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Immunotoxins: Hybrid Molecules Combining High Specificity and Potent Cytotoxicity

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I. INTRODUCTION

A *Basic idea*

Biological activities of enzymes, hormones or antibodies are induced only after recognition of their specific targets. This selective activity is obtained in Nature with molecules which possess at least two different functions, recognition and biological activity, in general performed by different domains of the same molecule. Specificity of antibody activity is obtained by the sequential involvement, first of the binding unit, which then activates the effector function, i.e. complement-binding antibodies only activate the complement system and destroy target cells if they are first bound to their specific antigen.

The idea of applying specific cell lysis by antibodies to passive immunotherapy of tumors has been very attractive for many years. However, the capacity of antibodies to destroy tumors in animals or man has always been limited, and it has often been observed that specific antibodies can enhance tumor growth (enhancement phenomenon). As a result, a series of attempts have been made to render the effector function of antibodies more potent by attaching either anticancer agents to these antibodies, a tentative step first described by Mathé et al. (1958), or toxins, as initiated by Moolten & Cooperband (1970). Higher potency, however, will only be beneficial for tumor therapy if it is specific for the target tissue, in the sense that the effector function

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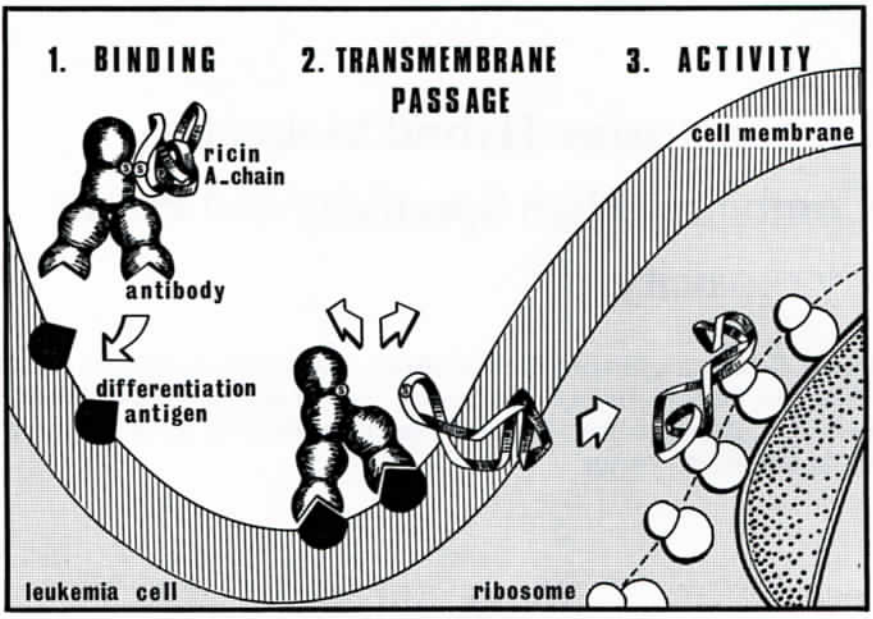

Figure 1. Hypothetical mechanism of sequential activation of the binding and effector functions of IT.

remains inactive during transport in the body and becomes activated only after binding of the antibody to the target cells. The development of such anti-tumor agents seems to be possible today with hybrid molecules consisting of antibodies, chemically bound to the toxic subunit of toxins, which we called immunotoxins (ITs) (Jansen et al. 1980). Toxins, such as ricin, consist of two chains, the A-chain or active chain and the B-chain or binding chain. The A-chain inhibits protein synthesis on ribosomes and the B-chain binds to galactose residues of the cell membrane and thereby helps the A-chain to penetrate into the cytoplasm to reach the ribosomes. Therefore, if A-chain alone is coupled as effector molecule on specific antibodies, it remains inactive during transport. Through its concentration on the target cell by the antibody, it becomes capable of penetrating into the cytosol and only then can it inactivate ribosomes (Fig. 1). High and specific efficiency could be obtained with such ITs *in vitro* and may, after further development, be applicable to the treatment of cancer.

