only the protein-binding part of the antibody, called the variable region. They do this by cleaving off this region with a protease (making a Fab fragment), or by cloning the variable region into bacteria and expressing as a single-chain antibody. Proteolysis however does not easily yield molecules smaller than a Fab fragment, and microbial expression of single chain Fv (scFv) is currently the favoured method of production. In scFv, the variable (V_H and V_L) domains are stably tethered together with flexible polypeptide linker. Smaller antibody fragments such as Fab or scFv exhibit better pharmacokinetics and also provide full binding specificity because antigen-binding surface is unaltered.

ii. Toxins Used:

Toxins used in immunotoxin constructs are derived from bacteria, fungi, and plants, and most function by inhibiting protein synthesis. Bacterial toxins commonly used in immunotoxins include Diphtheria toxin (DT) and the toxin from *Pseudomonas* exotoxin (PE). Plant toxins utilized in immunotoxins include the A chain of ricin (RTA), and the ribosome inactivating proteins (RIPs) gelonin, pokeweed antiviral protein, and dodecandron. Because it is an enzyme, one toxin molecule can work on many substrate molecules, having a devastating effect on the cell. Toxins such as diphtheria toxin (DT) and *Pseudomonas* exotoxin (PE) prevent protein synthesis by an effect on elongation factor 2 (EF-2). In order to be effective, however, immunotoxin must be internalized and route to the appropriate intracellular compartment for translocation of their attached toxin into the cytosol. The targeting moiety and toxin are joined by a cross linker which is stable extracellularly but labile intracellularly so that the toxin can function in the cytosol.

PRODUCTION OF IMMUNOTOXINS:

Immunotoxins are 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. 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. The other method is that harvesting the protein from cytoplasm or cell lysate and then using an affinity column to capture the dilute protein.

MECHANISM OF IMMUNOTOXINS:

The mechanism by which immunotoxins work to kill diseased cells in the body is quite simple. Using AIDS therapy as an example, let's say we have developed an immunotoxin to kill HIV-infected cells by raising antibodies that bind to GP120, a viral protein found on the outside of only HIV-infected cells. Once an AIDS patient has been treated, the immunotoxin floats around in the bloodstream until it binds to a GP120 molecule on the outside of an infected cell. Once bound, the GP120-immunotoxin complex gets pulled inside the cell by endocytosis, where it is either localized to an acidified endosome (if DT is the toxin), or the endoplasmic reticulum (ER) and trans-golgi apparatus in the cell. Inside these organelles, the linker holding the toxin to the antibody is cleaved. Usually the linker is made with an internal disulfide bond, so that it is stable in the oxidizing atmosphere outside the cell and cleaved by reduction in the reducing environment inside the cell. Once freed from the antibody, the toxin now catalytically inactivates the protein synthesis machinery of the cell. The bacterial toxins perform this by inactivating the ribosome accessory protein elongation factor-2 (EF-2). The plant RIPs accomplish their task by cleaving a single adenine base from the ribosomal RNA so that it can no longer bind EF-2. Either way, the inactivation of protein synthesis leads to the death of the cell [7].

Table 1: Immunotoxins under Development

Sr. No.	Immunotoxin Name	Target	Disease Indication	Toxin	Reference
1.	Anti-B4-blocked ricin	CD19	Lymphocytic leukemia	Ricin	[8]
2.	IL13PE	IL13Rα2	Pancreatic cancer	PE	[9]
3.	H22ETA	CD64	Arthritis	PE	[10]
4.	scFv35-ETA	Acetylcholine receptor-γ	Rhabdo-myosarcoma	PE	[11]
5.	Affitoxin	HER2	HER2[+]cancers	PE	[12]
6.	VB6-845	EpCAM	Various cancers	Bouganin	[13]
7.	FR betaPE	Folate receptor β	Inflammation	PE	[14,15]
8.	26292(Fv)-PE38	CD123/IL3Rα	Acute myeloid leukemia	PE	[16]
9.	SS1P	Mesothelin	Lung cancer	PE	[17]
10.	BL22	CD22	Hairy cell leukemia	PE	[18]
11.	CD19-ETA	CD19	Lymphocytic leukemia	PE	[19]
12.	scFv(MUC1)-ETA	MUC1	Breast cancer	PE	[20]

ADVANTAGES AND DISADVANTAGES:

Immunotoxins, together with all antibody-based therapeutics, are a growing field in targeted cancer therapy. New and better antibodies are developed now a days and toxins are being improved to better fit their target.

