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CYTOTOXIC CONJUGATES CONTAINING TRANSLATIONAL INHIBITORY PROTEINS

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INTRODUCTION

Cytotoxic drugs administered during conventional chemotherapy are taken up by sensitive nontarget organs besides the tumor cells and this often leads to unwanted side effects. These problems have initiated studies during the past decade to develop novel methods in which the therapeutic reagents could be directed to specific cell populations to widen the therapeutic window. The concept of targeting drugs was proposed nearly a hundred years ago by Paul Erlich, who envisioned the possibility of transporting toxic substances to tumor cells by carrier molecules (magic bullets). This novel approach became a reality with the advent of tumor selective monoclonal antibodies (Mabs) produced by somatic cell hybridization. Both cytotoxic drugs and toxin polypeptides have been chemically linked to Mabs. During the past decade various molecules have been produced and tested in a number of model systems (reviewed in 1–10). The range of targets includes (*a*) ex vivo treatment of bone marrow grafts to remove contaminating leukemic cells and alloreactive T cells, (*b*) administration of conjugates to inhibit tumor growth

in restricted anatomical spaces such as the peritoneum, and (*c*) systemic application of conjugates to inhibit diffused tumors and solid tumors. Success of the last two applications depends largely on the pharmacological and toxicological properties of the chimeric molecules. This article focuses on the most recent developments in targeting toxin polypeptides.

Immunotoxins are composed of a cytotoxic molecule linked to a carrier molecule by either a reducible or a nonreducible bond. The cytotoxic moiety is often a toxin polypeptide that catalytically inhibits a vital biosynthetic pathway. In general, the cytotoxic moiety used in targeting is a translational inhibitory protein but some investigators have also used enzymes such as phospholipases to destabilize the integrity of the tumor cell plasma membrane. Various carrier proteins have been investigated to deliver the toxin molecules, including tumor selective monoclonal antibodies, growth factors, and lymphokines.

CHOICE OF TOXIN MOLECULES

Three types of toxin polypeptides are used in the preparation of immunotoxins (IT)[1]: (*a*) bacterial toxins (*b*) plant toxins and (*c*) fungal toxins. All three groups of molecules catalytically inhibit protein synthesis in eukaryotes but each at a distinct step in translation. Most of the work on the bacterial toxins was carried out on two highly toxic molecules, namely diphtheria toxin (DT) and pseudomonas exotoxin A (PE). Detailed studies on the structure/function of these two molecules are primarily responsible for the advances made in preparing highly effective chimeric molecules against tumor cells. Based on their biochemical characteristics, plant toxins can be grouped as Type I, single chain polypeptides that enzymatically inhibit translation, and Type II (e. g. ricin and abrin), which are heterodimers. The A chains of Type II toxins are the true toxic moieties, whereas the B chains contain binding sites for carbohydrates through which the A chain gains access to the interior of the cell. Fungal toxins such as alpha sarcin are also single chain proteins but are functionally different from Type I polypeptides. Alpha sarcin, for example, is a phosphodiesterase while the Type I toxins are N-glycosidases.

[1] Abbreviations used: CDR: complementarity-determining regions; DT: diphtheria toxin; EGF: epidermal growth factor; Fc: fragment crystallizable; GVHD: graft versus host disease; HAMA: human anti-murine antibody; IT: immunotoxins; KDEL: endoplasmic retention signal sequence; LDL: low density lipid; PAP: pokeweed antiviral protein; PE: pseudomonas exotoxin A; PEG: polyethyleneglycol; RIP: ribosomal inhibitory proteins; SCA: single chain antibodies; SPDP: N-Succinimidyl 3-(2- pyridyl)dithiopropionic acid; TCS: trichosanthin; TFR: transferrin receptor; tPA: tissue plasminogen activator.

RICIN

Structural Features

Ricin is a toxic lectin isolated from castor beans. Its precursor is a single polypeptide composed of a signal peptide and two chains linked by a 12 amino acid joining peptide (J peptide). It is proteolytically cleaved to form the mature ricin of 62 kd containing an A chain (267 amino acids) and a B chain (262 amino acids) linked by a disulfide bond (11). The toxin is post-translationally glycosylated on both A and B chains with high mannose branched chains containing xylose and fucose. The A chain has two N-linked sugars at positions 10 and 236. The B chain has N-linked carbohydrates attached to residues 95 and 135. Heterogeneity in composition of sugars has been observed in all four sites (12). In the mature ricin molecule the A chain is linked to the B chain by a reducible bond between Cys 259 of the A chain and Cys 4 of the B chain. Ricin has been crystallized, and the three-dimensional structure has been elucidated by Robertus and colleagues (13, 14) at a resolution of 2.5 Å. Interaction between the subunits is mainly due to hydrophobic forces among various aromatic rings and aliphatic side chains.

The carboxy terminal of the B chain seems to be involved in association with the A chain. Three domains can be distinguished in the A chain by X-ray crystallography (14). Domain I consists of amino acids 1–117 dominated by a six-stranded B-sheet and two helices. Domain II encompasses residues 118–210 and is mainly made of alpha helices. The C-terminal end constitutes Domain III, and the striking feature of this part of the molecule is a stretch of hydrophobic residues located between positions 247 and 257. This region of the molecule is suggested to play a key role in the translocation of the toxic subunit into the cytoplasm.

The A chain of ricin is a specific N-glycosidase that catalytically hydrolyzes the glycosidic bond of adenosine residue 4324 in the 28S rRNA (15). Depurination results in irreversible inactivation of ribosomes and inhibition of protein synthesis. Ricin is such a potent toxin that a single A chain molecule can inactivate 1500 ribosomes per minute and once in the cytosol can kill the cell (16). The Km for reticulocyte ribosomes is about 0.1–0.2 μM and the Kcat about 1,500 min^{-1}. The depurination mechanism of ricin is predicted to be similar to that of adenosine monophosphate nucleosidase involving the formation of an oxycarbonium ion. Based on site-directed mutagenesis and X-ray diffraction studies, important residues of the A chain either in direct contact with the substrate or those involved in the actual catalysis have been identified. The catalytic site is present in a cleft; Tyr 80 and Tyr 123 are present at the top of the cleft. Neither is believed to be important for catalysis but both may contribute to the tight binding of the substrate. Substitution of

other residues present around the cleft such as Asn 209 and Trp 211 does not significantly change the enzymatic activity (17, 18).

Tight binding of the substrate (28S rRNA) is responsible for the partial breakage of the N-C bond (18). Two important changes involving either Glu 177 or Arg 180 have consistently been shown to dramatically decrease the biological activity of the A chain. The positive charge on Arg 180 is supposed to help in the partial breaking of the bond by ion pairing to the phosphate backbone. The destabilized N-C bond is then vulnerable to nucleophilic attack by a water molecule. Interestingly, intact ribosomes are better substrates than the synthetic oligomers homologous to the conserved region covering the depurination site. It is therefore likely that the ribosomal proteins may contribute to the proper presentation of the sensitive site in a manner facilitating catalysis or that some important regions of ricin A chain may interact with ribosomal proteins in stabilizing the enzyme-substrate complex. This issue is currently being investigated by genetic deletion analyses in many laboratories.

The B chain of ricin has two functions: (*a*) binding reversibly to galactose presented by cell surface glycopeptides and glycolipids, and (*b*) facilitating uptake of whole ricin by endocytosis and transport through the Golgi apparatus before translocation of the A chain into the cytosol (19). It is still contentious whether the two functions of the B chain are separable. Some studies indicate that toxin entry could be mediated by mechanisms independent of galactose recognition, but other evidence supports the galactose binding activity on the B chain as an important step in the entry to the cytosol (20–24).

Conjugates Prepared with Ricin and Ricin A Chain (RTA)

Many immunotoxins using intact ricin and monoclonal antibodies or growth factors have been developed (25, 26). They are extremely cytotoxic to relevant target cells, but the specificities are suboptimal since B chains may bind to surfaces on nontarget cells. Another problem is the rapid clearance of ricin IT from the bloodstream (27). This is due at least partially to the clearance of native ricin by oligosaccharide-mediated uptake by the liver cells (28, 29). To circumvent this problem, immunotoxins have been made with chemically/enzymatically deglycosylated A chain (30, 31) or recombinant A chain. Chemical treatment destroys the mannose and other sugar residues on native A chain while recombinant A chain expressed in bacteria is free of B chain and completely lacks oligosaccharide side chains. These constructs have better pharmacological properties than conjugates made with native ricin A chain.

Several groups have obtained ricin A chain (rRTA) by genetic engineering techniques. Shire et al (32) expressed a synthetic 842 bp RTA gene in

Escherichia coli at the level of 1.5 mg/liter of culture. O'Hare et al (33) cloned ricin A chain cDNA using vector pDs/3 and expressed the recombinant A chain with ten extra amino acids at the N-terminus in *E. coli* at the level of 2–3 mg/liter of culture. Piatak et al (34) obtained a correct 267 amino acid A chain from *E. coli* using several different host/vector systems at the level of 1–2% to 6–8% of total cell proteins. Frankel et al (35) used an expression system to produce a fusion protein containing ricin A chain linked to the enzyme β-galactosidase via a collagen fragment. Treatment of the fusion protein with collagenase released free ricin A chain that could be recovered from the enzyme by selectively absorbing the latter on an affinity column. In our laboratory, the coding region of the ricin A chain gene was cloned into the pET3b vector. The cloned fragment is flanked by the T7 promoter and terminator in this construction and expressed in *E. coli*, BL21(DE3). The production of purified A chain protein with 11 extra amino acids at the N-terminus was 80–90 mg/liter of culture or 24–26% of total cell proteins.