B *Historical development*

In the first attempts of Gorer & Amos in 1956, antitumor antisera were injected into tumor-bearing mice, but only minor results were obtained. This approach

was often repeated and has been greatly improved during the last year with the use of monoclonal antibodies against differentiation antigens present on tumor cells. Although some encouraging results were obtained with high doses of monoclonal antibodies in an animal model (Kirch & Hammerling 1981,) their efficiency depended in other models on the injection of heterologous complement (Bernstein et al. 1980). In man a transient decrease of tumor cells could be induced (Ritz et al. 1981, Miller et al. 1981, Royston et al. 1980) and even remissions were achieved, but tumor cells could not be eradicated (Miller & Levy 1981).

The insufficient capacity of antibodies justifies, retrospectively, the parallel effort which has been made to increase the effector function of antibodies by attaching anti-cancer drugs, radioisotopes, enzymes with cell surface activity, or toxins (Ghose et al. 1976, Ghose & Blair 1978). However, radioisotopes or enzymes carried by specific antibodies represent an example of effector molecules which are always active during their transport in the body and damage all tissues with which they come into contact; on the other hand low molecular weight drugs, such as chlorambucil, act stoichiometrically and need more than 20,000 alkylations of DNA per cell to induce cell death (Crathorn & Roberts 1966).

Higher potency was expected when polypeptide toxins were coupled to antibodies (Moolten et al. 1976). Toxins such as diphtheria toxin have the principal advantage that one molecule, once it has penetrated into the cytoplasm, enzymatically inactivates all ribosomes and thereby induces cell death. As few as 19 molecules of diphtheria toxin or 1800 ricin molecules bound to the cell surface seem to be sufficient to obtain the penetration of at least one A-chain into the cytoplasm, which is sufficient to kill the cell (Olsnes & Pihl 1981). The first antibody-toxin conjugates indeed showed high potency, but only marginal specificity (Moolten et al. 1976). By improving the chemical linkage higher specificity could be obtained (Thorpe et al. 1978, Youle & Neville 1980). Such conjugates nevertheless retained considerable nonspecific toxicity. If the nonspecific binding by the B-chain of the toxin is not completely inhibited by steric hindrance through the chemically linked antibody, such hybrid molecules have the same chance of being bound to the cell surface by the specific antibody or by the nonspecific toxin binding site. In both cases the binding induces the intracytoplasmic penetration of the A-chain. Conjugates of whole toxins with antibodies are therefore an example of effector molecules which become active after binding, but binding is not only due to the antibody. With whole toxins, high specificity of IT can only be obtained if the nonspecific binding site is irreversibly inactivated.

When we became involved with antibody-toxin conjugates in 1975 we thought that it was more difficult to inactivate the binding site of the B-chain than to eliminate the B-chain completely and we projected conjugates in which the B-

chain of the toxin was replaced by an antibody. Ricin was preferred to diphtheria toxin, against which almost everybody is immunized. According to the description by Olsnes et al. (1974) the A-chain of ricin was estimated to be poorly toxic for whole cells. Its concentration on the cell surface by the antibody should then enhance its penetration into the cytoplasm. Antibody-A-chain conjugates (ITs) should therefore represent an example of inactive effector molecules which become active after the specific binding of the antibody, enhancing intracytoplasmic penetration. Fab or F(ab')2 fragments should be preferred to whole antibodies because they are devoid of nonspecific Fc receptor binding.

The success of an IT depended on two essential conditions: a) the A-chain must be sufficiently nontoxic to avoid any nonspecific activity of ITs and b) the antibody must effectively replace the B-chain of a toxin and render the A-chain active in a specific way.

We managed to fulfill these conditions on four tumor models. With an artificial model we showed that an antihapten IT specifically killed hapten-modified tumor cells *in vitro* and inhibited their tumor growth *in vivo* (French Patent no. 78.27.838, 1978; Jansen et al. 1980). Higher *in vitro* specificity was obtained with a monoclonal antibody against the Thy 1.2 antigen and a mouse leukemia cell line (Blythman et al. 1981). *In vivo* activity, however, was less satisfactory. With a more tumor-selective IT directed against a human melanoma-associated antigen (Brown et al. 1980) high specific activity was found on a human melanoma cell line *in vitro.* In the presence of ammonium chloride IT was more potent than ricin and killed the last plated tumor cells (Casellas et al. 1982). Finally, we report here on an IT against a human T-lymphocyte antigen, which was highly specific and much more potent than the toxin ricin on a human leukemia cell line. Several other laboratories independently reported that antibodies conjugated to the A-chain of toxins show specific activity in different cell models (Masuho et al. 1979, Krolick et al. 1980, Raso & Griffin 1980, Gilliland et al. 1980, Miyazaki et al. 1980, Thorpe et al. 1981).

