Immunotoxins and Other Conjugates: Preparation and General Characteristics

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Abstract: Targeted toxins represent an invaluable tool offering a wide range of potential applications, both in experimental models and in the clinics. Here we will review several aspects related to the preparation and properties of carrier molecule-toxin heteroconjugates and fusion toxins.

Keywords: Toxins, Ribosome Inactivating Proteins, Immunotoxins, Targeting.

1. BACKGROUND

In the late 1800s, dyes injected into animals were found specifically to stain different tissues. Paul Ehrlich postulated in 1906 [1] that this tissue-specific affinity of dyes could be coupled with the toxicity of certain metals to generate a new class of tissue-specific and pathogen-specific drugs. This concept has been successively expanded and exploited in the past years to develop cell-specific reagents that have found their widest application in the elimination of unwanted target cells, particularly in the domain of cancer therapy.

The design of such cytotoxic agents is conceptually simple: attach a toxic substance or a mediator of toxicity to an appropriate vehicle molecule and you have a "magic bullet" that can find and eliminate the one-in-a-billion cells that have the requisite marker. The vehicle molecule provides recognition and binding capacity, while the associated toxic component effects cellular alterations leading to cell death.

The approach to constructing carrier-toxin heteroconjugates for selective elimination of target cells has taken a number of forms. The first conjugates were synthesized using polyclonal and later monoclonal antibodies with toxins that were able to block protein synthesis at the ribosome level. Biospecific agents other than monoclonal antibodies have also been employed in developing cell-targeting conjugates. The targeting component in these systems consists of any molecule that can function as a ligand having specific affinity for some receptor molecule on the surface of the tumour cells and that can be internalized by receptor-mediated endocytosis. Such an affinity molecule might be a hormone, a growth factor, a cytokine, an antigen specific for binding particular antibodies projecting from B cell surfaces, transferrin, α2-macroglobulin, or anything else able to specifically interact with the targeted cells.

The polypeptide toxins used are very powerful, and because they function catalytically, few molecules reaching the cytosol of the target cell are enough to be lethal. Unlike chemotherapeutic agents, toxins kill both quiescent and proliferating cells. Therefore, they may be targeted against non-cycling cells that are not attacked by chemotherapy.

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In the present chapter carrier-toxin conjugates of various compositions will all be referred to as Immunotoxins (ITs) for the sake of simplicity.

Toxins of different types can be used to create effective IT conjugates, including plant, bacterial and fungal toxins. Here we will focus on a family of plant toxins called Ribosome Inactivating Proteins (RIPs) [2]. RIPs are plant enzymes that damage ribosomes in an irreversible manner. They can be divided into type-1 RIPs (RIPs-I) which are single-chain proteins, and type-2 RIPs (RIPs-II), which are heterodimeric proteins consisting of an enzymatically active A-chain connected by a disulfide bond to a B-chain. ITs can be made with either RIPs-I or with RIPs-II. The resulting ITs, however, will be endowed with different properties. Shown in Fig. **1** is a schematic representation of RIPs-I, RIPs-II and of some different types of Immunotoxins.

The linkage of the carrier molecule to the toxin can be accomplished in one of the following ways: 1) chemical cross-linking, 2) indirect linking, and 3) gene fusion. The general properties and functions of the ITs are obviously greatly affected by the method used for obtaining them. Other factors that may intervene in influencing the overall cytotoxic potential of ITs include the affinity and valency of binding to the cell surface target, the number of target molecules, the spatial configuration of the epitope recognised, the internalisation and routing processes, the intracellular distribution of the internalized conjugate, the cell type, and other variables. To overcome limitations of some types of ITs (e.g. weak cytotoxicity, slow kinetics of cell intoxication) intracellularly acting potentiating substances can be used.

The ultimate goal of ITs development is to obtain *in vivo* effective therapeutic biomolecules. However, their therapeutic impact is dependent on several factors more directly affecting their *in vivo* behaviour. Among these are physico-chemical properties (e.g. size, glycosylation), the type of tumour, the host immune response against the conjugate components, the possible side effects.

Many of the aspects briefly summarized above will be reviewed in the next paragraphs in greater detail.

2. CARRIER MOLECULES

Several types of targeting agents can be employed to direct a cytotoxic moiety against target cells, among these

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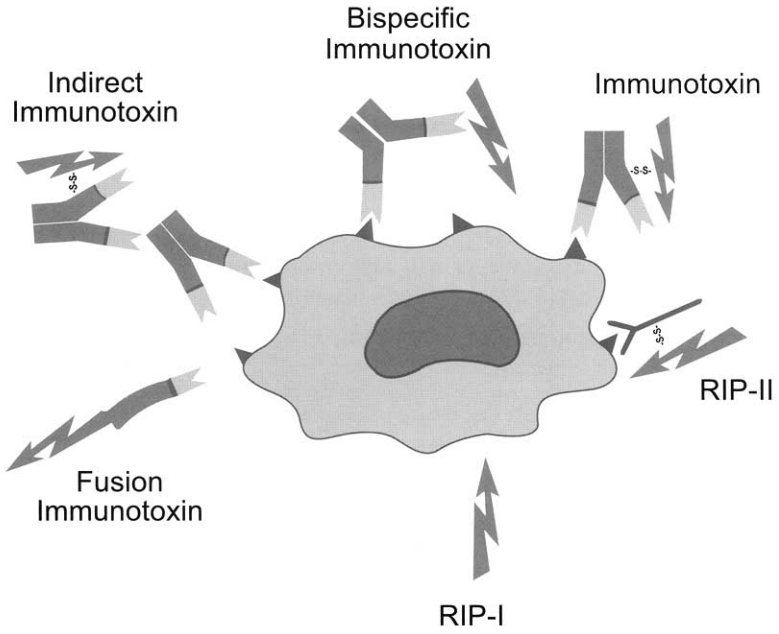

Fig. (1). Toxins can be directed to target cells by various modalities.

are antibodies, cytokines, growth factors and soluble receptors. A very large number of carrier molecule-RIPs-II and carrier molecule-RIPs-I conjugates have been studied in the past years, and it would be impossible to discuss each one of them, therefore in the present review we will summarise important aspects, advantages and drawbacks that have been highlighted in the *in vitro* and *in vivo* use of such targeting agents, with particular emphasis on anti-tumour ITs, which represent the largest part of all anti-target cell ITs produced and investigated so far.

Antibodies

Because of their selective targeting properties antibodies and their derivatives are by far the most utilised toxin carrier molecules. The first ITs were made by linking polyclonal antibodies (antiviral, antithymocyte) [3,4] to intact diphtheria toxin. In the following years cell-reactive monoclonal antibodies (mAbs) became the most frequently used carrier molecules. Applications of antibody-toxin conjugates have ranged from ex-vivo purging of bone marrow to the treatment of hematological and solid neoplasias.

In the case of tumour cells, many mAbs used are directed to differentiation antigens shared also by normal cells. In fact, unique tumour-specific antigens have not been identified yet for most human tumours. Because tumour cells often express higher levels of these differentiation antigens, the tumour cell may be preferentially killed. In other instances, cell surface structures ubiquitously distributed (e.g. the transferrin receptor, TfnR) [5,6] may become operationally useful tumour targeting markers (e.g. tumour cells bearing an abundant amount of TfnR in the central nervous system, CNS). Thus, even in cases where tumour-reactive mAbs react with some normal tissues, crossreactivity does not necessarily prevent their use. Low antigen density, anatomical barriers or poor endocytosis could interfere with the efficient killing of normal cells bearing cell surface structures shared with tumour target cells. Conversely, some cross-reactions not detectable by conventional techniques can damage life-sustaining tissues [7]. Therefore appropriate animal models in which the mAbs react with the animal antigen are desirable to test the safety of an IT to be developed for the use in humans.

The cytotoxic potential of RIPs-based ITs directed to true tumour-specific antigens (i.e. idiotypes) has also been investigated [8-11]. However, it must be noticed that true tumour-specific antigens may not be suitable targets for IT-mediated cell killing (see also below).

One drawback in using mAbs as the targeting agents is that they are usually of mouse origin and are therefore immunogenic in humans. If previous therapeutic treatments are themselves immunosuppressive or when patients are immunocompromised as a result of the ongoing disease, this is less problematic. In cases where the patient is able to mount an immune response against the IT, the use of less immunogenic chimeric or humanised mAbs is being explored [12-18] (see also paragraph 4.3). Non-Ab targeting molecules (e.g. cytokines/growth factors) may be of human origin and therefore non immunogenic in humans (see also below). Bispecific antibodies (molecules combining two different antigenic specificities, one directed towards cell surface structures, the other directed to the toxic component) are also being developed as carriers of cytotoxic agents (see also paragraph 4.2).

Only a proportion of mAbs make potent ITs. Several features of the mAb-target antigen interaction (e.g. antibody valency, affinity, epitope recognized, internalization, routing, presence of shed antigens interfering with binding) may be relevant to determine the efficacy of target cell intoxication by an IT. These aspects will be considered in paragraph 6 ("Factors affecting the potency of Immunotoxins").

Cytokines/Growth Factors and Hormones

Other ligands utilized for preparing ITs are cytokines/growth factors. Although these bind also to normal cells, cytokine/growth factor receptors are often upmodulated during cell activation, differentiation or tumour progression, making it possible to selectively target discrete cell populations. Cytokines and growth factors are effective targeting agents because their affinity for their cell surface receptors can be several orders of magnitude higher than that of monoclonal antibodies, and their receptors are able to internalise the respective cytokine/growth factor-based ITs with great efficiency by receptor-mediated endocytosis [19]. Moreover, being of human origin they are non immunogenic. A further advantage is the availability of cloned cytokines/growth factors genes for generating fusion proteins. Possible drawbacks of using cytokines as carrier molecules is their rapid clearance *in vivo* and the agonistic effects often exerted by the cytokine/growth factor linked to the toxic moiety, which could promote proliferation of the targeted cell population when the amount of ITs bound are insufficient to kill the target cell. A further disadvantage is the presence of circulating ligands or soluble receptors that compete for the IT. These effects have been observed with ITs targeting the receptors of the cytokines IL-2, IL-4 and IL-6 [20-22]. RIP-based ITs using the cytokine TNF as the carrier molecule have been also studied [23, 24]. Other cytokine/growth factors that were used as targeting agents are epidermal growth factor (EGF) [25-31], and its homologous HER2/neu [32], fibroblast growth factor (FGF) [33-36], granulocyte-macrophage colonise stimulating factor (GM-CSF) [37,38], transferrin (Tfn) [39-44], and nerve growth factor (NGF) [45-48]. The latter has been widely employed to investigate the functional role of discrete CNS regions by immunolesioning strategies.

