Chapter 6
Challenges for Therapeutic Application of *Pseudomonas* Exotoxin-Based Immunotoxins

Vladimir Dergachev and Itai Benhar

Abstract Immunotoxins are therapeutic molecules that belong to a class of biopharmaceuticals called "Armed antibodies". Immunotoxins are based on very potent toxins of bacterial or plant origin that lack target-cell specificity. To make them target-cell-specific, the non-specific cell binding domains of the original toxins are replaced with a target-cell-specific binding protein, in most cases a monoclonal antibody or a recombinant antibody fragment. The most clinically-advanced immunotoxins are currently being evaluated in phase II and III clinical studies. Like other targeted and non-targeted therapeutics, immunotoxins too suffer from several limitations that may hinder their therapeutic efficacy. Such limitations include, but are not limited to immunogenicity, modification of the extracellular target to which the targeting antibody binds, modification of the intracellular target upon which the toxin acts to cause cell growth inhibition, and insufficient potency as single agents and off-target toxicity, where non-target cells and organs are affected by the immunotoxin, severely impairing its therapeutic index. This chapter is devoted to a group of immunotoxins in which the toxic moiety is derived from exotoxin A (PE) of the bacterium *Pseudomonas aeruginosa*. The limitations to the efficacy of PE-based immunotoxins, as well as potential solutions for overcoming such limitations, will be presented. Chapter 2 of this book: "Resistance of tumor cells against antibody-targeted protein toxins" by Ulrich Brinkmann et al. is focused on factors that influence the sensitivity or potential resistances of cancer cells towards recombinant immunotoxins which carry truncated and/or mutated derivatives of Pseudomonas exotoxin as cytotoxic payloads.

Keywords Immunotoxin(s) · Pseudomonas exotoxin A · Immunotoxin · Monoclonal antibody · Immunogenicity · De-immunization

Abbreviations

ADC	Antibody-drug conjugate
APCs	Antigen presenting cells

I. Benhar (✉) · V. Dergachev
Department of Molecular Microbiology and Biotechnology, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, 69978 Ramat Aviv, Israel
e-mail: benhar@post.tau.ac.il

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ATL	Adult T-cell leukemia
BBB	The blood brain barrier
CED	Convection-enhanced delivery
CLL	Chronic lymphocytic leukemia
CTCL	Cutaneous T-cell lymphoma
dsFv	Disulfide-stabilized Fv fragment of an antibody
DT	Diphtheria toxin; *E. coli*, *Escherichia coli* bacteria
EGFR	Epidermal growth factor receptor
GBM	Glioblastoma multiforme
HCL	Hairy cell leukemia
IL-13	Interleukin 13
IL-13R	Receptor for IL13
IL-4	Interleukin 4
IL-4R	Receptor for IL4
IT	Immunotoxin
Le^Y	$Lewis^Y$ carbohydrate antigen
mAb	Monoclonal antibody
NSAIDs	Non-steroidal anti-inflammatory drugs
PBMCs	Peripheral blood mononuclear cells
PDAC	Pancreatic ductal adenocarcinoma
PE (or ETA)	Pseudomonas exotoxin A
PEG	Polyethylene glycol
RICs	Antibody-radionuclide conjugates (radioimmunoconjugates)
RIP	Ribosome-inactivating protein
scFv	Single-chain Fv fragment of an antibody
VLS	Vascular leak syndrome

6.1 Introduction

Many organisms living in nature are known to produce and secrete poisonous substances to their local environment. These toxins appear as secondary metabolites and virulence factors originating from animals/plants/bacteria and usually play notable roles in defense/prey strategies, necessary for maintaining the fitness and survival of the organism. Natural toxinsvary, with a wide range of chemical composition and molecular size, aiming at different cellular targets and operating in mechanisms of action. They have been perfected during evolution for efficient harming of potential enemies; some of them are lethal even in minute doses.

The majority of natural protein toxins can be classified into three groups by their mechanism of intoxication: (1) disruption of cell integrity, (2) disruption of electrical activity of the cell, and (3) disruption or interference with cellular processes mediated by enzymatic activity.

During the last three decades, monoclonal antibodies (mAbs) have become major promising “players” for anti-cancer therapy. Most of the approved mAbs, a little

over 30, inhibit growth of target cells by recruiting immune effector mechanisms or by interfering with a cell-survival signal transduction pathway, However, the majority of antibodies does not show sufficient cell-killing effects in their native "naked (un-armed)" form, and, thus, can only be used as "guided missiles", delivering a lethal cargo to the target cells to enhance killing activity [1]. These antibody-drug complexes (immunoconjugates) may bear a variety of potential killing agents and are usually classified into four groups: (1) antibody-radionuclide conjugates (RICs)—designed to deliver a sufficiently high dose of radiationlocally to eradicate the tumor while sparing the surroundingnormal tissue; (2) antibody—small-molecule drug conjugates (ADCs)—designed to selectively deliver potent anticancer drugs and, thus, improve their therapeutic index; (3) antibody-protein toxin conjugates (immunotoxins, ITs)—recombinant fusion proteins composed of antibodies and plant or bacterial-derived toxins; and (4) antibody–enzyme conjugates—designed to be administrated with prodrugs to provide them the required metabolism and activate the drug locally [2].The combination of antibody-provided specific targeting and a highly potent cytotoxic agent in a single molecule enables the crucial discrimination between healthy and cancer cells within the body. Another prominent advantage of immunoconjugates over the free drug is in their large molecular dimensions that provide them with in vivo stability, leading to a prolonged therapeutic effect [3]. In general, immunoconjugates suffer from several limitations such as immunogenicity, sub-optimal pharmacokinetics and biodistribution properties or decomposition before being delivered (premature cargo release)—all these are only several examples of challenging limitations. Thus, currently only three mAb-based immunoconjugates have been approved by the FDA: two murine radiolabeled anti-CD20 mAbs for treatment of B-cell lymphomas (ibritumomabtiuxetan and tositumomab) and humanized anti-CD33 antibody-drug conjugate for the treatment of leukemia (gemtuzumabzogamicin) [4].

In this chapter, we will focus on representatives from the immunotoxins group with a focus on the most clinically-advanced group, *Pseudomonas* exotoxin-based immunotoxins.

6.2 Immunotoxins

ITs are targeted toxins in which a specific target or, usually a monoclonal antibody or an antibody fragment, replaces the non-specific cell-binding domain of a bacterial or a plant toxin [5]. Many toxins were evaluated as candidates for development as ITs, still, the field is dominated by two bacterial ADP ribosylating toxins, Pseudomonas exotoxin and diphtheria toxins, and by one representative of the plant ribosomal-inactivating proteins (RIPs), ricin toxin. All of these toxins, once their catalytic domain reaches the cytosol, inhibit protein synthesis either by inhibiting translation elongation (ADP ribosylating toxins) or by inactivating eukaryotic ribosomes (RIPs), eventually leading to cell death [5].

The first generation of ITs, created in the mid to late 1980s, were composed of intactIgGs that werelinked to full-length toxins by disulfide bonds. These ITs

suffered from an enormous size, heterogeneous composition and lack of specificity, making them limited in their ability to penetrate into solid tumors. The second generation of ITs, investigated from the mid 1980s to the early 1990s, were composed of truncated versions of toxins that lack a cell binding domain that were chemically conjugated to a targeting moiety. The development of the third generation of ITs (recombinant ITs, RITs), started in the early 1990. This development was made possible due to break through in recombinant DNA and protein engineering technologies, and consisted of atoxin-encoding DNA sequence fused to the antigen-encoding DNA sequence (or antibody fragment, particularly variable region of the antibody engineered as a single chain Fv) on the same Expression vector and were mostly expressed in *E.coli* bacteria. These conjugates were much smaller in size and homogeneous [3]. Early recombinant RITs used mAb variable regions of the heavy and light chains in the single chain format (scFv) that were connected by a 15-amino acid peptide linker [6]. Later, to improve stability and binding activity, the peptide linker was replaced by a disulfide bond between the heavy and light chain Fv fragments (dsFv), and binding affinity was improved by in vitro affinity maturation [7].

The critical features of ITs are: (1) the cell-binding domain of the native toxin is replaced with an antibody fragment [8, 9]; (2) general size minimization is done by removing unnecessary parts of the toxin and by using small antibody fragments rather than full-size IgGs—this significantly improves the ability to penetrate into solid tumors and also protects Its from degradation by proteases [10, 11]; and (3) reducing or eliminating immunogenic epitopes that maybe recognized by the immune system and contribute to rapid neutralization and elimination of the therapeutic molecule [9, 12].

The most commonly used toxins may be divided into two groups by their origin: (1) bacterial exotoxins—diphtheria toxin from *Corynebacterium diptheriae* (DT) and Pseudomonas exotoxin A from *Pseudomonas aeruginosa* (PE); and (2) plant toxins—ricin (the prominent member of this group), saporin and pokeweed antiviral protein (PAP). All of the above have been tried in targeting a variety of tumor-associated surface markers [13, 14].

As this chapter is being written, searching PUBMED using “immunotoxin” as a query word returned 5364 hits. There are hundreds of publications related to PE-based ITs. Our chapter, focusing on limitations to clinical applicability of PE-based immunotoxins, cannot be comprehensive enough to allow the citation of most of them. We apologize to the authors of studies that were not cited in this chapter.

6.3 Brief Historical Overview of PE-Based ITs

Pseudomonasexotoxin A (abbreviated PE or ETA) is one of the virulent factors that are naturally secreted by the *Pseudomonas aeruginosa* bacterium and helps it invade animal tissues, including the tissues of humans. PE is a polypeptide composed of 613 amino acid residues and it belongs to the ADP-ribosylation toxins family.