The specificities of the IT made from nRTA or rRTA are excellent due to the absence of nonspecific binding of B chain (27, 36–39). However, the cytotoxicities of these conjugates were relatively less (40) than intact ricin IT due to the loss of translocation functions asssociated with the B chain (40–43). Some of the immunoconjugates prepared with ricin A chain were not cytotoxic, whereas the same antibody linked to ricin was effective against target cells in the presence of lactose. Translocation signals associated with the B chain of ricin have been effectively used in potentiating ricin A chain conjugates. The B chain can be either delivered directly to cell bound A chain conjugates or indirectly by a second antibody homing onto the same cell. In both approaches however, lactose has to be included to prevent nonspecific toxicity. Alternatively, intact ricin could be modified (blocked ricin) by chemical modifications (41–43) that retain the translocation properties without the nonspecific toxicity mediated by the sugar binding sites. The cell binding, enzymatic specificity, and mechanism of cytosol entry between bacterial toxins and plant toxins are very different.

SINGLE CHAIN RIBOSOMAL INHIBITORY PROTEINS (TYPE I RIP)

This class of proteins is used as an alternative in immunotoxin preparations and has many advantages in the treatment of several clinical diseases. Perhaps the best-studied single chain toxins are pokeweed antiviral proteins (PAP) isolated from the plant *Phytolacca americana,* saporin 6, isolated from the plant *Saponaria officinalis* and gelonin purified from *Gelonum multiflorum.* Similar proteins have been isolated from other plants (reviewed in 44) and are

generally basic proteins of about 30 kd. Most are nonglycosylated although recent evidence suggests the presence of simple sugars. The mechanism of action of Type I RIP seems to be similar to ricin in depurinating a single adenine residue at position 4324 of 28S rRNA.

A major problem in using dual chain toxins (Type II) is that they also bind to saccharide residues present on normal cells. Before they can be used in IT preparations, these toxins must either be modified to prevent binding or must have their B chain removed. Single chain toxins, on the other hand, do not bind to any cell surface components. Structurally and evolutionarily, Type I RIP are related to Type II heterodimeric toxins. They usually do not contain free sulfhydryl groups (SH); these are often introduced to facilitate conjugation to antibodies. SH groups can be introduced by modifying the epsilon amino group of lysine that, in certain instances, has led to a reduction in biological activity. Modification of lysine residues with reagents such as SPDP results in the loss of a positive charge on the molecule. Alternative thiolation procedures using 2-iminothiolane retain the positive charge and do not significantly reduce the biological activity of the toxins.

Pokeweed Antiviral Protein (PAP)

Three different forms (PAP-I, PAP-II, and PAP-S) of RIP have been isolated from the plant *P. americana* (45, 46). PAP-I and II were found in the leaves, and PAP-S was purified from the seeds. Pokeweed plants express the various forms of PAP at different seasons (46). Amino terminal sequences of these proteins are homologous with minor variations and there is limited immunological crossreactivity between these proteins. Recent studies have shown that PAP inactivates ribosomes in a manner similar to ricin A chain (47). IT prepared with PAP have been highly specific in cytotoxicity to target cells (48–50). These conjugates proved useful in bone marrow purging and showed minimal toxicity to pluripotent progenitor cells.

PAP-linked conjugates could be potentiated severalfold by ammonium chloride and monensin (51), which indicates that the conjugates were routed through acidic intracellular compartments. Since PAP does not have complex sugars, it is relatively more stable than native ricin A chain conjugates (52). Free PAP, however, is rapidly excreted from the circulation. PAP-IT directed to CD40 proved to be effective against clonogenic cells from acute lymphoblastic leukemia and non-Hodgkin's lymphoma in the absence of potentiators. PAP-IT specific toward CD7 were only effective against a T cell acute lymphoblastic leukemia lineage (53). A recent study showed that Type I RIP including PAP are useful in inhibiting phagocytic parasites when targeted via antibodies (54).

Saporin

Saporin is resistant to proteolysis, has a pI greater than 10, and has no carbohydrate chains. Yoshikawa et al (55) have described the separation of two distinct saporins from the seeds, N(1) and P(2). When conjugated to an anti-CD4 antibody, saporin kills CD4 cells effectively. Neither CD4 nor CD8 cells internalize RTA-IT made with anti-T4 or anti-T8 specificities; the cells are not killed by either of these IT (56). Another study included three saporin IT that recognized CD5, CD2, or CD3. All three IT bound T cells and were effective in inhibiting protein synthesis in cell-free systems. The anti-CD2 IT, however, had weak toxicity to whole cells, whereas the other two were quite potent. The anti-CD5 IT was marginally potentiated by amantadine (57).

Saporin conjugated to the transferrin receptor (TFR) suppresses leukemic stem cell generation. Progenitors of the erythroid lineage were more sensitive than those of the myeloid lineage. There was no effect in vitro on normal or leukemic human hematopoietic progenitors. Since TFR expression is associated with proliferation, it would be expected that the most susceptible cells would be those that are actively dividing. The antibody itself did not block TFR function (58). Similarly, Barbieri et al (59) found that saporin IT with antibodies directed toward plasma cell antigens bound and killed RAJI and U266 cells. Both had a high toxicity toward bone marrow, but only one inhibited differentiation of myeloid precursors. Bifunctional antibodies used to deliver saporin to specific tissues were tenfold more potent if the portion binding the toxin was obtained from a polyclonal mixture rather than a monoclonal source. This was shown for guinea pig leukemic cells (60).

Trichosanthin

Trichosanthin (TCS) is characterized by high content of Asn, Asp, Glu, Gln, and no Cys residues or carbohydrates. Its sequence is homologous to that of RTA, as is the tertiary structure. TCS loses over 90% of its activity when modified with SPDP; treatment with 2-iminothiolane resulted in the addition of 1.5 sulfhydryl groups per molecule on average. When conjugated to an anti-hepatoma antibody, the IT had a 50-fold higher toxicity than native TCS. Cytotoxicity of the IT was time dependent. Trichokirin, isolated from a related plant, is homologous but not completely identical to TCS (61). Trichokirin linked to an anti-Thy1.2 antibody was cytotoxic against T2 cells (62). Conjugation with dimethyl 3, 3'-dithiobis propionimidate (DTBP) resulted in only a small loss of activity; derivatization with SPDP and 2-iminothiolane reduced the biological activity significantly. The ability to inhibit cell-free translation was comparable to that of RTA and had the same enzymatic target. NH_4Cl had a negative dose-related effect on toxicity, characteristic of a requirement for passage through an acidic cellular compart-

ment. Monensin potentiated the trichikirin-IT fivefold. Clearance of the IT was slower than RTA-IT and had a biphasic pattern with half-lives of 0.5 and 8 hours.

A closely related toxin, β-trichosanthin, can be effective against cells in the lymphocytic and monocytic lineages and can also block HIV replication in acutely and chronically infected cells. A rank order potency for inhibition of protein synthesis is β-TCS, β-momorcharin, luffaculin > α-momorcharin > TCS > momorcochin. These toxins are homologous in amino acid composition, tertiary structure, have molecular weights between 28 and 32 kd, and have N-terminal Asp residues (63).

Other Single Chain RIP

Anti-Thy1.1 antibodies conjugated to momordin or bryodin with SPDP were effective against Thy 1.1 positive target cells in the low nanomolar range. Bryodin IT were slightly more toxic. A two- to fivefold loss of activity was seen following SPDP treatment. This loss can possibly be prevented by treatment with 2-iminothiolane instead. The positive charge in vivo (high pI) may shield the disulfide bond and confer higher stability in circulation (64).

Alpha Sarcin

The toxin α-sarcin is also a basic nonglycosylated protein, but has a molecular weight of 17 kd. The smaller size of this peptide could prove useful in penetration of solid tumors and be easier to use in clinical settings. It has no sequence similarity to plant RIP but is homologous to restrictocin and mitogellin. α-sarcin is an example of single chain toxins that hydrolyze the phosphodiester bond in the 28S rRNA. An α-sarcin-IT had similar pharmacokinetics as other RIP with the same antibody and was stable for 4 days at 37°C, much better than RTA-IT. The activity of the α-sarcin IT was 800-fold better than for α-sarcin alone and approached the potency of RTA-IT. The α-sarcin IT had a longer biological half-life in serum, with clearance following the biphasic pattern common to RTA-IT (0.8 and 6 hr half-lives). IT with gelonin or momordin behaved similarly (65).

The gene for α-sarcin has recently been sequenced (66) and shown to encode a stretch of 27 amino acids at the amino terminus that could potentially be cleaved. This could afford the cell protection against its own product. Restrictocin is homologous to α-sarcin and its gene has also been cloned and expressed, showing a single 52-base intron, possible regions for transcription signals, and polyadenylation sites flanking the open reading frame. An immature form of restrictocin may be produced that could undergo activation during secretion. Both genes show homology to the U2 ribonuclease. IT made with restrictocin were toxic in vitro (67). Other proteins have been used in IT (68–72).

PSEUDOMONAS EXOTOXIN A

Structural Features

Pseudomonas exotoxin A (PE) is a single chain protein toxin produced by *Pseudomonas aeruginosa*. The molecular weight of PE is 66,000 daltons, and in its native form PE has four intrachain disulfide bridges, no titratable sulfhydryl groups, and is devoid of sugars and other posttranslational modification (73). Structural analysis of PE has indicated that it is composed of three domains when interacting with mammalian cells. Domain Ia located at the N-terminal end of the molecule (aa 1–252) is responsible for cell binding. Domain II located in the middle of the molecule (aa 253–364) is suggested to be associated with translocation of the toxin moiety across cell membranes, and Domain III located at the C-terminal end (aa 405–613) possesses the ADP ribosylation activity. ADP ribosylation of elongation factor 2 irreversibly inhibits the synthesis of proteins and leads to cell death. The role of Domain Ib (aa 365–404) has not yet been determined (74). Domain II contains important information for protein secretion (75); the absence of Domain I shortens the half-life of OVB3-PE immunotoxin in mice (76). Once inside the cell, native PE undergoes proteolytic cleavage at a site rich in arginine residues. Primary sequence analysis indicates the presence of a consensus sequence homologous to the endoplasmic retention signal sequence (KDEL) at the C-terminal end. Amino acid substitutions at this region affect translocation of PE to the cytoplasm (77).

