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IMMUNOTOXIN THERAPY OF HEMATOLOGICAL MALIGNANCIES*

Laura Pasqualucci,* Leonardo Flenghi,* Adelmo Terenzi,* Andrea Bolognesi,° Fiorenzo Stirpe,° Barbara Bigerna,* Brunangelo Falini*

**Institute of Hematology, University of Perugia, Perugia; °Department of Experimental Pathology, University of Bologna, Bologna, Italy*

Several years have passed since the potential for unconjugated monoclonal antibodies (mAbs) to selectively target malignant cells was first described. Although the concept behind serotherapy is theoretically simple, the overall clinical results in these studies have often been disappointing and numerous obstacles have emerged due to the difficulty in penetrating tumor masses, the limited inherent cytotoxic activity of many mAbs, and the large amount of antibody often required for therapeutic efficacy with consequent prohibitive costs.[1,2] Over the last decade, progress have been made in rendering native mAbs more effective by coupling them to drugs, toxic agents or radionuclides. In this review we will limit our consideration to toxin-antibody conjugates and their possible application in the treatment of hematological malignancies.

Immunotoxins (ITs) are synthetic hybrid molecules consisting of a highly potent toxin moiety linked to a mAb selectively directed toward specific cell targets.[3-8]

Essential prerequisites for the clinical use of ITs are: a) a high specificity and affinity of the Ab component for the target molecule, which should be consistently expressed on neoplastic cells and have minimal distribution in normal human tissues; b) a high density of the target antigen on the surface of neoplastic cells; c) the potential for the IT to be internalized after antigen binding and routed to the appropriate intracellular compartment for translocation of the attached toxin to the apparatus where it exerts its effect.

Efficient ITs have been prepared using several toxins of plant or bacterial origin (Table 1). This paper will review only the ribosome-inactivating proteins from plants (RIPs).[9] These molecules exist in nature in two forms: type 1 and 2 RIPs. Type 2 RIPs are holotoxins containing two polypeptide chains, namely A and B (Table 1). The A chain is the enzymatically active one and is linked, through a disulphide bond, to a sugar-binding B-chain that recognizes the galactosyl-terminated receptors present on the surface of most mammalian cells and promotes the translocation of the A chain into the cytoplasm. Among type 2 RIPs, ricin has been the one most widely used in preclinical and clinical studies. Predictably, ITs containing native ricin and other type 2 RIPs lack specificity since thay bind not only to target cells, but virtually to any other cell via the B chain. This problem has been successfully circumvented by altering or deleting the binding domain through two different approaches: 1) by separating the functionally active A chain from the B chain and deglycosylating the A chain (dgA)[10] (this latter procedure prevents liver toxicity due to glycosylated residues on the A chain which recognize parenchymal and non parenchymal cells in the liver, causing hepatotoxicity and poor biodistribution[11,12]); 2) by attaching affinity ligands to the sugar-binding sites of the B chain (blocked ricin).[13,14] However, the easiest way to overcome this problem is to purify type 1 RIPs that lack the B chain and are present in large amounts in several plant species.[9] This family of a plant hemitoxins includes saporin (SO6), momordin (MOM) and pokeweed antiviral protein from seeds (PAP-S) (Table 1).

RIPs 1 act catalytically on eukaryotic cells by irreversibly inactivating the 60S ribosomal subunit and by impairing its binding to elongation factor 2 (EF2) (Figure 1); protein synthesis is

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Correspondence: Prof. Brunangelo Falini, Istituto di Ematologia, Policlinico Monteluce, Perugia, Italy.
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consequently arrested and cell death occurs.[15] Due to their catalytic mechanism of action, these toxins are extremely potent; it has been estimated that a few molecules of ricin in the cytoplasm are enough to kill a cell.[16] Moreover, since their mechanism of cell killing is different from those of standard chemotherapeutic agents, it is reasonable to expect that they could exert an efficient antitumor activity against chemoresistant and/or resting neoplastic cells without cumulative bone marrow toxicity.

As shown in Table 2, immunotoxins prepared with type 1 RIPs are highly specific for their designated target cells *in vitro* but vary in potency depending on the affinity of the ligand, the cell surface molecule and the ability of the conjugate to enter an intracellular compartment that is favorable for their use.[1,9] Compared to solid tumors, which have a poor blood supply and large interstitial pressures, hematologic malignancies appear to be a more suitable target for immunotherapy since they are more eas-

Toxin	*Toxin receptor*	*A-chain action*
Plant holotoxins		
Ricin	Galactose	N-glycosidase for 28S ribosomal RNA
Abrin	Galactose	N-glycosidase for 28S ribosomal RNA
Plant holotoxin A subunits		
Ricin A chain	None	N-glycosidase for 28S ribosomal RNA
Abrin A chain	None	N-glycosidase for 28S ribosomal RNA
Plant hemitoxins		
Saporin	None	N-glycosidase for 28S ribosomal RNA
Momordin	None	N-glycosidase for 28S ribosomal RNA
Pokeweed antiviral protein (PAP)	None	N-glycosidase for 28S ribosomal RNA
Gelonin	None	N-glycosidase for 28S ribosomal RNA
Bacterial holotoxins		
Diphtheria toxin	Heparin-binding EGF-like precursor	ADP-ribosylation of elongation factor 2
Truncated diphtheria toxin (DAB 486)	None	ADP-ribosylation of elongation factor 2
Pseudomonas exotoxin A	α_2-macroglobulin receptor-like molecule	ADP-ribosylation of elongation factor 2