The toxin itself contains three main structural and functional domains: (1) the N-terminal receptor ® binding domain Ia (aa 1-252) is required for target cell recognition; (2) domain II (aa 253–364) is responsible for retrograde translocation (T) of the toxin through cell membrane into the cytosol; and (3) the catalytic (C) domain III (aa 405–613), together with the last amino acids of domain Ib (aa 365–404),is the catalytic unit of the protein [8, 15]. After the toxin reaches the blood, carboxypeptidases remove the C-terminal lysine (#613) and expose the REDL sequence (that functions as a KDEL, ER retrieval sequence). Next (see Fig. 6.1), the toxin binds to CD91 (alpha2-macroglobulin receptor) on the cell surface via its cell-binding domain Ia, followed by the toxin's internalization via clathrin-coated pits into early endosomes. Acidification of the endosome leads to PE dissociation from its receptor, a conformational change and finally cleavage of the toxin by the cellular protease furin (within the furin-sensitive loop in domain II). This cleavage results in two products linked by an intradomain disulfide bond. This disulfide bond undergoes reduction and the C-terminal part, comprising part of domain II, domain I band domain III travels to the trans-Golgi network, binds to the KDEL receptor

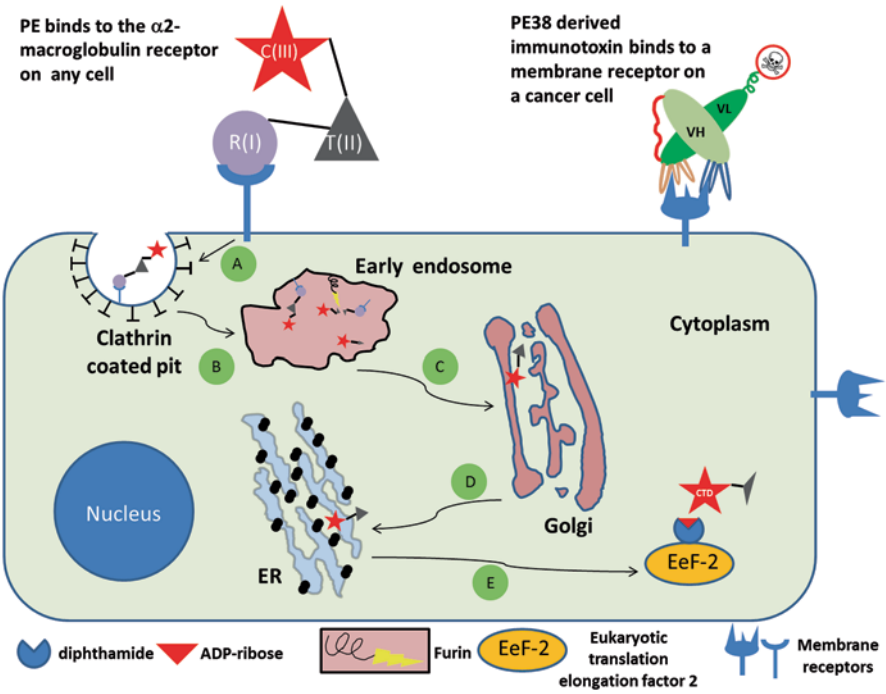

Fig. 6.1 How PE and PE-based ITs intoxicate cells. **a** PE or IT binds to their respective cell surface receptors. **b** They internalize into clathrin-coated pits that fuse with early endosomes, where they undergo furin-mediated cleavage and reduction of the interdomain disulfide bond. **c** The C-terminal part (CTD) retro-translocates via Golgi to the ER (**d**). **e** The CTD is further translocated from the ER to the cytosol where it binds to a diphthamide residue on the translation elongation factor 2 (EeF-2) and ADP ribosylates it. This step irreversibly-inactivates EeF-2, leading to arrest in protein synthesis and eventually to apoptotic cell death

(via its exposed REDL sequence) and finally routes to the endoplasmic reticulum (ER).In the ER, sequences in domain II mediate the translocation of the 37 kDa fragment to the cytoplasm via the Sec61p translocon. Once in the cytosol, the catalytic domain III blocks the eukaryotic translation elongation factor 2 (EF2) via an ADP-ribosylation mechanism, causing arrest of protein synthesis. This event initiates the apoptotic cascade by lowering Mcl-1 levels and unleashing Bak to promote apoptotic death [16–18].

In the early 1980s, Ira Pastan's group at the NIH pioneered the development of PE-based immunotoxins. They were the first to propose the possibility of targeting PE to cancer cells by using antibodies. Like other first generation ITs, the first attempt to attach PE to antibody was done chemically, by treatment with iminothiolane, which reacts with lysine residues on proteins and generates free sulfhydryl groups that are used in the coupling reaction. PE was attached to anti-transferrin receptor and anti-Tac antibodies that binds to CD25 on T-cells and T-cell malignancies [19]. Because these antibody-toxin conjugates contained the non-specific cell-binding domain I, they remained capable of binding healthy cells, thus producing side effects in animals, and due to severe hepatotoxicity, clinical trials were little pursued. A first-generation PE-based immunotoxin that was tried in patients was OVB3-PE [20, 21].

Deletion of domain I was the first step in making the second generation, PE-based immunotoxins. Structure-function studies of PE that were carried out during the same time period have shown that a large part of domain Ib (a.a.365–380) can be deleted without effecting cytotoxicity, resulting in a smaller version of the modified toxin called PE38 (the name denotes the molecular weight of the protein, i.e. the truncated derivate is 38 kDa, while the domain I-deleted toxin is 40 kDa) [22]. To facilitate efficient site-specific chemical coupling, a small-lysine containing peptide was appended at the amino terminus (a molecule that was named LysPE38). A prototype second domain immunotoxin was a conjugate between the anti Lewis^Y (Le^Y, a carbohydrate antigen widely presented in colon, breast and many other epithelial cancers) carbohydrate antigen B3 mAb and LysPE38. This immunotoxin, designated LMB-1, has shown a much better therapeutic window in animals and was subsequently tested in phase I clinical studies. Such a clinical trial has shown a complete response in a patient with breast cancer and a striking partial response in a patient with colon cancer with extensive metastases to retroperitoneal lymph nodes [23]. This was a milestone study, since it demonstrated for the first time an antitumor response to an immunotoxin in epithelial tumors. In that study, the maximum tolerated dose (MTD) of LMB-1 was 75 μg/kg given intravenously three times every other day. The major toxicity was vascular leak syndrome—an example for off-target toxicity that will be further discussed below.

Enter the third generation immunotoxins; the immunotoxins that have been developed since the early 1990s were mostly designed and produced by using molecular biology techniques and thus were called recombinant immunotoxins (RITs). This enabled reducing the size from ~200 kDain chemical conjugated immunotoxins to 63 kDain RITs, and, in this manner, significantly increased penetration into solid tumors. The two RITs that were the first to be selected for clinical development

were B3 (Fv)-PE38 (LMB-7), targeting Le^Y, and anti-Tac (Fv)—PE38 (LMB-2), targeting CD25.

LMB-7 completed a long series of pre-clinical studies that will be discussed below. It underwent a single phase I clinical study (study NCT00003020 in WWW. clinicaltrials.gov)to testthe effectiveness in treating patients who have leptomeningeal metastases. During its preclinical development, this prototypic third generation immunotoxin demonstrated limitations that are typical of such molecules, such as immunogenicity, instability and off-target toxicity, which will be further discussed below. As for Le^Y as a therapeutic target, a PE-based IT developed by Bristol-Myers Squibb, BR96 sFv-PE40, was evaluated preclinically, but later abandoned [24].

Anti-Tac (Fv)—PE38 (LMB-2) is based on mAb Anti-Tac that binds the alpha subunit of the IL2 receptor (CD25, the Tac antigen) with high affinity. Many hematological malignancies express this receptor at a high level ranging from several hundred to a few thousand sites/cell. Daclizumab (trade name Zenapax) is a therapeutic humanized anti-Tac mAb antibody that is FDA approved to prevent rejection in organ transplantation and is undergoing clinical evaluation for treatment of multiple sclerosis [25–27]. ITs based on anti-Tac antibody fragments have been extensively studied by Ira Pastan's group and their collaborators since the early 1990s and have been evaluated in a number of clinical studies. Clinical trials were conducted in patients with hairy cell leukemia (HCL), chronic lymphocytic leukemia (CLL), Hodgkin's disease and cutaneous T-cell lymphoma, showing partial and complete responses. Due to the problems of instability and aggregation at 37 °C, RITs were designed in away that light and heavy chains of the Fv are held together by an engineered disulfide bond. Anti-Tac sdFv-PE38 RITs were also evaluated following such stabilization (see below).

BL22 is an example of a disulfide-linked immunotoxin where the Fv of the anti-CD22 antibody (RFB4) was linked to PE38. Many B-cell malignancies express the CD22 antigen on their cell surface. The development of anti CD22, PE-based ITs began with RFB4 (Fv) PE38 [28], which underwent "disulfide stabilization", resulting in BL22. BL22 has shown remarkable activity in drug-resistant HCL in phase 1 clinical trials.

Another example for a disulfide-stabilized IT is SS1P [SS1 (dsFv) PE38], an anti-mesothelin RIT. Mesothelin is a 40-kDa cell surface membrane glycoprotein and its biological role in normal mesothelial cells is not clear. While showing limited expression in normal human tissues, it is known to be a solid tumor antigen that undergoes up-regulation in a number of epithelial cancers such as pancreatic, ovarian, lung and others [29]. This offers it as an attractive candidate for targeted therapy. SS1P was produced by the fusion of anti-mesothelin Fv (SS1) to PE38, a 38-kDa portion of *Pseudomonas* exotoxin, improved by affinity maturation and disulfide-stabilization [30–32]. The mechanism of action involves binding to the target, internalization by endocytosis and cell death by arrest of protein synthesis [9]. It is currently being evaluated in a phase I clinical trial in patients with mesothelin positive tumors. Currently, two anti-CD22-*Pseudomonas* exotoxin (PE) immunotoxins designed for treatment of B-cell malignancies are undergoing clinical phase II evaluation: BL22 [RFB4- (dsFv)-PE38] and its affinity matured version HA22

(moxetumomab pasudotox) that was developed due to low activity of the original BL22 in some other B-cell malignancies (i.e., CLL, ALL and non-Hodgkin's lymphoma). HA22 has a higher binding affinity for CD22 and greater in vitro potency. Phase I trials in patients with hairy cell leukemia ($n=32$) showed that moxetumomab pasudotox has a better complete response rate comparing to BL22 (31 vs. 25 %, respectively). Expanding its application to other hematological malignancies is also under evaluation [33].

While the study of PE-based RITs in the continental USA was carried out primarily by Ira Pastan's group and their collaborators, several groups in Europe have also studied similarly constructed RITs. Most of these studies were carried out in in vitro or in animal models and will not be discussed in detail here (a partial list of such studies can be found in references [34–40]. Winfried Wels and collaborators have been studying RITs that target ErbB2 and EGFR since the early 1990s. While most of their studies were pre-clinical [41–44], they did carry out a phase I clinical study with the RIT called scFv (FRP5)-ETA. ScFv (FRP5)-ETA is a recombinant single-chain antibody-toxin fusion protein with binding specificity for ErbB2/HER2. Previous studies from their group demonstrated potent antitumor activity of the molecule against ErbB2 overexpressing tumor cells in vitro and in animal models. The clinical study of scFv (FRP5)-ETA was reported in 2003, summarizing case reports from four different clinical centers. Eleven patients suffering from metastatic breast and colorectal cancers and from malignant melanoma were treated on a compassionate use basis by intratumoral injection of scFv (FRP5)-ETA into cutaneous lesions once daily for 7–10 days. Treatment caused the injected tumors to shrink in six of the tencases evaluated (60 %). Complete regression of injected tumor nodules was accomplished in four patients (40 %), and partial reduction in tumor size in another two patients (20 %). The authors suggested that their results demonstrated that local therapy with scFv(FRP5)-ETA can be effective against ErbB2 expressing tumors, justifying further clinical development of this reagent [45].