Pseudomonas exotoxin is a lethal toxin with an ID_{50} for a 20-gram mouse in the range of 0.1–0.2 μg. Liver toxicity is a major cause of the death in mice (78–80). Intact PE has been coupled to a variety of tumor-binding monoclonal antibodies (for example 260F9, 454C11, 280D11, JL1, 106A10, 245E7, 520C9) to produce potent IT, with ID_{50} in the 0.01–0.1nM range after a 24 hr exposure of cells to IT (8). In addition to killing cancer cells in culture, PE-containing IT are also effective in inhibiting tumor growth in mice. However, PE conjugates cannot be given in large amounts to animals or patients because of residual binding of PE-IT to normal cells (74).

To minimize this undesirable side effect, Chaudhary et al (75, 81) used genetic engineering to construct a plasmid containing codons for amino acids 252–613 of PE and changed the glutamic acid at position 252 to lysine by site-directed mutagenesis. This construct was expressed in *E. coli* and produced a protein of 40,000 daltons (Lys PE40) that was 100-fold less toxic to mice than was intact PE and lacked Domain Ia of PE (74). An extra lysine residue at the N-terminus of PE40 also facilitated derivatization with cross-linking reagents (75, 81). Lys57 (in Domain Ia) was shown to be involved in the cellular binding of PE (82) and, when mutated to arginine, decreased the

toxicity to murine cells in vitro and also was less toxic to mice. Use of PE^{arg57} as the toxic moiety in IT also reduced liver damage.

Cytotoxic Conjugates Made with Native PE and Genetically Engineered PE

Pai et al (76) coupled a monoclonal antibody OVB3, which reacts with many human carcinomas, to native PE, lys PE40, and PE^{arg57}, and compared the characteristics of the three IT. Each was cytotoxic to human tumor cell lines expressing the OVB3 antigen on their surfaces. All three IT caused complete regression of $50mm^3$ tumors with no toxic effects to the animals at therapeutic doses. The half-life in blood of OVB3-PE and OVB3-PE^{arg57} was 20 hr, whereas the half-life of OVB3-LysPE40 was 4 hr. But the single dose LD_{50} value for OVB3-lys PE40 (200μg) was much larger than for OVB3-PE or OVB3-PE^{arg57} (2.5 μg and 3.5 μg). This means OVB3-lys PE40 can be administered in higher doses to treat cancer more efficiently (76). In a recent study, PE linked to an antibody directed to human cervical carcinoma was evaluated for tumor growth inhibition in vivo. The $F(ab)_2$ fragment of the antibody was chemically conjugated to PE via a reducible or nonreducible thioether bond. Both conjugates were tumor suppressive when injected 4 to 14 days after tumor transplantation (83).

Immunotoxins made by chemically coupling protein toxins to specific antibodies are faced with low yields, formation of heterogenous products, and difficulties in large-scale production. To circumvent some of these problems, various chimeric toxins have been prepared by genetically fusing the coding sequences of toxin moieties to carrier proteins (84). Chaudhary et al (85) constructed a single chain antibody toxin fusion protein by ligating the DNA fragment of anti-Tac (Fv) with the gene of PE40. Anti-Tac is a monoclonal antibody to the P55 subunit of the human interleukin-2 receptor. A variable domain is the smallest binding unit of an antibody. The fusion protein anti-Tac (Fv)-PE40 was highly cytotoxic to two IL-2 receptor-bearing human cell lines but was not cytotoxic to receptor-negative cells (85). PE-40 was also linked to the HIV-binding portion of the human CD4 molecule to create a hybrid protein, CD_4-PE40. CD_4-PE40 displayed selective toxicity toward cells expressing the HIV envelope glycoprotein gp120 and could therefore be used as a therapeutic agent for the treatment of Acquired Immune Deficiency Syndrome (86).

In addition, many receptor-specific chimeric toxins have been constructed by fusing cDNAs encoding cytokines (TGFα, IL2, IL4, IL6) to the gene of PE40. Each chimeric protein was specifically cytotoxic to the appropriate receptor-bearing cells (87–90). Interestingly, the position of the ligand moiety, i.e. cell recognition element, in the chimeric molecule might be important. When the N-terminus of PE40 was fused to the C-terminus of TGF-α

(TGF-α-PE40), the conjugate was 30-fold more potent than a fusion between the C-terminus of PE40 to the N-terminus of TGF-α (PE40-TGF-(α). The former construct recognized and bound with about the same affinity as EGF. In native PE the cell recognition domain (Ia) is attached directly to the translocation domain. The close association of the receptor recognition and translocation domains may possibly facilitate movement of toxin into the cytosol (88).

DIPHTHERIA TOXIN

Structural Features

Diphtheria toxin (DT) is a single polypeptide exotoxin secreted by *Corynebacterium diphtheriae* and is lysogenic for bacteriophage Beta^{tox+} that carries the gene for DT (91, 92). Intact DT has two disulfide bridges. After mild trypsin digestion ("nicking") and reduction of the disulfide bonds, DT can be separated into an N-terminal 21.17-kd fragment A of 193 amino acids (1–193) and a C-terminal 37.20-kd fragment B of 342 amino acids from position 194 to 535 (8, 93, 94).

The A chain enzymatically ADP ribosylates a unique amino acid (diphthamide) of elongation factor-2 (EF-2), resulting in irreversible inactivation and termination of cellular protein synthesis (95). The B chain has two functions: binding cell surface receptors and translocation of the A chain to the cytosol. Binding activity is located in the 8-kd C-terminal fragment of the B chain, and the last 50 amino acids are confirmed to be required to form the receptor binding domain (94, 96). The translocation activity is in the N-terminal three fourths of fragment B. From amino acids 270 to 370 and amino acids 420 to 440 in fragment B there are four extremely hydrophobic regions. During the endocytosis process, exposure to pH below 5.5 promotes a conformational change that exposes the hydrophobic domains (97) and promotes both toxin insertion into the endosomal membrane and eventual translocation to the cytosol (98, 99). These two functions of the B chain can be separated. Greenfield et al (100) identified a point mutation of amino acid 508 that blocks binding but allows cytosol entry.

In addition, the C-terminal 50 amino acids stabilize the conformation of DT and its binding function. Removal of these 50 amino acids makes DT a better target for proteases (94, 101). Bishai et al demonstrated the necessity of the disulfide bridge between Cys461 and Cys471 for toxicity. The point mutation at Cys471 (converting Cys to Tyr) reduced toxicity by 1000-fold when compared to native DT (96, 99). Mekada et al determined that one amino acid residue substituted at position 52 (from Gly to Glu) in fragment A altered binding properties of DT to cells. Binding of the mutant is significantly affected by nicking and unaffected by ATP, in contrast to wild-type DT (102).

DT is a very potent toxin. One molecule can inactivate 100 EF-2 molecules per minute in vitro, and one molecule in the cytosol can kill a cell. DT is toxic to animals and humans at a dose as low as 100 ng/kg (101, 103, 104). Mice are somehow resistant to DT. Antibodies directed against cell surface antigens have been linked to intact DT (105–107). These IT were very potent but were not specific enough for use in humans because of the presence of fragment B (107–109). Several immunotoxins containing only the A chain of DT have also been made (105, 110, 111) that were relatively less cytotoxic to target cells because the translocation function of the B chain was absent.

IT Prepared with DT and Truncated DT Molecules

Ideal conjugates should have potent cytotoxicity and high specificity. Since the binding and the translocation functions of B chain can be separated, genetic engineering techniques facilitated the design and construction of new conjugates or fusion proteins containing the A chain and the portion of the B chain involved in translocation, or of DT mutants lacking the binding function (112).

Greenfield et al constructed the fragment coding for the N-terminal 382 amino acids of DT linked to a synthetic oligonucleotide spacer arm with a Cys at the C-terminus. The protein produced by this gene, MspSA, included the A chain and three hydrophobic regions of the B chain but deleted the fourth hydrophobic region and binding region (8, 113). Colombatti et al linked the intact A chain of DT and MspSA to the monoclonal antibody UCHT1, specific for the T3 antigen on human T cells, and identified that UCHT1-DTA and UCHT1-MspSA were 10,000-fold and 100-fold less potent than UCHT1-DT, respectively. This finding indicated that the three hydrophobic regions of the B chain potentiated UCHT1-MspSA activity 100-fold but could not confirm if another 100-fold potency reduction was associated with the fourth hydrophobic region or binding function, or both (8, 108).

Based on structure-function studies, novel chimeric proteins have been made by using a truncated form of DT. Murphy et al constructed two fusion proteins: DAB_{486}-αMSH (α-melanocyte stimulating hormone) and DAB_{486}-IL2. They selected an SphI site, the first unique restriction enzyme site in the gene that is 3' to the codon for Cys471, as the fusion junction between DT and the hormone sequence. The resulting fusion protein was composed of the A chain, the portion of the B chain up to Ala_{486} including the four hydrophobic regions but not the binding domain, a linker, and αMSH. The chimeric toxin was expressed in *E. coli* and found to be selectively toxic for αMSH receptor-positive human melanoma NEL-M1 cells and not effective against αMSH receptor-negative CHO-K1 or CN-1 cells (114). In a parallel study, a DAB_{486}-IL-2 fusion protein was made with a strategy analogous to that used for the DAB_{486}-αMSH (96, 114). The fusion protein, in which the binding

domain was replaced with a fragment of IL-2, selectively inhibited protein synthesis in T cell lines expressing high-affinity IL-2 receptors. Cell lines devoid of high-affinity IL-2 receptors were resistant to the DAB_{486}-IL-2 fusion toxin (114). Since the high-affinity IL-2 receptors appear to be largely limited to activated T cells and recently activated B cells, DAB_{486}-IL-2 may have application in a variety of disorders, e.g. in adult T cell leukemia, allograft rejection, and certain autoimmune diseases (115). Over 60 patients have recently received this fusion toxin. The results showed that DAB_{486}-IL-2 was well tolerated; antitumor effects were seen in about 40% of patients. The most interesting observation was that this toxin selectively eliminated HIV-1 infected cells from mixed cultures of infected and uninfected cells and inhibited production of viral proteins and infectious viruses.