Also hormones have been utilised to direct RIPs against target cells. Conjugates of hormones and toxins are typically called "hormonotoxins" [49,50].

One of the earliest hormone-RIP conjugate obtained by cross-linking the beta chain of human chorionic gonadotropin to ricin A-chain was described by Oeltmann and Heath [51,52]; other conjugates were later described made with RIPs cross-linked to corticotropin-releasing factor [53], and to leuteinising hormone [49,50,54-57].

Among the non-antibody targeting molecules also CD4 [58], the receptor for acetylcholine [59-62] and thyroglobulin [63] should also be considered.

3. TOXINS

RIPs utilised for the construction of ITs will be here only briefly described. A more comprehensive exposition on RIPs properties and characteristics will be found elsewhere in this book.

RIPs-II

RIPs-II (e.g. ricin, abrin) are heterodimeric proteins of molecular mass of approximately 60-65 kDa, consisting of an enzymatically active A-chain (A = active) connected by a disulfide bond to a B-chain (B = binding). The latter has the properties of a lectin specific for terminal galactose and N-acetylgalactosamine and binds to galactosyl-terminated receptors on the cell surface, thus allowing the A-chain to enter the cytoplasm. It is likely that the B-chain also facilitates the intracellular routing of the A-chain and its translocation to the cytosol across the membranes of intracellular organelles [64]. Consequently some RIPs-II are potent toxins, the best known being ricin, while others are much less toxic.

RIPs-I

RIPs-I are functionally analogous to the A-chains of abrin and ricin but, lacking a B-chain, are much less toxic then RIP-II. The RIPs-I show sequence homology suggesting that they share a common evolutionary ancestry [2]. A great degree of structural similarity has been observed between ricin and RIPs-I or other RIPs-II [65-67]. Differences between RIPs-I and ricin may be attributed to the evolution of the former without a B-chain partner [66].

Ribosome-inactivating proteins are rRNA N-glycosidases, because they are able to remove a single adenine residue from rRNA (A^{4324} from rat liver rRNA) [68]. More recently, it was observed that some RIPs remove more than one adenine from rRNA and that some act also on poly(A), while all RIPs remove adenine from DNA [69]. For a protein to be identified as a RIP, it is necessary to ascertain that it inhibits protein synthesis and possesses rRNA N-glycosidase activity. This can be detected from the modification of rRNA or can be measured by determining the adenine released.

Ribosome-inactivating proteins have been linked to antibodies or other carriers (e.g., hormones, growth factors, cytokines) to form ITs or conjugates specifically toxic to target cells.

RIPs-II as such cannot be used for this purpose; they must be modified to block the sugar-binding site, otherwise their B-chains would bind to ubiquitous nontarget cells (see also paragraph 4.1). More frequently, ITs and other conjugates have been prepared with the A-chains of RIPs-II, that of ricin being the most widely employed (i.e. Ricin Toxin A-chain, RTA), or with RIPs-I. These have some advantages with respect to A-chains obtained from RIPs-II: they are more stable, simpler and safer to prepare, and sometimes give more active conjugates. Also, they are diverse and often immunologically distinct, and consequently can be employed to overcome the immune response elicited by the administration of ITs. With these regards, it was found that human clonal T-cell responses against RTA are not cross-reactive with RIPs-I [70]. RIPs-I have molecular mass in the region of 30 kDa, are strongly basic proteins (with pI 9.5 or higher), and are generally resistant to proteases and various denaturating agents. Resistance to proteases might be related to their low lysine content [71]. These properties are exploited in the purification of these proteins and render them tolerant to the treatments for the insertion of linkers to obtain chemical conjugates.

RIPs-I have relatively low toxicity to cultured cells and animals because they lack the equivalent of a toxin B-chain by which they bind to cells. Examples of RIPs-I are gelonin from the seeds of *Gelonium multiflorum* [72], saporin from the seeds of *Saponaria officinalis* [73], momordin from the seeds of *Momordica charantia* [74], bryodin from the seeds

of *Bryonia dioica* [75] and pokeweed antiviral protein (PAP) from the seeds and leaves of *Phytolacca americana* [76]. They all inactivate eukaryotic ribosomes in a similar fashion to the A-chains of abrin and ricin and with a similar potency.

Several RIPs have been cloned, and chemical or fusion ITs have been prepared with appropriate carriers [77-86].

4. PREPARATION OF IMMUNOTOXINS

4.1 Chemical Immunotoxins

Three types of ITs are obtained by cross-linking the vehicle molecule and the toxin component of the conjugate by means of appropriate heterobifunctional cross-linkers.

Briefly, the linkage used to join the antibody and toxin component must meet three criteria:

(i) It should not impair the antigen-binding capacity of the antibody; in practice, antigen binding is generally unaffected by the introduction of only one to two cross-linking groups per antibody molecule.

(ii) It must allow the active A-chain component or RIP-I to enter the cytosol and kill the cell; this is thought to require release of the toxic component from the antibody carrier or, in the case of ITs containing intact toxin, from the B-chain moiety.

(iii) For *in vivo* therapy, the linkage must be stable enough to remain intact while the IT is passing through the tissues of the animal to its intended site of action.

The methods for generating linkages with these properties are different for ITs containing intact toxins and ITs containing isolated toxin A-chains or RIPs-I and will be outlined separately below. Detailed methods for the preparation and purification of ITs have been described [87,88].

Immunotoxins Containing Isolated Toxin A-Chains

A-chain ITs are usually prepared by linking the A-chain of the RIP-II toxin to the antibody or antibody fragment by means of a reducible disulphide bond. Such ITs often possess a cytotoxicity to target cells that approaches that of the RIP-II toxin itself. In contrast, ITs in which the A-chain is attached to the antibody via a thioether bond are usually much less cytotoxic than those made with disulphide linkages [89-91] suggesting that splitting of the disulphide bond is required for the toxic component to gain access to the cytosol. Failure to split the bond linking the enzymatic moiety to its B-chain or to the vehicle molecule results in inactive conjugates, likely due to steric hindrance by the B-chain or the vehicle component. The synthesis is generally accomplished by modifying the antibody or $F(ab')_2$ fragment with a heterobifunctional reagent that introduces an activated disulphide group and then mixing the antibody derivative with reduced toxin A-chain. The free thiol group in the A-chain displaces the leaving group from the activated disulphide group and forms a disulphide linkage with the antibody. One of the most frequently used heterobifunctional reagents is N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), which reacts with amino groups on the antibody via a stable carboxamide bond to introduce a dithiopyridyl group [92] (Fig. **2**). Another commonly used reagent is 2-iminothiolane hydrochloride which produces a similar linkage. However, its thiol group must be first activated by treatment with 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) before conjugation. After the conjugation reaction, initial purification is achieved by gel permeation chromatography. This procedure removes unreacted A-chain, high molecular weight aggregates and much of the unreacted antibody. However, the protein fraction, which contains mostly a single molecule of antibody linked to a single molecule of A-chain is contaminated with free antibody and with ITs containing two or more molecules of A-chain per antibody molecule. Ion exchange [93] or affinity chromatography [94] methods are available that eliminate the free antibody and, to some extent, also the ITs with two or more A-chains. Fab' antibody derivatives, prepared by reducing $F(ab')_2$ fragments, normally contain a single free thiol group. Toxin A-chains have been attached to this group after activating the thiol group in either the antibody or the A-chain component with DTNB. This method produces a homogeneous IT containing one molecule of A-chain coupled to one molecule of Fab' [95].

RTA isolated from the seeds of *Ricinus communis* bears mannose-terminating oligosaccharides. When using RTA-based ITs, it has been found that chemical deglycosylation of the subunit prevents its non-specific binding to mannose receptors on cells of the reticuloendothelial system [96-98]. Thus, ITs containing deglycosylated RTA (dgA) have been shown to survive longer *in vivo* and are more efficient at reaching their intended target cells. In addition, if the antibody component does not contain Fc region, but consists of only $F(ab')_2$, Fab', Fab, or smaller Fv fragments, then non-specific binding of the IT *in vivo* will be reduced to a minimum.

Stability of SPDP or 2-iminothiolane however, may not be satisfactory for use *in vivo*. Thus, new coupling agents were synthesized for making ITs containing disulfide bonds with improved stability *in vivo*: sodium S-4-succinimidyloxycarbonyl-alpha-methyl benzyl thiosulfate (SMBT) and 4-succinimidyloxycarbonyl-alpha-methyl-alpha(2-pyridyldithio)toluene (SMPT). Both reagents generate the same hindered disulfide linkage in which a methyl group and a benzene ring are attached to the carbon atom adjacent to the disulfide bond and protect it from attack by thiolate anions (Fig. **2**). An IT consisting of monoclonal anti-Thy-1.1 antibody (OX7) linked by means of the SMPT reagent to chemically deglycosylated ricin A-chain had better stability *in vivo* than an IT prepared with 2-iminothiolane hydrochloride, which generates an unhindered disulfide linkage [99].