Despite success in treating hematologic malignancies, the therapeutic application and, in fact, the FDA approval of RITs has been hindered by a number of obstacles: immunogenicity of the murine antibody, immunogenicity of the protein toxin and of the targeting antibody, rapid clearance from the blood stream and systemic toxicity at very low doses (the maximum tolerated dose achievable with such immunotoxins is about 0.05 mg/kg). The combination of rapid clearance and low dose limits them from use in solid tumors. Biodistribution studies show that only negligible amounts of intravenously administrated RITs reach the tumor tissue (< 0.01 % of the injected dose per gram of tumor) and, thus, it is unlikely that therapeutic concentrations of such RITs can be delivered to solid tumors. In addition, recent trials have shown that despite toxin-mediated inhibition of protein synthesis, the action is not absolute, and in some cases mammalian cells that were treated by RITs appear to survive the treatment [46]. This phenomenon, referred to here as insufficient potency, points to the existence of some not fully understood/described toxin-resistance mechanisms. Current effort is directed at better understanding the complex regulation machinery at different stages of the pathway by which immunotoxins kill cells. This knowledge may be helpful to enhance the killing effect of these immunotoxins, or even to expand them to the treatment of different malignancies [47, 48].

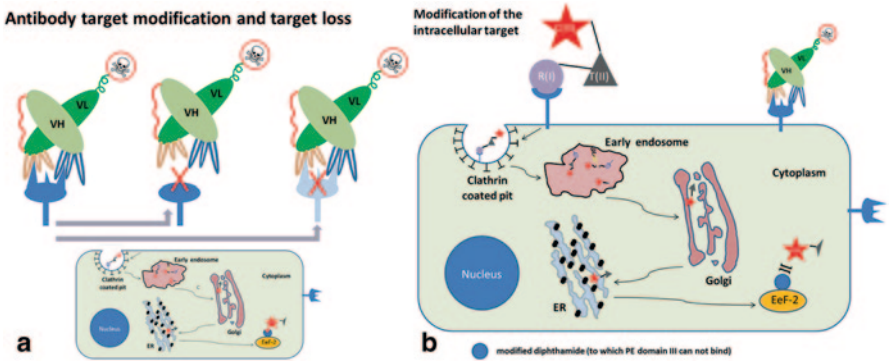

Fig. 6.2 How cancer cells may become resistant to RITs. **a** Cancer cells may down-regulate, mutate or lose altogether the cell-surface protein to which the RIT binds. This will result in the cells becoming resistant to the RIT. **b** Modification of the intracellular target leading to the cell becoming resistant to the toxin. In the case of PE, when EeF-2 is mutated in a way that it no longer binds the PE domain III, the cells become totally resistant to PE intoxication. Fortunately, such resistance has been observed only in the cell culture and not in the clinical setting

In summary, the major limitations of RITs are: immunogenicity, limited stability, insufficient potency, off-target toxicity and sub-optimal PK/PD profile. Additional obstacles that are also typical of other antibody-based therapeutic approaches are loss of target antigen on the cancer cells, receptor shedding and modification of the intracellular target molecule upon which the toxin acts (see Fig. 6.2). These limitations, and solutions that have been suggested or attempted to overcome them, are the subject of the following sections.

6.4 Immunogenicity of PE-based RITs and Solutions for Reducing it

Immunogenicity of protein therapeutics is presently considered a major obstacle to their successful clinical application. Immunogenicity is generally manifested by the appearance of anti-drug-antibodies (ADAs) and finding ways to "deimmunize" immunogenic biopharmaceutical (biologics) is a burgeoning field of study [49–52]. Therapeutic monoclonal antibodies, being the largest group of biologics, are no exception to that rule, and a lot of effort has and is still being invested in reducing their immunogenicity [53–56].The leading approach for reducing the immunogenicity of biologics involved the identification of B- or T-cell epitopes by computational or be experimental means (or combinations thereof) and eliminating amino acid residues that correspond to these epitopes while trying to maintain the activity of such "de-immunized" proteins [56–59].

The immunogenicity of PE-based RITs was recognized since the very early days of RIT development (see Fig. 6.3). This was in the early 1990s when the term "de-immunization" was net yet coined. Still, several studies were carried out to identify mouse, primate and human antibody epitopes of PE and PE-based RITs [60–62].

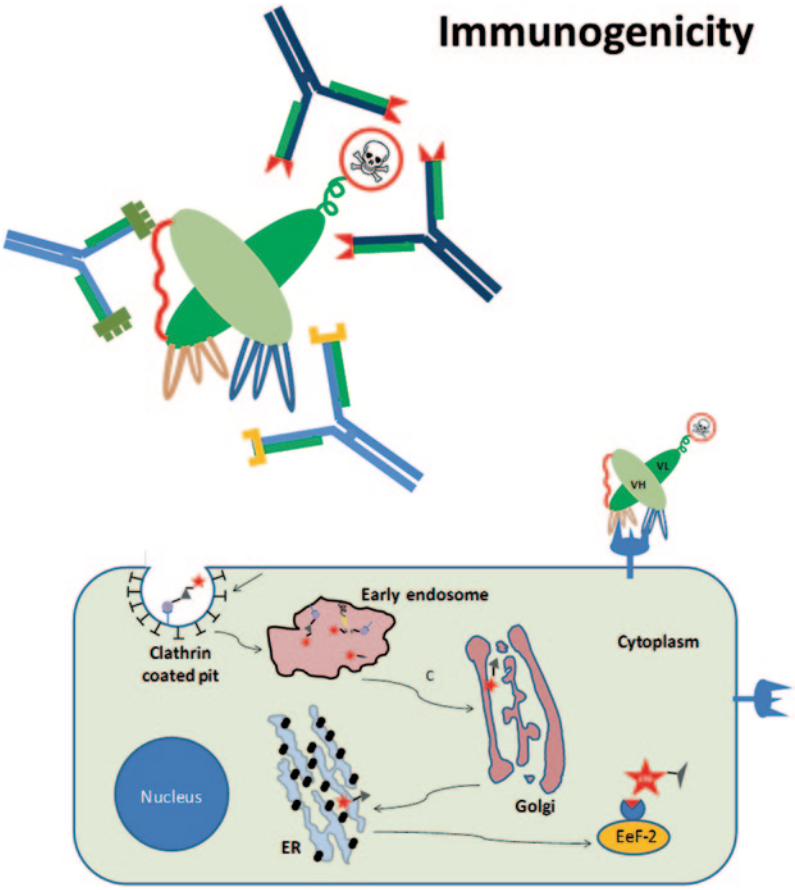

Fig. 6.3 Immunogenicity. When an IT is injected into an animal or human patient with an intact immune system, antibodies that recognize immunogenic epitopes on the targeting antibody and (primarily) on the toxin lead to IT neutralization upon repeated administration. This makes subsequent treatment not useful

Well before deimmunization of PE-based RITs was attempted, other approaches to reduce their immunogenicity were carried out. Initially, the attachment of polyethylene glycol (PEGylation) to PE and RITs was considered. PEGylation of biologicals as a means of modulating their PK/PD and/or their immunogenicity is known for over 30 years [63–65]. A number of studies were carried out to evaluate the effects of PEGylation on PE and RITs. Initially, detailed mapping of surface-exposed residues that can be mutated to cysteine (to facilitate chemical conjugated to PEG) on PE domains II and III were carried out [66, 67]. In these studies, a large number of residues were identified that could be mutated to cysteine with minimal loss of potency. Several PE cys domain III mutants that were conjugated to monomethoxy-polyethylene glycol (mPEG) via a disulfide or a thioether bond retained high cytotoxic activity. However, when a 20-kDa mPEG was used, there

was a decrease in cytotoxic activity with the disulfide-bonded molecules being more active. Positions 522 and 604 were good sites for PEGylation, but position 490 was not. The authors also found that PEGylation of PE 522C prolonged its in vivo circulation time in mice [67]. When PE domain II was studied, each of the five most exposed surface amino acids (H276, E282, N306, R313, and E327) were mutated to obtain PE-cys proteins that retained most of their cytotoxic activity. When the PE-cys proteins were conjugated with ovalbumin, using a cleavable disulfide linkage, cytotoxicity was retained, but it was lost with a non-cleavable thioether linkage. In contrast, cytotoxicity was maintained when PE-cys mutants were coupled to 5- or 20-kDa mPEG, using either a disulfide or a thioether linkage. Pharmacokinetic studies on one of the PEG-conjugated molecules (R313C) showed that the mean residence time ($t_{1/2}$) was prolonged to 72 min, compared to 20 min for un-PEGylatedPE-cys (R313C). The authors suggested that those studies showed that it is possible to derivatize PE at specific residues in domain II, maintain significant cytotoxic activity, and alter pharmacokinetics. Those studies also suggested that large mPEG molecules can be translocated to the cytosol while still attached to domain II of PE [66]. These two early studies of PE PEGylation did not evaluate the effect on immunogenicity.

Six years later, another study evaluated the effect of site-specific PEGylation, this time of a RIT. To make a PEGylated RIT with improved therapeutic properties, the authors prepared a mutant of anti-Tac (Fv)-PE38 (LMB-2). For site-specific PEGylation of LMB-2, one cysteine residue was introduced into the peptide connector (ASGCGPE) between the scFv and the toxin. This mutant LMB-2 (cys1-LMB-2), which retained full cytotoxic activity, was then site-specifically conjugated with 5 or 20 kDa of polyethylene glycol-maleimide. When it was compared with unmodified LMB-2, both PEGylated RITs showed similar cytotoxic activities in vitro, but superior stability at 37 °C in mouse serum, a 5- to 8-fold increase in plasma half-lives in mice, and a 3- to 4-fold increase in antitumor activity. This was accompanied by a substantial decrease in animal toxicity and immunogenicity [68].