It would be desirable to delete the binding site on DT without affecting the translocation properties of the B chain. This was achieved by point mutations blocking the B chain binding site. Laird & Groman mutagenized *Corynebacterium* with nitrosoguanidine and ultraviolet irradiation and identified point mutations CRM103 (Ser^{508}-Phe) and CRM107 (Ser^{525}-Phe) that blocked binding and decreased the toxicity 1000 to 10000-fold (8, 116). After linking CRM103 and CRM107 to a monoclonal antibody, full toxicity was restored, indistinguishable from the DT-IT. Selectivity was increased four orders of magnitude over the 100-fold selectivity seen in native DT-IT (8).

In spite of these exciting achievements, the application of DT-based reagents is limited because the general population is immunized against DT. Exceptions are the DT-IL-2 conjugate or fusion protein that may prevent a secondary immune response against the reagent (8). Genetic engineering of this toxin may lead to the broad application of DT by identification and deletion of immunogenic regions in DT or domains not essential for DT B chain function. Such toxin molecules would not be recognized by preexisting antibodies in circulation (99).

OTHER TOXIN MOIETIES

Single chain toxins such as Shiga-like toxins (117) and mistletoe lectin A chain (118) have also been used in the construction of active immunotoxins. Shiga toxins are structurally related to ricin A chain. The translocation efficacy of Shiga toxin was improved by genetic fusion with DTB. A tripartate fusion (reviewed in 1) generated a chimeric molecule containing Shiga toxin A chain fused with DT-B and IL-2. The resultant protein had a specific cytotoxicity to IL-2 receptor-bearing cells at picomolar concentrations. The fungal toxin restrictocin has also been used as a cytotoxic conjugate (119). The relative in vivo efficacies of these conjugates has yet to be investigated.

CELLULAR PROCESSING OF IMMUNOTOXINS

Binding

Antibodies used in immunotoxins (IT) can be directed toward various cell surface molecules. The receptor for transferrin notably is present in high quantities on many cells and thus can bind a large amount of IT. Other cell surface molecules that are not expressed in high quantities can potentially be upregulated in a cell-specific manner to increase the amount of binding relative to other cells.

Within a given antigen, differences among epitopes can be measured in terms of the efficacy of immunotoxins directed against them. Of several RTA-IT tested with specificity toward CD2, one antibody in particular had a 100-fold higher potency than the others. Cross blocking experiments showed that the most effective IT bound Region II of CD2, as defined by Peterson & Seed (120); this is the only domain of CD2 not involved in T cell activation. IT reactive with Region II were also retained longer by the cells before degradation. The other antibodies bound epitopes farther from the cell membrane belonging to Region I of CD2 (121). A similar result was obtained with antibodies raised against mouse IgD heavy chains. The resulting antibodies reacted with various epitopes along the length of the delta chains. RTA-IT most effective against murine B cells included antibodies directed against regions of the heavy chain closest to the cell membrane (122). The effect of epitope recognition by these IT appears to influence potency by altering the intracellular trafficking of the conjugates (123).

RTA-IT using antibodies to CD10 affected surface expression of the antigen depending on which domain was recognized. One induced down regulation of CD10 and was less effective in a nude mouse model than a different antibody that did not modulate CD10 (124). Modulation of surface antigen density could account in part for the differences in toxicity observed in other studies.

CRM107, a mutant of DT, has two amino acid substitutions in the B chain that together reduce its binding 8000-fold and toxicity 10000-fold. When CRM107 was conjugated into an IT, full toxicity was restored but nonspecific B chain binding remained blocked (125). IT directed against glycolipids can potentially be useful since native ricin, cholera, and tetanus toxins can bind these molecules before endocytosis from uncoated membranes. Coated pit uptake has also been observed from glycolipid complexes. Binding is probably due to the lectin properties of these toxins (126).

Uptake of Immunoconjugates

After binding to a cell, IT are usually internalized by receptor-mediated endocytosis into a network of endocytic compartments. Some toxins, DT and

PE for example, bind diffusely along the entire surface of cells and are later actively clustered to a high density at clathrin-coated regions of the plasma membrane (127, 128). It seems likely that IT also are bound and concentrated in this manner. An unknown sequence of events triggers invagination at the clathrin-coated sites, followed by pinching off of the membrane to form cytosolic vesicles. Both the endosomal (129) and lysosomal networks may consist of vesicles as well as tubular membranous structures best visualized by rapid freezing techniques (130). An alternative method of entry into the cell seen with some RTA-IT is a nonspecific temperature-sensitive fluid phase uptake (8). The acidic pH found in endosomes and lysosomes is not a requirement for vesicular budding in either mechanism.

No strict correlation between antigen density and the amount of IT internalized was noted in T cells; T cell lines with a high density of CD8 could sometimes take up only a small amount of IT (131). Given surface markers may have intrinsic efficiencies for internalization as shown by the rank order by degree of internalization CD7 > CD3 > CD5 (131) compared to surface expression on T cells in the order CD5 > CD3 > CD2 (121). Furthermore, the anti-CD8 antibodies used in this study did not induce antigen modulation. Handling of RTA-IT varied between cells tested, and lysosomotropic amines such as NH_4Cl had no effect on the amount of IT internalized (131).

Binding by itself does not necessarily lead to ligand uptake. Goldmacher et al (132) grouped the receptors for transferrin (TF), low density lipid (LDL), and epidermal growth factor (with EGF as a ligand but not with an anti-EGFR antibody) together as proteins that can endocytose ligands to a higher intracellular concentration than is found outside the cell. Those that can endocytose to a lower concentration inside include Thy1, T3, CD10, ricin-binding sites, the mouse T cell receptor, and the IL-2 receptor. The receptors exhibiting more effective uptake would probably have the highest potency when targeted with IT. Antibodies binding B4 were only internalized when conjugated to gelonin (132), which implies involvement of the toxin moiety in IT endocytosis.

Internalization from receptor-ligand complexes is slow if the receptors are not clustered, but coated pits can form from a flattened membrane within 20 sec. For a typical cell with at least 1000 coated pits, this can account for very rapid uptake of extracellular materials (133). Using an IT with mistletoe lectin A chain, Weidlocha et al (118) found that most clathrin-mediated uptake is of glycoproteins and that specific amino acids may serve as recognition sites. Wargalla & Reisfeld (134) reported that the correlation between internalization and toxicity is valid only if degradation is not a major factor in the lifetime of the IT.

In T47D cells, RTA-IT are concentrated in patches and endocytosed by coated pits. A second slower pathway begins with membrane invagination,

vesicle formation, and fusion with lysosomes. IT used in these studies were directed to the MAM-6 carcinoma antigen. Regardless of the internalization route, IT cross into the cytosol intact, without separating into antibody and toxin components (135). Experimental separation of clathrin-dependent (i.e. TF) and independent uptake can be accomplished with cytochalasin D and colchicine, agents that disrupt microtubules and filamentous actin (136). Nonclathrin-mediated endocytosis can be selectively blocked in Vero cells by cytochalasins but stimulated in A431 cells by phorbol esters and EGF (118).

Vesicular Traffic

Some endocytosed vesicles immediately return to the cell surface and release their contents to the extracellular fluid (137). More likely though, the endosomes become acidified in a process that can be monitored from the extracellular pH of 7.4 to less than 5.0 upon fusion with a lysosome. A low pH environment is necessary for the dissociation of receptor ligand complexes and for activity of intact toxins such as DT (8). However, reduction of disulfide bonds is optimized at neutral pH (138), which is important for most chemically linked toxin-antibody conjugates.

The toxin and antibody moieties of an IT can have different destinations within the cell, or one can be returned alone to the cell surface. Between early endosome and lysosome there is room for variability in processing. If passage through additional compartments can extend the life of an IT before lysosomal destruction, then increased cytotoxicity could be attained using carrier molecules that might direct the toxin to these extra organelles. Both PE and ricin pass through the Golgi apparatus (139, 140), whereas DT does not (141). PE-IT entry to the Golgi can be prevented by NH_4Cl or methylamine (139). Recycling of receptors or IT can be blocked using colchicine, which interferes with microtubule mediated exocytosis (135).

Ricin can be recycled to the plasma membrane in a biphasic manner, implying two mechanisms for this process (137). These could involve recycling directly after endocytosis or after additional fusion steps with Golgi components. Of the total amount of ricin entering a cell, about 5% is directed to the trans Golgi, arriving there via endosomal vesicles. Sorting of receptor and ligand occurs in endosomes followed by passage through the Golgi, as shown by experiments with desialated TFR; these receptors are recycled to the cell surface in a sialated form. The enzymes responsible for sialation are localized to the Golgi (142).

The kinetics of internalization have been investigated using ricin, modeccin, DT, and PE. An initial lag phase was observed followed by approximately first order inhibition of protein synthesis in each case. In broad terms, the lag phase would include all steps from toxin binding to translocation into the cytosol, with the transmembrane movement being the rate-limiting step.