Table 1. Toxins most frequently employed for preparation of immunotoxins

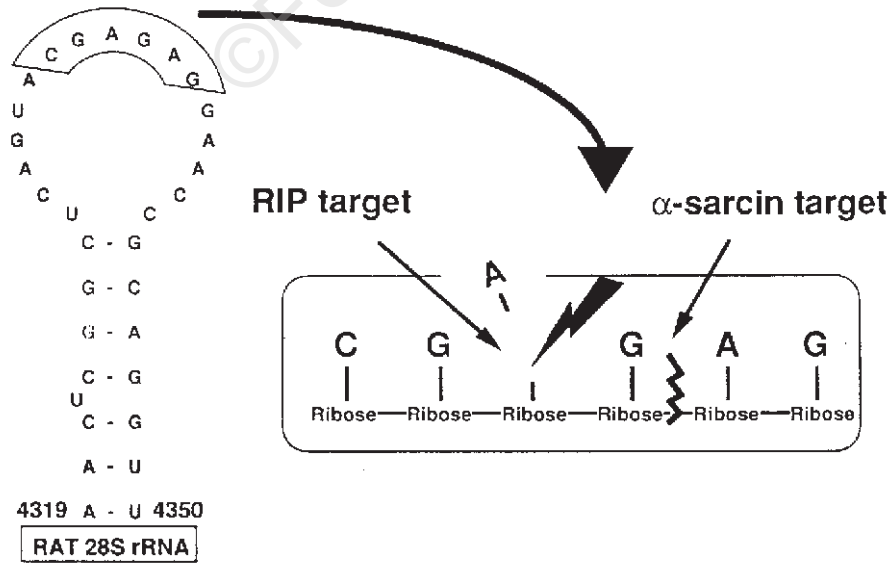

Figure 1. The mechanism of action or RIPS. They have a specific N-glycosidase activity which cleaves a single adenine base (A4324 in rat) from ribosomal RNA of the large subunit and causes complete inactivation of the ribosome. The site of attack is adjacent to the α-sarcin site and is contained in an exposed loop termed the α-sarcin domain. The latter toxin cleaves the phsophodiester bond between G4325 and A4326 in rat 28S rRNA which also results in loss of ribosome function. (*Reprinted from Stirpe F, Barbieri L, Battelli MG, Soria M, Lappi DA. Ribosome-inactivating proteins from plants: present status and future prospects. Biotechnology 1992; 10:405-12).*

Table 2. Immunotoxins prepared with type 1 RIPs.

RIPs (type 1)	*Toxicity (IC_{50})*	
	Target cells	Non target cells
Saporin	0.003-0.1	> 300
Momordin	1-2.8	> 300
Pokeweed antiviral protein	0.7-3	> 300
Gelonin	0.001-3	> 300
Bryodin	0.01-0.04	> 300

ily penetrated by the IT. To this end, several animal models are being devised for preclinical determination of IT efficacy and phase I-II clinical trials are currently in progress.

Preclinical studies

B-cell malignancies

The B-cell restricted antigens CD19 and CD22 are particularly attractive target molecules for immunotherapy of B-cell lymphomas/leukemias with specific mAbs. Ghetie *et al.*[17-19] developed a SCID/Daudi model of disseminated human Burkitt's B-cell lymphoma for preclinical evaluation of two ITs consisting of dgA coupled to either the monomeric Fab' fragment or the whole IgG of the mAbs RFB4 (anti-CD22) and HD37 (anti-CD19). In their studies, a dose corresponding to 40% of the LD_{50} significantly prolonged the survival of mice when the ITs were administered 24 hours after tumor challenge, and excellent anti-tumor effects were obtained by using a combination of the two ITs or a combination of anti-CD22-dgA and native anti-CD19 antibody.[19] Similarly, an anti-CD19 (B43)/PAP IT showed potent antitumor activity *in vitro* and *in vivo* against the leukemic cell lines RS4;11 and NALM 6-UM1,[20,21] extending the event free survival of SCID mice challenged with a tumorigenic dose. Unfortunately, none of the above treatments was curative; eradication of advanced and disseminated disease was achieved only when the above ITs were administered together with cyclophosphamide or other chemotherapic agents, thus providing evidence for synergy between ITs and standard chemotherapy.[22,23]

We recently obtained similar results in SCID/Daudi mice treated sequentially with three different anti-CD22 1 ITs (anti-CD22/saporin, anti-CD22/momordin and anti-CD22/PAP).[24] In this experimental model, we compared the therapeutic efficacy of two different treatment schedules (single course vs sequential administration of 3 ITs) and demonstrated that the latter was significantly more effective in terms of event free survival. Furthermore, mice treated with a combination of IT plus cyclophosphamide were tumor free at 120 days of follow-up, suggesting that combination treatment with IT plus chemotherapy might induce better therapeutic effects without cumulative toxicity (unpublished results).

Finally, the recent demonstration that B43-genistein conjugates can induce apoptosis in CD19-bearing tumor cells by inhibition of signal transduction pathways[25] opens new horizons in the field of immunotoxin therapy.