Immunotoxins Containing RIPs-I

As with A-chain ITs, RIP-I ITs in which the RIP-I is linked to the antibody via a disulphide bond show the highest cytotoxic activity. Since RIPs-I do not contain a free thiol group, one must first be introduced using a thiolating reagent such as 2-iminothiolane (Fig. **3**). Thereafter the procedures are similar to those used to prepare A-chain ITs. However, chemical derivatization of the RIP-I may affect its ribosome-inactivating activity depending on the type of cross-linker employed. For example, thiolation of gelonin

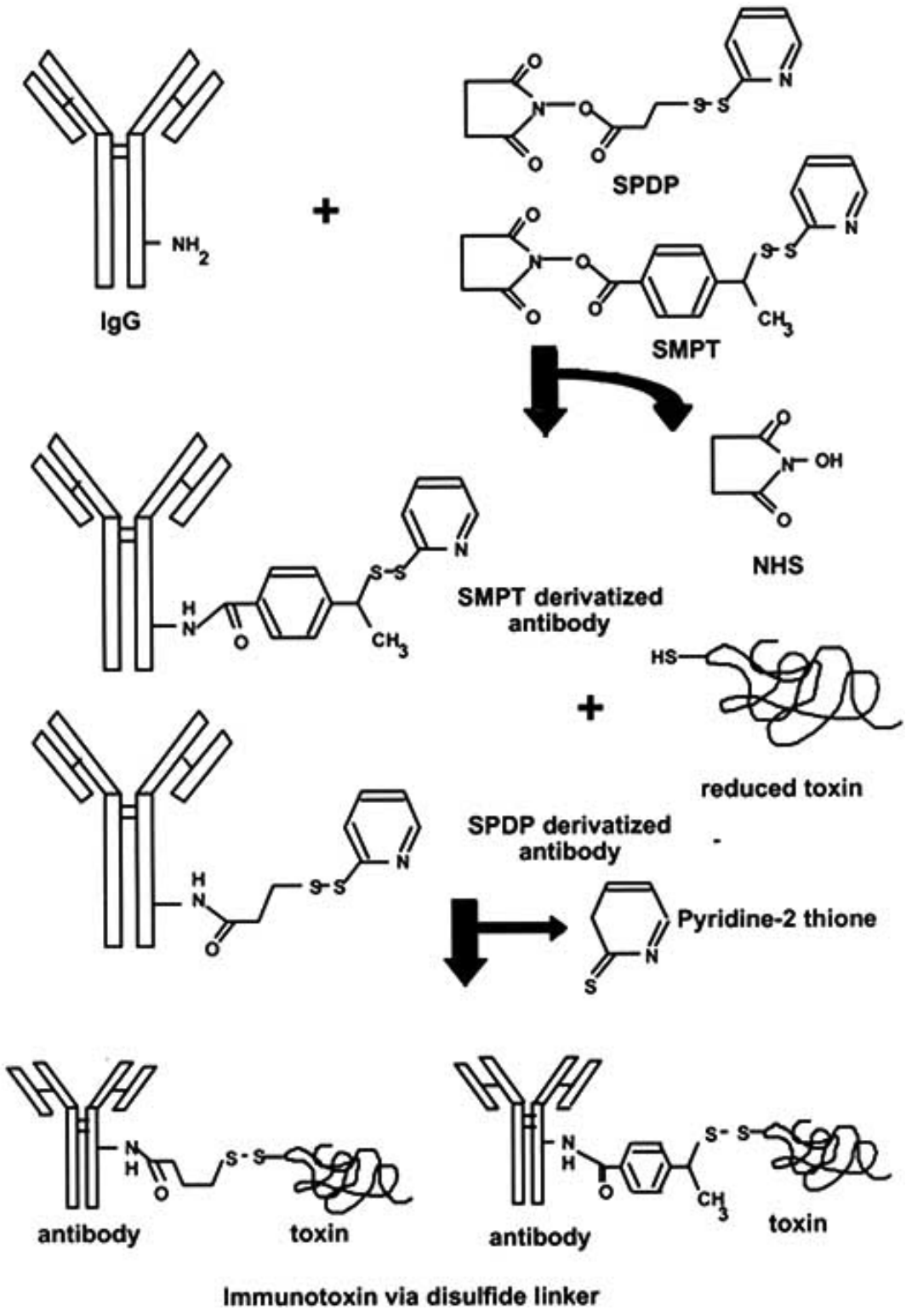

Fig. (2). Immunotoxin made by linking antibody and toxin via a disulfide bond. The antibody was first derivatized with SMPT or SPDP and then linked to the reduced toxin.

by treatment with SPDP followed by reduction with dithiothreitol reduced its inhibitory activity on protein synthesis in reticulocyte lysates by 10-fold whereas thiolation with 2-iminothiolane had no apparent effect [100]. This problem does not arise with A-chain ITs because the native A-chain is released by reduction irrespective of the type of cross-linker used.

RTA or RIPs-I-containing ITs, however, may not be quite as cytotoxic as conjugates formed from RIPs-II molecules [101].

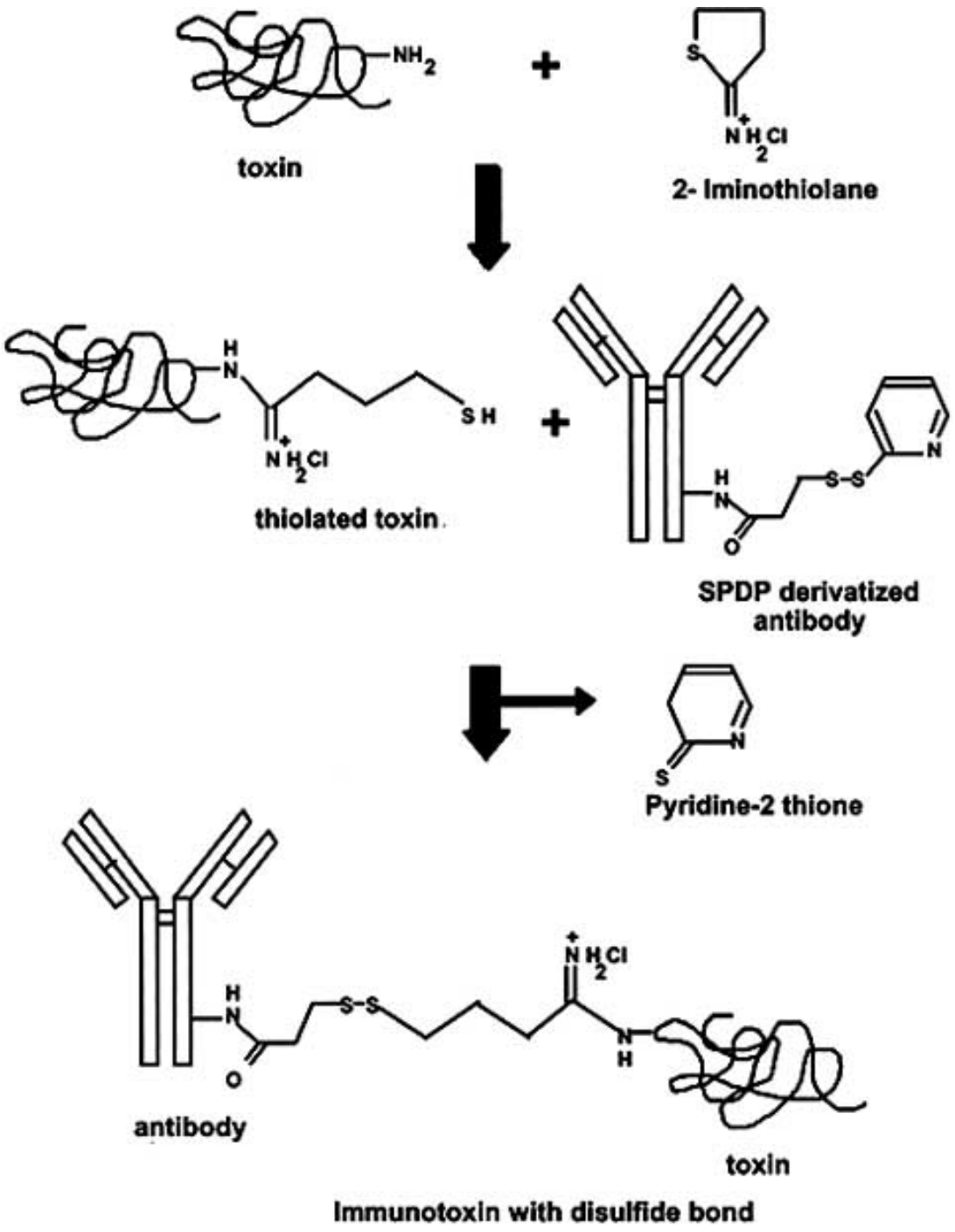

Fig. (3). Toxin with no free sulfidryl groups available for direct conjugation to carrier molecules are first activated with 2-iminothiolane or SPDP and then cross-linked to derivatized carrier molecules.

Immunotoxins Containing RIPs-II

RIPs-II-based ITs are often more powerful than corresponding ITs made with RIPs-I or isolated A-chains. This is likely due to the potentiating function exerted by the B-chain (see also paragraph 7). ITs made with RIPs-II are usually prepared by reacting antibody into which a free thiol group has been chemically introduced, with toxin into which an alkylating function has been introduced. The thiol and the alkylating groups then react to produce an IT in which the toxin is attached to the antibody via a thioether linkage (Fig. **4**). This linkage is stable to reduction and precludes dissociation of the intact toxin from the antibody. However, such ITs are cytotoxic because they retain the natural disulphide bond between the A- and B-chains, which permits release of the active A-chain inside the cell.