First order kinetics would apply to enzymatic ribosomal inactivation. Endosomal acidification was necessary only during the lag phase for the latter three toxins (143). The internalization kinetics of IT using RTA or intact ricin have similar one hour lag times before significant accumulation of toxin is observed; the complete process takes from one half to four hours. Some of this uptake could be nonreceptor mediated. If ricin B chain alone or coupled to an antibody is given in conjunction with RTA-IT, the toxic effects are increased and appear to be independent of surface binding or antigenic modulation (144).

Chinese hamster ovary cell mutants deficient in endosomal acidification were developed by Ghosh & Wu (145). The major route of modeccin intoxication is via low pH endosomes, consistent with the resistance of the mutant cells. Nigericin (10 nM) activates an alternative uptake for modeccin but blocks the main pathway, possibly by interfering with vesicular fusion. No protection of the cells from DT was obtained with this concentration of nigericin. Ricin was released from endosomes more efficiently in the acidification mutants than in wild-type cells (145). Cain et al (146) described a major role of the sodium/potassium ATPase as a regulator of endosomal pH. In a preparation of turtle bladder epithelium, two ATP driven mechanisms are proposed for creation of the transmembrane pH gradient. Neither is sensitive to ouabain or oligomycin, selective blockers of the Na^+/K^+ ATPase and the mitochondrial F0/F1 pump respectively. They can be distinguished by inhibition by vanadate; the vanadate-sensitive pump requires the presence of vesicular potassium and is conceivably a K^+/H^+ exchanger. The H^+ gradient can be increased with this mechanism by the stimulation of valinomycin in KCl-based medium.

The fluorescent label FITC is quite useful in following the acidification of intracellular vesicles. Its fluorescence intensity drops linearly with a decrease in pH from 7.3 to 4.0, and this also correlates with the toxicity of various IT. With this method, Chignola et al (146a) have shown that anti-CD3-DT is endocytosed to vesicles with lower pH than anti-CD5-DT. IT composed of anti-Thy1.2 and intact DT are slow and can be traced to nonacidic compartments, in contrast to the normal route through endosomes. An in vitro system was developed by Shi et al (147) using endosomes from kidney proximal tubules; the sensitivity of FITC-dextran allowed measurement of acidity to a resolution of 0.2 pH units.

Nature of Chemical Linkage and Potentiation of Immunotoxins

Reduction of disulfide bonds is a two-phase process that probably begins at the cell surface. Feener et al (144) used the reagents DTNB and pCMBS to

modify free sulfhydryl groups. Since these reagents are not bound to membrane receptors, they should be sorted from internalized IT and expelled from the cell. Their intracellular concentrations should therefore be near zero, but significant labeling of proteins was observed. Endosomes or lysosomes are most likely not the site of the second phase of disulfide cleavage because reduction is favored at a neutral pH. The three enzymes known to mediate disulfide reduction each have a pH optimum of 7 and have been localized to the Golgi (144). The second phase probably occurs in the Golgi and is not due to recycling of conjugates to the cell surface.

Immunoconjugates linked by disulfide bridges are more effective if the two components are able to dissociate. Since disulfide bonds have increased stability at acidic pH, compounds that raise the pH of intracellular compartments could enhance the potency of IT linked in this fashion (148). Cation ionophores such as monensin neutralized acidic endosomes tenfold better than did lysosomotropic amines (149). This effect required the passage of IT through an acidic vesicle. Lysine residues were used for disulfide linkage after derivitization by SPDP, but these conjugates were not affected by monensin treatment. On the other hand, monensin did potentiate IT linked by thioether bonds even though they had an intrinsically low toxicity (148). The calcium channel blockers verapamil and nifedipine had different effects on IT activity: the first enhanced PE-IT toxicity (150), but the second had no effect on RTA-IT (148). Verapamil treatment increased IT accumulation in lysosomes while preventing degradation. The mechanism of action depended on the concentration of verapamil but is probably not related to calcium antagonism (151). In vivo use of calcium channel blockers is precluded by significant cardiac toxicity (152).

The direction of intracellular trafficking of a toxin is determined in part by the molecule to which it is conjugated. RTA, for example, passes through the Golgi if linked to RTB (as intact ricin) or an anti-TFR antibody, but not if linked to TF itself. RTA also goes through an acidic compartment only if linked to TF. In a similar way, the carrier protein can determine the effects of monensin on toxin routing. Monensin acts proximally to the pre-Golgi and thereby may short-circuit the requirement of RTA to reach this organelle for activity (129). Golgi compartments, however, are more sensitive to low monensin concentrations than are endosomes (142).

A common method for following the progress of endocytosed materials between organelles involves coupling to colloidal gold and tracing the marker by electron microscopy. Care must be taken in interpreting results from this procedure, as illustrated by a comparison in trafficking of TF with and without the label (133). TF normally is recycled in tubular elements of the endosomal network, but when coupled to 15nm colloidal gold it is targeted to

lysosomes. There is apparently no vesicular fusion with mitochondria or other double membrane organelles.

In most IT with a ricin component, the B chain is removed to prevent nonspecific binding to galactose residues on the cell surface. The B chain additionally seems to influence trafficking of vesicles within the cell: RTA-IT have a greater chance of being directed to lysosomes than those made with intact ricin. Monensin alters vesicular traffic, causing RTA-IT to traffic as ricin-based IT. The Golgi is the main site of trafficking arrest in monensin-treated cells (153). The effect of NH_4Cl probably also lies in modification of vesicular traffic (131).

An interesting demonstration of intracellular vesicle trafficking is shown by a study using saporin conjugated to antibodies against Thy1. These IT were injected into the vagus nerve or caudate nucleus and destroyed neurons that project axons to the area of treatment. The agents were taken up and carried by suicide transport towards the cell body and killed the cell. A disulfide linkage was necessary for complete transport. If injected alone, the antibody itself was axonally transported, but IT were more toxic than saporin alone. Rat neurons express high levels of Thy1 but the IT could be absorbed by fluid phase uptake as well. Conjugates using modeccin or volkensin were effective in suicide transport although they were more nonspecific in toxicity (154). This experiment illustrates the ability of IT to remain in an endosome long enough to reach the cell body. If the IT were to enter the cytosol rapidly, it would have to passively diffuse the entire length of the axon and would probably be much less effective.

A cell-free system developed by Wessling-Resnick & Braell (155) made use of avidin-β-galactosidase and biotin-TF. Receptors were separated from their ligands at low pH and directed to tubular endosomes; the ligands were sent to lysosomes. In this system, vesicular fusion required ATP, was not pH dependent, and was sensitive to the alkylator NEM. The effects of NEM of vesicle fusion were reversible. The lysosomotropic amines chloroquine and NH_4Cl did not block sequestration of receptor and ligand.

Translocation

The cytotoxic effect of most of these molecules is mediated by inactivation of the 60S ribosomal subunit to block protein synthesis. This function can only be attained if the catalytic portion of the toxin reaches the cytosol. Considering that it is energetically unfavorable for a soluble protein to cross a hydrophobic lipid bilayer, this is generally the least efficient step of the process (143). If the immunotoxin cannot enter the cytosol, it will eventually be degraded by hydrolytic enzymes when its vesicle fuses with a lysosome.

The B chain of DT undergoes a conformational change and likely forms a

pore through which the A chain can pass (8). As the best-studied toxin, DT can serve as a model for designing IT modifications that could assist in transporting the toxin across the membrane, either from acidic endosomal vesicles or from neutral organelles such as the Golgi. Many pore-forming proteins have been studied, especially in the context of transport into the endoplasmic reticulum during protein synthesis and secretion of bacterial products. There are currently no reports of investigations into this matter with conjugates although it represents a large potential for improving the potency of existing IT.

Efficacy of Immunotoxins in Vivo

Most IT constructed from various toxins and antibody molecules have been effective in vitro in inhibiting the proliferation of tumor cells. However, only a few conjugates were effective in vivo and inhibited the tumor growth in experimental tumor model systems. In general, injection of IT at the time or immediately following tumor transplantation has led to decreased tumor incidence and/or slower growth of the tumor graft. A delay in the onset of treatment has met with only limited success in arresting the growth of the transplanted tumor. Even coadministration of potentiating reagents such as monensin, which had been previously found to increase the IT activity in cytotoxicity assays by three orders of magnitude, has failed to improve in vivo efficacy. This apparent difference between in vitro and in vivo effects is attributed to the pharmacological and toxicological properties of the IT. Specifically, the biological half-life, stability of the bonds between toxin and antibody molecule, sequestration by sensitive organs such as liver, inability to penetrate the interior of the tumor, and side effects related to capillary leakage are said to limit the application of IT in vivo.

Stability of Immunotoxins in Circulation

The disulfide bond between antibody and toxin is not stable in vivo. IT are cleared from systemic circulation in a biphasic manner. An initial rapid phase of clearance is followed by a prolonged, slower, beta phase. The beta phase accounts for the slow reduction of disulfide bonds between antibody and toxin molecule. Most conjugates are removed from circulation in 24 hr (156). The pharmacokinetic data suggest a two-compartment open model for IT clearance. Blakey et al (157, 158), Shire et al (32), and Wawrzynczak et al (27) compared the clearance of unconjugated monoclonal antibody immunotoxin made with native ricin (nRTA), dg-RTA, and rRTA from a synthetic gene or cDNA of RTA. The results indicated that the clearance of dg-RTA and rRTA-containing IT were very similar and far slower than that of the IT made with intact ricin or native RTA, but much more rapid than unconjugated

monoclonal antibody (159, 160). The latter means that clearance of RTA-based IT is not entirely attributable to the presence of sugar side chains (32).