The anti mesothelin RIT SS1P was also studied as a PEGylated RIT. The authors have modified this immunotoxin using several PEGylation strategies employing releasable linkages between the protein and the PEG polymers, and observed superior performance of these bioconjugates when compared to similar PEG derivatives bearing permanent linkages to the polymers. The releasable PEGylated RITs exhibited increased antitumor activity in A431-K5 xenografts in mice, with a diminished animal toxicity. Pharmacokinetic analysis of the releasable PEGylated derivatives in mice demonstrated an over 80-fold expansion of the area under the curve exposure of bioactive protein when compared to the un-modified (un-PEGylated) RIT [69].

To preserve potency, PEGylation of RITs by conjugating PEG to lysine residues should be site-specific and link the PEG to residues so that potency is not compromised. A mutant of PE with no lysine residues within PE38, designed for site-specific chemical conjugation was described in 1994 [70] (see also Sect. 5, below). However, in a RIT, the targeting antibody also contains lysine residues and accidental conjugation of PEG to some of them may compromise binding affinity.

Two studies looked at mutating lysine residues of scFvs that were used to target RITs. In the first study, the effect of chemical modification of lysine residues of the scFv of the anti Le^Y RIT B3 (Fv)-PE38 was carried out to study which of the scFv lysine residues could tolerate chemical modification while preserving biological activity. The authors found that derivatizing lysine residues of B3 (Fv)-PE38 causes a marked loss of specific target cell cytotoxicity and/or immunoreactivity. They also showed that two lysine residues in the antibody-combining region of B3 (Fv)-PE38 can be replaced with arginine residues, with only a small loss of cytotoxicity and no change in specificity. This mutant molecule is 3-fold more resistant to inactivation by derivatization with succinimidyl 4-(N-maleimidomethyl)cyclohexane 1-carboxylate (SMCC) or Bolton-Hunter reagent [71].

In the second study, to determine if a RIT could be produced with a diminished number of lysine residues so that chemical modification could be restricted to certain regions of the protein, the authors chose the RIT anti-Tac(dsFv)-PE38 that has 13 lysine residues in the Fv portion and 3 in the toxin. They prepared a series of mutants with 0–12 lysines in the Fv and 0 or 3 in the toxin. Almost all of these molecules retained full biological activity. Those data suggest that replacement of lysine residues can be achieve without loss of biological potency. These molecules could be a useful starting point to carry out site-specific PEGylation experiments of RITs [72].These studies demonstrated that chemical modification of RITs in general and their PEGylation in particular are very dependent on the antibody used for targeting, with some antibodies being more tolerant than others. As for PE itself, it can be chemically modified at a large number of positions with full preservation of potency. Presently, we are not aware of attempts to introduce PEGylated PE-based RITs into the clinic.

Regarding immunogenicity of PE-based RITs, it can originate from the targeting antibody, from the toxin or from artificial linkers used to connect together the parts of the molecule. PE itself has long been known to be a highly immunogenic protein and high titers of anti-PE binding, as well as neutralizing antibodies, were generated in rodents, primates and humans that were injected with RITs, following even a single injection [61, 73, 74]. Antibodies against the targeting Fvs also appeared in most cases, but their titers were much lower that the anti-toxin titers. In general, it is well documented that HAMA, human anti mouse antibodies that appear in humans injected with murine antibodies, are mostly directed at the antibody constant domains and less against the antibody variable domains.

There is a single study where a murine scFv used to target a RIT was humanized. In that study, the humanization of the scFv of the anti Le^Y RIT B3(Fv)-PE38 (LMB-7) by “framework exchange” was reported. The variable domains of the heavy (VH) and light (VL) chains were aligned with their best human homologs to identify framework residues that differ. Initially, 11 framework residues in VH and six in VL were changed by site-specific mutagenesis to human framework residues and introduced simultaneously into a preassembled single-chain Fv expression cassette. Six VH and five VL residues that differ were not changed because they were buried in the interdomain interface, or previously found to result in decreased affinity when mutated. As in many naïve initial attempts at antibody humanization [75], this

basic design resulted in some 20-fold loss of activity. To recover affinity, VL residues at the interdomain interfacial position 100 and at the buried position 104 were changed to the human sequence, which resulted in increasing the activity 8-fold. Changing the VH residue at position 82b from the human sequence back to that of the mouse restored the activity 2- to 3-fold to the full binding and cytotoxic activity of the mouse sequence. Humanized B3(Fv)-PE38 lost immunogenic epitopes recognized by sera from monkeys that had been immunized with B3(Fv)-PE38 [76].

Currently, no special effort is being invested in humanizing the scFvs or dsFvs that are used in clinical studies. The antibodies used to target LMB-2, SS1P, BL22 and HA22 are murine. As described below, most of the efforts for RIT deimmunization were focused on the toxin. We believe that now, when toxin deimmunization has met with apparent success, more attention will be directed at the targeting antibody, and future RITs, directed at new targets, will be based on humanized or fully human antibodies.

The largest strides toward reducing the immunogenicity with practical implications towards clinical development have been made during the past 10 years in a series of studies that were designed to identify and eliminate the immunogenic epitopes of PE itself. Because PE is of bacterial origin, it is highly immunogenic to animals and humans. As a result, all PE-based ITs are also highly immunogenic proteins. The recently conducted clinical trials include three RITs based on a 38-kDa fragment of PE, PE38 (Moxetumomab pasudotox, SS1P, and LMB-2) which is made up of domains II and III. Both domains II and III contain immunogenic B-cell epitopes [62]. Of note, over half of the patients with drug resistant HCL that were treated with BL22 achieved complete remission after 3–10 cycles of treatment [77]. However, patients having a normal immune system respond with production of anti-toxin antibodies after approximately 3 weeks, significantly limiting the number of cycles that could be applied for these patients. It should be noted that patients with leukemias and lymphomas (like in the case of drug resistant HCL) have a shattered immune system due to previous chemotherapy treatments, making their immune response to RITweaker than that of carcinoma patients. Destruction of immune cells by tumor cells infiltrating into the bone marrow also contributes to the weak immune system of such patients. However, to increase efficiency and expand immunotoxin therapy to other types of cancers, deimmunization is a necessary move to enable multiple cycles of treatment [58]. Clinical observations from patients treated with different derivatives of PE38 have shown that most of the antibodies were directed against PE38 and rarely to the Fv. It was understood that finding efficient ways to reduce the immunogenicity of PE38 have a crucial importance for clinical applications of PE38-based RITs in the future.

To briefly recount the order of events in the development of an anti-protein immune response: high affinity antibodies are produced and undergo affinity maturation in B cells. Initial antigen recognition happens between the antigen and a surface-displayed immunoglobulin, the B-cell receptor on pre-B cells. This is followed by internalization of the antigen, digestion to peptides and its further presentation on the surface by major histocompatibility complex class II. Next, specific helper T cells bind to those B cells that together promote class-switching, affinity

maturation and production of high affinity antibodies by mutual stimulation. This process is mediated by different intracellular signals and co-receptors, and occurs in secondary lymphoid organs, such as lymph nodes [78, 79]. It should be stressed that the location of B cell epitopes does not fully overlap with the locations of the T cell epitopes on an immunogenic protein [80].

Ira Pastan's group at the NIH has been working for about a decade exploring the field of PE38 deimmunization while applying several approaches. The leading approach that was evaluated first is based on identifying B-cell epitopes on the protein and their elimination by mutagenesis. One should bear in mind that to preserve killing abilities in mutated RITs, several features of the toxin should be preserved: (1) cleavage by furin, (2) translocation to the ER, (3) translocation from ER to cytosol, (4) binding to NAD, (5) binding to EF2 and (6) transfer of ADP ribose to EF2. Onda et al. performed a series of studies aimed to deimmunize the anti CD22 RIT HA22. These studies combined structural and functional analyses with identification and removal of B-cell epitopes. First, they isolated a panel of anti-PE38 mouse mAbs and categorized them into seven major epitope groups and 13 subgroups. Their working hypothesis was that changing large, surface-exposed, hydrophilic residues that are commonly involved in antibody binding, such as arginine, lysine, glutamine, and glutamate, to smaller residues such as alanine, glycine, or serine will reduce or eliminate the antibody reactivity with the mutated RIT. The derived RIT mutants were found to have a reduced immunogenicity in mice and retained antitumor activity [81]. Subsequently, in a study aimed at stabilizing the toxin by eliminating protease cleavage sites (carried out on SS1P, an anti-mesothelin RIT see Sect. 5, below), a large part of domain II was removed, resulting in the RITHA22-LR; HA22-lisosomal resistant). Since many B-cell epitopes are located in domain II, HA22-LR hada diminished immunogenicity compared to the parental molecule HA22-PE38. [10, 62, 81, 82]. In the more recent studies, the authors performed a detailed bioinformatic analysis and revealed a small number of discrete, putative B-cell epitopes that may be important for antibody recognition and are all located on the surface of domain III. Subsequent 8 point mutations enabled to abolish the identified epitopes. The obtained deimmunized HA22-LR-8M did not induce a primary or secondary response when repeatedly injected intravenously into mice, yet retained excellent cell killing of $CD22^+$ cells and antitumor activity in a mouse xenograft model. Since (until such RITs will enter clinical evaluation) it was not possible to evaluate the immunogenicity in humans, an antigenicity study that is closely related was done instead (binding of HA22-LR-8M to preexisting anti PE38 antibodies that were obtained from patients that were treated with RITsLMB-9 or SS1P during their clinical evaluation as a surrogate measure of the immune response [62]). HA22-LR-8M demonstrated consistently lower antigenicity than HA22-PE38 with patient serum samples in the competition assay as well as with mouse anti PE38 antibodies, hinting that mice and humans may share some B-cell epitopes [83].

To further reduce the immunogenicity of PE-based RITs, despite the fact that the previously obtained HA22-LR-8M with eliminated major mouse B-cell epitopes demonstrated no significant response with human serum, it was obvious that since not all human and mouse epitopes are identical additional human B-cell epitopes

need to be identified and eliminated. Liu et al. focused on the production of further improved variants of HA22-LR by identifying and silencing additional human B-cell epitopes. The authors constructed a phage-display library containing Fvs that react with the native PE38 toxin. RITs were then point mutated to locate B-cell epitopes reactive with these phage-displayed human Fvs. Using this approach, six human B-cell epitopes were identified in domain III. These epitopes were eliminated by mutations, and the best clone, HA22-LR-LO10. had a total of seven point mutations in domain III, had much better cytotoxic activity in vitro (when tested for killing $CD22^+$ lymphoma cell lines) compared to its predecessor HA22-LR, and same anti-tumor activity in an in vivo mouse xenograft model in which CA46 Burkitt's lymphoma cells were implanted subcutaneously. Antigenicity was measured by a competition assay and showed that binding of HA22-LR-LO10by sera of 22 out of 25 patients was reduced up to 10.000-fold.

