Review

Prospects of Bacterial and Plant Protein-based Immunotoxins for Treatment of Cancer

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Abstract. *Bacterial- and plant-derived immunotoxins have documented potential for treatment of cancer. We discuss Anthrax toxin, ribosome inactivating-toxins, such as saporin and ricin, and ADP-ribosylating toxins such as Diphtheria toxin and Pseudomonas exotoxin, with focus on the latter, which has been most thoroughly investigated. Regarding their potential as anticancer agents, critical issues such as immunogenicity and toxicity are outlined. We describe different generations of immunotoxins, the pathways for the delivery of the cytotoxic 'warheads', molecular parameters modulating efficacy, and combination therapy with other anticancer agents. Finally, we discuss deimmunization strategies based on the removal of B- and T-cell epitopes from the cytotoxic component, and highlight promising clinical proof-of-concept studies.*

Development of anticancer agents is based on the hallmarks of cancer and the identification of corresponding molecular targets (1, 2). The hallmarks of cancer are properties such as constitutive proliferative signaling, de-regulation of cellular energetics, resistance to cell death, evasion of growth suppressors, avoidance of immune destruction, enabling of replicative immortality, tumor-promoting inflammation, activation of invasion and metastasis, genomic instability and mutation, as well as induction of tumor angiogenesis. Based on these properties, in addition to cytotoxic agents, target-specific therapies were established. Thus, some types of

cancer can be medicated by inhibition of driver mutations (3, 4) and others might be treated in the future by induction of synthetic lethality (5, 6). Interference with tumor angiogenesis has resulted in clinical benefit for several types of cancer, especially in combination with chemotherapy (7, 8). Activation of an antitumor immune response by antibody-based therapy is another promising approach with documented clinical benefit (9, 10). However, targeted therapies are often hampered by the activation of compensatory pathways leading to treatment resistance (11, 12). Paul Ehrlich brought-up the idea of a "magic bullet" for a disease-relevant antigen which does not hit normal cells of the human body (13). Bacteria- or plant-derived toxins, such as Pseudomonas exotoxin (PE), Diphtheria toxin (DT), ricin, saporin and others, can inhibit protein synthesis and induce apoptosis efficiently by only a few intracellularly-processed molecules (14). Since it is not possible to define a therapeutic window for these toxins, directing these agents to tumors with appropriate ligands is necessary; such targeting moieties can be cytokines or antibody-related entities.

In the present review, we discuss prospects, critical issues and clinical proof-of-concept studies for these powerful agents. We focus on toxins exerting their function through cleavage and inactivation of kinases of the mitogen-activated protein kinase (MAPK) family, *N*-glycosidase or adenosine di-phosphate (ADP)-ribosylation activities. Progress is based on the development of new formats for targeting immunotoxins (ITs) (Figure 1) and understanding of the trafficking pathways of ITs (Figure 2).

Anthrax Toxin-based Approaches

The Anthrax toxin derived from *Bacillus anthracis* is the causative agent of anthrax in animals and humans. It is composed of protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa) and edema factor (EF, 89 kDa) (Figure 3A). It belongs to a family of toxins in which LF or EF must be

combined with PA in order to elicit toxicity (15-17). The proteolytic activation of PA has been exploited for tumor therapy. PA can bind to either of two receptors: tumor endothelial marker 8 (TEM8) and capillary morphogenesis protein 2 (CMG 2) (18, 19). Cell surface proteases, such as furin, cleave PA into an N-terminal PA63 (63 kDa) and a C-terminal PA20 (20 kDa) fragment. Receptor-associated PA63 oligomerizes into a ring-shaped heptamer, which can bind up to three molecules of LF or EF in any combination (20). The resulting complexes are referred to as lethal toxin or edema toxin. They are internalized *via* clathrin-dependent receptor-mediated endocytosis and undergo a conformational change, which leads to the insertion of the complex into the membrane of the endosome and finally delivery of LF and EF into the cytoplasm. Once in the cytoplasm, LF can function as a zinc metalloprotease, which can inactivate MAPK through cleavage. EF is a Ca/calmodulin-activated adenylate cyclase (AC) involved in the elevation of intracellular adenosine mono-phosphate (AMP) or cyclic adenosine mono-phosphate (cAMP) and thus interferes with the balance of intracellular signaling. Replacement of the furin cleavage site of PA with cleavage sites for proteases which are overexpressed in tumors, such as matrix metalloproteinase-2 (MMP2) and MMP9, allows tumor-specific activation of PA. Making use of LF and MMP-activated PA, antitumor activity was observed not only in tumor xenografts derived from human melanomas bearing the V600E v-Raf murine sarcoma viral oncogene homolog B (BRAF) mutation, but also in other tumor xenograft models with non-mutated *BRAF* (21). Furthermore, Anthrax toxin can inhibit tumor angiogenesis by interference with endothelial proliferation, migration and tube formation for which MAPK plays an essential role (22, 23). Overexpression of urokinase-type plasminogen activator (uPA) in many types of tumors has also been exploited in this context by replacing the furin cleavage site of PA with an uPA-cleavage sequence (24). The specific processing of PA has also been used for the delivery of other toxins, such as the ADP-ribosylating moiety of PE. Cleavage site-modified PA in combination with a recombinant fusion protein, consisting of amino acids 1-254 and the ADP-ribosylation domain of PE, referred to as FP59, was shown to be activated on the surface of tumor cells and to mediate efficient tumor cell binding activity *in vitro* and *in vivo* (25). This combination of proteins selectively kills MMP-overexpressing tumor cells, whereas no toxic effect was noted in non-tumorigenic cells. Combined inhibition of tumor growth and angiogenesis by LF has been documented by independent investigations (26, 27). Critical issues of Anthrax-based toxins, as described above, are the expression of MMPs and uPA in non-malignant tissues, and the lack of a tumor-specific targeting component challenges the feasibility to translate these agents into clinically-validated anticancer agents.