An investigation into the clearance of IT made with various toxins found that all IT had a shorter half-life than their parent antibodies. The initial phase was probably due to receptor-mediated endocytosis by the mannose chains on ricin. IT with gelonin had no obvious alpha phase of elimination; the saccharides of gelonin apparently are not bound in the liver. The rapid elimination points to an alternative method of removal. An IT with the abrin A chain had the greatest longevity, and its disulfide linkage was the least susceptible to reduction by serum glutathione. The beta phase of elimination was comparable for IT with RTA, gelonin, or momordin, while that for abrin A chain IT was much slower. It is possible that the disulfide linkage in the abrin A-IT is protected from reduction, leading to an extended beta phase half-life (69).

Greenfield et al studied the tissue distribution of IT which had been labeled internally to avoid artifacts introduced in metabolic clearance by radiation-induced damage to proteins. It became apparent from these elegant studies that the disulfide bond between toxin and antibody is labile and the rate of clearance is not altered by retaining charge densities on the toxin moiety similar to the native protein (161). However, the pharmacokinetic properties of the IT were significantly altered when the derivatization was carried out by an analog of 2-iminothiolane containing a methyl group. Such modifications in the linkage strategies are believed to hinder the reduction of disulfide bonds by thiol exchange.

Novel conjugation strategies were developed by Thorpe et al (162) to establish a hindered disulfide that would increase the biological half-life of IT. When toxin molecules were linked to antibodies by nonreducible bonds, the stability of the conjugates was improved. However, the biological activity was substantially lower when compared to homologous IT containing disulfide bonds. Conjugates made from Type I RIP, for example, using a thioether bond showed longer beta phase with no change in the alpha phase of clearance (163). Similarly, IT made with cross-linking agents such as 4-succinimidyloxycarbonyl-α-(2-pyridyldithio) toluene (162) had a significantly longer beta phase of elimination. This was due to the presence of a hindered disulfide bond between the antibody and toxin molecule.

The presence of carbohydrates also contributes to the clearance from circulation. Mannose binding cells in the liver are implicated in the rapid removal of glycoproteins. Since the circulatory half-life affects tumor localization and the efficacy of immunotoxins, investigations were carried out to improve the half-life by removing carbohydrates by treatment with glycosidases (164). Alternatively, recombinant ricin A chain expressed in prokaryotes (devoid of carbohydrates) could be used. In this respect, Type I RIP are advantageous.

A comparison of IT prepared from Type I and II RIP indicates that certain structural features of the toxin moiety could significantly alter the biological half-life. Blakey et al (68) reported that a saporin-IT recognizing the Thy1.1 antigen had a greater effect than the comparable RTA-IT in a murine tumor model. The former IT was not cleared from the blood as quickly but was 5-fold more toxic to the liver. They proposed the existence of an alternative means of uptake for the saporin-IT. Parenchymal cells were 30-fold more sensitive to saporin than to RTA but were only 6-fold more sensitive to saporin-IT than to the RTA-IT. The saporin-IT also was 13-fold weaker in cell-free protein synthesis than was the RTA-IT. Siena et al (57) found that a single nontoxic injection of saporin-IT retained its biological activity after 48 hr in circulation. Abrin A chain conjugates, for example, were cleared slowly with a beta phase of 13.3 hr, whereas ricin A chain and gelonin had a beta phase half-life of 7.5 and 8.0 hr, respectively. Momordin, a Type I RIP, linked to the same antibody (Fib 75) showed a half-life of 0.71 hr (alpha phase) and 8.6 hr (beta phase).

One factor that may be responsible for the differences in in vivo stability is the pI of the toxin molecules. Abrin A chain is acidic (pI 4.6) whereas other RIP such as ricin A chain, gelonin and momordin are basic (pI 7.5, 8.15, 8.6, respectively). Local electrostatic interactions between antibody and toxin moiety could lead to the relative inaccessibility of the disulfide bond for reduction. Using a solid tumor model system, Sung et al (165) have recently evaluated the pharmacokinetic and tumor penetration properties of immunoconjugates made with DT. Compared to intact toxin molecules, the IT was slower in permeability. Blood-to-tumor transfer efficiency was 0.13 ml/min/g for IT compared to 0.29 ml/min/g of tissue for DT (165). Despite the slower transfer efficiency of IT, the plasma-to-tissue transport of IT was 60–100% higher in the tumor tissue than in muscle. Such a difference is attributed to the increased permeability of the tumor vasculature. Furthermore, the interstitial fluid flow was also higher for the tumor than that for muscle. Transfer of macromolecules from the plasma to tumor is not only dependent on the molecular size, chemical nature, and characteristics of the chemical linkage, but also is affected by the size of the tumor and its location (subcutaneous, intradermal, intraperitoneal, and capsulated or noncapsulated).

CLINICAL TRIALS

Phase I clinical trials are underway in many centers using IT made from different toxin moieties and carrier molecules. One trial is aimed at determining the utility of anti-T cell IT in preventing graft versus host disease (GVHD) and in the removal of residual leukemic cells from bone marrow grafts. A

second group of trials is directed to tumors such as breast and ovarian cancer. Significant progress was made in identifying dosage regimens that could give sustained levels of IT in circulation. Antibody-ricin A chain (native) conjugates directed to CD5 were administered at a dose of 200 μg/kg/day over a period of 10 days to patients with cutaneous T cell lymphoma (166, 167). In another study, anti-CD7-ricin A chain conjugates were used in patients with T cell leukemia. Both conjugates had previously been shown to be highly effective and specific in in vitro cytotoxicity studies. Only a partial response was seen in a limited number of treated patients. Invariably, anti-toxin and anti-murine IgG antibody responses were observed in all subjects.

Other clinical trials include (*a*) 260F9-ricin A chain (recombinant) for breast cancer (Duke University Medical Center, Durham), (*b*) 454A12-ricin A chain (recombinant) for ovarian cancer (Fox Chase Cancer Center, Philadelphia), (*c*) OVB3-PE in ovarian cancer patients (National Cancer Institute, Bethesda, and (*d*) native ricin A chain linked to melanoma-specific antibodies. All the trials noted mild to severe side effects including nausea, mild pain, chills, and fever. Some of the patients treated with 260F9-RA developed peripheral neuropathy, which was later found to be due to cross reaction of the antibody to neuronal components (168). This trial is currently suspended pending the development of second generation immunotoxins that are likely to have higher specificity. The trial conducted at Fox Chase Cancer Center used an antibody conjugate reactive to human transferrin receptor. Although transferrin receptor is ubiquitous in distribution, it was believed that the tumor cells may express a relatively high number of them when compared to normal cells and the route of administration of conjugates (intraperitoneal) may limit the toxicity in normal tissues. Two patients treated with this conjugate developed neurological complications, including paralysis. Extensive pathological studies have shown selective leakage of capillaries in certain areas of the brain leading to the neurological complication.

The last trial used an ovarian cancer-selective monoclonal antibody, OVB3 linked to PE (169). This construct was very effective in tumor model systems in inhibiting the growth of transplanted ovarian cancer cells. However, administration of this conjugate also had the side effects of neuropathy. It is believed that ovarian epithelium and neuronal tissues may share some antigenic components and that the selection of noncrossreactive antibodies may be critical in determining the therapeutic index. From the limited number of clinical trials it has become apparent that the toxicity associated with IT treatment is not due to the toxin moiety itself but to the crossreactivity of the antibodies to sensitive tissues.

Other reagents under development for clinical applications are (*a*) anti-transferrin receptor antibody linked to DT or the binding mutant of DT (CRM 107), (*b*) use of anti-B cell antibodies linked to deglycosylated ricin A chain,

e.g. anti-CD 19 and anti-CD 22 reactive antibodies (170), and (c) anti-T cell antibodies linked to blocked ricin (171). Vitetta et al (172) treated 15 patients with refractory B-cell lymphoma with an immunotoxin consisting of anti-CD22 antibody linked to deglycosylated ricin A chain. To improve the pharmacological behavior of the conjugate, the monovalent Fab' fragment of the antibody was used. In this Phase I study of dose escalation, the maximum tolerated dose was determined to be about 75 mg/m^2. A partial response was seen in 38% of the evaluable patients and lasted between 1 and 4 months. A phase II clinical trial is underway with one of the immunotoxins directed to malignant melanoma cells. These studies confirm that the application of IT in clinical settings has yet to be perfected. The early trials have been encouraging, but to achieve total response may require a combination of therapies along with IT. Additionally, biological response modifiers can be used to improve tumor localization and the therapeutic efficacy of IT.

SECOND GENERATION IMMUNOTOXINS

Antibodies are multifunctional molecules composed of four polypeptide chains: two light chains of about 220 amino acids and two heavy chains of about 440 amino acids. The integrity of the chains is maintained by noncovalent forces and disulfide bridges. Antigen binding domains form the Fab portion of the antibody; the constant region (Fc) is responsible for effector functions such as Fc receptor binding, complement fixation, catabolism, and placental transport. The framework and hypervariable regions that form the variable domains of the antibody play an important role in antigen binding. The framework regions are responsible for correct folding of the β-pleated structure and for interchain interactions that hold the domains together. The hypervariable regions or complementarity-determining regions (CDR) form loops adjacent to the β-sheets. These domains are actually responsible for antigen binding. Sequence comparisons show that each domain of heavy and light chain has three CDR flanked by four conserved framework regions (173). Antibodies are glycosylated molecules and usually carry sugar residues in the CH2 domain, an exception being murine IgA. The pattern of glycosylation seems to vary between antibodies from different species. Rarely, glycosylation sites are observed either in the VH (174) or VL domain (175). In general, antibody glycosylation does not influence antigen binding, but lack of glycosylation does affect some effector functions.