Hodgkin's disease and CD30-expressing lymphomas

Animal models of Hodgkin's disease (HD) and $CD30^+$ anaplastic large cell lymphoma (ALCL) have been used to investigate the antitumor activities of different ITs, including anti-CD30/dgA,[26,27] anti-CD30/saporin (SO6),[28] anti-CD25/dgA[29] and IRac/dgA.[30] In all these studies ITs produced excellent tumor regression but failed to cure animals, mainly due to the following mechanisms of resistance to treatment: a) emergence of mutant clones, i.e. antigen-deficient mutants,[27,30] and b) inability of the IT to reach the tumor target.

We investigated the antitumor activity of an anti-CD30 IT (Ber-H2/SO6)[31] *in vitro* ($CD30^+$ cell lines COLE, L428 and L540 and JB6), and *in vivo* using our newly established SCID mouse model of human xenografted $CD30^+$ anaplastic large cell lymphoma (ALCL).[28-32] *In vivo*, a 3-day treatment with this IT at a dose corresponding to 50% of the LD_{50} induced lasting complete remissions (CRs) in about 80% of the animals injected 24 hours after tumor transplantation and significantly delayed the

Table 3. Immunotoxin therapy for lymphomas and leukemias.

Target antigen	*Immunotoxin*	*Disease*	*No. of patients*	*MTD*	*Side effects*	*Response*	*Reference*
CD22	Fab'-RFB4-dgA	NHL	15	75 mg/m^2 IV bolus q48hr	VLS, myalgias	5 PRs	34
CD22	IgG-RFB4-dgA	NHL	26	20 mg/m^2 IV bolus q48hr	As above	1 CR, 5 PRs	35
CD22	IgG-RFB4-dgA	NHL	18	19.6 mg/m^2/196hr	VLS	25% response rate	36
CD19	B4-bR	NHL	23	250 mg/kg	↑ liver transaminases, thrombocytopenia, fever, hypoalbuminemia, pleural effusion	1 CR, 2 PRs	37
CD19	B4-bR	NHL	34	350 mg/kg	↑ liver transaminases, thrombocytopenia, fever, nausea, myalgia, VLS	2 CRs, 3 PRs, 11 transient responses	38
CD19	B4-bR (post ABMT)	NHL	61	280 mg/kg	↑ liver transaminases, thrombocytopenia, fever, myalgias, hypoalbuminemia	most patients remain in CR	39, 40
CD19	B43/PAP	ALL	26	not reached at 250 µg/kg/d x 5d IV bolus	VLS, myalgias	6CRs, 3PRs	33
CD30	Ber-H2/SO6	HD	12	0.8 mg/kg IV bolus	↑ liver transaminases, VLS, fever, myalgias	4 PRs, 2 MRs	43, 44
CD25	RFT5-dgA	HD	15	15 mg/m^2 IV bolus on d 1,3,5,7 x 1-4 cycles	VLS, fatigue, myalgias	1PR,1MR	33

Legend: NHL: Non-Hodgkin's lymphomas; ALL: acute lymphoblastic leukemia; HD: Hodgkin's disease; VLS: vascular leak syndrome.

tumor growth rate when administered at later stages of tumor growth (subcutaneous tumors of 40 to 60 mm^3 volume).[28] Notably, the efficacy of treatment in this study, as well as in other reports employing anti-CD25 ITs,[30] correlated with the size of tumor masses, suggesting that ITs exert their maximum effect when the tumor cell burden is small.[28] This finding probably reflects the difficulty of these high molecular-weight molecules to penetrate large neoplastic masses.

In conclusion, *in vitro* and *in vivo* experimental data strongly suggest that ITs directed against the CD30 or CD25 molecule and other lymphoid-associated antigens (CD19, CD22) are likely to be more effective when used to treat minimal or low burden disease. Anti-tumor activity could also be improved by the simultaneous administration of immunotoxin constructs directed against different surface antigens (e.g. CD25 and CD30) on H & RS cells in order to ensure delivery of the agent to those tumor cells that are single antigen negative and would otherwise escape destruction.

Clinical trials

The pharmacokinetics, immunogenicities, toxicities, and anti-tumor efficacies of a variety of ITs for treatment of leukemias or lymphomas refractory to conventional therapy, have been evaluated in several phase I clinical trials (Table 3). The toxins investigated include dgA, ìblockedî ricin, saporin, pokeweed anti-viral protein (PAP-S), and truncated forms of diphtheria toxin (DAB 389). Toxins have been linked to mAbs directed against the CD19, CD22, CD25, and CD30 antigens. Although the antitumor efficacy of these immunoconjugates cannot be completely ascertained from phase I dose-

escalation trials, encouraging objective clinical responses have been recorded in 12 to 50% of the refractory patients participating in these studies (Table 3). These results have prompted the initiation of phase II and phase III trials that should more clearly define the response rates and clinical utility of these compounds.