Ribosome-inactivating Protein (RIP)-based Immunotoxins

RIPs are a family of toxins derived from plants, bacteria, fungi or algae with *N*-glycosidase activity which inactivate ribosomes by cleaving an *N*-glycosidic bond of a specific adenosine residue (A4324) within the *28-S* rRNA subunit, thereby rendering ribosomes unable to interact with eukaryotic elongation factor-2 (eEF2), and thus inhibiting protein synthesis (28). Type I RIPs are single-chain proteins with enzymatic activity as described above, while type II RIPs are heterodimeric proteins which consist of an enzymatically active A-chain linked to a B-chain with lectin properties (29-32). Saporin, pokeweed antiviral protein (PAP) and gelonin are prototypic class I RIPs, while ricin and abrin are typical class II members (Figures 3B and C). Ricin has been thoroughly investigated. It binds to cell surface galactose or *N*-acetylglucosamine residues on glycoproteins or glycolipids through the B-chain, is internalized by endocytosis and is routed backwards from the Golgi apparatus to the endoplasmic reticulum (ER). In this compartment, the disulfide bond linking the two chains is reduced and the catalytic A-chain is translocated into the cytoplasm to excert its *N*-glycosidase activity. The *28-S* RNA modification compromises ribosomes to interact with eEF2, resulting in the inhibition of protein synthesis and ultimately cell death (33) (Figure 2). In contrast, the class I RIP saporin does not seem to rely on Golgi-mediated retrograde transport and is probably translocated from the endosomes into the cytoplasm (32). Ricin-based conjugates directed against cluster of differentiation-5 (CD5), CD19, CD22, CD25 and CD30 were evaluated clinically in several hematological malignancies. Here, several complete and partial responses were observed, however, vascular leak syndrome emerged as a dose-limiting toxicity (34-36). In order to reduce non-specific uptake by receptor-associated carbohydrates, especially by reticuloendothelial cells in the liver, the enzymatically-active A-chain was chemically de-glycosylated (dgA). With regard to saporin, impressive *in vitro* and *in vivo* efficacy of saporin-based ITs was observed in several models of hematological and solid tumors (37). CD2, CD3, CD5, CD7, CD19, CD20, CD22, CD30, CD38, CD80 and CD86 were explored as targets for hematological malignancies (37). Epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), CUB- domain containing protein 1 (CDCP1), prostate-specific membrane protein (PSMA), high-molecular weight melanoma-associated antigen (HMW-MAA) and activated leukocyte cell adhesion molecule (ALCAM) were evaluated for the treatment of solid tumors (37). Depending on the target, corresponding ligands or antibody-derived entities were conjugated or genetically fused with saporin. A cleavable adapter inserted between EGF and saporin was shown to reduce non-specific cytotoxicity of this
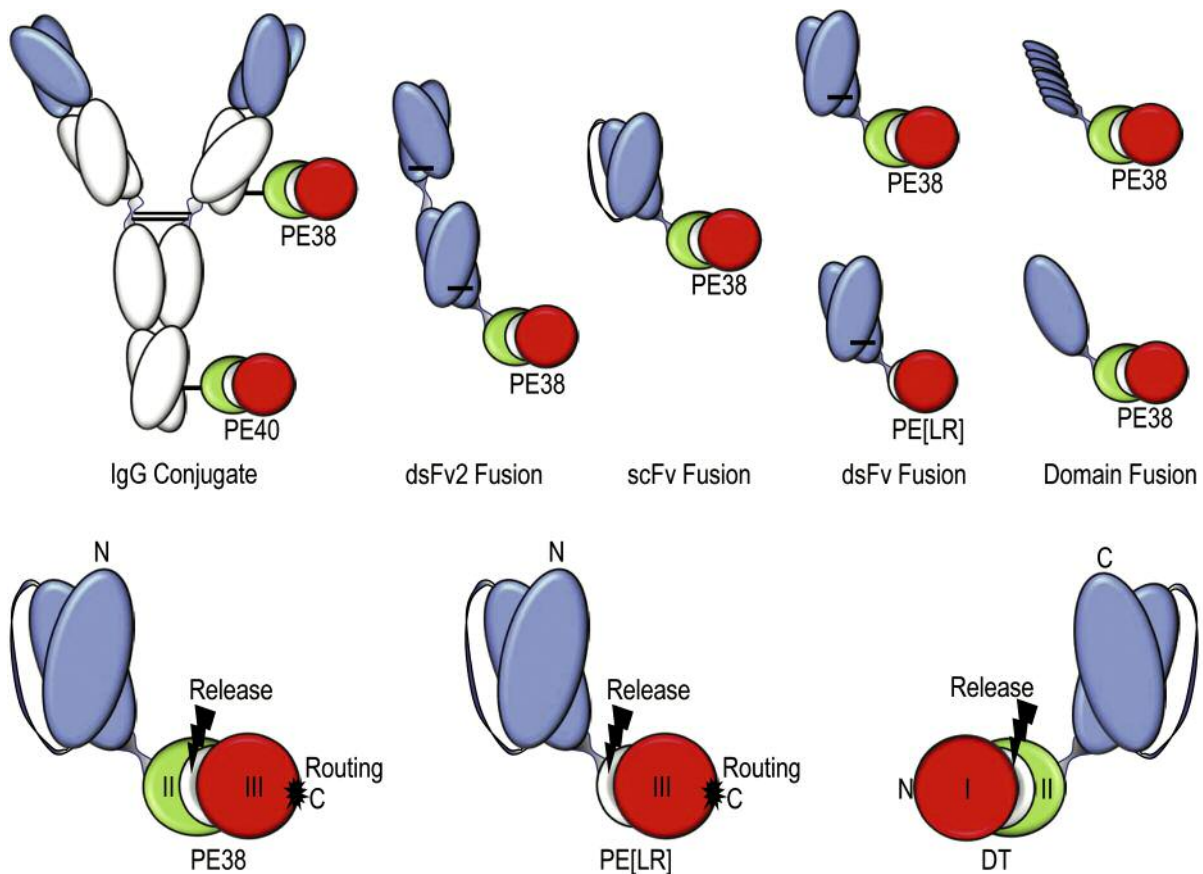
Figure 1. *Composition of immunotoxins. The composition of various formats of immunotoxins is shown exemplarily for derivatives of Pseudomonas exotoxin A (PE) and Diphtheria toxin (DT). Upper panel: Similar design principles are applied for conjugates and fusions with different payloads, including Ribosomal inactivating proteins such as ricin A chain or bouganin. Payloads are truncated toxin derivatives, which lack their own cell-binding domains (PE40 or PE38). These can be chemically conjugated at lysines (N-hydroxysuccinimide chemistry) or cysteines (maleimide chemistry) of whole IgG (IgG-conjugate, upper left panel). Truncated toxins can be recombinantly-fused to single-chain or disulfide-stabilized Fvs (scFv- or ds-Fv fusions). It is also possible to attach two recombinant Fvs to one toxin (dsFv2-fusions), or to mediate cell binding with single domain-binding entities such as VH derivatives or scaffold-based (e.g. Darpin) modules (domain fusions, upper right panel). Lower panel: The first generation of PE-derived immunotoxin fusions (PE40 and PE38 derivatives) consisted of truncated toxins whose N-terminal cell-binding domain I was deleted and replaced by recombinant antibody derivatives (shown is a Fv). N-Terminal fusion of the binding moiety is mandatory for PE-derived entities because the free C-terminus harbors a routing signal that directs the toxin to the ER. Domain II contains a processing site which becomes cleaved by the protease furin, releasing the toxin from its targeting moiety. Second generation toxins, such as PE[LR], are further decreased in size, with most of domain II deleted except for the processing site. DT-derived immunotoxins are composed in a similar manner, also containing a furin-cleavable processing site that enables the release of toxin from targeting entities. The toxin domain of DT derivatives is in the N-terminal region of the protein located (in contrast to PE). Binding entities are therefore fused to the C-termini of DT-derived immunotoxins.*
Antigen-binding moieties are shown in blue, other antibody-related moieties are shown in white, translocation domains in green, protease cleavage (release) modules in grey and catalytic domains in red. Routing modules are highlighted as black stars.