The smaller size of antibody Fab fragments makes them useful molecules in the development of immunodiagnostic and immunotherapeutic applications. Moreover, they penetrate tissue boundaries more effectively and have lower antigenicity and improved pharmacokinetic properties compared to whole antibodies (176). Fab fragments of antibodies can easily be prepared by

proteolytic cleavage with the antigen-binding activity similar to that of the parental molecule. The binding constants of a recombinant Fab fragment are similar to those of the proteolytic material (177). An interesting difference between Fab and Fv fragments is that the former include the variable and constant portion of light and heavy chains (CH1 domain only), whereas the latter refers only to the variable portion of the HC and LC.

A major milestone in antibody engineering was the coexpression of light and heavy chains in a single microbial host (178). The antibody fragment initially was expressed as a cytoplasmic protein (179) followed by its expression as a cleavable fusion protein, thereby protecting it from proteolytic degradation (180). This was followed by the simultaneous secretion of the antibody chain into the periplasm of *E. coli* resulting in correct folding in vivo. Fv fragments have been expressed in other systems including mammalian cells (181), plants (182), yeast (183), *E. coli* (177, 184), and baculovirus-infected insect cell lines (185). The two chains can be linked to form a secreted single chain Fv molecule, and major improvements in vector and purification strategies have been described (186–190). The synthesis and expression of variable regions of immunoglobulin has significant potential in clinical and basic research.

Gene technology was beneficial in creating Fv fragments resistant to proteolysis. The binding constants of Fv fragments were similar to those of Fab or whole parental molecules (191). The VH and VL domains associate only weakly and tend to dissociate upon dilution. Several strategies have been attempted to overcome this problem by covalently linking the two chains. Initially, chemical cross-linking agents such as glutaraldehyde were used, followed by intramolecular disulfide bridging (191). The use of peptide linkers to connect the two domains resulted in single chain Fv fragments. Initially, they were obtained as insoluble inclusion bodies but were later modified and expressed as functional secreted molecules.

Single chain antibodies (SCA) are an alternative to Fab and Fv fragments and have already proved to be valuable tools in studying structural and functional relationship between heavy and light chains. SCA construction involves de novo bacterial synthesis of a single gene encoding the variable regions of a monoclonal antibody (VH and VL) spliced with a peptide linker. By virtue of their smaller size, they are beneficial in imaging and therapy. Proper folding of VH and VL is facilitated by connection through a peptide linker. A computer-assisted method (192) was used to design the composition and length of linker. Modeled linker consisted primarily of alternating Gly and Ser with Glu and Lys residues to enhance solubility (193). The orientation of the linker (C-terminus of VH connected to the N-terminus of VL or vice versa) did not alter the binding activity. Both constructs gave rise to fragments that could refold and exhibit similar binding activity (193, 194). A flexible

hydrophilic linker was chosen compatible with secretion and in vivo folding (191). Alternatively, a fragment known to connect residues at a similar distance and orientation was taken from the protein structure data base. The role of linkers in folding of light and heavy chains and their effect on binding affinity has not been completely evaluated.

Cloning of Antibody Fv Fragments by PCR

The limiting step in rapid construction of antibody molecules is cloning of the sequence encoding the variable region. Conventional methods of antibody cloning require intensive time and effort. The polymerase chain reaction became effective for rapid cloning and sequencing of variable region of rearranged immunoglobulin. PCR has been used for genomic and cDNA cloning and also for cloning of highly polymorphic genes such as those found in the major histocompatibility complex. Basically, PCR involves repeated rounds of denaturation, annealing, and extension from a primer specific for regions at the ends of each gene. Incorporation of restriction sites within the primers facilitates forced cloning of amplified DNA. A unique property of Taq polymerase in priming a PCR reaction leaves a single deoxyadenosine residue that enables direct ligation into a vector. This process overcomes the necessity of having restriction sites in the primer and subsequent subcloning.

A phage system has been developed to clone and express a combinatorial library of Fab fragments of mouse antibodies in *E. coli* (195). The colonies of interest are screened for antigen binding and modified by site-directed mutagenesis. However, this method requires a sensitive assay to detect the Fab of interest and an efficient expression system. Winter & Milstein (196) proposed an alternate strategy to express a VH library since an appreciable amount of binding and affinity energy comes from the heavy chain domain. Isolated light chains exhibit little antigen affinity (197). The VH library can be combined with unique LC to form a new catalytic antibody. Conversion of selected Fab fragments (50 kd) from a combinatorial library to the small SCA form (26 kd) facilitates structural analysis and mutagenic alteration of these proteins. Gibbs et al (198) successfully converted Fab fragments from a combinatorial library into a SCA.

Another strategy to construct SCA requires the determination of variable region sequences; this was found to be the rate-limiting step. Recently, Larrick et al (199, 200) and Orlandi et al (201) have used PCR for direct rapid cloning and sequencing of human and mouse Ig variable regions. Although sequence variability exists among antibodies even for the most conserved regions, PCR can tolerate and prime the reaction allowing a certain percentage of mismatches. Hence, PCR has simplified the process of constructing SCA by using degenerate primers or mixtures of primers (202). However, this method also requires knowledge of the N-terminal amino acid sequence in

order to design upstream primers. Recently, a general approach to amplify the variable region of Ig from mouse and human hybridomas was demonstrated based on the use of mixed primers complementary to the relatively conserved leader sequences of HC and LC (198, 199). This system was so sensitive that it was possible to amplify the sequence of interest from a single hybridoma cell line or from a wide variety of antibodies irrespective of their isotypes (198).

Bifunctional Antibodies

Bispecific antibodies offer great promise as therapeutic agents. Antibodies with two distinct binding activities can be generated to deliver radionucleotides, cytotoxic drugs, or host cytotoxic cells to target tissues (203). Bispecific antibodies can be prepared by chemical conjugation, hybrid hybridoma, or by construction of single-peptide bispecific antibodies using a peptide linker between variable domains of two distinct monoclonal antibodies. Murine anti-Tac-anti-CD3 and anti-Tac-anti-CD16 bifunctional agents used in conjugation with peripheral blood mononuclear cells killed target cells expressing the IL-2 receptor (204). Human T cells have been targeted by bifunctional antibodies to treat established human carcinoma in a nude mouse model. Bispecific antibodies have also been used in nontumor systems such as enhancement of thrombolysis by targeting tissue plasminogen activator (tPA) with specific antibodies to tPA and fibrin (205). Raso (206) showed bispecific antibodies to have specificity for ricin A chain and surface Ig of a lymphoma cell line. Different types of bispecific antibodies for targeting with various pharmacological agents have been used. One Fab specific for a surface idiotype of guinea pig lymphoblastic leukemia and a second Fab recognizing the plant RIP, saporin, have been successfully used in vitro and in vivo (207). Bifunctional antibodies can redirect cytotoxic T cells to cells infected with viruses (208).

Humanizing Antibody Molecules

Murine monoclonal antibodies, though useful in human therapy, are immunogenic and induce a human anti-murine antibody (HAMA) response (209). Human immunoglobulins are widely used both as prophylactic and microbial agents, but it is difficult to make human monoclonal antibodies by the conventional route of immortalization of human plasma cells. Gene technology offers an alternative by generating a chimeric molecule in which a hypervariable region of a mouse antibody can be grafted onto the framework region of a human antibody, thereby "humanizing" the antibody. The humanization of rodent monoclonal antibodies is currently the most practical approach. It allows access to a vast pool of rodent antibodies with good affinity and specificity.

Humanized antibodies produced by this approach have reduced binding affinity as compared to the original parental molecules. Queen et al (210) used computer modeling to identify amino acids in mouse frameworks that are likely to interact with CDR and retain those amino acids in humanized antibodies. The catalytic rate of an immunoglobulin is determined by the CH2 domain of the Fc region, and the survival rate of humanized antibodies probably reflects the replacement of murine IgG CH2 domains with their human counterparts (211). Human antibodies can be produced in SCID-hu mice (212). Antibodies produced from such methods have not yet been therapeutically evaluated.

Gene technology has to be fully exploited to explore the potential of other approaches, especially construction of transgenic mice with V, D, J, and human "C" regions (209). Such technology should allow production of human antibodies directly from hyperimmune mice or by using SCID-hu mice. Immortalization with EBV and cell fusion can be improved to incorporate ideas and techniques involving DNA manipulation. Hypervariable regions can be recycled on different human frameworks for the development of anti-idiotype vaccines. Though clinical trials have shown that smaller size fragments of antibodies have some advantages, rapid clearance from the circulation may prove to be a problem. Stable Fv fragments can be engineered by linking domains with hydrophilic and flexible peptides (209) or by introducing disulfide bonds. Single VH domains are likely to be useful in generating smaller recognition molecules by introducing hydrophilic residues at the interface and thereby preventing interactions with the light chain.

TARGETING TOXINS TO VIRUS INFECTED CELLS

Antiviral Action of the Native Proteins

As early as 1925, antiviral activity was noted in ribosome inactivating proteins (RIP) isolated from plants (reviewed in 213). Over the years, the pokeweed antiviral protein (PAP) has also been shown to be effective against a number of plant and animal viruses (213). Animal viruses inhibited by PAP include the herpes simplex virus (214), influenza virus (215), poliovirus (216), and recently the human immunodeficiency virus (217, 218). Other single chain ribosome inhibitory proteins isolated from plants such as tritin, gelonin, and dianthin also inhibit virus replication (213). A number of dimeric ribosome inactivating proteins such as ricin, modeccin, and abrin also possess antiviral activity (219, 220). Recently, we evaluated the anti-HIV activity of two RIP (PAP-S and Luffa ribosome inhibitory protein) and also a recombinant form of the ricin A chain. These proteins effectively inhibited HIV replication at concentrations that were not toxic to uninfected cells (217).