The experience with four different IT models allows review in this article of:

1. the high specific activity of conjugates between antibodies and the A-chain of ricin, which can reach higher potency than ricin and kill the last plated tumor cells;
2. the essential requirements for optimal *in vitro* IT activity;
3. the advantages and limitations of ITs *in vitro* and *in vivo* today.

In the following sections the three subunits of IT: the toxin, the antibody and the chemical linkage will be described. After the presentation of the cellular models follows the demonstration of *in vitro* and then *in vivo* IT activities. Finally some conditions necessary to compare ITs of different origin and the possible applications of IT today are listed.

II. RICIN AND RICIN A-CHAIN ACTIVITIES

The coupling of isolated A-chain of ricin, as effector molecule, to antibodies requires complete elimination of all B-chain contaminations, otherwise B-chain could recombine with A-chain and recover residual nonspecific toxicity similar to ricin. Therefore A-chain was highly purified and examined for *in vitro* and *in vivo* activities.

A. In vitro *properties*

1. *Methods*

The toxin activity was measured in two ways, by inhibition of protein synthesis in an acellular system, allowing direct access of the toxin to ribosomes, and by a cellular system which requires the intracytoplasmic penetration of the toxin.

The acellular method consisted of a cell-free protein synthesis system of rat liver. In the presence of the artificial messenger poly-U the incorporation of ^{14}C phenylalanine into proteins was measured. If ricin was dissociated in its two chains by β-ME it showed a 100-times higher activity as compared to the undissociated form, suggesting that the A-chain is sterically hindered by the B-chain in intact ricin (Vidal 1981).

The cellular method determined the inhibition of the uptake of ^{14}C leucine into newly synthetized cell proteins. However this method was rendered much simpler by measuring only the ^{14}C uptake into the cell, which paralleled exactly the overall uptake of radioactivity into the proteins of the cell (Jansen et al. 1980).

Ricin and its B-chain also have the ability to agglutinate erythrocytes. This was measured by agglutination of human O erythrocytes (Olsnes et al. 1974).

2. *Purification*

Ricin (MW 60,000) has affinity for galactose-containing structures and can easily be purified from non-lectin proteins of a castor bean extract by adsorption on agarose-containing gels (Tomita et al. 1972). However, it is contaminated by the castor bean agglutinin (MW 120,000) which has higher affinity for agarose. Nevertheless, by careful stepwise elution with a low galactose solution very pure ricin is recovered first with less than 1% agglutinin contamination (Vidal 1981).

After dissociation of ricin with 2.5% β-ME, the A-chain can be separated from the B-chain by ion exchange chromatography (Olsnes & Pihl 1973). However, this method was inadequate to eliminate all B-chain contaminations in A-chain preparations. We therefore combined ion exchange with affinity chromatography using DEAE-Sepharose (Table I). Thereby, A-chain preparations became more active in an acellular system and higher values were obtained

TABLE I

Decrease of A-chain toxicity by the combination of affinity chromatography with ion exchange chromatography during A-chain isolation or isolation plus concentration

A-Chain purification: combination with affinity chromatography for		A-Chain activities				
		acellular system	cellular system			
Isolation	Concentration	Prot. synth. IC_{50}(nM)	agglutin. µg/ml	Tox. *in vivo* LD_{50} µg/mouse	Tox. *in vitro* HeLa IC_{50} (nM)	Tox. *in vitro* A-Chain/ricin
−	−	0,25	17	3	2	20
+	−	0,12	>1200	450	130	1300
+	+	0,12	>1200	450	500	5000
ricin		0,25	2,5	0,3	0,1	−

for hemagglutination thresholds, *in vitro* toxicity on cells and LD_{50} in mice. A second opportunity to combine ion exchange with affinity chromatography was offered by the concentration of A-chain preparations on CM-Sepharose gel. A subsequent additional decrease of toxicity was obtained, but could only be demonstrated by *in vitro* toxicity on intact cells, which seems to be the most sensitive test system. A-chain purified in this way no longer contained any B-chain contamination. This was demonstrated by absorbing it on erythrocytes to which B-chain can bind. Absorption of a highly purified A-chain preparation did not diminish toxicity, suggesting that the remaining toxicity was no longer due to B-chain but represented the inherent toxicity of the A-chain, which was therefore able to enter the cytoplasm on its own if it was highly concentrated.