IT without affecting *in vitro* cytotoxicity and *N*-glycosidase activity (38). Further optimization can be achieved by introducing cytosolic cleavable peptides, membrane transfer peptides and endosomal cleavage peptides. Tumor angiogenesis-related saporin-based ITs were also evaluated. Endosialin (CD248), expressed in stromal cells, endothelial cells and pericytes of various tumors and TEM8, an integrin-like surface protein which is up-regulated in tumor-associated blood vessels, were efficiently targeted by saporin ITs (39, 40). Saporin ITs were evaluated in clinical trials in a limited number of patients (36) with Hodgkin's lymphoma, non-Hodgkin's lymphoma and B-cell lymphoma (37). Nine partial responses and 13 stabilization of disease were observed. The formation of antibodies against the toxin component, vascular leak syndrome and hepatotoxicity were identified as critical parameters for successful clinical application of these types

of ITs. Other RITs are based on gelonin (rGel) which does not bind efficiently to cell surfaces (41). A vascular endothelial growth factor (VEGF121)-rGel fusion protein was shown to inhibit vascular endothelial growth factor receptor 2 (VEGFR2)-mediated angiogenesis and as a consequence prostate cancer progression in an *in vivo* model by targeting osteoblasts, osteoclasts and vasculature simultaneously (42, 43). B-Lymphocyte stimulator (BLys-rGel) fusion protein targeting malignant B-cells expressing the B-Lymphocyte stimulator BLys receptors, B cell activating factor receptor (BAFF-R), transmembrane activator and calcium-modulating cyclophilin ligand (CAML) interactor (TACI), and B-cell maturation antigen (BCMA) (44) of diffuse large B-cell lymphoma, mediated growth inhibition *in vitro* and *in vivo* by up-regulation of B-cell lymphoma-associated X (BAX) and caspase 3, inhibition of signal transducer and activator of transcription-3 (STAT3) signaling and down-regulation of the interleukin-6 (IL6) receptor (45). Recombinant rGel-single-chain (scFv) fusion proteins targeting human epidermal growth factor receptor-2 (HER2), efficiently inhibited growth of a model of ovarian xenograft tumor (46). Another approach is based on the overexpression of FGF-inducible protein 14 (Fn14), the cell surface receptor for human tumor necrosis factor-like weak inducer of apoptosis (TWEAK) in various types of human tumors (47). A recombinant fusion protein composed of rGel and a humanized, dimeric single-chain antibody directed against Fn14 was highly cytotoxic to a panel of melanoma cells and significantly inhibited growth of MDA-MB-435 xenograft tumors (48). VB6-845, an anti-epithelial cellular adhesion molecule (EpCAM) IT containing a T-cell epitope-depleted variant of the plant toxin bouganin (Figure 3D) showed *in vitro* potency greater than many commonly used chemotherapeutic agents, and in tumor xenograft models the majority of the mice treated with this type of molecule were tumor-free at the end of the study (49).

DToxin-based ITs

DT (50, 51) is the prototype of an ADP-ribosylating protein which is secreted by pathogenic strains of the bacterium *Corynebacterium diphtheria* and binds to heparin-binding epidermal growth factor precursor (HBEGF) on the cell membrane. A single molecule of DT can be lethal to the host cell (50). DT consists of 535 amino acids and is composed of fragments A and B (Figure 4B). Fragment A mediates the cytotoxic enzymatic activity, fragment B confers cell entry and is sub-divided into a translocation domain (T) and a receptor-binding region (R). The catalytic domain (C) is located at the *N*-terminus at amino acids residues 1-193 and is composed of 7α-helices and 8β-strands; the T-domain and the R-domain are located at the *C*-terminus. The T-domain (amino acids 202-378) has nine α-helices and is required for translocating the C-domain from the endosome to the cytosol.

The R-domain consists of amino acids 386-535, comprises of 10β-strands and binds to HBEGF on the cell membrane. After binding, DT is cleaved by furin or furin-like proteases, leaving the two protein chains still linked by a disulfide bond between cysteines 186 and 201 (14), and finally undergoes receptor-mediated endocytosis. In the endosome, at an acidic pH, the loop connecting the T- and C-domains is cleaved by furin resulting in a conformational change of the T-domain, which is able to insert into the endosomal membrane due to the exposure of hydrophobic amino acids. After the subsequent formation of a channel to translocate the C-domain into the cytosol, the disulfide bond linking the C- and T-domains is reduced, releasing the free C-domain. Once in the cytosol, the C-domain exerts its toxic activity by transferring ADP-ribose from nicotinamide dinucleotide (NAD) to diphthamide, a modified histidine residue at position 699 of eEF2{2-[3-carboxyamido-3-(trimethylamino)propyl]histidine (His699)} (14, 50, 51) resulting in translation inhibition and ultimately cell death (Figure 2). Cytotoxic fusion proteins have been constructed partially or entirely by removing the R-region and substituting it with ligands specific for receptors overexpressed on tumor cells. This manipulation does not alter ligand binding to its receptor, internalization and endocytosis of the IT, translocation into the cytosol and subsequent ADP-ribosylation of eEF2. Fusion of the ligand to the *C*-terminus of DT was shown to be the most successful strategy. The most advanced approaches are targeting of the IL2 receptor by denileukin diftitox (Ontak) and the granulocyte-macrophage colony stimulating factor receptor by DT388-granulocyte macrophage-colony stimulating factor (GM-CSF). Denileukin Diftitox was constructed by the genetic fusion of a truncated form of DT, composed of 389 amino acids of DT followed by the sequence of human IL2, which replaces the receptor-binding domain (52-54). This IT was approved for the treatment of advanced cutaneous T-cell lymphoma (CTCL) in 1999. A 30% overall response rate and a 10% complete response rate were observed. However, serious side-effects, such as infusion reactions, vascular leak syndrome and loss of visual acuity were noted (53, 54). In DT388 GM-CSF, human GM-CSF was fused to the truncated DT toxin (DT388), thus replacing the natural receptor binding-domain (55, 56). Thirty-one patients with chemotherapy-refractory acute myeloid leukemia (AML) were treated, and one complete remission and two partial responses were seen after *i.v.* infusion of DT388 GM-CSF. On the other hand, in two patients, liver failure and transient hepatic encephalopathy were observed possibly due to release of cytokines from Kupffer cells.