SS1P, anti-mesothelin RIT, was a subject for additional attempts to deimmunize PE-based RITs. The clinical utility of SS1P is limited by inducing an immune response and by causing dose-limiting capillary leak syndrome (CLS) in patients. With SS1P treated patients that immunogenicity problem is more acute than HA22 treated patients as SS1P patients are not immunosuppressed, thus, they all mount an antibody response to the RIT already following the first administration. Weldon et al. tried to overcome these obstacles by redesigning the SS1P molecule. The authors implemented earlier observations that were made during the development of the much less immunogenic variant HA22-LR, where two major mouse B-cell epitope groups and antigen processing sites were removed from PE38, while in vitro activity on patients' CLL cells was dramatically increased [62, 82]. When those changes were introduced into SS1P, the LR-adapted SS1P, named SS1-LR/GGS/8M, carried the following modifications: (1) domain II was removed; (2) a Gly–Gly–Ser short peptide linker was added after the furin cleavage site; and (3) eight highly solvent exposed residues were replaced in the catalytic domain III of PE. SS1-LR/GGS/8M had significantly improved properties compared to its parental SS1P, had increased anti-tumor activity, and could be given in much higher doses to rats and mice without production of toxic side effects. Its immunogenicity was diminished greatly, suggested by lowered reactivity with human anti-sera against SS1P [84].

Taking into account the pivotal role of T cells for the immune response and the formation of neutralizing antibodies in particular, it was hypothesized that removal of T-cell epitopes may be also required to better meet the challenge of reducing the immunogenicity of PE-based RITs. Following this line of reasoning, Mazor et. al conducted a study that investigated $CD4^+$ T-cell epitopes in PE38 and used these data to produce RITs that do not stimulate T-cell responses in a majority of human donors. To identify peptides that result in T-cell activation, donor peripheral blood mononuclear cells (PBMCs) were incubated with RIT for initial stimulation, in order to allow processing of the RIT by antigen-presenting cells (APCs) and further presentation of its peptides to T cells. Then, these activated T cells were exposed to overlapping synthetic peptides corresponding to the PE38 sequence. T-cell response was measured by ELISpot assay for IL-2 secretion. The result was that samples from all 50 healthy donors (that had never been exposed to PE38)

responded to at least one peptide. This promiscuous HLA class II DRB1-restricted highly immunodominant epitope was found in 46% (23/50) of the donors with different HLA alleles and located in domain II of PE. Alanine-scanning mutagenesis revealed two amino acids that were found responsible for establishing of this T-cell immunodominant epitope, and their deletion/alanine exchange yielded elimination of this epitope. Subsequently, mutations in discovered positions were introduced into HA22. The obtained HA22- L297A and HA22-Y298A mutants were evaluated on four $CD22^+$ cell lines, showing a small decrease in cytotoxicity compared to the parental HA22 RIT. Novel mutant derivatives preserved the RIT stability and did not stimulate a T-cell response as was shown by in vitro expansion with whole protein (not immunogenic in 34% of donors and less immunogenic in an additional 42% of the donors). To rule out the possibility that by mutating PE new T-cell epitopes were generated, alanine mutagenesis was used to reduce the binding of peptides to HLA molecules. The authors discussed their plans to continue effort to find epitopes in domain III as well and combine them with already achieved deletions in domain II. This, they suggested, may yield a RIT with very low immunogenicity characteristics to apply in humans with a normal immune system [85].

Recently, the same approach was applied to prepare a further deimmunized HA22 RIT. HA22-PE38 (also known as Moxetumomab Pasudotox) is currently undergoing phase III clinical trials for the treatment of refractory hairy-cell leukemia (http://www.clinicaltrials.gov/ct2/show/NCT01829711?term=Moxetumomab+Pasudotox&rank=1). To perform a high-resolution mapping of the T-cell epitopes on PE38, Mazor et al. applied the same approach described in the previous paragraph [85]. It enabled the discovery of seven additional T-cell epitopes located on the domain III, additionally to the already known immunodominant epitope on the domain II. This knowledge was used to construct an RIT named LMB-T18 based on the HA22-LR scaffold (that lacks domain II) with six additional point mutations in domain III (R505A, R494A, L477H, R427A, L552E, and F443A) and incorporated a GGS peptide linker after the furin cleavage site. The authors found that the cell killing efficiency of the mutated RIT, evaluated in vitro in four $CD22^+$ cell lines, proved to bevery potent (IC_{50}s less than 10 pM). In vivo evaluations were carried out using SCID mice implanted with lymphoma cell xenografts showing complete remissions. LMB-T18 was also evaluated for killing cells freshly isolated from seven HCL and six CLL patients and found that it was extremely active. The T-cell response had a decrease of 90% compared with HA22-PE38 (tested with PBMCs from naive donors). The authors concluded that the next logical step wouldbe to produce RITs with combined B-cells and T-cells eliminated/mutated epitopes [86].

The RIT deimmunization studies described in this section involved bioinformatics tools as well as experimentation. In the B-cell epitope elimination campaign, the bioinformatics part was restricted mainly to using the 3D structure of PE to identify the putative surface-exposed residues that may be a part of the B-cell epitopes. There are no generally accepted algorithms for predicting B-cell epitopes. The situation is different with regard to predicting T-cell epitopes. One such approach was recently demonstrated in a report by King et al. who described a computational protein design method that can predict T-cell epitopes and maximize the content of human

peptide sequences without affecting protein stability. This method incorporates host genome information and MHC-binding prediction tools. Interestingly, when applied to predict T-cell epitopes of PE38, mutations that were predicted by the theoretical method partially matched the mutations observed in previous deimmunization experiments of Mazor and colleagues. To experimentally verify the computational predictions, the approach was applied on superfolderGFP and on PE38, resulting in successful prediction and elimination of known immunodominant T-cell epitopes. The work was focused only on eliminating the most immunoreactive epitopes for a given set of MHC alleles, therefore, to cover a breadth of HLA allotype diversity and testing of a larger number of patients should be tested [87].

To conclude this section, immunogenicity of PE-based immunotoxins, arguably the largest hurdle for their progress to clinical approval, has been addressed in a most impressive tour-de-force of epitope identification and elimination. One should also be at awe to the remarkable robustness of the PE protein that can tolerate so much "abuse"; deletion of large parts and many combined point mutations and still retain potency. We are confident that these, what we shall perhaps call "fifth generation PE-based RITs", will soon enter clinical studies. Such deimmunized RITs should be more effective in cancer treatment because more treatment cycles can be given.

6.5 Limited Stability of PE-Based RITs and How it Was Overcome by Antibody and Toxin Engineering

When the first third generation PE-based RITs were constructed they were based on single-chain antibody fragments (scFvs) fused to PE38. It soon became clear that scFv-based RITs suffer from limited stability, with a tendency to aggregate in solution fairly rapidly. It was quite clear that PE itself was not toblame, since recombinant forms of PE were already produced and had shown excellent stability. It was also known that scFvs are in general un-stable antibody fragments. Solutions for stabilizing Fv fragments were offered by the group of Andreas Plückthun already in 1990 [88]. In that publication, the authors compared how well the Fv of the mAb McPC603 can be stabilized by chemical crosslinking, by a peptide linker (as a scFv) or by the introduction of an artificial disulfide bond facilitated by mutating VH-VL interfacial residues to cysteines. In that study, disulfide stabilization proved to be very efficient in improving stability. However, the disulfide-stabilized McPC603-derived Fvs suffered from some loss in binding affinity.

About two years later, a "disulfide stabilization" campaign was initiated by Ira Pastan at NIH and collaborators. They undertook a systematic analysis of antibody structural analysis for designing the positions of the introduced cysteine mutations to fulfill in particular two major criteria: (1) that the selected positions would be universal interfacial positions, with a Cα-Cα distance suitable for the formation of the artificial disulfide bond, and (2) that the engineered disulfide bond would be distal from the CDR loops, so the binding affinity would not be compromised.

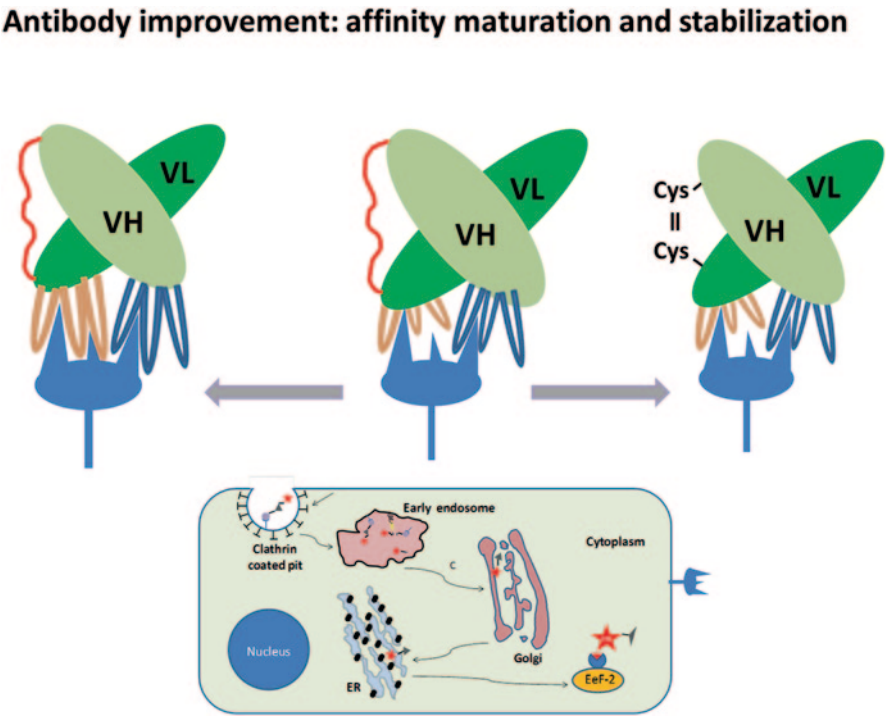

Fig. 6.4 Affinity maturation and disulfide stabilization. Affinity maturation of the targeting antibody (simulated on the *left* by increasing contact complementarity with the antigen) contributes to higher affinity, leading to increased potency. Stabilization of the targeting Fv (disulfide stabilization, simulated on the *right*) also contributes to improved RIT efficacy

The first article describing disulfide-stabilized Fvs (that became known as dsFvs, see Fig. 6.4) in the context of RITs was published in 1993 [89]. A series of articles followed, further evaluating several pairs of interfacial positions for disulfide stabilization, and testing the generality of the approach by constructing and evaluating RITs based on different antibodies [31, 90–96]. In those studies, it was found that all the tested antibodies were more stable as dsFvs in comparison to the corresponding scFvs, and that in almost all cases the affinity had not been compromised. As a result, disulfide stabilization became the "norm" for antibody fragments used as targeting moieties of RITs. In fact, all the PE-based RITs that were clinically evaluated since the mid-1990s, which include BL22, HA22 and SS1P (LMB-2 is the only one still based on a scFv) are all dsFv-based RITs [77, 97–99]. An additional RIT that has been evaluated (mostly preclinically, but soon to be tested in patients) as asdFv-PE38 fusion protein is MR1–1 [100], directed against a mutant form of the EGF receptor.