To maintain antibody specificity in intact toxin conjugates toward only one cell type (and thus prevent non-specific cell death), all cell binding capability within the

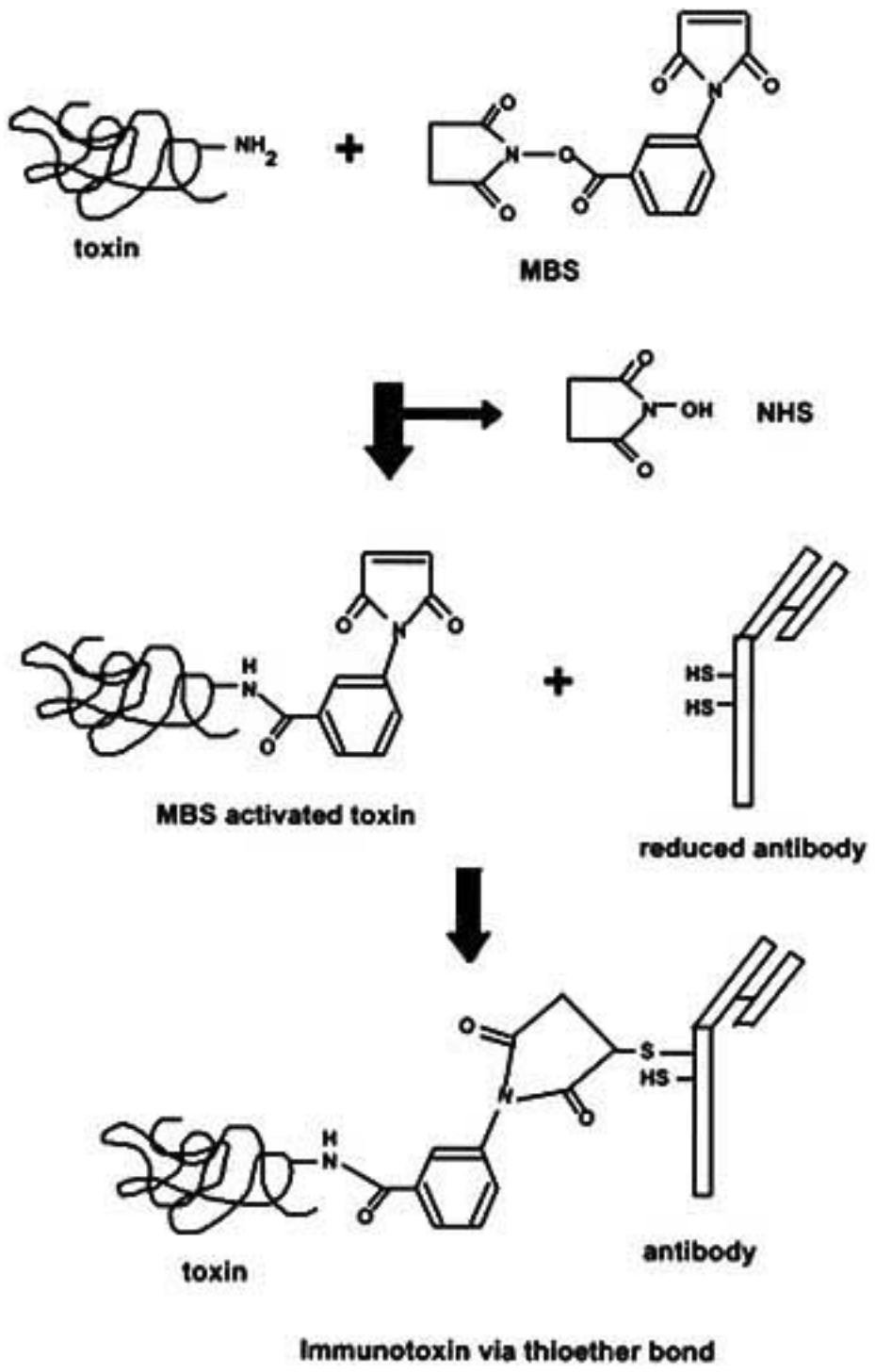

Fig. (4). RIPs-II can be cross-linked to carrier molecule by means of a thioether linkage using the cross-linker MBS.

toxin itself must be removed. Fortunately, a large proportion of the binding sites on the B chains usually are blocked during the conjugation process, and the galactose binding potential is significantly impaired. Further purification to remove conjugates that have remaining galactose binding potential can be done on an acid-treated Sepharose column (which contains galactose residues) or on a column of asialofetuin bound to Sepharose [88]. Conjugate fractions that do not bind to both affinity gels contain no non-specific binding potential toward non-targeted cells.

More elaborate methods of blocking or eliminating the B-chain galactose binding site can also be devised. Blocked ricin is a derivative of native ricin, in which the galactose-binding sites of the B-chain are blocked by covalent modification with affinity ligands prepared from N-linked oligosaccharides of fetuin. This modification impedes the binding function of the B-chain, while sparing its ability to facilitate the entry of the toxic subunit of ricin, the A-chain, into the cytoplasm. The mechanism by which RTB helps RTA entry into the cytosol of the target cell is still under

investigation. Several possibilities have been explored in the past few years (see also above), among these: (i) the insertion of RTB hydrophobic domains within the membrane may facilitate, similar to B chain of DT, the passage of RTA to the cell's cytosol. Partial unfolding may be necessary for this step to occur [102,103]; or (ii) RTB may help RTA to progress along a route and to reach an intracellular site whence RTA may easily escape to its cytosolic site of action. Support for the latter mechanism also comes from a study by Timar *et al.* [104] using an anti-bladder carcinoma RTA-IT (Fib75-ss-ricin) *in vitro* in the presence of RTB as a potentiator; (iii) more recently, it was proposed that RTB interaction with intracellular chaperones (i.e. calreticulin) facilitates its routing towards Golgi-E.R. [64]; another recent study by Morlon-Guyot *et al.* [105] demonstrated that residues from RTA and RTB concur in creating a catalytic site with lipase activity, which may ease the passage of RTA across cellular membranes. ITs prepared with blocked ricin approach the cytotoxic potency of native ricin with antibody-dependent specificity [93].

Availability of cloned ricin B-chain (RTB) has allowed to genetically alter the B-chain galactose binding sites in the attempt to obtain ricin-containing ITs with low or no non-specific recognition of non target cells. However, when recombinant B-chains with complete or partial deletion of galactose binding sites were reassociated *in vitro* with RTA the resulting heterodimer showed greatly impaired or absent cytotoxicity, thus supporting the notion that the ricin B chain galactose-binding activity plays a role not only in cell surface binding but also intracellularly for ricin cytotoxicity [106]. On the other hand, Frankel et al., [107] found that an IL2-lectin-deficient RTB-RTA, conjugate intoxicated IL2 receptor-bearing cells as well as ricin or IL2-wild- type RTB-RTA, thus suggesting that high-avidity intracellular galactose-binding may not be required for ricin intoxication, at least in the case of IL2 receptor-targeted molecules.

4.2 Non-covalent Immunotoxins

To obtain ITs that could be directed against multiple targets, non-covalent methods of carrier-toxin conjugation were developed. Thus, streptavidin-biotin-ricin (SA-BR) conjugates were synthesized by biotinylation of whole ricin, which was then complexed with streptavidin [108]. This SA-BR conjugate was used in an indirect cytotoxicity assay. The assay involved sensitising of target cells with biotinylated monoclonal antibody followed by treatment with dilutions of SA-BR conjugate. The assay demonstrated a specific antibody-directed cytotoxicity. Therefore, a single conjugate could be used in conjunction with a panel of biotinylated monoclonal antibodies to selectively target phenotypically different cell types. Dosio *et al.* [109] synthesized a panel of ITs made by a non-covalent interaction between a monoclonal antibody derivatized with a dichlorotriazinic dye and six different RIPs-I. The non covalent linkage did not affect significantly the toxic activity of the glycosyated RIPs and suggested a method that could be generalised to prepare ITs with various RIPs for repeated administrations in patients and avoid immune response.

Bispecific antibodies (BsAb) are prepared by chemically linking two different monoclonal antibodies or by fusing two hybridoma cell lines to produce a hybrid-hybridoma. Both of these approaches present challenges with respect to yield and purity that molecular genetic approaches have partially solved. BsAb have been used to demonstrate that specific surface molecules can trigger leukocytes to either phagocytose or kill tumour cells, viruses, parasites, and infected cells. BsAb have been used also to localise toxins to tumour sites in experimental models as well as *in vivo* in humans.

A panel of bispecific F(ab')2 antibodies were constructed for delivering the ribosome-inactivating protein saporin to CD19, CD22, CD37 or immunoglobulins to human B cell lymphoma. It was found that pairs of anti-CD22 BsAb, which recognised different non-blocking epitopes on the saporin molecule were able to bind saporin more avidly to the target cell and, as a consequence, increased cytotoxicity by at least an additional 10-fold with respect to individual anti-CD22 BsAb [1110]. A synergistic effect was also observed by Sforzini *et al.* [111] using two anti-CD30/anti-saporin BsAb produced by hybrid hybridomas, which reacted against non-cross-reactive epitopes of the saporin molecule.

Duke-Cohan *et al.* [112] have developed a bispecific antibody that recognises the CD4 and CD29 antigens simultaneously and that was examined for its ability to target CD4+CD29^{bright} T cells. Binding through both antigens would facilitate endocytosis and intracellular delivery of the toxin. Immunoconjugates incorporating blocked ricin preferentially killed CD4+CD29^{bright} cells *in vitro* by a factor of 25 in comparison with killing of total CD4+ cells in functional assays. Similar results were obtained also with anti CD4-CD26 BsAb [113].

A BsAb recognising both carcinoembryonic antigen (CEA) and RTA induced significant RTA cytotoxicity against MKN45 gastric carcinoma cells, which express high levels of CEA, using RTA at a concentration below that known to be intrinsically cytotoxic. The addition of ricin toxin B chain (RTB) also potentiated cytotoxicity of RTA [114].

Flavell *et al.* [115,116] investigated the efficacy of a F(ab'gamma)$_2$ BsAb with dual specificity for the CD7 or CD38 saporin, for delivering saporin to the acute T-cell leukaemia cell lines HSB-2 or HPB-ALL. Saporin titration experiments revealed that sAb increased the toxicity of saporin several hundred-fold.

Animal studies demonstrated that BsAb can be applied for the delivery of toxins *in vivo* [10,117].

Patients affected by low-grade, end-stage, B-cell lymphoma were treated with BsAb having one arm directed at saporin and one at the CD22 on target B cells. All patients showed a rapid and beneficial response to treatment. While these responses were mainly short-lived with tumour progression once the treatment was stopped, their speed and magnitude, and the relative lack of associated toxicity warranted further development of this type of treatment strategy [118,119].

4.3 Recombinant and Fusion Immunotoxins

Recombinant DNA technology was applied in several instances to produce recombinant RIPs and constructs and ITs containing recombinant or native RIPs.

Fusion toxins have been prepared by fusing and expressing the genes for the toxin and targeting ligand. When bacterial toxins (e.g. Pseudomonas exotoxin A, PE, or Diphtheria toxin, DT) are used, the fusion protein will contain the proteolytic cleavage site that is normally present in the native toxin [120,121]. The enzymatic domain can therefore be separated from the targeting moiety by proteolysis once the IT is internalized. When a plant toxin is used (which has no cleavage sites in its native sequence), nucleotides that encode a cleavage site must be introduced between the targeting and toxic moieties [122].