Bispecific DT-based ITs have also been described (57, 58). One of them targets the human IL13 receptor and uPA receptor, the other one, HER2 and EpCAM. These bispecific ITs were more active *in vivo* than the monospecific ones.
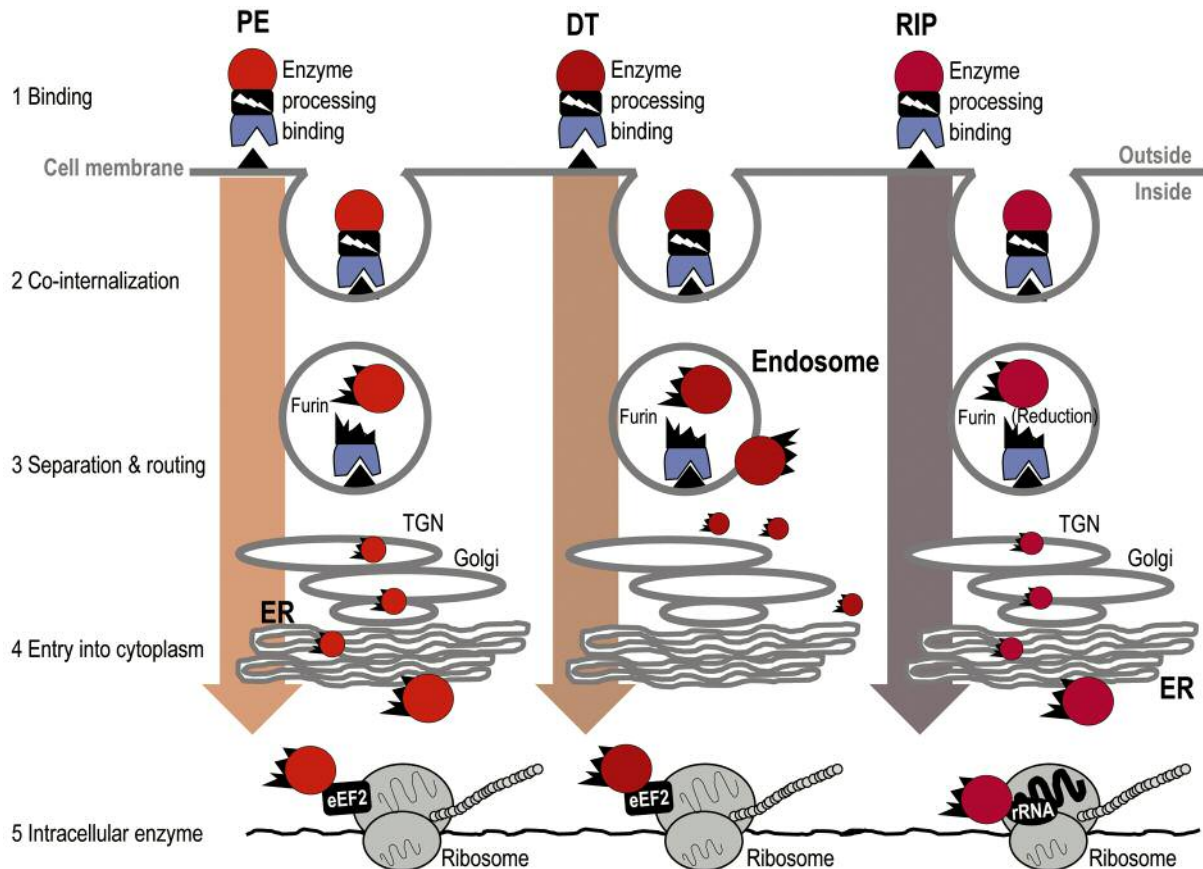
Figure 2. *How immunotoxins enter and kill cells. The mode by which immunotoxins enter and kill cells is similar for most attached payloads. Shown are the subsequent steps of entry pathways for Pseudomas exotoxin (PE), Diphteria toxin (DT) and Ribosomal-inactivating proteins (RIP) (e.g. bouganin-derived immunotoxins), which inhibit the protein synthesis of eukaryotic cells. Step 1: Immunotoxins (applies to all payloads) bind to cell surface antigens and hence accumulate on target cells. Target antigen specificity determines the degree of accumulation on desired (cancer) cells and potential undesired accumulation on antigen-expressing normal cells. Step 2: Internalization of immunotoxins (frequently via clathrin-coated pits) is triggered by internalization of the bound target antigen. Internalization is necessary for subsequent entry into the cell. Therefore, non-internalizing antigens make poor targets for immunotoxin therapy. Step 3: Once internalized, toxins need to be released from their targeting moieties to evade degradation and to enable their independent route within the cell. In the case of recombinant fusion proteins, release from targeting domains is achieved by proteolytic cleavage in endosomal compartments (e.g. via furin for DT, PE and bouganin fusions). Payloads of conjugates can also become released by proteolysis or disulfide reduction. Step 4: Intracellular routing and distribution of processed toxin domains are no longer influenced by bound target antigen. PE derivatives contain a C-terminal routing signal by means of which they enter the endoplasmic reticulum (ER), and subsequently translocate directly into the cytoplasm. Translocation of RIP derivatives also follows the same route. DT derivatives can translocate directly into the cytoplasm from endosomal compartments. Step 5: Toxins are in fact enzymes that inactivate intracellular targets. Protein synthesis inhibition by PE and DT is a consequence of ADP-ribosylation of eukaryotic elongation factor-2 (eEF2) at its diphthamide residue, leading to cell death. RIPs block protein synthesis and kill cells by cleaving ribosomal rRNA and hence inactivating the ribosome. eEF2, rRNA, ribosomal RNA; TGN, trans-Golgi network.*