Acute leukemias

Anti-CD19 monoclonal antibody (B43) conjugated to pokeweed anti-viral protein (PAP-S) has been used for treating relapsed B-lineage acute lymphoblastic leukemia (ALL).[33] Thirty-seven cycles of therapy were administered to 26 patients with multiply-relapsed ALL, resulting in 6 CRs and 3 PRs. A correlation was observed between the response to therapy and the attainment of a high peak serum IT concentration, a high serum IT *area under the curve*, low IT clearance, and a low peripheral blast count. Vasculary leak syndrome (VLS) was the main dose-limiting factor. Current studies are investigating combination immunochemotherapy of relapsed ALL with B43-PAP and high-dose cyclophosphamide prior to bone marrow transplantation.

Non-Hodgkin's lymphomas (NHL)

Fabi and intact (IgG) anti-CD22 (RFB4) linked to dgA have been tested in patients with refractory B-cell NHL. Both immunotoxin constructs were administered as an IV bolus for 2-6 doses to two groups of 15 and 26 patients, respectively.[34,35] No differences in clinical findings emerged from these studies: partial remissions (PRs) were achieved in 38% of evaluable patients and were of short duration. Major side effects included VLS, fever, anorexia and myalgias. The serum half-life of the ITs was 86 minutes for Fab' RFB4 and 10.8 hours for IgG RFB4, while the maximum tolerated dose (MTD) was reached at 75 mg/m^2 and 20 mg/m^2, respectively; the latter value probably reflects the extended half-life of the intact IgG IT. VLS (pulmonary edema), aphasia and rhabdomyolysis defined the dose limiting toxicity.

Sausville *et al*.[36] recently reported that the MTD of the RFB4-IgG-dgA IT given by continuous intravenous infusion (19.6 μg/m^2/192 hr) is similar to that of the same IT administered by bolus injection (20 mg/m^2). VLS was the main dose-limiting factor even with this infusion schedule. This study also suggested that serious toxicities were more frequent in patients without circulating tumor cells who experienced high peak serum IT concentrations (>100 μg/mL at day 3-4). Thus, individualization of the IT dose based on the number of circulating tumor cells or peak IT concetration may be necessary to optimize therapy and minimize toxicity.

Grossbard *et al*.[37] administered escalating doses of anti-CD19 (B4) linked to blocked ricin (B4-bR) to 25 patients with NHL by daily bolus infusion for 5 consecutive days. One CR and 2 PRs were observed. Potentially therapeutic serum levels were only transiently achieved in the majority of patients treated at the MTD (50 μg/kg/d; total dose = 250 μg/kg). The dose-limiting toxicity was defined by a grade 3 increase in liver transaminases; other significant toxicities included fever, thrombocytopenia and hypoalbuminemia. In an attempt to achieve more sustained therapeutic IT serum levels, 34 patients were treated with the same anti-CD19 IT on a 7-day continuous infusion schedule.[38] Two CRs, three PRs and 11 transient responses were observed, primarily in patients with low disease burden. The MTD, as defined by grade 4 reversible elevation in hepatic transaminases plus grade 4 thrombocytopenia, was reached at 50 μg/kg/d (total dose=350 μg/kg). The relatively greater nonspecific toxicity observed in this trial likely correlated with sustained serum levels of the IT.

Dr. David Scanned has recently reported the results of a phase I trial involving the administration of B4-bR following combination chemotherapy with the m-BACOD regimen for AIDS-related lymphomas.[33] Twenty-eight of 46 patients achieved remissions (12 CRs and 16 PRs) with the chemotherapy component of the program. Twenty-six patients received 20 μg/kg/d B4-bR for 7 days × 1-2 cycles after completion of chemotherapy. Median survival was 10.5 months for the 26 patients receiving the IT.[33]

Although mAbs have been the preferred carrier for targeting toxins to tumor cells, ligand

molecules (e.g. interleukin 2) can also be exploited for this purpose. Interleukin-2 (IL-2) fused to truncated diphtheria toxin (DAB389-IL2) was administered to 73 patients with lymphomas expressing the high affinity IL-2 receptor.[33] Thirty-four percent of patients with cutaneous T-cell lymphomas responded to this treatment (including 14% CR). Clinical responses were also observed in 18% of patients with other types of NHL, but not in cases of refractoty Hodgkin's disease. The MTD of DAB389-IL2 was 27 μg/kg/day and fatigue was the dose-limiting toxicity. VLS was not a significant problem. Expression of the IL-2 receptor α chain appeared to be essential for response to this treatment.[33]

The above clinical results, when taken together with the experimental data, suggest that a major obstacle to effective immunotherapy may be inadequate delivery of immunotoxins to lymphoma cells, especially when the tumor burden is high. Based upon these concepts, sixty-one patients with low-grade NHL were treated with B4-bR for 7 days by continuous IV infusion using an outpatient infusion pump for 2 cycles after autologous marrow engraftment (administered a median of 109 days after bone marrow transplantation). The relatively low MTD (40 μg/kg/d) observed in this study may reflect the higher serum levels achieved in these patients, who lacked circulating normal and neoplastic cells able to bind the IT. Dose-limiting toxicities were grade 4 thrombocytopenia and elevation of liver transaminases. Most of these patients have remained in CR after therapy.[33] Notably, immunotherapy was able to induce, in at least a percentage of cases, a conversion from PCR^+ (for the *bcl-2* translocation) to PCR^-, strongly suggesting that possible eradication of minimal residual disease had occurred. Based on these promising results, a phase III randomized trial was recently initiated at 48 Institutions to rigorously assess the value of B4-bR after bone marrow transplantation for patients with relapsed B-cell lymphomas.[33]