Fab fragments are universally known to be more stable than scFvs. A few Fab-based immunotoxins have been produced and evaluated pre-clinically [101, 102]. When third generation PE-based RITs were first constructed, their size was kept to a minimum to facilitate improved tumor penetration. Hence, scFvs and later dsFvs

were the targeting molecules of choice. More recently, smaller derivatives of PE were developed as part of the effort to deimmunize PE and to make it more resistant to proteolysis [10]. While sdFv-PE38 RITs are about 63 kDa in size (about the kidney infiltration size), the smaller RITs are cleared more rapidly from the circulation. Thus, their size is sub-optimal and will likely be re-increased by using Fabs to target them instead of dsFvs.

PE itself is a very robust protein with exceptional stability and solubility. This can be appreciated from one of the studies where the stability of scFvs and dsFvs was compared. In that study by Reiter et al., the stability of scFvs and dsFvs was compared by incubating them at temperatures from 25 ºC to 50 ºC or in the presence of increasing concentrations from 0–8 molar of the denaturing agent urea and measuring residual activity following the incubation. PE itself was included as a control. The results that were obtained clearly showed the increase stability of dsFvs compared to the corresponding scFvs. Still, even the more stable dsFvs were inactivated at some point while PE itself remained fully active even at the harshest tested conditions [94].

Nevertheless, PE underwent many modifications to make the derivatives suitable for particular challenges. Early studies compared the potency of PE derivatives in which the C-terminal REDLK ER retrieval sequence was changed to other sequences, including the "canonical ER retrieval sequence" KDEL, and their potencies in cell killing were compared. ER retrieval is a key step in the retrograde transport that PE (and PE-based RITs) undergoes on the route from receptor binding to cytosolic localization. This is because PE molecules that do not enter the pathway leading to the ER are delivered to the lysosome and destroyed. In such studies, it was found that PE and RITs that have the C-terminal sequence KDEL are more potent (by about 10 fold) than the REDLK, REDL of RDEL sequences [103, 104]. However, such toxins had a much higher liver toxicity in mice and, therefore, a potentially reduced therapeutic window. As a result, RITs that progressed to clinical development carry the wild-type C-terminal sequence.

An additional useful modification of PE was the removal of all the lysine residues in PE38 to make it (after the addition of an N-terminal peptide that contains a single lysine residue) most suitable for site-specific lysine-directed chemical conjugation. A few derivatives were made, like PE38QQR and PE38QQΔ, in which lysine at PE positions 590 and 606 were mutated to glutamine and the C-terminal lysine was either mutated to arginine (QQR) or deleted (QQΔ) [70].

As described above, during intracellular trafficking, PE and RITs can berouted to a "productive" route ending in the lysosome or a "destructive" route ending in lysosomal degradation. Several studies evaluated the possibility of producing PE derivatives that are less susceptible to lysosomal degradation. Notably, studies by Weldon et al. were focused on mapping residues within PE38 that, when mutated or deleted, reduce the extent of lysosomal degradation. The authors have investigated the proteolytic susceptibility of PE38 immunotoxins to lysosomal proteases and found that cleavage sites were clustered within a limited segment of PE38. Specifically, the lysosomal protease cleavage sites occurred between residues 260–261, 265–266, 297–298, 341–342, 342–343, 351- 352, 352–353, 353–354, 364–381, 390–391, and 391–392. All these clustersare located within domains II and Ib. Subsequently, RIT

deletion mutants were generated in this region using HA22, an anti-CD22 Fv-PE38 RIT currently undergoing clinical trials for B-cell malignancies. One of these mutants, HA22-LR (for "lysosome resistant), lacked all the identified cleavage sites (essentially most of domain II, leaving the "furin cleavage loop"), was resistant to lysosomal degradation, and retained excellent biological activity. HA22-LR killed CLL cells more potently and uniformly than did HA22, suggesting that lysosomal protease digestion may limit immunotoxin efficacy unless the susceptible domain is eliminated. Finally, a remarkable observation that was made during the study is that mice tolerated doses of HA22-LR at least 10-fold higher than lethal doses of HA22, and these higher doses exhibited markedly enhanced antitumor activity. The authors concluded that HA22-LR advances the therapeutic efficacy of HA22 by using an approach that may be applicable to other PE-based immunotoxins [10]. It is expected that PE-LR will be the toxin-of-choice for the RITs that will be developed in the future.

A bonus that resulted from the generation of HA22-LR is, as described above in the section discussing immunogenicity, that many B- and T-cell epitopes of PE are mapped to domain II. Thus, the LR version of PE-based RITs is inherently less immunogenic than PE38 [86].

In a follow-up study, Liu et al. tested the hypothesis that increased stability may result in reduced immunogenicity of RITs. The authors introduced a disulfide bond into domain III by identifying and mutating two structurally adjacent residues to cysteines at sites suggested by computer modeling. This RIT, HA22-LR-DB, displayed a remarkable increase in thermal stability and an enhanced resistance to trypsin degradation. In addition, HA22-LR-DB retained cytotoxic and anti-tumor activity, while exhibiting significantly lower immunogenicity in mice [105].

6.6 Insufficient Potency and Combining Therapies to Enhance Potency

In oncology, drug combinations are the mainstay of therapeutic intervention. One of the major potential strategies for the problem of insufficient potency is by combination of RITs with agents that enhance cell killing. In general, in cancer treatment, synergistic effects caused by combination therapies usually considered to be more effective comparing to single agents.

A case-in-point of overcoming insufficient potency can be demonstrated in the case of the anti-mesothelin RIT SS1P [SS1(dsFv)PE38]. SS1P is currently evaluated clinically in patients with mesothelin positive tumors. Despite successful phase I clinical trials, recent studies show that SS1P alone is limited in its efficiency [99, 106]. In fact, the major obstacle for treating patients with SS1P is the immunogenicity of PE, which was discussed above in Sect. 4. Still, these observations led scientists to examine SS1P in combination with other agents to improve its efficiency against different tumors. So far, such combination therapy studies were carried out mostly in vitro and in animal models.

In one study, SS1P was evaluated in combination with taxol (Paclitaxel, a drug whose action involves stabilization of cellular microtubules. As a result, it interferes with the normal breakdown of microtubules during cell division). Immuno-deficient mice were implanted with A431/K5 tumors expressing mesothelin antigen and were treated with SS1P alone, taxol alone, or the two agents together. The results showed that the combination treatment had a strong synergistic anti-tumor effect in the mice, but not in vitro [107]. An additionalstudy by the same group investigated the mechanism of synergy and comparedtaxol-sensitive and taxol-resistant KB tumors (both equally sensitive to SS1P alone). It turned out that KB tumors have high levels of shed mesothelin in their extracellular space (receptor shedding, see Fig. 6.5). Taxol treatment significantly lowered shed mesothelin levels in drug-sensitive but not in the drug-resistant KB tumors. The shed form of mesothelin antigen competes with the membrane form of the antigen for binding of SS1P, and as a result decreases its anti-tumor activity. Taxol-induced reduction in shed antigen levels can explain the synergy of immunotoxin and taxol in taxol-sensitive tumors and lack of synergy in taxol-resistant tumors [108].

An additional study focused on studying the effect of modulating the concentration of shed antigen on RIT potency was carried out using LMB-2. While RITs have been shown with high effectiveness in malignancies where most of the tumor burden is suspended in the peripheral blood or spleen (like in case of HCL), they are very limited in their effectiveness against aggressive solid tumors [109].This study evaluated the potential to obtain a synergistic effect of LMB-2combined with the chemotherapeutic agent gemcitabine (a nucleoside analog) in case of ATL. LMB-2 was previously evaluated in patients with relapsed and refractory hematologic

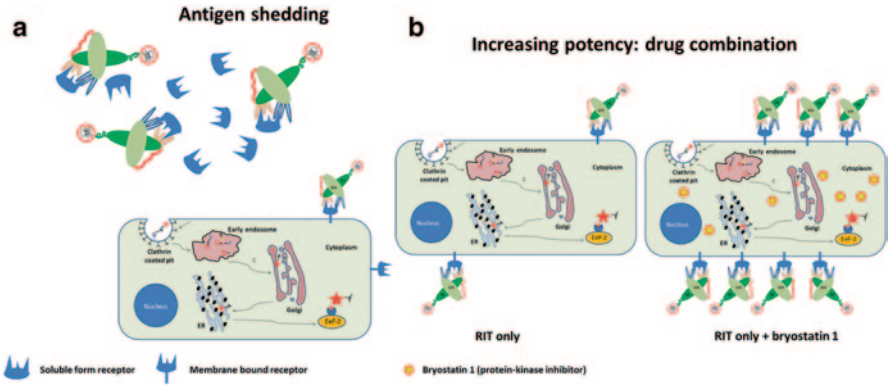

Fig. 6.5 Increasing potency by drug combination. **a** Many cell surface receptors, including RIT targets, shed the extracellular domain to the tumor interstitium and on to the circulation. The shed form of the RIT target competes with the membrane form for binding of the RIT, and as a result decreases its anti-tumor activity. In one example described in the text, when the anti-mesothelin RIT SS1P was combined with taxol, the taxol treatment significantly lowered shed mesothelin levels in drug-sensitive but not in the drug-resistant KB tumors. **b** A drug combination may increase the antigen density on the target cells, as is described in the case where BL22 RIT was combined with bryostatin 1, which led to an increase of CD22 expression on difficult-to-treat target cells, resulting in their becoming sensitive to RIT treatment

malignancies with most promising results obtained in Hodgkin's lymphoma, CTCL, HCL, CLL and ATL [98, 110]. However, its clinical benefits were limited because of immunogenicity and rapid disease progression, particularly in ATL. The authors proposed that in the case of solid tumors, the presence of high concentrations of soluble target antigen CD25 (sCD25) may potentially block the circulating LMB-2 and lower the effective concentration that can bind the tumor cells. To study if this is indeed the case, the levels of sCD25 in $CD25^+$ ATAC-4 tumor xenografts in nude mice were measured before and after administration of gemcitabine and then determined whether gemcitabine and LMB-2 would show in vivo or in vitro synergy. It was found that levels of interstitial sCD25 within the tumors were higher by100-fold than in the serum and that gemcitabine could reduce them by 10-fold. Additionally, a synergistic antitumor activity in vivo was shown by combination of gemcitabine and LMB-2, while in vitro their combined effect was only additive [109]. These two studies highlight the obstacle placed by the presence of soluble target proteins that compete with the tumor cells for RIT binding and the importance of treatments that reduce the concentration of soluble targets on potentiating RITs.