PE and Variants

Pseudomonas aeruginosa exotoxin A (PE) is one of the virulence factors of this bacterial strain and is a secreted bacterial toxin composed of 613 amino acids. Similar to DT, it is a member of the family of ADP-ribosylating toxins. X-ray crystallographic structure analysis revealed three domains (59). Domain I is the receptor binding-domain and is subdivided into nonsequential, but structurally adjacent domains, Ia and Ib. *Via* this domain I, PE binds to its receptor, CD91, also called α2-macroglobulin receptor/low-density lipoprotein receptor-related protein (α2MR/LRP). Domain II has a crucial function in translocation, and domain III is the catalytic subunit, which inactivates eEF2 through ADP-ribosylation (60-62) (Figure 2 and 4A). After secretion, PE is processed by carboxypeptidases, removing the C-terminal lysine residue, exposing the ER localization signal (REDL). After binding to CD91, PE is internalized by
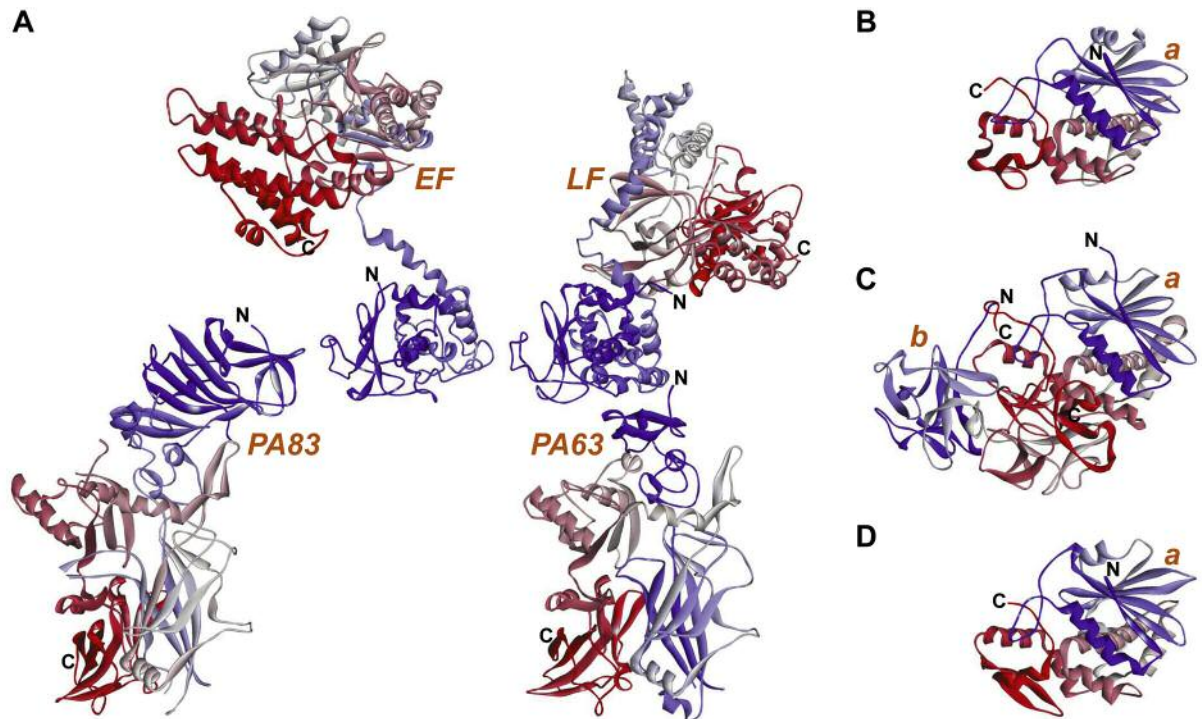
Figure 3. *Structure of Anthrax toxin, saporin, ricin and bouganin. The proteins or protein assembly are displayed as ribbons with a color code ranging from N- (blue) to C-terminus (red). Structural data from the Protein DataBank (PDB, Oct. 2013) (124) were extracted and superimposed (125) when required to reveal the functional motifs. A: Anthrax toxin has three components: the pro-antigen protein PA83 (pdbcode: 1ACC) recognizes its receptor and a 20-kDa fragment is cleaved from PA83's amino terminus. The remaining receptor-bound portion of the pro-antigen, PA63 (pdbcode: 3KWV), assembles into a ring-shaped oligomer forming a translocase pore. The exposed new PA63 N-terminal part binds up to three to four lethal factor proteins (LF, pdcode: 1JKY) edema factor proteins (EF, pdcode: 1Y0V) and mediates endocytosis. Both EF and LF exhibit enzymatic activity, leading to cellular death. B, C and D: The ribosome-inactivating proteins (RIPs), saporin (B, pdbcode: 1QI7), ricin (C, pdbcode: 2AAI), and bouganin (D, pdbcode: 3CTK). They are structurally very similar with respect to their enzymatic domain. The type II RIPs such as ricin have a B chain with lectin-like properties conferring cytotoxicity in addition to the catalytic domain a. Amino- and carboxy-termini of the proteins are marked with N and C.*

receptor-mediated endocytosis in clathrin-coated pits. Within the acidic endocytic compartment, PE dissociates from its receptor and is cleaved into two fragments by the endoprotease furin. In the trans Golgi network the REDL sequence binds to the lysine-aspartic acid-glutamic acid-leucine (KDEL) intracellular sorting receptor. The *C*-terminal fragment complex of domain III and part of domain II is transported to the ER, probably due to the REDL sequence. Subsequently, the toxin translocates to the cytoplasm, mediated by sequences located in domain II, where it ADP-ribosylates eEF2 (Figure 2). Several residues in domain III were shown to have a crucial function in catalysis, such as Glu 553, His 440, Tyr 481 and Tyr 470 (63). Diphthamide of eEF2 has been identified as crucial for the ADP-ribosylation function of PE (64, 65). The ADP-ribosyl group is derived from NAD^+ and is transferred to the N3 atom of the imidazole moiety of diphthamide. NAD^+ is cleaved to produce nicotinamide, which is released, and an ADP ribosyl oxycarbenium containing a positively charged