Hodgkin's disease

Optimal *in vivo* targeting of H and RS cells can be achieved in all tumor sites by injecting low doses (15 to 40 mg) of native anti-CD30(Ber-H2).[41,42] This is most likely due to one or more of the following:[41,42] a) restricted expression of the CD30 molecule in normal human tissues; b) low levels of soluble CD30 in the serum; c) low percentage of tumor cells in Hodgkin's disease-involved tissues. The demonstration that native Ber-H2 may efficiently penetrate tumor sites prompted us to start a phase I clinical trial on 12 patients with advanced, refractory HD using the Ber-H2-saporin IT.[41] A single IV injection of the anti-CD30 IT at doses ranging from 0.2 to 0.8 mg/m^2 induced a rapid reduction in tumor masses (50% to >75%) in about 40% of patients[43,44] (Figure 2). The average response duration was 8 weeks. It should be noted that duration of response is generally short even after aggressive chemotherapy in patients with advanced refractory HD.[45,46] Main side effects in about 70% of patients included fever, myalgias, grade I VLS and a 4-5 fold tran-

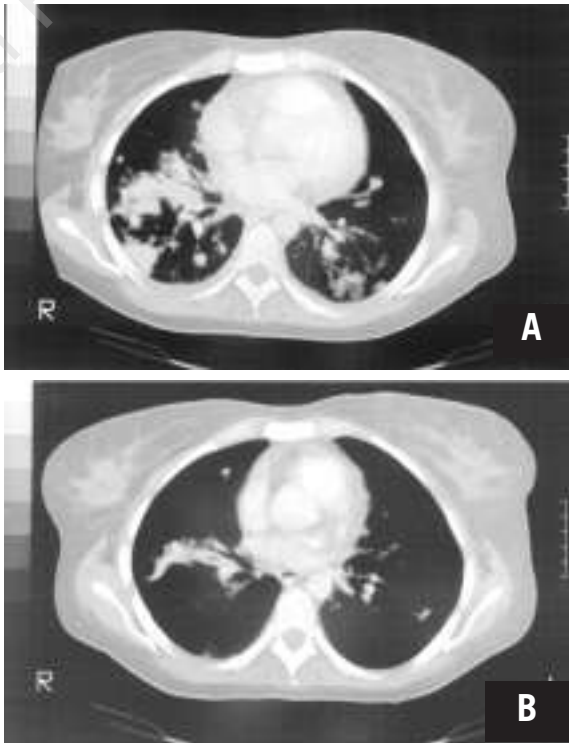

Figure 2. Refractory Hodgkin's disease (chest CT scan). A) Before immunotoxin therapy. Numerous lung tumor nodules are present. B) Seven days after injection of a single dose of Ber-H2/saporin (0.4 mg/kg). Lung tumor nodules have almost completely disappeared.
(Reprinted with permission.[42])

sient increase in ALT and AST.[44] The MTD, as defined by a grade III reversible VLS, was reached at 0.8 mg/kg IT (as single dose) and was characterized by pericardial effusion and pulmonary edema. Current trials are studying the effects of Ber-H2-saporin after either combination therapy or autologous bone marrow transplantation, as well as the potential utility of prophylactic methylprednisolone for preventing VLS.

The anti-CD25 IT RFT5-dgA was selected by Engert *et al.*[33] to treat 15 patients with refractory HD; the IT was administered IV over 4 hours for 7 days at doses of 5 to 20 mg/m^2. Serum half-life of the IT averaged 4.8 hours and the MTD was defined by a severe myalgia with increased levels of CPK. Other side effects were related to mild VLS. Minor/partial responses were obtained in 2 patients and disease stabilization in five.[33] An immune response against the toxin was observed in 6/15 patients.

No clinical responses were reported in any of the 21 patients with Hodgkin's disease enrolled in a multicenter dose-escalation study using the recombinant fusion toxin DAB_{389}-IL-2.

Table 4. Main immunotoxin problems and proposed solutions.

Problem areas	Possible solutions
Difficulty in penetrating tumor masses	
1. Antigen competition	– MAbs with restricted reactivity with normal tissues
	– Target anbtigens that are not shed
2. Large size of IT	– Single chain Fv IT
3. Difficult access	– Intracavitary therapy
	– Treatment of minimal residual disease
Toxicity	
1. Specific	– Preclinical immunohistological screening
2. General	
a) vascular leak syndrome	– Steroids
b) hepatotoxicity	– Deglycosylated toxins
Immunogenicity	– mPEG derived IT tolerization
	– Immunosuppression
	– Humanized mABs
	– Use of antigenically different IT
	– Naturally occurring toxins (ribonucleases)
	– Prevent acute allergic reactions by appropriate pre-medication
Inability to kill all tumor cells	
1. Heterogenous target antigen expression radioimmunotherapy	– Cocktail of ITs
	– Immunotoxin plus radioimmunotherapy
	– Immunotoxin plus chemotherapy
2. Emergence of antigen-negative tumor cells	– As above

Conclusions

The problems related to immunotoxin treatment and the possible solutions are summarized in Table 4. ITs can be administered safely to patients with tolerable, reversible toxicity. Side effects do not include damage to rapidly dividing normal cells; nevertheless, toxicity to non-target tissues still occurs. Hepatotoxicity and VLS have frequently been observed with RIP-containing ITs. Other side effects included fever, anorexia and myalgias. The increase in liver transaminases is likely due to clearance of the IT by the reticuloendothelial system or to nonspecific binding of the IT to serum proteins that have receptors in the liver. This problem could be prevented by using dgA-containing ITs.