Kim and Weaver [123] engineered a recombinant plasmid containing the coding regions for a functional fragment of staphylococcal protein A (PA) and the entire RTA in tandem in the same reading frame. Such a toxic fusion protein could be directed against IgG mAb bound to any type of cell surface target structure. This approach was followed up by O'Hare *et al.* [122], who prepared constructs consisting of N-terminal RTA and C-terminal PA (RTA-PA) or N-terminal PA and C-terminal RTA (PA-RTA). These constructs were capable of binding to immunoglobulin G (via PA) and of specifically depurinating 28S ribosomal RNA (via RTA). However, neither fusion protein was cytotoxic to antigen-bearing target cells in the presence of an appropriate monoclonal antibody, presumably because the RTA could not be released from the PA within the cytosol. To overcome this, a short amino acid sequence from DT was engineered between the RTA and PA to produce a disulfide-linked loop containing a trypsin sensitive cleavage site [122]. Cleavage of this fusion protein with trypsin converted the RTA-DT-PA to the two chain form consisting of RTA linked by a disulfide bond to PA. The cleaved fusion protein was highly toxic to Daudi cells coated with anti-immunoglobulin antibody suggesting that the RTA could be released from the PA by reduction within the cytosol.

Several fusion ITs containing RIPs have been constructed with single chain recombinant antibodies (scFv) as targeting moiety. scFv are composed of the variable light and variable heavy chains of a monoclonal antibody. Routinely, to hold these two chains together, a 15 amino acid flexible peptide linker is used to tether the COOH-terminal of one chain with the NH_2-terminal of the second chain [124,125]. While this approach facilitates expression of the recombinant antibody from a single transcript, the product is not always stable. Unstable single chain antibodies can be stabilised by the introduction of novel disulfide bonds into the framework segments of the variable chains [126].

Recombinant scFV-IT might have a larger “therapeutic window” due to enhanced stability and tissue penetration. The major advantages of recombinant scFv-IT over biochemically synthesized ITs is that the former are homogeneous, genetically modifiable, have enhanced tissue penetration, and the site and manner in which the scFv and the toxin moieties are joined can be exactly controlled.

Fully recombinant RTA-ITs could be also obtained by separate expression of mAb and RTA followed by chemical linkage of the two constructs [127]. Better *et al.* [127] used genetic engineering to obtain secretion of anti-human CD5 antibody fragments from *E. coli* for conjugation to RTA. An IT was prepared with Fab' by directly coupling to the unique free cysteine on RTA. These ITs efficiently killed a CD5+ T-cell line and human T cells from peripheral blood. Attempts at producing fully recombinant RTA-ITs in eukaryotic cells, however, did not succeed [128].

D’Alatri *et al.* [129] created a recombinant IT (scFv(MGR6)-Cla), composed of the Fv region of an anti ErbB2 monoclonal antibody (MGR6) fused to clavin, a single-chain toxin from *Aspergillus clavatus*. The recombinant IT was expressed at high levels in E. coli and assayed for activity after renaturation. Cell-free protein synthesis inhibition and binding assays showed that both clavin and scFv(MGR6) maintained their properties after refolding

Bryodin 1 (BD1) is a potent RIP-I isolated from the plant *Bryonia dioica* and is 20-30- fold less toxic in animals than commonly used toxins [75]. A single-chain IT composed of BD1 fused to the single-chain Fv region of the anti-CD40 antibody G28-5 (ntBD1-G28-5 sFv) was expressed in tobacco tissue culture as a soluble protein and was specifically cytotoxic toward CD40 expressing non-Hodgkin's lymphoma cells *in vitro* [78]. These data also indicate that tobacco tissue culture is a viable system for soluble expression of BD1 and BD1-containing ITs [78] and might also be extended to other types of recombinant ITs.

An immunoconjugate consisting of a humanised anti-CD33 monoclonal antibody (HuM195) linked to recombinant gelonin was toxic to acute myelogenous leukaemia (AML) cell lines and primary AML blasts obtained from patients and exposed to the IT *in vitro*. This IT was therefore proposed for clinical testing in Phase I trials [130].

ITs containing recombinant human-derived single-chain fragment variable (scFv) reagents against CTLA-4 (CD152) linked to the RIP-I saporin, were prepared and tested on CD3/CD28-activated T lymphocytes, MLRs, CTLA-4-positive cell lines, and haemopoietic precursors. The results showed the possibility of targeting CTLA-4 to kill activated T cells [131].

In some cases, however, fully recombinant ITs utilising RIPs may not provide satisfactory results. Wang *et al.* [132] utilised two methods to produce an anti-CD19 IT containing a single-chain Fv (scFv) FVS191 and RTA. The first method produced the recombinant protein FVS191CDRTA from a fusing gene containing sequences encoding FVS191, cathepsin D proteinase digestion site (CD), and RTA. FVS191CDRTA did not show CD19 antigen binding and cytotoxic activity. The second method generated a disulfide-linked FVS191cys-dgRTA from a FVS191cys, the FVS191 with an additional C-terminal cysteine, and a deglycosylated RTA (dgRTA). The protein synthesis inhibition assay revealed that FVS191cys-dgRTA was toxic to CD19 positive cell lines, but it was less potent than the intact antibody-conjugated B43-dgRTA.

Recombinant DNA technology offered also the opportunity to create novel recombinant toxins. Li and Ramakrishnan [133] prepared a chimeric protein by genetically fusing the coding region of RTA and Diphtheria toxin A-chain (DTA). The hybrid protein (RTA-DTA) expressed in bacteria retained the N-glycosidase activity of the RTA and the ADP-ribosylation activity of the DTA. An IT made with the hybrid toxin was about 100- and 1000-fold

more effective than RTA or DTA conjugate, respectively, in inhibiting tumour cell growth *in vitro*. Hybrid toxins could be useful in preparing potent IT with better antitumour cell activity (Fig. **1**).

5. ASSAYING IMMUNOTOXIN CYTOTOXICITY

The cytotoxic potential of ITs can be assayed *in vitro* and *in vivo* in various ways. In this paragraph we will briefly outline the principles of the most common assays employed to assess the cytotoxic effects of ITs *in vitro*. In other parts of this book methods to evaluate the *in vivo* cytotoxic impact of ITs will be described.

Incorporation of Radiolabelled Aminoacids

This assay directly measures the effects of toxins on the proteosynthetic activity of the target cells. In this assay after an appropriate time of treatment in the presence of the toxin or IT under study, a radiolabelled aminoacid (e.g. ^{3}H-Leucine, ^{14}C-leucine) is added to cells cultured in medium deprived of cold leucine, cells are harvested and the incorporated radioactivity measured in a β-spectrometer. Mock-treated controls will supply the incorporation values corresponding to 100% protein synthesis.

Incorporation of ^{3}H-Thymidine

This and similar assays (e.g. incorporation of bromodeoxyuridine) measure cell proliferation. The effects of toxins in a cell population, leading to cell killing and to a reduction in the total number of cells, negatively affect the incorporation of nucleic acid precursors. Although the effects of the toxins are in this case not measured directly, the overall activity of a toxin or an IT on a target cell population can be extrapolated from data obtained in this kind of assay. Briefly, after an appropriate time of treatment with a given toxin/IT, ^{3}H-TdR is added to cells, the incubation continues for a further 8-12 hr, the cells are then harvested and the incorporated radioactivity measured in a β-spectrometer. Mock-treated controls will supply the incorporation values corresponding to 100% ^{3}H-TdR incorporation.

Measurement of the Plating Efficiency

This assay evaluates the capability of a treated cell population to originate colonies. Also in this case the cytotoxic effect of a given toxin/IT is measured indirectly. This assay, however, has the advantage to supply useful information concerning the overall cytoreductive effects of a toxin/IT. After treatment with a toxin/IT, target cells are washed and plated in decreasing numbers (e.g. from 10^{4} cells/well to 1 cell/well) in the wells of a microtiter plate. The medium is replaced at regular intervals and after an appropriate time (15-30 days), wells containing growing cells are scored as positive under a microscope. The fraction of surviving cells is determined by comparing the plating efficiency of the treated cells to that of control cells. Plating efficiencies are then estimated by statistical analysis (e.g. Poisson analysis) of the proportion of wells without growth at different limiting cell concentrations.

3-D Cell Cultures

To investigate the cytoreductive potential of toxin/IT against 3-D structures one can take advantage of the model represented by Multicellular Tumour Spheroids (MTS). These 3-D cultures mimic the biologic behaviour of small avascular metastases or of intervascular regions of larger tumours and can supply valuable information as regards the ability of toxin/IT to exert cytoreductive effects against 3-D structures. MTS can be obtained with established cell lines as well as with explants from primary tumours, although not all cell lines or explants may be able to take as MTS *in vitro*. Spheroid volume and growth curves are based on measurements of spheroid diameters with a reticule inserted into the eyepiece of an inverted microscope. Other more automatic methods for assessment of growth curves are also available. The effect of therapeutic compounds in MTS can then be quantitated with the Gompertz growth equation by considering the growth delay (measured as time needed to reach a given size) induced by the different treatment modalities.

Viability

Other assays can be used to measure directly the viability of treated cells. In these cases, vital dies (e.g. trypan blue) are utilised to discriminate between living and dead/dying cells. This type of assays, however, is somewhat less accurate than more quantitative methods described above. Moreover, cells doomed to die because of toxin/ITs intoxication can exclude vital dies for long times and can be erroneously counted among living cells.

6. FACTORS AFFECTING THE POTENCY OF THE IMMUNOTOXINS

Antibody Affinity and Valency

The initial step in RTA-IT-mediated cell killing involves binding to the target cell Ag through the antibody moiety. One of the factors influencing this is the affinity of the antibody component for the target cell Ag because this will, in part, determine the number of IT molecules bound to a cell at a given IT concentration.

Ramakrishnan and Houston [91] attached either an anti-Thy-1.1 antibody (19E12) or its $F(ab')_2$ fragment to pokeweed antiviral protein and found that the intact antibody immunotoxin was 45-fold more cytotoxic against Thy-1.1 expressing AKR SL3 cells. They concluded this difference was due to the 10-fold lower affinity of the $F(ab')_2$ fragment compared with the intact antibody.