ribosyl group that reacts with N3 of the imidazole moiety of diphthamide (66, 67). Several lines of evidence point to a dual mode of action of PE, including inhibition of protein synthesis and induction of apoptosis. However, it is not precisely known how ADP-ribosylation of eEF2 and induction of apoptosis are interconnected. In cell lines derived from hematological malignancies, hallmarks of apoptosis such as cleavage of poly(ADP)-ribose polymerase and DNA laddering, were noted (68, 69). In essence it was shown that PE-mediated killing is often facilitated, but not completely dependent, on apoptosis. More recently it was shown that PE-mediated apoptosis is dependent on B cell leukemia 2 (BCL2) homologous antagonist/killer (BAK) and is preceded by the degradation of myeloid cell factor-1 (MCL-1) (70). Regarding killing efficacy, it was shown that as few as 1000 molecules of PE per cell can induce cell death *in vitro*, and 400-750 molecules per cell can mediate tumor regression *in vivo* (71). Using labeled PE, it was shown that less than 1% of the internalized PE may
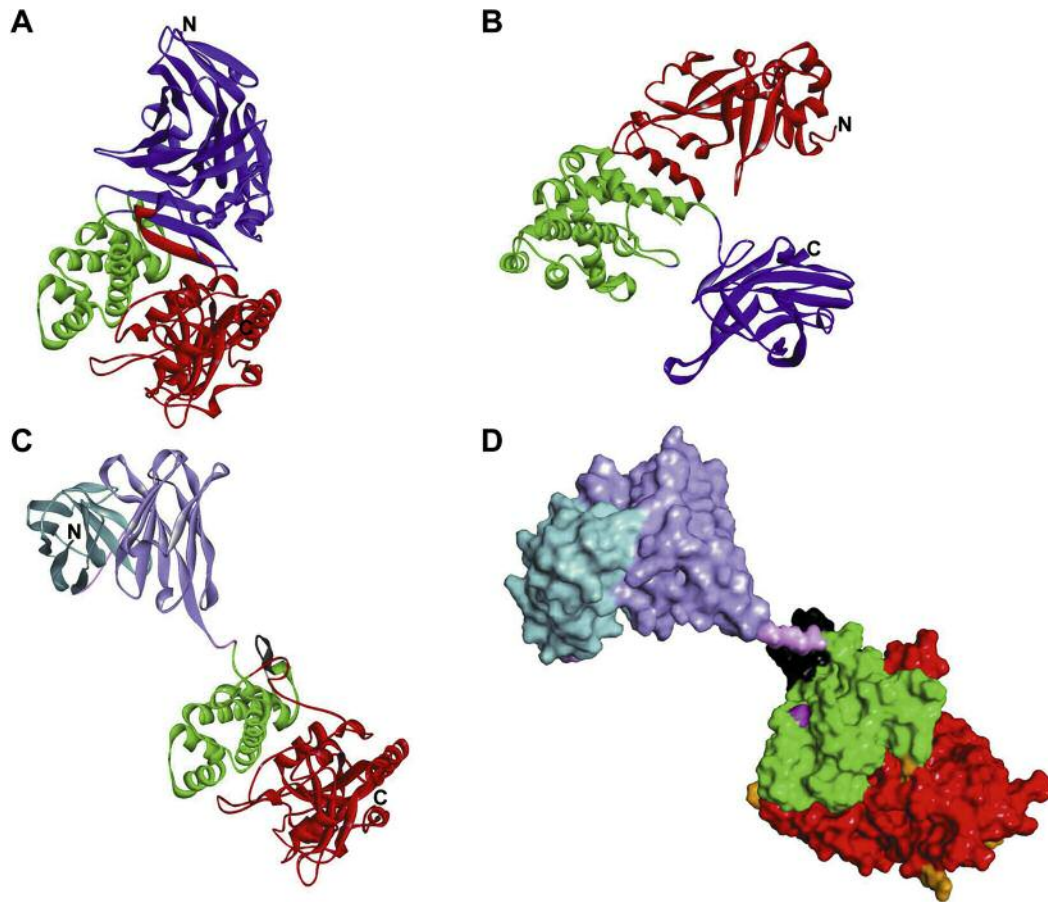
Figure 4. *Structure of Pseudomonas exotoxin variants and Diphtheria toxin. A: The Pseudomonas aeruginosa exotoxin (pdbcode: 1IKQ) contains three successive domains for receptor binding (blue), translocation into the cell (green), and enzymatic activity (red). B: Diphteria toxin (pdbcode: 1SGK) also consists of three domains with similar functions compared to PE toxin but in the reverse order. C: A shortened variant of the Pseudomonas exotoxin (PE38) is coupled to a single-chain variable domain fragment (scFv) anti-mesothelin targeting moiety. The immunoglobulin variable domains (cyan and violet) are linked together and to PE38 with flexible linkers (pink). The PE38 translocation domain (green) contains the protease cleavage site (black) and is linked directly to the catalytic domain (red) (101). D: Modified PE38 includes one mutation removing a T-cell epitope (magenta) and eight mutations removing B cell epitopes (orange). Four B-cell epitopes are located on the shown protein surface. The structural models were generated based on available structure data of domains or entities and are assembled and minimized using DiscoveryStudio40 (124, 125). Amino- and carboxy-termini of the proteins are marked with N and C.*

ultimately reach the cytosol and based on observations with DT, one single toxin molecule in the cytosol may be sufficient to kill a cell (72, 73).

PE derivatives. Recombinant immunotoxins are chimeric molecules that fuse antibody-based moieties to fragments of PE (Figure 1). The most frequently used are PE38 and PE40 (14, 61), truncated versions of PE, consisting of amino acids 253-334 and 318-613 or amino acids 381-613, respectively. Recently, a smaller version with a more extensive deletion in domain II, referred to as PE[LR] was described. PE[LR] encompasses amino acids 274 to 285 and 394 to 613 (62) (Figures 4C and D). Several deimmunized ITs are derived

from this version and are described under de-immunized ITs later in more detail.

PE-based recombinant ITs. First-generation ITs were antibodies chemically-coupled to toxins with often disappointing efficacy in animal models due to non-specific killing of normal cells. In the second generation, a modified version of PE with a deleted cell-binding region was chemically attached to antibodies. These molecules were much better tolerated in animals and some of them were evaluated in phase I studies in patients with cancer. Some clinical activity was observed, but these agents were abandoned due to heterogeneity of composition and low tumor penetrance due