Another study of combination therapy involved mesothelin-expressing pancreatic cancers that are known to be resistant to most of the chemotherapeutic agents. The SS1P RIT inhibited protein synthesis in two of the pancreatic cancer cell lines, but did not significantly affect cell death. The resistance to RIT treatment was contributed to low levels of the pro-apoptotic protein Bak. The authors demonstrated that combining TRAIL or an anti-TRAIL receptor 2 agonist antibody, HGS-ETR2, with SS1P caused an effective synergistic effect leading to cell death and reduction of tumor size in xenograft-bearing nude mice [111].In yet another study, SS1P was applied in combination with several activated protein kinase (PKC) inhibitors. PKC enzymes contribute to cells survival, proliferation and angiogenesis and this is why the therapeutic application of PKC inhibitors are considered a potential strategy to improve cancer treatment [112]. Enzastaurin, but not two other tested PKCs in the study, has shown significant enhancement of SS1Pimmunotoxin action in combination treatment of cells that exhibited partial resistance to SS1P alone. Reductions of ATP levels, caspase activation and loss of attachment from culture dishes finally resulted in apoptotic cell death. This synergistic effect was concentration-dependent in the range of 4–10 μM enzastaurin and showed a 10-fold enhancement of immunotoxin action of KLM1 cells [113]. This combination treatment resulted in greater general reductions in protein synthesis and even in the complete loss of activation of caspases 3 and 7, and of several proteins, such as Mcl-1, Bcl2, AKT, considered pivotal in many immunotoxin-cell death models. The nature of this additional inhibitory action and the contribution of each component are not fully clear. However, the authors note that like many other kinase inhibitors, enzastaurin is known to be a multi-kinase inhibitor (not sufficiently specific) and it will be a problem to achieve required-for-treatment concentrations in patients with mesothelin-positive tumors. They suggested that looking for more specific kinase inhibitors that produce synergistic effects with RITs is a challenge for future studies.

In a recent study, the authors hypothesized that protein tyrosine kinases may have important roles in affecting their susceptibility of cancer cells to RITs. Tyrosine

kinases are known to be major players of protein phosphorylation within cells, and are hyperactivated during cancer processes. The anti mesothelin RIT SS1P and the anti CD22 RIT and HA22 (moxetumomab pasudotox) were chosen for the study. To examine their hypothesis, the authors used siRNAs to knock down the expression of 88 known tyrosine kinases in cancer cells while testing their response to SS1P or HA22. Only five of the siRNAs (that knocked down the expression of *INSR*, *HCK*, *SRC*, *PDGFRβ*and *BMX*) were found to enhance the activity of SS1P. Further investigation of the enhancement mechanism showed that *HCK* knockdown stimulated SS1P processing by furin-mediated cleavage, lowered levels of the anti-apoptotic protein Mcl-1 and raised the expression level of the pro-apoptotic protein Bax. Additionally, the authors demonstrated that *SRC* family inhibitors could mimic the effect of tyrosine kinase knockdown, resulting in a significant increase of SS1P and HA22 killing activity in A431/H9 and CA46 cells, respectively. One of these SRC inhibitors, SU6656 has been successfully evaluated in mouse xenograft tumor models, where it demonstrated a synergistic antitumor effect with both SS1P and with HA22 [114].Another recent study looked for additional agents that could induce an effective cell apoptosis of pancreatic ductal adenocarcinoma (PDAC) cell lines that are resistant to SS1P despite high mesothelin expression. In that study, SS1P was combined with the BH3-mimetic ABT-737 (which selectively targets and neutralizes three BCL2 family pro-survival proteins BCL-XL, BCL-2, and BCL-W, but now Mcl-1 [115]). The combination led to a significant increase in cell death, while the studied cells lines were resistant to each component alone. The effect had a variable extent in different cell lines (KLM-1, BxPc-3, Panc 3.014). The authors showed that RIT-mediated protein synthesis inhibition and the capability to down regulate Mcl-1 and Bcl2A1 were major factors that affected the efficacy of the combination treatment [116].

The following study is a case in point where drug combination was used to expand the utility of an RIT for treating additional malignancies than originally intended. BL22 is an RIT targeting CD22 molecules presented on the surface of certain B-cell malignancies such as lymphoma and leukemia [117, 118]. BL22 has shown significant therapeutic potency in patients with HCL [119], but failed in the treatment of less indolent leukemias and lymphomas [77], particularly CLL. This failure was apparently due to lower expression of the target CD22 on the surface of tumor cells of the unresponsive malignancies. In a recent study, it was found that pre-activation of primary CLL cells with the macrocyclic lactone bryostatin 1 (a potent modulator of protein kinase isolated from the marine bryozoan *Bugulaneritina*) overcame this issue. Primary CLL cells that were treated with bryostatin 1 followed by BL22 treatment showed significant induction of apoptosis. It turned out that bryostatin 1 works in two distinct pathways: (1) it strongly upregulates the surface expression of CD22 receptors on leukemic cells that cause a "hairy cell phenotype" in CLL cells, and (2) it depletes protein kinase C-β2. Additionally to CLL cells, the authors showed that BL22 and bryostatin 1 combined treatment exerts a strong apoptotic effect in large B-cell lymphomas and mantle cell lymphoma cells. The authors concluded that this drug synergism should be tested in vivo to evaluate if it can be a feasible therapeutic approach for CLL and B-cell malignancies [48].

6.7 Potentiation of RITs by Affinity Maturation of the Targeting Antibody

The antigen binding affinity of antibodies is one of the key factors contributing to-their therapeutic efficiency. In vitro affinity maturation of therapeutic antibodies is a commonly applied practice in their clinical development (see Fig. 6.4) [120–124].

Accordingly, affinity maturation of the targeting antibodies used to construct RITs may significantly improve their antitumor activity. There are several strategies for in vitro affinity maturation of antibodies. These include "non-targeted within the V-gene" approaches such as error-prone PCR, mutator *E. coli* strains, chain shuffling and DNA shuffling and "targeted within the V-gene approaches" such as CDR randomization, CDR walking, hotspot mutagenesis, PCR-based mutagenesis, parsimonious mutagenesis and saturation mutagenesis. Each approach has its own advantages and disadvantages. The affinity maturation approach introduces sequence diversity into the antibody genes, creating a repertoire of mutants derived from the original antibody. One of several display technologies is then applied to isolate the highest affinity clones for further study [125, 126].

Affinity maturation of an scFv as a part of a RIT was first reported in 1999. In that report, Chowdhury and Pastan reported the affinity maturation of the anti mesothelin scFv SS1. The process that was applied was called "mimicking somatic hypermutation *in vitro*", which may be classified as a "targeted within the V-gene" approach. In that study, DNA sequences were identified in the antibody variable domains that are naturally prone to hypermutations (as evident from the fact that they are frequently mutated during the natural in vivo affinity maturation process that antibodies undergo). The authors selected a few hot spots encoding non-conserved amino acids, and introduced random mutations to make libraries with a size of under 10^4 independent clones. Affinity selection of the hot spot libraries by phage display yielded several mutants with a 15- to 55-fold increase in affinity [32]. The best affinity matured clone later became the dsFv used to target the SS1P RIT [99].

Another study focused on the improvement of the anti-CD22 RIT BL22 for B-cell malignancies. In the previous section, it was already mentioned that BL22 was much less effective against CLL compared to HCL. Thus, to improve its affinity, hot spot mutagenesis combined with phage display using CD22-positive Daudi cells for affinity selection was carried out. The best affinity improved clone contained mutations in HCDR3, specifically amino acid residues Thr-His-Trp (THW) in place of Ser-Ser-Tyr (SSY) at positions 100, 100A, and 100B of the Fv and had an affinity improved from 85 nM to 6 nM. The THW mutant (that was named HA22) had a 5- to 10-fold increase in activity on various CD22-positive cell lines and was up to 50 times more cytotoxic to cells from patients with CLL and HCL [97, 127]. Later, in order to achieve a more productive intracellular trafficking and reduced immunogenicity (described above), most of the PE domain II was deleted, resulting in an RIT named HA22-LR. HA22LR was much more effective than HA22-PE38 in killing CLL cells, showing less general toxicity. In an attempt to further improve the affinity of HA22, Kawa et al. applied a different approach instead of the hotspot

mutagenesis that was used in HA22 evolution. In that case, a different antibody fragment format was used, consisting of an scFv further stabilized byan artificial disulfide bond (according to the dsFv approach). The resulting HA22 (scds) Fv-LR had the same cytotoxicity as that of HA22 (dsFv) LR. In order to identify functionally important residues for antigen binding, alanine scanning mutagenesis was implemented. Single asparagine to alanine exchange (N34A) in VL CDR1 resulted in a substantial increase in affinity and activity. Cell viability assays showed that the N34A mutanthad a 10-fold improvement in activity toward CD22-positive cell lines. The authors suggested that this may constitute a clinical benefit with a lower dose and in turn lead to a decrease in nonspecific toxicity in patients [128].

Recently, Kuan et al. isolated a high-affinity scFv against the glycoprotein NMB and showed how affinity maturation can improve its potency as a targeting antibody of an RIT. The glycoprotein NMB (GPNMB) is a transmembrane glycoprotein specifically associated and overexpressed in malignant gliomas, such as common primary adult brain tumor glioblastoma multiforme (GBM). The median survival after diagnosis of GBM is 14 months [129]. Glycoprotein NMB and other antigens presented in GBM are promising targets for immunotherapeutic cancer treatments. Initially, the anti-GPNMB scFv (G49) isolated from a human synthetic phage display library, showed high affinity to GPNMB-expressing cells and once converted to an RIT form was cytotoxic to GPNMB-positive glioma cells. The authors conducted in vitro affinity maturation (by a random mutagenesis approach) and introduced mutations into CDR3 of the light chain and CDR1 of the heavy chain. This introduction of sequence diversity combined with phage display made it possible to isolate a mutant scFv (902V) with an 11-fold increase in affinity. Clone 902V was further improved by sequence randomization throughout the whole scFv by error-prone PCR, and one mutant, F6V, was selected by yeast-surface display. Finally, this affinity-matured scFv was fused with PE38. The obtained F6V-PE38 targets HGGs, medulloblastomas, and melanomas; it has shown significant activity in in vitro cell-killing assays and in vivo models of GPNMB-expressing xenografts in nude mice [130].