Multiple epitopes on carcinoembryonic Ag have been mapped providing a range of mAb of known specificity. These have been used to show that the cytotoxicity of an RTA-IT directed to carcinoembryonic Ag is potentiated by the simultaneous use of mAb recognising different epitopes. The potentiating antibodies also increased the level of target cell binding of the mAb (mAb 228) used to synthesize the IT. They did so by modifying the binding affinity of the mAb 228 thus increasing the half-life of antibody at the cell surface [134]. Changing the valency of ITs from bivalent (i.e. IgG or their $F(ab')_2$ fragments) to monovalent (i.e. Fab' or Fab fragments) has often been found to reduce their effectiveness. Masuho *et al.* [90] found that an $F(ab')_2$-RTA IT was 5-fold more cytotoxic than its Fab' counterpart. Variations in entry rates appear to account for the fact that a Fab-RTA IT directed against the common acute lymphoblastic leukaemia Ag (CALLA) was 70-fold less

cytotoxic to target cells than its divalent $F(ab')_2$ counterpart [135]. Although the levels of binding of the two types of ITs were the same, in 24 h assays 85% of the bivalent IT versus 30% of the monovalent IT disappeared from the target cell surface. It was suggested that this was because the bivalent IT induced modulation and internalisation of CALLA much more rapidly than the monovalent IT. When polyclonal rabbit anti-human IgG was linked to RTA, it was found that the bivalent form was internalized by Daudi cells much more rapidly than its monovalent counterpart [136]. In this instance, however, the bivalent and monovalent ITs were approximately equipotent. It was hypothesized that the similar potency observed might be due to the fact that the two ITs entered different intracellular compartments with different competency for RTA translocation to the cytosol. In agreement with this hypothesis, Metezeau *et al.* [137] observed that monovalent Fab' bound to membrane Ig on mouse B-splenocytes was internalized and then rapidly recycled to the cell surface, whereas bivalent IgG was internalized 4-fold faster but was routed to the lysosomes. Other Authors have also observed that divalent ITs are more effective than monovalent ones [138].

Successive studies evidenced that due to the intervention of different mechanisms the contention that divalent ITs are endowed with greater potency might not always hold true. The *in vitro* killing of the human CEM cell line was studied [139] by using RTA-IT constructed with either the whole IgG or the Fab and $F(ab')_2$ fragments of the same T101 (anti CD5) mAb. The efficacy of the anti-CD5 IT was greatly improved when fragments were used. In fact, at a saturating dose, a cytoreduction of three orders of magnitude was obtained with the fragment IT versus less than one order of magnitude for the whole IT, as assessed in clonogenic assays. This enhancing effect was related to better cell killing kinetics, because with a similar amount of RTA molecules bound per cell, T101 fragment IT achieved a two-fold faster protein synthesis inhibition rate than the corresponding whole IgG IT. No significant difference in activity was instead found between monovalent (Fab) and divalent ($F(ab')_2$) forms of IT. This observation was further supported by Chiron *et al.* [140], who compared the cell killing potency of a whole Ig RTA IT (T101 IgG-RTA) against its Fab fragment counterpart (T101 Fab-RTA). T101 Fab-RTA was significantly more toxic to both CEM cell line and fresh leukaemia cells than T101 IgG-RTA. A possible interpretation of these observations is that the Fc fragment of the IgG hinders cell entry of the IT or of its toxic component.

Antigen Density

Studies using various antibodies coupled to RTA have shown that Ag density correlates with the cytotoxic potency of the IT. This was approached by Casellas *et al.* [141] using an RTA-IT directed against the melanoma-associated Ag p97. They found that melanoma cell lines expressing over 78,000 p97 Ag/cell were efficiently killed by the IT whereas those expressing fewer than 5000 sites/cell were not killed. However, the Authors found no correlation between the cytotoxic potency of the IT and Ag density in those instances where the number of p97 Ag/cell was above 78,000. By comparing the sensitivity of four sublines of CEM T-cell leukaemia cells with different amounts of CD5 Ag to a T101 (anti CD5)-RTA IT, Laurent *et al.* [142] found that the cytotoxic potency of the IT and the rate at which protein synthesis was inhibited correlated with Ag density in three of four cell lines. However, a fourth cell line having an intermediate CD Ag expression did not fall into this pattern. Therefore, additional factors other than the Ag density on the cell's surface must play a role in determining the potency of an IT.

Against the conclusion that IT potency correlates with Ag density at the cell surface is the work by Preijers *et al.* [143], who studied the parameters responsible for the variations observed in IT potency. RTA was linked to mAb to CD3, CD4, CD5, CD7 and CD8. When Ag density and cytotoxicity were plotted for all CD Ag, no correlation could be found. They concluded that the degree of Ag expression might not be so important as the absolute amount of antibody internalized in predicting the efficacy of IT. This was further supported by another work by Prejiers *et al.* [144], who observed that the cytotoxicity of various RTA-IT (anti CD3, anti CD5 and anti CD7) on human T-cell lines (GH1, CEM, HPB-ALL and Jurkat) appeared to be closely related to the Ag density and internalisation rate of the IT; normal unstimulated T cells appeared to be rather insensitive to IT not due to a low Ag density or to a decreased internalisation. Stimulation of T cells with PHA prior to treatment with the IT considerably increased the sensitivity to IT treatment. Thus, normal unstimulated T-cells may appear to be less sensitive to ITs with respect to T-cell lines or to activated normal T-cells because of a lower protein synthesis rather than to lower Ag expression.

Modulation of the Ag-IT complex from the cell surface has also been held responsible for variations in the cytotoxic potency of ITs. Manske *et al.* [145] evaluated the modulation of the anti-CD5 T101 IT and of free T101 antibody from the surface of normal and leukaemic cells to determine whether the presence of toxin on the antibody affected Ag modulation. Their findings show clearly that: (1) the presence of toxin on antibody does not inhibit (and may actually enhance) CD5 Ag modulation; (2) T101-IT are internalized, not shed from the surface; (3) the lack of toxicity of T101-RTA is not attributed to inability to modulate. In another investigation [146] two isotype-matching mAb, SN5d and SN5, which are directed against two distinct epitopes of the common acute lymphoblastic leukaemia Ag (CD10), were compared for their antitumour activity after conjugation to RTA. The Authors found that SN5d-RTA was much more effective than SN5-RTA. This probably derived from the marked differences found between SN5d and SN5 in the induction of Ag modulation and in the regulation of Ag biosynthesis and expression. Binding of SN5 to NALM-6 leukaemia cells caused strong Ag modulation and strong downregulation of Ag biosynthesis and cell surface expression of newly synthesized Ag. In contrast, binding of SN5d to target cells caused little modulation of overall cell surface expression of CD10.

Route of Entry

The IT route of entry into a cell after binding to the target Ag is probably one of the most important factors affecting its cytotoxic potency. Receptor-mediated endocytosis of the IT is generally considered to be an advantageous route of entry, inasmuch as RTA coupled to

Tfn, asialofetuin or EGF (all entering the cell following binding to cell surface receptors) is highly toxic to cells that bind and endocytose rapidly these vehicle molecules [147,148].

The relationship between the rate of IT internalisation and cell intoxication was investigated by Wargalla and Reisfeld [149], who examined the relationship between the cellular internalisation of an anti-ganglioside GD2 mAb and the toxic effects of its RTA-IT. The capacity for ligand uptake correlated with the cytotoxic activity of the IT against melanoma and small cell lung cancer (SCLC) cell lines. The Authors demonstrated that the consequence of internalisation of the IT is the intracellular release of undegraded RTA from the mAb. They concluded that the rate of internalisation is a quantitative parameter that plays a key role in predicting the cytotoxic potency of an IT. Using the F(ab')2-T101-RTA-lT, directed against the CD5 Ag expressed on CEM leukaemic T cells Ravel and Casellas [150] found that during the first hours of cell intoxication internalisation is not the rate limiting step of IT cytotoxicity. Internalisation becomes limiting in cell intoxication only when the entry rate is low. Braham *et al.* [151] approached the study of the role of internalisation and intracellular routing in RTA-IT-mediated cell killing by applying a mathematical model, and concluded that cell intoxication induced by ricin or RTA requires two processing steps and that although the cytoplasmic internalisation of ricin might be a slow process, it is nevertheless, compensated for by an extremely fast enzymatic inactivation of ribosomal activity.

The routing of internalized IT towards intracellular compartments competent for their translocation into the cytosol or else towards compartments where the IT can be degraded or recycled to the extracellular environment appear to be other essential mechanisms in determining the efficiency of the cell intoxication process. Press *et al.* [152] synthesized RTA-IT against CD5, CD3 and CD2. Anti CD2 RTA-IT had minimal effects on target cells. Ineffective ITs were more rapidly delivered to lysosomes than effective ITs. These data support the hypothesis that there might be several distinct pathways for internalization of ITs and that the ability of RTA to reach and inactivate ribosomes may depend upon the specific membrane receptor involved in binding a given IT, its route of internalisation, and the rate of entry of the IT into lysosomes [152]. That intracellular degradation may be one of the factors limiting the effectiveness of RTA-IT-mediated intoxication was also suggested by van Oosterhout *et al.* [153] who compared the cytotoxicity of an anti CD3 IT (WT32-RTA) with the rate of internalisation and intracellular degradation of the IT during continuous exposure. Using post-embedding electron microscopy (EM) Calafat *et al.* [154] studied the binding and intracellular routing of an mAb-RTA IT directed to the carcinoma associated Ag sialomucin. The IT was internalized into the cell by two different pathways: one via coated pits-coated vesicles followed by transport to the lysosomes and one via large enclosed invaginations of the plasma membrane, which apparently fused with lysosomes. This internalisation was similar to that of the mAb alone. During transport via both pathways, the IT remained intact until it reached the lysosomes. Moreover, in areas of abundant endocytic vesicles the labels for both IT moieties (mAb and toxin) were also found in the cytosol, suggesting that intact IT is translocated from the vesicles into the cytosol. The role of the Golgi in toxin processing was studied [155] using Brefeldin-A (BFA), a fungal metabolite which blocks Golgi function. At concentrations that inhibit secretion of IL-2, BFA enhances the toxicity of two RTA-IT targeted against distinct cell surface determinants. Based on their results Hudson and Grillo [155] concluded that Golgi function is tightly linked to IT translocation and that BFA has effects on vesicular routing in addition to the block of Golgi function in secretion.