to their large size (61) (Figure 1). In the third generation of recombinant ITs the cell attachment region of PE was replaced by scFv in which heavy chain variable domain (VH) and light chain variable domain (VL) are either connected by a linker or antibody Fv in which VH and VL domains are linked by disulfide bond stabilized Fv (dsFv). The antibody moiety is followed by the translocation and cell killing domains. In another version of recombinant ITs, the cell-binding region of the IT was replaced by a ligand for a receptor overexpressed on tumor cells. Transforming growth factor-α (TGFα), IL2, GM-CSF, IL4, IL13 are ligands for which ITs were evaluated, with excellent *in vivo* efficacy (61). However, it should be kept in mind that these agents were evaluated in immune-compromised mice, bypassing the immunogenicity issues of the ITs under evaluation. Targets for scFv- or dsFv-based recombinant ITs were CD22, CD25, Lewis Y, mesothelin and others (61). Some of these agents are discussed in greater detail later. Lewis Y is a glycoprotein-based antigen overexpressed in adenocarcinomas. However, vascular leak syndrome and gastric and renal toxicity have hampered further development of a recombinant IT targeted to Lewis Y (74, 75). The specific subunit of the IL3 receptor (IL-3Rα, CD123) is strongly expressed on various leukemia blasts and stem cells. There is evidence that relapsed AML is due to leukemic stem cells which express CD123 (76). A recombinant IT [26292(Fv)-PE38] killed CD123$^+$ cells of lines TF1, Molm-13 and Molm-14 with an inhibitory concentration 50 (IC$_{50}$) of 40 nM (76). More recently, a recombinant IT targeting malignant B-cells expressing receptor tyrosine kinase-like orphan receptor 1 (ROR-1) was evaluated (77). ROR-1 was shown to be overexpressed in chronic lymphocytic leukemia and mantle cell lymphoma (78, 79). The corresponding recombinant IT consisting of the VH and VL fragments of an antibody to ROR1 and PE38 was partially internalized by chronic lymphocytic leukemia and mantle cell leukemia cells and induced apoptosis in the latter which expressed ROR-1 *in vitro* with IC$_{50}$ between 16 pM and 16 nM, paralleling ROR-1 expression levels among these cell lines, but did not affect ROR-1-negative cell lines (77). H22scFv-PE is a human scFv-based fusion protein directed against CD64 (80) with the ability to kill AML-derived cell lines and primary cells derived from patients with AML. In a U937 cell-based severe combined immunodeficient (SCID) mouse xenograft model for AML, the IT eliminated CD64$^+$ cells in mouse organs, and treatment prolonged overall survival.

Parameters Affecting Recombinant IT-based Cytotoxicity

Since many steps are involved from binding of the IT to the cell surface until its final release into the cytoplasm and subsequent cell killing, several parameters can have an impact on the efficacy of IT-based cytotoxicity. Antigen density on the cell surface, selectivity of the target, affinity of the antibody to its target antigen and stability of the antibody-antigen complexes are crucial parameters for selectivity and efficacy. PE-based recombinant IT targeting CD19, CD22 and mesothelin showed better efficacy after improving affinity which resulted in longer cell surface retention (81, 82). Another parameter affecting efficacy of recombinant ITs is the epitope recognized by the corresponding antibodies. Recombinant ITs directed against different epitopes of an antigen showed significant differences in their *in vitro* potency (81-83). A correlation between target antigen density and apoptosis induction has been observed for several recombinant ITs (77, 82-85). However, downstream effector molecules such as BCL2 levels and the levels of other anti-apoptotic proteins such as MCL1, survivin and inhibitor of apoptosis proteins were reported to modulate the efficacy of recombinant ITs. Enhanced killing of CD22$^+$ leukemia cells was observed for a combination therapy with BCL2 inhibitor ABT-737 (86, 87). Along these lines, it was shown that low BAK expression could cause resistance to recombinant IT treatment and that combining recombinant IT with TNFα-related apoptosis-inducing ligand or their agonists was able to alleviate resistance (88). A further parameter, modulating the efficacy of recombinant ITs is the internalization rate of their complexes. This is supported by a comparative analysis of recombinant ITs directed against CD19 and CD22 (89). All steps of the intracellular routing and processing potentially influence efficacy. In addition, it was recently shown that the insulin receptor negatively regulates intracellular processing of a recombinant IT directed against mesothelin (90). siRNA knock-down of insulin receptor enhanced the cytotoxic activity of native PE and mesothelin-targeted recombinant IT in several human cell lines, but did not affect the response to cytotoxic agents such as TNFα-related apoptosis-inducing ligand (TRAIL), etoposide or cycloheximide. Making use of recombinant IT constructs which are targeted to the endocytic recycling compartment *versus* those which are delivered through the late endosomes, it was shown that the trafficking route *via* specific organelles was an important factor modulating the efficacy of LMB2, a recombinant IT composed of PE38 and an Fv directed against the IL2 receptor (91).

Deimmunization

Recombinant ITS are highly immunogenic proteins (92). Thus, removal of B- and T-cell epitopes is a prerequisite for reducing their immunogenicity (92). In general, the generation of high-affinity antibodies is initiated by the interaction of cell surface immunoglobulin on B-cells with corresponding B-cell epitopes of the antigen. These complexes are subsequently internalized and processed, and

peptides derived from these antigens associated with major histocompatibility complex (MHC) class II molecules are presented to CD4^{+} helper T-cells, which are essential for the generation of high-affinity antibodies (93, 94). B-Cell epitopes are mostly discontinuous and conformation-dependent (95-97). In contrast, T-cell epitopes are continuous epitopes which are displayed on MHC molecules. These peptide-MHC complexes in turn are bound by T-cell receptors (98, 99). Since T-cells are essential for antibody responses to proteins, removal of T-cell epitopes also has to be part of de-immunization strategies. In the case of PE, B-cell epitopes were identified by using a panel of antibodies derived from immunized mice. These epitopes were also recognized by human antibodies present in the sera of patients treated with PE38-based recombinant ITs (100). A mutual competition assay making use of the native antigen and 60 monoclonal antibodies involving pair-wise competitions was used to identify seven major epitope groups and 13 subgroups (101). The exact location of the epitopes was determined by introducing 41 individual mutations by alanine replacement of bulky amino acids and subsequent loss of binding analyzing a panel of monoclonal antibodies. Finally, the thus-identified epitopes of PE38 were combined into one molecule, with the final molecule containing eight amino acids replacements. The resulting RIT(HA22-8X) targeting CD22 on leukemia cells showed similar anti-tumor activity as the non-mutated HA22 in xenograft models (102). HA22-8X induced much lower antibody responses compared to HA22 after repeated *i.v.* injections into mice. Immunogenicity was further reduced by removing a large part of PE38 domain II, resulting in HA22-LR-8M (103). HA22-LR-8M did not induce antibodies in mice when given repeatedly by *i.v.* injections and did not induce a secondary response when administered to mice previously exposed to HA22. HA22-LR-8M maintained cytotoxicity against CD22^{+} leukemia cells and anti-tumoral activity in xenograft models. Further modification resulted in HA22-LR-010 (104). Fvs derived from B-cells of patients with antibodies against recombinant ITs were isolated and a phage display library containing the recombinant IT-binding Fvs was established. Based on the epitope information thus obtained, HA-22-LR-010 was constructed. HA-22-LR-01 possesses low reactivity to human anti-sera while maintaining *in vitro* and *in vivo* cytotoxicity (104). Furthermore, an immunodominant T-cell epitope in PE-based recombinant ITs was identified and eliminated. This was achieved by incubation of peripheral blood mononuclear cells with an IT to stimulate T-cell activation, subsequent re-stimulation to overlapping peptides derived from P38, and quantitation of the responses in an IL2-enzyme-linked immunospot assay. The identified epitope was recognized by T-cells of 46% (23/50) of the donors. A mesothelin-targeting recombinant IT (SS-LR/GGS/8M) with greatly reduced immunogenicity after removal of the B-cell epitopes, as described above, was functionally evaluated (105) (Figure 4C and D).