The single chain plant RIP, trichosanthin, has received much recent attention due to its ability to inhibit HIV-1 replication. Trichosanthin is a 26-kd

basic protein isolated from the root tubers of *Trichosanthes kirilowi*. Both the primary sequence and conformation of this protein are strongly homologous to the ricin A chain (221, 222). GLQ233 is a formulated preparation of trichosanthin that effectively inhibits HIV-1 replication in both lymphoid cells and the monocyte-macrophage lineage. This compound reduced viral replication at concentrations that showed little inhibition of total protein and DNA synthesis in uninfected cells. The fact that this RIP exhibits anti-HIV activity in monocytes makes it particularly appealing since many of the potential anti-HIV agents have been targeted primarily to CD4-bearing T cells. Since the monocyte-macrophage lineage is relatively resistant to the cytopathic effects of the virus, these cells are involved in chronic HIV infection and act as viral reservoirs. Therefore, anti-HIV agents that can be targeted to these cells are desirable. GLQ233 was recently used in clinical trials on patients with AIDS or AIDS-related complex (ARC). In the phase I dose-escalation study using a single infusion of GLQ223 no change occurred in CD4 cells or HIV antigen levels (223). However, when GLQ233 was injected three times over a 9–21 day period, a decrease in virus antigen and increase in CD4 cells was observed (224). The most common side effects included headache, mylagia, fever, and fatigue. The most serious side effect was neurological toxicity that was presented as dementia and resulted in coma in two patients.

Another single-chain RIP with anti-HIV activity, TAP 29, has recently been isolated from *T. kirilowii* (225). This protein is distinct from trichosanthin in size, N-terminal amino acid sequence, and is less toxic to normal cells. Although TAP 29 and trichosanthin exhibit similar anti-HIV activity, the reduced cytotoxicity of TAP 29 results in a higher therapeutic index than that of trichosanthin. Trichosanthin and other RIP need to be evaluated further to establish the therapeutic potential of these anti-HIV agents.

The mechanisms by which unconjugated RIP and toxins are selectively cytotoxic to infected cells are not yet completely understood. One mechanism of selective inhibition of virus-infected cells may be due to alterations in the cell membrane caused by the viral infection. The infection process may increase membrane permeability and thus allow the RIP to enter the infected cells and inactivate the ribosomes. This phenomenon has been observed with a number of viruses wherein the plant toxins were able to enter the cells immediately following viral infection (226). Another mechanism by which the RIP and toxins could be selectively toxic to infected cells is by the selective inhibition of viral DNA synthesis or viral proteins. Additional studies are needed to determine the anti-HIV mechanisms of these compounds.

Immunoconjugates as Antiviral Agents

Although native RIP and toxins have shown substantial antiviral activity, their specificity may be improved by coupling these agents to various carrier

molecules such as monoclonal antibodies specific for viral antigens present on the cell surface. The first antibody-toxin complex was directed against viral antigens (227): it contained DT coupled to an antibody to the mumps virus and was selectively toxic to mumps-infected cells. The use of chimeric toxins as antiviral agents was generally ignored until the past few years when a variety of immunotoxins has been constructed to target HIV-infected cells. The HIV virion contains two glycoproteins within its viral envelope. The 120-kd glycoprotein (gp120) projects from the virion surface and is anchored by a 41-kd transmembrane glycoprotein (gp41). The major cellular receptor for the HIV virus is the CD4 antigen with which gp120 can bind. Once the virus attaches to the cell, fusion is mediated by gp41. Both gp120 and gp41 are expressed on the surface of infected cells. Therefore, toxins that target these viral antigens may act as effective anti-HIV agents.

A variety of carrier molecules and monoclonal antibodies have been used to target HIV-infected cells. The first generation of immunotoxins directed against HIV was based on the soluble form of recombinant CD4 (rCD4) as the carrier molecule (228, 229), since it was shown that rCD4 alone could inhibit HIV replication by competitively binding to the virus (230). Although much diversity exists within gp120, the CD4 binding site is highly conserved among the different isolates (231) and thus different formulations of soluble truncated CD4 molecules can inhibit the replication of a variety of HIV strains. CD4-PE has recently been used in combination with reverse transcriptase inhibitors (232). The RT inhibitor can block viral replication but it cannot kill cells that are already infected. The CD4-linked toxin is only toxic to those cells that display gp120 on their cell surface. When these two agents were administered simultaneously, the virostatic action and elimination of established HIV-infected cells resulted in effective elimination of HIV.

Numerous immunotoxins have been directed against HIV envelope proteins. Antibodies against the neutralizing loop and CD4 binding site of gp120 have been conjugated to PAP, ricin A chain, and PE (233–235). One potential drawback of directing the IT to gp120 is that this envelope glycoprotein varies in different HIV isolates. This extensive variability is not seen in gp41. Thus, IT prepared with anti-gp41 antibodies may be more effective against a larger number of HIV isolates (236). Recently, Pincus et al compared the anti-HIV activity of a number of IT directed to the HIV envelope proteins (237). In their study, they demonstrated that one of the most effective IT against HIV was prepared from an anti-envelope polyclonal antibody obtained from the sera of HIV patients. This polyclonal conjugate had the highest specific activity and could also target diverse strains of HIV isolates. One potential advantage of any of the IT directed against the viral envelope is their ability to neutralize free virions. However, the recognition of free viral proteins may also lead to some complications, e.g. if viral proteins such as gp120 bind to uninfected cells, the immunotoxin could erroneously kill a normal CD4 cell.

Finberg et al (238) described a unique type of immunotoxin in which IL-2 was used as a carrier to HIV-infected cells. They constructed the fusion toxin, DAB_{486}-IL-2, by replacing the diptheria toxin receptor binding domain with human IL-2 sequences. When HIV-1 interacts with CD4, cytokine production and IL-2 receptor expression is increased in both T cells and monocytes. The construct was shown to selectively kill cells expressing the high-affinity IL-2 receptor at 10^{-8} M whereas viability of uninfected cells was not reduced until 10^{-7} M of DAB_{486}-IL-2. These results demonstrate that targeting cellular activation antigens may be a useful method of inhibiting early stages of HIV infection since it does not require the expression of viral proteins.

The success of anti-viral therapy using IT depends on the presence of preformed neutralizing antibodies to either the toxin moiety or the target antigen and the immune response to IT that will limit the frequency of administration of conjugates. Different types of antibodies against viral proteins arise during the progression of the HIV disease. Since many IT are directed against viral proteins, the action of the IT may be inhibited by these antibodies. Some of the problems associated with preformed anti-toxin antibodies can be overcome by using immunologically noncrossreactive toxins in the IT preparations. However, antibodies to viral proteins would still pose a problem. Under these circumstances, CD4-linked toxin conjugates can be used. Since CD4 is a self antigen, antibodies to CD4 will not be produced. The second constraint on the repeated use of IT is the immune response to the injected conjugates. Antibody responses to both the murine IgG and toxin polypeptide have been noticed in many clinical trials and in experimental therapeutic model systems. Antigen specific immunosuppression or immunological unresponsiveness has been induced by (*a*) coadministering antibodies to T4 differentiation antigen, (*b*) linking inert molecules such as polyethyleneglycol (PEG) to mask immunogenic determinants, and (*c*) humanizing the antibody molecules to prevent anti-mouse IgG antibody responses. These methods have not yet, however, been evaluated under clinical settings.

FUTURE DIRECTIONS AND PROSPECTS

Over the past two years significant advances have been made in understanding the factors contributing to the in vivo efficacy of IT. Reducing the toxicity by structural changes in the toxin moiety seems promising. Many toxins have been cloned and expressed. X-ray crystallographic data have permitted the molecules to be engineered by using recombinant DNA methods. Most toxin moieties currently in use are translation inhibitory proteins. A few investigators have used alternate proteins that can either act on the cell surface or at a distinct step in the biosynthetic pathway. A chimeric toxin was recently generated by fusing the gene for a protein with RNAse activity and PE (239).

Such hybrid molecules could be more effective since they would inhibit multiple biosynthetic pathways.

Similarly, progress has been made in identifying other carrier molecules which could be used in targeting. Growth factor conjugates offer unique opportunities to home in on tumor cells expressing higher densities of relevant receptors. Genetic fusions are used to produce IT containing a growth factor (TGF-α), single chain antibody (anti-TAC), and a toxin moiety (PE). Such tripartate fusion molecules have advantages over conventional constructs. An alternative strategy for the delivery of cytotoxic agents using monoclonal antibodies as carrier molecules is through enzymes to target them to tumor cell surfaces (240). The enzymes are chosen in such a way that they convert drug precusors injected parenterally into active antineoplastic drugs.

Tumor homing and penetration is a serious limitation in developing IT treatments for solid tumors. Use of biological response modifiers is investigated to facilitate tumor penetration. IL-2, for example, produces capillary leakage when admininstered in high doses. This side effect could be exploited with antibodies linked to IL-2. In a recent study, chemical conjugation of IL-2 to an antibody led to improved tumor localization (241). Such methods could be used to increase the delivery of IT to the interior of solid tumors. Similarly, TNF could be used to enhance IT efficacy in vivo. Coadministration of γIFN with IT and the combined use of chemotherapeutic drugs potentiates the biological activity of IT (242). Alternatively, chemical modification of ricin (blocked ricin) or genetic alteration of the galactose binding sites in ricin offer promise in preparing highly effective conjugates that can be used in bone marrow purging as well as in vivo.

Understanding the structural features that could improve capillary permeability of proteins would have a major impact on treatments with IT. Since the uptake of IT by sensitive organs is a potential risk, the development of decoy molecules to block binding to these organs would reduce toxicity and broaden the therapeutic window. Similarly, the expression of target antigens on the cell surface could be manipulated by pretreatment with biological response modifiers to enhance the activity of IT. If these changes could be incorporated, a new generation of cytotoxic conjugates could be generated with higher efficacy and lower toxicity.

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