VLS has been the major dose-limiting toxicity observed in almost all clinical trials. This syndrome is characterized in humans by weight gain, increased vascular permeability, hypoalbuminemia and peripheral edema. The mechanisms underlying VLS are not known, although some evidence suggests that it might be related to an interaction of the toxin with the vascular endothelium, either directly or cytokine-mediated, leading to alterations in the endothelial cell barrier function. Our understanding of IT-mediated VLS is also hindered by a lack of appropriate animal models; for instance, mice generally lose rather than gain weight, and hepatotoxicity rather than VLS has been the dose-limiting toxicity observed in animal models (mice, monkeys). Finally, there are no *in vitro* tests available for predicting clinical toxicity. In fact, no correlation has been found between the toxic effects of ITs on human umbilical vein-derived endothelial cells (as assessed *in vitro*) and *in vivo* findings.

Some of the IT-related symptoms, such as fever and myalgias, can be abolished by concomitant steroid therapy (Falini B, unpublished results). However, it is still unclear whether glucocorticoids may prevent or limit VLS. Recently, Siegall *et al.* developed a rat model of IT-induced VLS by using high doses of BR96 sFv-PE40. Many experiments were performed to determine if dexamethasone administered prophylactically would inhibit the antitumor activity of the IT. In this rat model a slight decrease in

antitumor efficacy was noted when using suboptimal concentrations of the IT, but complete regression of tumor xenografts was observed with therapeutic doses of IT and prophylactic doses of dexamethasone (unpublished data).

To date, significant clinical success has rarely been observed in patients with bulky disease, although a large number of transient responses have been reported (Table 3). Nevertheless, these results are promising if we consider that in patients with advanced, bulky disease IT antitumor activity may have been hampered by inaccessibility to neoplastic cells. Moreover, most patients received only short courses of treatment with a single IT, sometimes at suboptimal doses due to the dose escalation schedule. The major limiting factor in delivering repeated courses of therapy has been the development of host immune responses to both the mAb and the toxin moiety. There is some suggestion that immune response may be less common if only the Fab' Ig fragment is used as the carrier molecule. In the future this obstacle could be circumvented by sequential administration of different, non cross-reacting toxins linked to *humanized* antibodies; this view is supported by our experimental data on animals treated with three different anti-CD22 ITs at 21 day intervals.[24]

Finally, the selection of tumor antigen-deficient mutants could be overcome by using cocktails of ITs directed against different antigens expressed on the membrane of the malignant cell (e.g. CD19 and CD38; Flavell *et al*, unpublished results) or by using a combination of ITs and radioimmunoconjugates.[47]

In conclusion, experimental and clinical studies indicate that ITs may have a role as adjuvant therapy in selected categories of patients with persistent minimal or low burden disease, following conventional or high-dose chemotherapy. Main efforts in the future should be focused on defining optimal administration regimens and on reducing immunogenicity and toxic side effects.

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Appendix

Abstracts from the Italian Society of Experimental Hematology, *Discutiamone Insieme* Meeting, held in Florence, April 6, 1995.

IMMUNOTOXINS CONTAINING RIBOSOME-INACTIVATING PROTEINS

Bolognesi A, Falini B*
*Dip. di Patologia Sperimentale, Univ. di Bologna; *Ist. di Ematologia, Univ. di Perugia, Italy*