Many common disadvantages still need to be improved, as many unwanted side effects are caused due to immunotoxin use. Of course, many therapeutic drugs, especially in the field of cancer therapy, cause side effects. Some of which are relatively "minor" like diarrhea, nausea or mild fever, but some are harsh and dose-limiting. As opposed to chemotherapy, immunotoxins are considered specific to their target cells, and therefore they can deliver highly potent toxic moieties that could not be given systematically. Moreover, the dosage could be less limited by systematic poisoning. Unfortunately, other characteristics of the immunotoxins prevent them from being of benefit because of these disadvantages. One problem that should be referred to is immunogenicity. Immunotoxins, as opposed to chemotherapy, are constructed from proteins that the human immune system cannot ignore. The immune system reacts to both the murine antibody fragment and to the protein toxin, by generating HAMA and human anti-toxin antibodies (HATA). When antibody levels in the blood are high, the patient can no longer receive the treatment, as in the case of N901-bR, XomaZyme-Mel and XomaZyme-791. This problem is addressed by the new generation of immunotoxins that are being constructed from humanized or human antibody fragments and efforts are also being made to minimize toxin immunogenicity. Another major

disadvantage of immunotoxins is the cause of CSL or VSL. Immunotoxins are usually given intravenously, and therefore encounter epithelial cells surrounding the blood vessels, this can cause VLS that can be severe and even cause death. Other severe side effects were observed, like hepatotoxicity, which brought about the termination of some clinical trials. The antibody moiety's size also affects the potency of immunotoxins. Although the abnormality of angiogenesis in cancer tumors increases their leakiness and cause accumulation of high molar weight molecules in the tumor area, the permeability of solid tumors is size-limited. Therefore, the potency of immunotoxins may be increased by lowering its molar weight. Since immunotoxins are defined as chimera of two proteins, their minimization is limited to about 45kDa.This fact led to the development of many immunotoxins for the treatment of non solid tumors where tumor penetration is not an issue.

IMMUNOTOXINS IN THE TREATMENT OF HIV:

Immunotoxins with their outstanding targeting and destroying capabilities, provide the patient with enough time to recoil or fight back by re-building the fire wall against the constant invasion of the HIV virus and its family. Thus, they help in weeding out most of the other infections which enter the body by an express-way during the attack by the HIV. Keeping that in hold, they firmly increase the viral mortality when used along with effective anti-retroviral drugs. Performing these many tasks they are humble and are highly specific when designed to

perfection. These are highly versatile in serving the humans [21].

RICIN-A-CHAIN IMMUNOTOXINS:

In recent years, antibody - ricin-A-chain immunotoxins have been investigated as antineoplastic agents. To achieve *in vivo* therapy it is necessary that the immunotoxin remains in circulation at a sufficiently high level for a sufficiently long time to allow binding to tumor cells to occur. Therefore, examination of the pharmacology of immunotoxins may elucidate the reasons for the poor *in vivo* antitumor effect of immunotoxin described before. The studies carried over the plasma clearance of antibody-ricin-A-chain immunotoxins have been shown that after intravenous injection in animals of different species, immunotoxins are rapidly eliminated from the bloodstream. Neither the properties of the antibody moiety nor the nature of the linkage binding rich A-chain to antibody account for the disappearance of immunotoxin from the plasma. On the other hand, it has been found that the rapid clearance of immunotoxin is due to the mannose residues on the rich A-chain moiety which are specifically recognized by liver cells. The coadministration of yeast mannan with immunotoxin enhances the level of active immunotoxin in circulation by inhibition of liver uptake, which drastically improves the anti-cancer efficacy of immunotoxin *in vivo [22]*.

PROBLEMS 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.

i. Immunogenicity:

One of the problems with monoclonal antibodies is their mouse, or murine, origin. A human patient's own immune system will recognize the murine antibody as foreign, and will clear the antibody from the bloodstream quickly, greatly reducing the immunotoxin's effectiveness. To combat this, laboratories have engineered "humanized" antibodies where the part of the antibody that the human immune system identifies as of mouse origin, called the constant region of the antibody, is swapped out for a human constant region. The method most useful for other biologic agents, such as interferon and L-

asparaginase, is PEGylation, which not only blocks immunogenicity but also prolongs half-life.

ii. 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 Vascular leak syndrome (VLS). Studies have shown that RTA binds directly to endothelial cells, while truncated PE requires a ligand that cross-reacts with the endothelium. 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. Such studies led to a mutant form of RTA that shows less VLS in an animal model. Hepatotoxicity, a typical side effect of recombinant immunotoxins, is due to the binding of basic residues on the Fv to negatively charged hepatic cells. Hepatoxicity appears to be related to cytokine production, possibly by the Kupffer cells of the liver. Although recombinant immunotoxins that specifically bind to antigens expressed on the liver are not well tolerated systemically, recombinant immunotoxins like LMB-2 and BL22 that cause transaminase elevations are not associated with decreased hepatic function. Renal toxicity due to immunotoxins could be nonspecific because the kidneys are the dominant route of excretion of recombinant immunotoxin.

CONCLUSION:

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 small recombinant fusion toxins that contain either growth factor or Fv fragments as ligands. Future development may include combinations of immunotoxins with other anticancer therapies in order to overcome problems of tumor penetration, toxicity, and immunogenicity. A successful immunotoxin therapy is obtained by the careful study and analysis of biology of tumour cells, choice of the ligand and toxin and their mode of delivery.

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