The most recent study involved other common cell surface targets for glioblastoma; gangliosides 3′-isoLM1 and 3′,6′-isoLD1. Gangliosides are a group of sialic acid-containing glycosphingolipids with extending extracellular section. They are overxpressed in over 60% of glioblastomas, making them attractive therapeutic targets for brain malignancies [131]. Piao et al. developed a novel RIT, DmAb14m-(scFv)-PE38KDEL (DmAb14m-IT) that specifically binds these gangliosides. First, the authors isolated ganglioside-targeting mAbs as murine hybridomas. Next, they cloned DmAb14 that exhibited high reactivity towards both 3′-isoLM1 and 3′,6′-isoLD1. Since the original hybridoma was of the IgM isotype, which is not ideal for fusion with ITs, cloning of its VH and VL domainsas a scFv was carried out. Subsequently, the DmAb14-scFvs were subjected to in vitro affinity maturation by CDR hotspot random mutagenesis combined with phage display. The best resulting clone was fused with PE38KDEL (PE38 that carries KDEL sequence at C-terminus to improve ER retrieval, leading to RIT potentiation). In vitro evaluation of the resultantRIT showed significantly improved abilities compared to the parental

molecule: increased cytotoxicity, better cell internalization and better affinity to the ganglioside targets. It showed potent killing activity on glioma cell lines and tumor xenograft-derived cells expressing 3′-isoLM1 and 3′,6′-isoLD1 [132].

6.8 Reducing Off-Target Toxicity and Overcoming Physical Barriers

In general, RIT dose-limiting toxicity is regarded in most cases as a part of "off-target" toxicity, resulting from interactions of the RIT with cells and tissues other than the target malignant cells. Regarding PE-based RITs, in the pre-clinical studies the dose-limiting toxicity in mice of PE and RITs was mostly liver toxicity [10, 133, 134]. Interestingly, it has been reported that the non-specific mouse liver toxicity of PE-based RITs can be significantly reduced by engineering the targeting Fvs to lower the isoelectric point of the Fv. The authors hypothesized that this may be due to modified pharmacokinetics of the differently-charged RITs, however, this has not been established experimentally. An additional modification of the PE component of RITs that reduced mouse toxicity was the deletion of most of the domain II in the context of HA22-LR (10-fold lower than HA22-PE38) [10]. The authors suggested that the observations that were made during the studies of lowering the toxicity by lowering the pI of the targeting Fvs probably did not account for the difference between HA22 and HA22-LR. This is because HA22-PE38 and HA22-LR have an identical Fv and the pI of HA22-LR is slightly increased relative to the pI of HA22 ($pI_{HA22\text{-}PE38} = 5.26$ and $pI_{HA22\text{-}LR} = 5.63$). In addition, the 2- to 3-fold difference in toxicity observed for this pI lowering strategy is also much smaller than the more than 10-fold difference between HA22 and HA22-LR [10]. It is still unknown whether the lower non-specific toxicity of lower pI RITs or using the LR toxin instead of PE38 is relevant to toxicity in humans [134, 135].

As for RITs that were tested clinically, dose limiting toxicity in humans varied between different RITs and so did off-target toxicities. In some cases the cause of the off-target toxicity was identified but in other cases it is still unknown. When the "second generation" IT LMB-1 (the anti Le^Y IgG B3 chemically conjugated to PE38) was tested in a phase I clinical study, the dose-limiting toxicity was mainly vascular leak syndrome (VLS, manifested by hypoalbuminemia, fluid retention, hypotension and, in one case, pulmonary edema) [23].Kuan et al. investigated the effects of several PE-based ITs on different human endothelial cell lines to elucidate the mechanism of VLS induced by ITs containing PE and found that anti Le^YITs, including LMB-1 and also the RIT LMB-7 (B3(Fv)-PE38 were toxic to several of the endothelial cell lines that were tested. This effect was unique to the B3-targeted ITs as other RITs that target different antigens were not toxic to endothelial cells. The authors further found that the cytotoxicity of B3-containing ITs is due to specific B3 binding to endothelial cells comes from the fact that the cytotoxicity can be blocked by excess free mAb B3 as competitor [136].

LMB-7 was also tested in a phase I clinical trial, in which, as in the case of LMB-1, VLS was also observed (although it was not dose limiting) [137]. In fact, in that clinical trial, two significant toxicities were observed. The first toxicity was a targeted toxicity: severe gastritis caused by the killing of normal cells that expressed Le^Y antigen in the stomach. This toxicity could be prevented by blocking acid secretion with the proton pump inhibitor Omeprazole accompanied by antacids. At higher dose levels renal toxicity developed and was dose limiting. This toxicity was probably because of the small amounts of Le^Y present on some tubular cells in the kidney [9].

The anti Le^Y RIT SGN-10 (BR96 sFv-PE40) was developed by Seattle Genetics and tested in a phase I clinical trial in 46 patients with Le^Y-positive metastatic carcinoma that was published in 2002. In that study, the dose limiting toxicities were gastrointestinal (including diarrhea, nausea, and vomiting) and VLS [138]. During the pre-clinical evaluation of SGN-10, it was tested in a rat VLS model. It was reported that intravenous administration of BR96 sFv-PE40 resulted in symptoms that closely resemble VLS seen in human immunotoxin trials. Prophylactic administration of the corticosteroid dexamethasone resulted in the prevention of VLS and survival of rats injected with what would otherwise be lethal doses of the RIT without changing the therapeutic efficacy [139, 140]. It should be noted that dose-limiting toxicities and adverse effects that were observed in most of the clinical trials used to evaluate RITs could be managed by simple medical interventions with i.e. blood-pressure lowering drugs or NSAIDs.

The anti mesothelin RIT SS1P was evaluated in two phase I study in which toxicity was also evaluated. In one study, SS1P was given by continuous infusion over 10 days. Continuous infusion was tested as an approach to increase tumor uptake. In the other trial, SS1P was given by a 30 min infusion every 2 days for 3–6 doses. The significant dose-limiting toxicity in both trials was pleuritis ascribed to the targeted killing of normal mesothelial cells in the pleura. VLS characterized by weight gain and a fall in serum albumin also occurred, but was not dose limiting [9, 141].

A group of ITs was developed in which PE38 or PE38KDEL is not targeted by an antibody but, rather, by a ligand (or part of a ligand) binding to a cell-surface receptor that is overexpressed in cancer. As a matter of fact, the only FDA approved IT (which is not PE-based) is denileukin diftitox (Ontak). Ontak, an IL-2-DT fusion protein, was approved in 1999 the for patients with persistent or relapsed CD25-positive cutaneous T-cell lymphoma (CTCL) [142].

As for ligand-PE fusion proteins, IL-4, IL-13 and EGFR were the tested ligands. Although overexpressed in malignancies, the receptors for these cytokines are present on many normal cells so that systemic therapy results in unacceptable toxic side effects. For example, in a phase I trial of IL-4(38–37)–PE38KDEL in patients with advanced solid tumors that expressed IL-4R, the dose-limiting toxicity was liver damage and no objective responses were observed. These agents are better suited for local therapy, and three of these agents that target the EGF, IL-4 and IL-13 receptors have been evaluated for the therapy of glioblastoma. During treatment, the proteins were slowly infused into or next to the brain tumor by continuous infusion over many hours. Phase I and II trials of IL-4(38–37)–PE38KDEL showed a few

complete and partial responses, but the associated toxicity was unacceptable (the dose-limiting toxicity was liver damage) and the development of this IT was abandoned [143]. By contrast, both TGFalpha–PE38 and IL-13–PE38QQR were much better tolerated and have shown complete responses in some patients during phase I and phase II trials. The development of these ITs is ongoing [144–146].

The toxic side effects of immunotoxins in animals and humans are of two types. One type arises from the targeted killing of normal cells that have the same antigen as the tumor cells. Unfortunately, the best solution to overcome this toxicity is to find a different target antigen that is not expressed on normal cells (and, of course, a different antibody). The second type of toxicity arises from undefined nonspecific binding to normal cells which is probably driven by the toxin itself or from physicochemical properties of the RIT. The studies that were described in this section highlight the importance of carefully assessing the target specificity of antibodies that are used to target RITs. Because RITs are so potent, differences in target expression level that may be sufficient for "naked" therapeutic antibodies (the anti EGFR mAb cetuximab and the anti ErbB2 mAb trastuzumab are such antibodies) may not provide a sufficient therapeutic window for RITs.

Finally, another critical issue for the success of treatment with immunotoxins is overcoming physical barriers within the body to gain access to the tumor target. Reducing the size of ITs was described in the introduction as a general solution for improving tumor penetration. The blood brain barrier (BBB) is the major barrier for systemically delivering chemotherapeutics from the circulation to brain tumors, for example the brain tumor glioblastoma multiforme (GBM) [147]. Traditional procedures like surgical resection and radiation therapy have little effectiveness for targeting brain tumor tissues due to their highly invasive nature. Catheter placement has many disadvantages in the context of drug delivery, such as leakage of the infusate into the interventricular and subarachnoid spaces and results in poor drug delivery and distribution [148]. As an alternative novel approach, convection-enhanced delivery (CED) was proposed to circumvent the BBB through direct intracerebral injection (by using a hydrostatic pressure gradient) of large drug molecules throughout the interstitial spaces of infiltrated brain tumors. The proposed advantages of CED drug delivery platform are: (1) minimizing the exit of the drug from the brain while enhancing the drug delivery and reducing systemic toxicity effects; (2) therapeutic agent distribution may be controlled by the applied pressure and, thus, it enables to deliver constant concentrations of the RITs for a predictable distance before a drop-off [149]; and (3) CED enables limited neurotoxicity yet provides effective drug therapy to the tumor upon accurate catheter placement [150]. As part of the need for more accurate methods of validation and drug distribution, Mehta et al. demonstrated the ability of monitoring CED of RITs in humans by in vivo imaging. The imaging for CED was performed by combined the infusion of the MR1-1 RIT [MR1-1(dsFv)-PE38KDEL] and the MRI contrast agents: iodine-labeled albumin (124I-HSA) and gadolinium conjugated diethylenetriaminepentaacetic acid in patients with supratentorial recurrent malignant gliomas. This method enabled high-resolution monitoring of large molecule distribution with the tissue [151]. The authors concluded that CED-infusion approaches offer a promising platform for therapy in patients with GBM.

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