Examples of Proof-of-concept Studies with PE-based Recombinant ITs

In the following, we highlight promising preclinical and clinical studies of PE-based ITs targeting CD22 and mesothelin. CD22 is a lineage restricted differentiation antigen expressed on B-cells and B-cell derived malignancies, which is internalized after binding by recombinant ITs (106). A disulfide-bridged Fv genetically fused to PE38, referred to as BL22 or CAT-3888, was found to be cytotoxic to cells. This required as few as 350 CD22-binding sites per cell and induced tumor regression in CD22^{+} xenografts (107, 108). The agent was also evaluated in patients with hairy cell leukemia by *i.v.* administration every other day three times in adults and every other day three (or six) times in children, with cycles repeated every 21 or 28 days. In a phase II study the overall response rate was 72%, including 47% complete responses (109). The dose-limiting toxicity (DLT) was completely reversible hemolytic uremic syndrome. Since BL22 was less active in malignancies with lower numbers of anti- CD22 binding sites, such as chronic lymphocyte leukemia, and with rapidly proliferating cells, such as acute lymphoblastic leukemia, HA22 (moxetumumab pasudotox) was generated (110). HA22 has a 14-fold higher affinity for CD22 due to a lower off-target rate and was more toxic towards hairy cell leukemia and chronic lymphocytic leukemia (110), but had similar animal toxicity and preclinical antitumor efficacy. A corresponding phase I study in patients with hairy cell leukemia, chronic lymphocytic leukemia, non Hodgkins lymphoma and acute lymphocytic leukemia is ongoing.

Another promising approach relies on recombinant ITs directed against mesothelin, a cell-specific receptor which is highly overexpressed in malignant mesothelioma, as well as pancreatic, ovarian and lung adenocarcinomas (111). The attraction of this target for recombinant IT based approaches is the dramatic overexpression of mesothelin in tumors as compared to normal mesothelial cells that line the pleura, peritoneum and pericardium (112). The IT SS1P consists of an anti- mesothelin Fv fused to PE38. SS1P has high affinity to mesothelin (Kd=0.72 nM), killed mesothelin -positive tumor cells efficiently and caused regression of mesothelin-expressing tumor xenografts (105, 112-114). In such xenografts, combination treatment with SS1P and paclitaxel resulted in increased antitumor activity with complete regression (115, 116). The increased efficacy was shown to be based on altered tumor architecture and inhibition of mesothelin shedding, and was restricted to paclitaxel-sensitive cells (115, 116). The most promising clinical results

were noted in clinical phase I studies evaluating a combination of SS1P with pemetrexed and cisplatin as frontline therapy in patients with advanced, unresectable mesothelioma. Of the 14 patients treated at all dose levels, seven had a partial response, three had stable disease and four had progressive disease (117). Of note, of the seven patients evaluable at the maximal tolerated dose, five had a partial response, one patient had stable disease and only one had progressive disease. Edema, hypo-albuminemia, fatigue and pleuritis were the main side-effects. A recombinant IT referred to as SS1-LR/GCS/8M, directed against mesothelin and re-engineered for high-affinity, low off-target toxicity and reduced antigenicity was described (82). Reduced off-target toxicity such as vascular leak syndrome was achieved by deletion of a large part of domain II of PE while maintaining the 11 residue furin cleavage site and including a Gly-Gly-Ser linker at the C-terminus of the furin cleavage site. Dramatically reduced immunogenicity was achieved in mice by introducing the above mentioned eight mutations that silence B-cell epitopes into domain III (82).

Critical Issues

Immunogenicity of recombinant ITs is a critical issue for repeated use. Based on numerous clinical studies, the incidence of an immune response after a single cycle is 0-40% for hematological tumors and 50-100% for solid tumors (118). Removal of B- and T-cell epitopes and immunosuppressive treatment are important approaches to address this issue. Another common toxicity problem in patients treated with recombinant ITs is vascular leak syndrome caused by binding of recombinant ITs to endothelial cells, and is characterized by edema, hypo-albuminemia and hypotension (119, 120). It was shown that the truncation of a large part of domain II of PE in a mesothelin-directed IT prevented this kind of toxicity (82). Hepatotoxicity caused by binding of basic amino acids to negatively-charged hepatic cells is another issue (119-121). Substitutions in the Fv part of the recombinant IT might solve this problem. Kidney toxicity was frequently observed after treatment of patients with recombinant ITs. The underlying mode of action has not been resolved yet, but could be due to the rapid kidney filtration of the recombinant ITs because of their small size, since kidney toxicity has not been observed with whole antibody-based recombinant ITs. Proteinuria, hematuria and creatinine release are hallmarks of kidney toxicity. Cytokine release syndrome with fever, chills, hypotension and bone pain was also frequently observed (121). In addition to these general toxicities, target-mediated side-effects were noted. With a recombinant IT-targeting Lewis Y antigen, severe gastritis and renal toxicity were observed. This is due to antigen expression by normal cells of the stomach and tubular cells of the kidney,

respectively (61, 122). Hepatotoxicity was noticed with a HER2-directed, PE-based recombinant IT (123) due to the expression of HER2 on normal hepatocytes revealed by immunohistochemistry.

Concluding Remarks

As outlined above, considerable progress was made by 'taming' ITs for cancer treatment. The impact of antigen density, internalization of antigen-recombinant IT complexes, efficacy of intracellular processing, including delivery into the cytosol, and the status of cancer cells with respect to expression of anti-apoptotic proteins needs further investigation for each IT and tumor type under consideration. Diphthamide modification of eEF2 seems to be a prerequisite for efficacy of PE- and DT-based recombinant ITs, but other additional parameters involved in modulation of efficacy of recombinant ITs need to be identified. In addition, involvement of changes of tumor architecture induced by chemotherapy or other antitumor agents and its impact on the *in vivo* efficacy of recombinant ITs deserves further investigation. The elimination of off-target effects by further genetic engineering of recombinant ITs is a promising avenue, as shown by the elimination of vascular leak syndrome caused by PE-based recombinant ITs through deletion of a large part of domain II of PE (62). The clinical performance of deimmunized recombinant ITs with B- and T-cell epitopes removed will be crucial for the future prospects of treatment of cancer with recombinant ITs.

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