Re-direction of internalized IT might also be an important phenomenon often explaining the low cytotoxicity of RTA-based IT. Ravel *et al.* [156] investigated the entry and subsequent intracellular fate of T101 mAb and T101-RTA IT directed against the CD5 Ag expressed on human leukaemic CEM cells. Both the mAb and the IT were internalized at a relatively low rate. This could be related to the partial recycling of the mAb/Ag or IT/Ag complexes. It was also found that lysosomal degradation and cleavage of disulfide-linked conjugates is a quantitatively minor phenomenon compared with the localisation of the internalized anti-CD5 IT in an endosomo-Golgi compartment, followed by their recycling to the cell surface. This could be one of the major factors explaining the low efficacy of anti-CD5 IT when assayed in the absence of potentiating substances (see below). The work by Ravel *et al.* [156] also suggests that the relevance of degradation phenomena may vary depending on the internalisation pathway.

Interestingly, also the plasma membrane might be greatly involved in the phenomena leading to RTA-IT cell intoxication. Byers *et al.* [157] found that papain treatment of target cells to remove IT from the cell surface indicated that the cell surface acts as a reservoir for continued internalisation of IT over several hours, but even so, in their model 50 % inhibition of cell survival was produced over the first 2-3-h period. Although RTA-based IT may be endowed with variable potency, the work by Sung *et al.* [158] highlights a considerable advantage that RTA-IT have with respect to their DT-based counterparts. Sung *et al.* [158] compared IT comprised of an mAb linked to rRTA or to a binding-defective form of DT with respect to their rates of protein synthesis inhibition and cytotoxic efficacy. At equivalent protein concentrations, the DT IT inhibited protein synthesis significantly more rapidly than the RTA-IT but contrary to previous predictions, achieved a significantly lower cell kill. Thus, the kinetics of protein synthesis inactivation do not necessarily correlate with killing efficiencies.A possible explanations for these results are that the effect of the DT IT on protein synthesis is partially reversible or that DT IT enters the cytosol at a faster rate than the RTA IT but is also degraded at a faster rate. Increased expression of the Multidrug Resistance (MDR) P-glycoprotein has been implicated in an increased routing to lysosomes of the IT HuM195-gelonin and in a marked resistance to this IT of P-glycoprotein overexpressing leukaemia cell lines [159].

Epitope

The "topography" of target epitopes might be also involved in regulating the efficiency of the RTA-IT cell intoxication process. Using anti sIgD RTA-IT against

human B cells May *et al.* [160] found that neither cross-linking nor rate of internalisation account for the different potencies of anti-Fc versus anti-Fd IT. Those ITs directed against epitopes within the constant region of the Ig, closer to the cell's lipid phase of the plasma membrane, were consistently more effective than those directed against the more distant Fab arm of the surface Ig. A possible explanation for this is that insertion of RTA into the membrane is facilitated when it is in close proximity to the membrane and that insertion in some way helps the RTA to enter the cytosol. An alternative explanation is that the position of the epitope may affect cross-linking of the surface Ig thus facilitating its internalisation and cytosol entry. However, when the relative cytotoxic activity of ITs reactive with different epitopes on the extracellular domain of the c-erbB-2 (HER-2/neu) antigen were assayed [161], it was found that cytotoxicity did not correlate with immunoglobulin isotype, binding affinity, relative position of epitopes or internalisation of the anti-HER-2 ITs and that both the most and the least effective ITs bound to epitopes in very close proximity.

Cell Type

The properties and biology of individual cell types can influence the susceptibility of RTA-IT target cells to a given IT. Raso *et al.* [135] assayed an anti CALLA RTA-IT on various human cell lines showing similar amounts of the CALLA Ag and observed considerable differences in their sensitivity. In a study using RTA-IT directed against various breast cancer Ag, it was found that in some instances a particular IT was cytotoxic to one breast tumour cell line but not to others, in spite of the fact that all cell lines bound similar levels of the IT [162]. Similar observations were obtained in another study where it was found that only two antibodies directed against the TfnR on myeloid leukaemia cells (HL60, KG1, U937 and K562) inhibited protein synthesis at relatively high concentration (IC_{50} at 10^{-8} M). The same ITs were highly toxic to non-myeloid cells which shared the target Ag. Fast and effective degradation in lysosomes could explain the poor susceptibility of myeloid cells to RTA IT [163]. Preijers *et al.* [144] observed considerable differences in sensitivity to the same RTA-ITs between human leukaemic cell lines and normal unstimulated T cells (see above). All these findings suggest that different cells may have individual mechanisms for the internalisation or intracellular routing of the same or different RTA-IT targeted against their cell surface Ag.

Cell Accessibility

The 3-D architecture of a solid tumour mass may influence the efficacy of RTA-IT cytotoxicity in several ways. Bulky macromolecules such as the ITs are not easily transported in the context of solid tumour masses and show heterogenous distribution within the tumour tissue. In addition, target Ag may be downregulated depending on the position of the target cell within the solid mass thus yielding an heterogenous distribution of target Ag within solid masses, with cells localised in the deep regions of the tumour expressing low or undetectable amounts of target Ag, which may be instead, highly expressed in monolayer cultures or in the outer cell layers of the solid mass. To approach these problems [164] others [165] and we [164] have used cell culture models (spheroids) mimicking the 3-D architecture and the properties of solid micromasses.

In a study by Kikuchi *et al.* [165], it was found that the cell-kill effects of an anti-melanoma IT were markedly delayed and reduced when target Minor cells were cultured as multicellular tumour spheroids (MTS) than when they were grown as monolayer (more than 100-fold lower cytotoxicity). The reduced cytotoxicity of the IT on melanoma cells in MTS as compared to cells grown in monolayer appeared to correlate with its inhomogenous distribution in the MTS. We [164] evaluated the sensitivity to IT of monolayer and of 200-250 μm MTS obtained with human breast (MCF7) and glioblastoma (U118) tumour cells and with rat glioblastoma (9L) cells. Monolayer MCF7 and U118 cells were highly sensitive to anti-transferrin receptor (TfnR) RTA-IT (Tfn-RTA and mAb OKT9-RTA) treatment in the presence of the intracellular RTA-IT enhancing agent HSA-Mo conjugate (see also below). A 790-2000-fold higher concentration of anti-TfnR-IT was instead required to reduce by 50% the volume of individually treated MCF7 spheroids. Binding studies performed with [^{125}I]-Tfn and FITC-labelled anti TfnR mAb revealed that 9L monolayers and MTS expressed 4.1-fold and 8.8-fold lower amounts of TfnR than MCF7 monolayers and MTS, respectively. These results indicated that the efficacy of IT against 3-D tumours may be also heavily influenced by the number of target Ag expressed by the tumour cells and that higher Ag expression in 3-D structures may lead to an "Ag barrier effect" [166] reducing the efficacy of IT treatment with respect to solid masses expressing lower Ag amounts.

7. POTENTIATION

7.1 Lysosomotropic Amines and Carboxylic Ionophores

Lysosomotropic amines and carboxylic ionophores are able to increase dramatically the cytotoxic potency of weakly cytotoxic ITs. In some instances even non-cytotoxic ITs may acquire considerable cytotoxic potency. These compounds accelerate the cell intoxication process and greatly reduce the number of IT molecules required for cytotoxicity.

They may act by several mechanisms: inhibition of lysosome hydrolases, traffic alteration along the endosome-Golgi route, inhibition of the extracellular recycling of internalized molecules, etc.

Ammonium Chloride

Ammonium chloride (NH_4Cl) is one of the most extensively studied reagents used for enhancing the ITs activity. Earlier kinetic studies using RTA-containing ITs showed that the rate of protein synthesis decreases according to a mono-exponential function, indicating a first-order process [167,168]. With increasing concentrations of IT a maximal rate of inhibition could be reached. NH_4Cl strongly increased the rate of protein synthesis inhibition by IT and increased the sensitivity of cells to the IT. Raising the pH within acidic organelles (e.g. lysosomes and endosomes) in which the ITs were taken up was considered to be one of the mechanisms involved in increased IT cytotoxicity [169]. Casellas *et al.* [168] found that this pH-sensitive process of IT activation is an all-or-nothing effect within an extremely narrow pH window of 0.7 pH units. The activation by NH_4Cl was abolished by lowering the pH, which in turn

lowered the free ammonium (NH_3) content of the medium. This suggests that the latter is the effective component in the activation of ITs. It is likely that the lipophilic NH_3 diffuses across the plasma and lysosomal membranes and becomes protonated to NH_4^+ within the intracellular organelles, where its entrapment determines the pH increase, thus inhibiting the function of acidic proteolytic enzymes. Ravel and Casellas [150] found that NH_4Cl acts on internalized molecules for a very short time, suggesting that this enhancer affects an early intracellular step. The cytotoxicity of gelonin-based ITs [170] and of a OKT1-saporin IT [171] were not influenced by NH_4Cl. In another instance, an anti CD30-saporin IT was instead somewhat inhibited by the presence of NH_4Cl [172].

Chloroquine

Chloroquine is a well-known drug used for the therapy of malaria and, being a clinical drug, might be more suitable for use in combination with ITs in patients. Cloroquine can enhance the cytotoxicity of RTA-ITs up to 2500-fold [168]. Various RIPs-I linked to human Transferrin (Tfn) were also potentiated by the addition of chloroquine during the assay [173], whereas other RIPs-I-based IT were not influenced by its presence [170,171,174].

Other Lysosomotropic Amines (Methylamine, Amantadine)

Poole and Ohkuma [169] have shown that also weakly basic substances can increase the intralysosomal pH in a concentration-dependent manner. Methylamine is a weak base, which influences the intralysosomal pH. A 10 mM concentration could enhance the activity of an anti-CD5 IT on CEM cells by 13,300-fold [168]. The drug 1-adamantanamine hydrochloride (amantadine) also is a potent enhancer of the cytotoxic activity of anti-CD5 RTA-IT against peripheral blood T cells. The treatment with the IT resulted in a 100-fold reduction of peripheral T cells *in vitro*; no adverse effects on the multipotential haemopoietic progenitor cells were observed through the use of amantadine [175]. Amantadine may be more advantageous than NH_4Cl because it is a licenced drug used for prophylaxis of influenza.