Immunotoxins are hybrid molecules composed of antibodies, acting as carriers, linked to toxic moieties. These molecules can be used to kill specific target cells, and are mostly used to eliminate malignant cells. Conventional antiblastic therapies kill rapidly dividing or metabolising cells, whether malignant or normal, but fail to kill non-dividing tumour cells. Moreover, conventional chemotherapy often lead to the development of multi drug resistance. Effective and highly selective immunotoxins can be obtained using monoclonal antibodies specific for surface antigens of neoplastic cells, not expressed or present at a very low level in normal tissues. The toxins employed in immunotoxins are proteins derived from bacteria, fungi and plants which finally produce irreversible inactivation of protein synthesis. Plant toxins referred to as ribosome-inactivating proteins (RIPs),[1] are RNA N-glycosidases that depurinate the major rRNA subunit. They are divided into single-chain proteins, type 1 RIPs, and two-chain proteins, type 2 RIPs. The A-chain of type 2 RIPs, with enzymatic activity, is linked by a disulphide bond to a B-chain, with lectin properties. These enzymes remove a crucial adenine residue from the 60S ribosomal subunit/needed for the binding of elongation factor 2 during protein synthesis, thus killing the cell. In spite of their apparently identical enzymatic activity, type 2 RIPs are much more toxic for animals and cultured cells. This type of toxins bindl via the B-chain, to galactose-containing glycoproteins and glycolipids present on the surface of all cell types. The toxin is then endocytosed and routed to the trans-Golgi network where the A-chain is believed to translocate to the cytosol. Type 1 RIPs, lacking the B-chain, enter into the cells through a much less effective fluid-phase endocytosis. Potent immunotoxins can be obtained using type 2 RlPs whose galactose-binding sites have been blocked either sterically or chemically. These procedures are complicated and do not completely avoid the aspecific binding of the B-chain to non target cells. Alternatively, using type 1 RIPs or the A-chain of type 2 RIPs it is possible to obtain immunotoxins highly specific for designated target cells, the potency of which depends mainly on the intracellular routing to a compartment from which the toxin can translocate to the cytoplasm. To join the monoclonal antibody and the single chain ribosome inactivating protein, cross-linkers are used that introduce a disulphide bond. This linkage is stable extracellularly (blood, culture medium etc.) but is easily broken by reduction inside the cell (i.e. lysosomal reductase system), allowing the toxic fragment to reach its intracellular site of action. Separation of the RIP is also essential for the correct interaction with the ribosome, owing to the steric hindrance of the large antibody moiety. During the last decade many ribosome-inactivating proteins have been tested in our laboratories, and some of them appeared to be particularly suitable for the preparation of immunotoxins (2). Momordin, PAP-S and saporin-S6 were found to be the most stable and active molecules, both during the chemical conjugation and the biological testing. Moreover, these protein do not cross-react with reciprocal antisera allowing for their sequential use during prolonged *in vivo* treatment. These ribosome-inactivating proteins conjugated with the monoclonal antibodies Ber-H2 (anti-CD30) and OM124 (anti-CD22), were extremely toxic and selective for specific target cells, both *in vitro* and in SCID mice. At the moment phase I/II clinical trial are under investigation in patients with advanced refractory Hodgkin's disease (anti-CD30 immunotoxins) (3) and B cell leukemias or lymphomas (anti CD22 immunotoxins).

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HEPATOTOXICITY OF IMMUNOTOXINS

L. Buonamici, M.G. Battelli, F. Stirpe
Dipartimento di Patologia Sperimentale, Università di Bologna, Italy

Immunotoxins containing ribosome-inactivating proteins (RIPs) are used in phase I/II clinical trials. Among side effects of the treatment, a transient hepatotoxicity is observed, as predicted from animal experiments. RIPs have a relatively low toxicity, but they can be rendered highly toxic to cells if targeted by specific carriers. However, their unspecific toxicity increases upon conjugation to antibodies. Necrotic lesions are present in liver, kidney and spleen of mice receiving lethal doses of free RIPs, whereas the histological damage is limited to the liver in the animals given the IgG-conjugates.[1] These differences in the organ distribution of necrosis could be due to the increased molecular size of the immunotoxins that prevents glomerular filtration and enhances blood half-life, as compared to free RIPs. This is also suggested by the evidence that homopolymers made with RIPs have blood half-life and kidney concentration intermediate to those of free and conjugated RIPs. Consistently, the liver uptake of saporin increases after conjugation to IgGs, at least in part, as a consequence of the larger size of the immunotoxin compared to that of unconjugated saporin.[2]

The study of the uptake of saporin conjugated to the monoclonal antibody Ber-H2 (anti-CD30) by rat liver parenchymal and non-parenchymal cells shows that: (i) the accumulation of Ber-H2/saporin, saporin and Ber H2, both *in vivo* and *in vitro* is considerably greater by rat liver non-parenchymal cells than by parenchymal cells, (ii) *in vitro* the immunotoxin is more toxic to non-parenchymal cells than to parenchymal cells. These results suggest that the sensitivity of liver cells to immunotoxin is proportional to the uptake and that the *in vivo* damage to parenchymal cells is at least in part mediated by the toxicity to non-parenchymal cells.[3] Liver damage often brings about changes in xanthine oxidase activity concerning: (i) the conversion from the dehydrogenase to the oxidase form, (ii) the leakage of the enzyme from injured cells into plas-

ma where it is rapidly converted into the oxidase form. Both alterations give rise to increased formation of free oxygen radicals by xanthine oxidase. We have determined the activity of xanthine oxidase in liver and blood of rats receiving lethal doses of Ber-H2/saporin, saporin and ricin. The administration of free or conjugated saporin induces an increment of serum xanthine oxidase without the conversion of liver enzyme. The treatment with ricin, on the contrary, induces the conversion of liver xanthine dehydrogenase to oxidase without serum increment of the enzyme. Consistently, the pattern of histological lesions induced by the precedent treatments are different. Animals treated with free or conjugated saporin show necrosis of isolated hepatocytes, whereas the treatment with ricin induces patches of necrosis in parenchymal cells which follows the destruction of sinusoidal endothelial cells and the consequent stasis. The fact that xanthine oxidase activity is increased in the serum of rats poisoned with saporin or saporin immunotoxin, but not in the serum of rats poisoned with ricin could mean that the enzyme is released from hepatocytes only in certain types of liver damage. If so, the determination of plasma xanthine oxidase could be a diagnostic tool for the differential diagnosis of liver disease, including liver damage in patients treated with immunotoxins containing RIPs. Therefore, we are developing an ELISA for xanthine oxidase suitable to measure the enzyme in human serum.