Carboxylic Ionophores

Carboxylic ionophores such as monensin (Mo) are well studied reagents for enhancing IT activity. Mo, grisorixin and lasalocid are all able to enhance the effect of RTA-IT, however, other ionophores such as nonactin, valinomycin and calcimycin have no effect on IT cytotoxicity. The present paragraph will focus essentially on the effects brought about both *in vitro* and *in vivo* by Mo, which is the most widely used and described carboxylic ionophore used for IT potentiation.

Mo is a molecule capable of ion complexation through a cyclic form stabilized by hydrogen bonding between the carboxyl and hydroxyl groups [176]. Mo is able to collapse Na^+ and H^+ gradients across cell membranes and may increase the pH of acidic vesicles like lysosomes through the exchange of Na^+ for H^+. Mo is a very effective RTA-IT potentiator [177], which can function at very low concentrations (nanomolar range) and can produce significant increase in the RTA-IT cytotoxicity with IC_{50} in the range 10^{-12}-10^{-14} M. Jansen *et al.* [178] suggested that Mo is approximately 10^5-fold more potent than NH_4Cl on a concentration basis and, indeed, Mo can potentiate to a great extent also RTA-based ITs that are only weakly or not enhanced by other compounds.

Lysosomotropic amines and carboxylic ionophores raise the lysosomal pH and so it has been suggested that they may act by reducing the rate of degradation of the IT [150,168]. However, Raso and Lawrence [177] and Jansen *et al.* [178] have shown that Mo markedly potentiates RTA-IT at concentrations, which do not affect lysosomal pH suggesting that an alternative mechanism may be operating. In fact, at μM concentrations Mo increases the pH in the lysosomes. However, vacuolization of the Golgi and enhancement of RTA-IT can be obtained at 100-fold lower concentrations of 50 nM [178]. Studies have shown that lysosomotropic amines can delay the delivery of ITs to lysosomes [179], keeping them longer in peripheral endosomes and possibly diverting them to other subcellular compartments, which facilitate escape to the cytosol. One possibility is that the intact IT or its toxin portion eventually arrives in the trans-Golgi region and that Mo and lysosomotropic amines prevent them from leaving this region. Support for this hypothesis has come from a study by Ravel *et al.* [156] (see also above) who investigated the entry and subsequent intracellular fate of an anti-CD5 T101 mAb and T101-RTA IT in human leukaemic CEM cells. They found that the effect of Mo or NH_4Cl was to slow or inhibit the recycling of the internalized IT outside the target cell. The Authors also showed that the presence of NH_4Cl or Mo, both dramatically enhancing the kinetics of IT cytotoxicity, did not affect the rate of internalisation or the intracellular localisation of the IT, suggesting that these activators could act at a post-endocytotic level on a limited number of IT molecules.

Several research groups have also approached the question of *in vivo* potentiation of RTA-IT using Mo. Conjugates of Mo and of the carrier protein human serum albumin (HAS) were therefore created in the attempt to facilitate *in vivo* delivery of Mo for IT potentiation. Jansen *et al.* [180] indicated that the use of the conjugate of Mo and human serum albumin (HSA-Mo) in combination with anti-human T cell IT could increase the survival of athymic mice bearing human T-cell leukaemia. Our group [43] evaluated the ability of Mo and disulfide-linked HSA-Mo to enhance the cytotoxicity of Tfn-RTA and anti TfnR mAb-RTA conjugates *in vitro*. HSA-Mo was 2-13-fold less toxic than Mo for cells *in vitro* but was active in the same concentration range as Mo in potentiating mAb-RTA and Tfn-toxin conjugates against different cell lines in monolayer cell cultures. Mo and HSA-Mo were also found to be active in 3-D tumour cell cultures to the same extent.

In spite of the promising properties shown *in vitro* by HSA-Mo conjugates, we [40,181] and others [178] found that factors present in the serum can block the ITs potentiating activity of Mo.

7.2 Antagonists of Ca^{++} Channels and Other Compounds

Ca++ channel blockers and their derivatives have been studied to evaluate their ability as IT enhancers. They can

often provide up to logs increase of IT efficacy. Their mechanism of action does not appear to be associated with the Ca^{++} channel function but might be related to the prevention of lysosomal degradation of the IT.

Verapamil and Its Derivatives

Verapamil was shown to actively enhance the cytotoxicity of anti EGFR RTA-ITs up to 40-fold [182]. Pirker *et al.* [183] evaluated four structural analogs of verapamil (D792, D595, D528 and Sz45) for their ability to enhance the *in vitro* activity of anti-human TfnR IT made with RTA or Pseudomonas exotoxin. The enhancing ability of the drugs did not correlate with their calcium-antagonistic activity. Enhancement ranged from 2 to over 60-fold and was dependent upon the cell line or the experimental conditions.

Verapamil and the analogs could delay lysosomal degradation of the ITs, thereby enhancing their activity. It was also suggested that verapamil may alter cellular membranes in a manner that independently affects the translocation of ITs and lysosomal function [182].

Perhexiline and Indolizines

Perhexiline maleate is another Ca^{++} channel antagonist and is able to enhance IT cytotoxicity. Jaffrezou *et al.* [184] evaluated perhexiline (Pex) and four structural analogs for their ability to enhance RTA-IT activity *in vitro*. Only Pex significantly enhanced the cytotoxic activity of an anti CD5 RTA-IT. Pex almost completely blocked RTA-IT intracellular degradation and profoundly modified its routing. These observations were linked to Pex-induced lipidosis via inhibition of sphingomyelinase activity. In a further study, Jaffrezou *et al.* [185] evaluated a novel class of calcium channel blockers (indolizines SR33557and SR33287) for their ability to enhance RTA-IT activity *in vitro* and *in vivo*. SR33287 had a significant impact on the intracellular routing of an RTA-IT and induced a greater than two-fold increase in intracellular intact IT. As shown for Pex also this effect on RTA-IT half-life may be linked to the inhibition of acid lysosomal sphingomyelinase by SR33827, leading to cellular lipidosis.

7.3 Retinoic Acid

In a study by Wu *et al.* [186] it was found that all-trans retinoic acid could specifically increase receptor mediated intoxication of RTA-IT more than 10,000 times, whereas fluid phase endocytosis of RTA alone or of RTA-IT was not influenced by retinoic acid. Retinoic acid receptor does not appear to be necessary for IT activity. Retinoic acid potentiation of IT is prevented by BFA indicating that in the presence of retinoic acid the IT is efficiently routed through the Golgi apparatus en route to the cytoplasm. Direct examination demonstrates that the Golgi apparatus undergoes morphological changes upon treatment with retinoic acid, suggesting that retinoic acid may alter intracellular routing.

7.4 Viruses

Viruses utilise specialized envelope structures to enter the cytosol of the infected cells. Following binding to cell surface receptors viruses are routed to acidic intracellular compartments (e.g. endosomes) where domains of the viral coat are activated, thereby triggering the interaction of viral proteins with the organelles' membranes and the disruption of the endosomal membrane.

Adenovirus has been used to enhance the cytotoxic effects of RTA coupled to anti TfnR antibodies [187]. The enhancement mechanism is probably related to the fact that both adenovirus and the IT entered the cells in the same intracellular vesicles and that the adenovirus disrupted the vesicles, allowing the passage of the IT into the cytosol. This finding was confirmed by Griffin *et al.* [188], who examined the effects of UV radiation-inactivated human adenovirus on RTA-IT cytotoxicity to the human colorectal adenocarcinoma cell line LoVo.

The study of the membrane disruption and fusion procedures that occur during viral entry and other important biological membrane events has led to the identification of amphipathic α-helical peptide sequences that are responsible for these membrane processes. The Influenza virus hemagglutinin structure is among those, which have been well studied in the past few years. Tolstikov *et al.* [189] have described the use of two related fusogenic peptides (HA23 and HA24, from influenza virus) to enhance the efficacy of anti-HIV ITs. The peptides were mixed with two different ITs. IT action was enhanced by both peptides, with HA24 providing greater enhancement. IT was then constructed by coupling HA23 or HA24 to the targeting antibody. Peptide HA23 enhanced the activity of the IT 4-5-fold. Greater potentiation was achieved by us [41] using a peptide derived from the vesicular stomatitis virsus (VSV). We obtained a chimeric protein by fusing together the RTA gene and a DNA fragment encoding a 25-aa N-terminal peptide derived from the envelope protein G of the VSV. The chimeric RTA (cRTA) retained full enzymic activity in a cell-free assay and was 10-fold less toxic against human leukaemic cells than either native RTA (nRTA) or unmodified recombinant RTA (rRTA). Conjugates made with cRTA and human Tfn showed 10-20-fold greater cell killing efficacy than Tfn-nRTA or Tfn-rRTA yielding a "specificity factor" of 100,000.

Lorenzetti *et al.* [190] obtained three chimeric proteins by fusing together the dianthin gene and DNA fragments encoding for the following membrane-active peptides: the N-terminus of protein G of the vesicular stomatitis virus (KFT25), the N terminus of the HA2 haemagglutinin of influenza virus (pHA2), and a membrane-acting peptide (pJVE). Chimeric dianthins (KFT25DIA, pHA2DIA and pJVEDIA) retained full enzymatic activity in cell-free assays and showed increased ability to induce pH-dependent calcein release from large unilamellar vesicles (LUVs). Conjugates made by chemically cross-linking KFT25DIA or pJVEDIA and human Tfn showed greater cell-killing efficiency than conjugates of Tfn and wild-type dianthin. Thus, genetic fusion of membrane-active peptides to enzymatic cytotoxins results in the acquisition of new physico-chemical properties exploitable for designing new recombinant cytotoxins and to tackle cell-intoxication mechanisms.

Potentiation not directly related to viral peptide structures but derived from interaction of virus coat proteins and its cell surface receptor is that described by Pincus and

McClure [191], who conjugated mAb specific for gp120 or gp41 or the HIV envelope protein gp160 to RTA and then evaluated their immunotoxic activities against HIV-infected cells in the presence or absence of soluble CD4 (sCD4). The efficacy of anti-gp41 IT was enhanced at least 30-fold in the presence of sCD4.

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ABBREVIATIONS

IT	=	Immunotoxin
RTA	=	Ricin toxin A-chain
Ag	=	Antigen
mAb	=	Monoclonal antibody
BsAb	=	Bispecific antibody
Tfn	=	Transferrin
TfnR	=	Transferrin receptor

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