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ANTI/CD22 RIPS IMMUNOTOXINS. EXPERIMENTAL MODELS AND CLINICAL STUDIES

Terenzi A,* Pasqualucci L,* Flenghi L,* Bolognesi A,° Martelli MF,* Stirpe F,° Falini B*

**Institute of Hematology, University of Perugia, Perugia; °Department of Experimental Pathology, University of Bologna, Italy*

We used a SCID mouse model of disseminated human B-cell Iymphoma for preclinical evaluation of immunotoxins (ITs) obtained by linking the anti-CD22 monoclonal antibody (mAb) OM124 to the ribosome inactivating proteins oftype 1 (RIPsl) Saporin (S06), Momordine (Mom) and Pokeweed anti-viral protein (PAP). In this model, a single intravenous injection with 5×10^6 Daudi cells induces disseminated B-cell lymphoma which infiltrates lymphoid organs and many extranodal sites, leading to death at day 39.2 after tumor challenge. The OM124/SO6 IT given intraperitoneally on days 1, 4 and 7 after cell inoculation (total dose 50% of the LD50 as S06) showed powerful antitumor activity, delaying the median survival time (MST) to 50.7 days (p=0.0001). We next compared the therapeutic efficacy of a single IT treatment with CD22/MOM versus a sequential treatment using anti-CD22 coupled to antigenically non cross-reacting ITs (OM124/SO6, OM124/MOM, OM124/PAP) given at three weeks interval. In each cycle of treatment, the above ITs were administered as a dose corresponding to 50% of the LD50. Mice receiving PBS or anti CD22 mAb alone served as negative control. Both treatment schedules showed significant antitumor activity extending MST by 59.1 and 72.6 days respectively, as compared with either PBS-treated (MST=39.2 days, p=0.0001) and OM124-treated mice (MST=44 days, p=0.0001). Notably sequential treatment with three ITs was significantly more effective than a single course treatment with CD22/MOM (p=0.0004). Furthermore, we combined ITs (CD22/SO6) with chemotherapy. The immunochemotherapy treatment markedly improved the survival of SCID mice (MST 117.5, 8/10 still alive at 120 days) compared with animals treated only with chemotherapy (MST 85 days, 2/10 mice still alive at 120 days) or with ITs (MST 86 days, 3/10 mice still alive at 120 days). Clinical data (Phase I studies) suggest that anti CD22/RIPs I ITs can be safely administered in humans and they are efficacious after ABMT (*Grossbard et al, Blood 81:2263, 1993*). The potentials of this therapy are: a) the treatment of minimal residual disease; b) the use in combination with radioimmunotherapy; c) prophylaxis and treatment of meningeal disease (B cell lymphomas and ALL).

ANTITUMOR ACTIVITY OF BER-H2/SO6 IMMUNOTOXIN IN VITRO AND IN SCID MICE XENOGRAFTED WITH HUMAN CD30 (KI-1)+ ANAPLASTIC LARGE CELL LYMPHOMA

Pasqualucci L,*° Wasik M,° Bolognesi A,# Stirpe F,# Martelli MF,* Kadin MF,° Falini B*

**Institute of Hematology, University of Perugia, Italy; °Department of Pathology, Beth Israel Hospital, Harvard Medical School, Boston, MA, USA; #Department of Eperimental Pathology, University of Bologna, Italy*

Ki-l $(CD30)^+$ anaplastic large cell lymphoma (ALCL) is a quite distinctive clinicopathologic entity which has been recently integrated into the updated Kiel classification as a high-grade lymphoma. To develop a novel adjunctive therapy, we evaluated the antitumor activity on ALCL cells of an immunotoxin (IT) constructed by coupling the plant emitoxin Saporin (S06) to the Ber-H2 monoclonal antibody (mAb) directed against the CD30 molecule, a newly recognized member of the TNF/NGF receptor superfamily. Tests were performed *in vitro* against the $CD30^+$ ALCL-derived cell line JB6 and *in vivo* using our newly established SCID mouse model of human xenografted $CD30^+$ ALCL. *In vitro*, Ber-H2/SO6 was selectively and highly toxic to the JB6 cell line, with an IC50 of 5×10^{-12} M as S06, far below the toxicity of irrelevant IT or the toxicity to irrelevant target. *In vivo*, a 3-day treatment with non toxic doses of Ber-H2/SO6 IT, corresponding to 50% of the LD50, induced lasting complete remissions in 80% of mice starting the treatment 24 h after tumor transplantation. When injected at later stages of tumor growth (mice bearing subcutaneous ALCL tumors of 40 to 60 mm^3 volume), Ber-H2/SO6 induced CR in 6 of 21 mice and, as shown in Table below, significantly delayed tumor growth rate (p<0.01). In contrast, neither the unconjugated mAb nor the toxin alone had any antitumor effect.

Groups	*Days to 500 mm^3*	*Tumor growth delay (days)*
Controls	18.28±0.99 (n=19)	
Ber-H2/SO6 IT	39.98±5.85 (n=21)	21.7±4.7
SO6	23.26±5.05 (n=7)	5.0±3.8
Ber-H2	17.97±0.67 (n=7)	

We conclude that Ber-H2/SO6 IT is an effective agent against $CD30^+$ ALCL growing in SCID mice, suggesting a possible therapeutic role in patients with CD30-expressing neoplasms refractory to conventional therapy.