3. *Sensitivity of different cell lines to toxin*

On intact Molt 4 cells 2000 times higher concentrations, on CEM cells 100,000 times and on BT20 cells 1,000,000 times higher concentrations of A-chain compared to ricin were needed to inhibit protein synthesis (Fig. 2). The different toxicity of ricin and A-chain therefore strongly depends on the cell line tested. In order to obtain a spectrum of ricin and A-chain sensitivity, 11 human cell lines were tested. When cell lines were arranged according to decreasing sensitivity to A-chain, no correlation could be found with their sensitivity to ricin, suggesting that ricin and A-chain follow different pathways for transmembrane passage (Fig. 2). Concentrations for extreme high and low sensitivity of the cell lines may vary by a factor of 100 for A-chain as well as for ricin sensitivity. The mean concentration of A-chain toxicity is about 2×10^{-7}M, the one for ricin 2×10^{-11}M. The mean ratio of A-chain to ricin toxicity corresponds to a factor of about 10,000. We must expect that *in vivo* normal tissues could also have a very

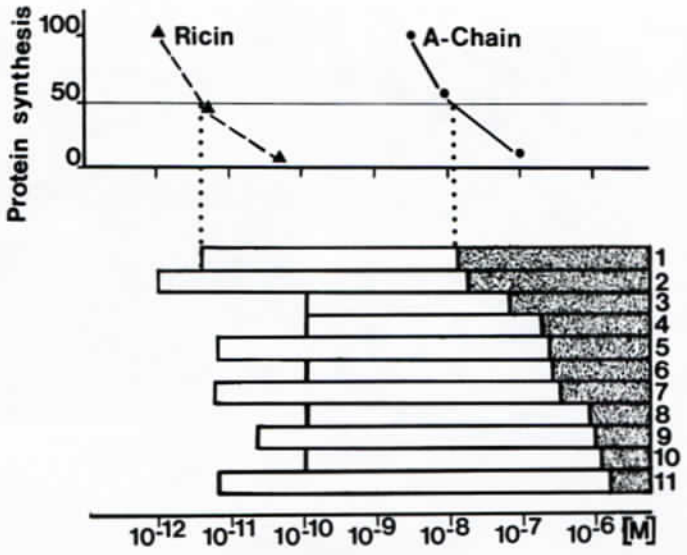

Figure 2. Activities of ricin and A-chain. Protein synthesis inhibition was measured at 50% inhibition, as indicated for the first cell line (JURKAT). The other cell lines are: 2. HPB-ALL; 3. AY-726; 4. Molt-4; 5. CEM; 6. – Me43; 7. RPMI-8402; 8. SK-MEL 28; 9. HeLa; 10. IgR.3; 11. BT-20.

different sensitivity to A-chain and that highly proliferative normal cells with a doubling time similar to the one of T-cell leukemia lines (JURKAT, HPB-ALL, MOLT-4) may possess a similar high sensitivity to A-chain (Fig. 2).

4. *Ultrastructural lesions*

The ultrastructural signs of ricin activity are a dissociation of polyribosomes into free ribosomes and the degranulation of the rough endoplasmic reticulum within a few hours after *in vitro* incubation (Fig. 3a, b). This is followed by an aqueous infiltration of the cell. The morphological aspect seems to follow an all or nothing rule for each cell. A good correlation was found between the percentage of cells with morphologically dissociated polyribosomes and the percentage of protein synthesis inhibition in a kinetic study. The morphological change seems to be a rapid consequence of the toxin activity. Although pinocytosis is very intense during incubation with ricin, pinocytotic vesicles do not come into contact with primary lysosomes. The absence of secondary lysosomes suggests that cellular defense mechanisms against the toxin are not very active (Carrière et al. 1980).

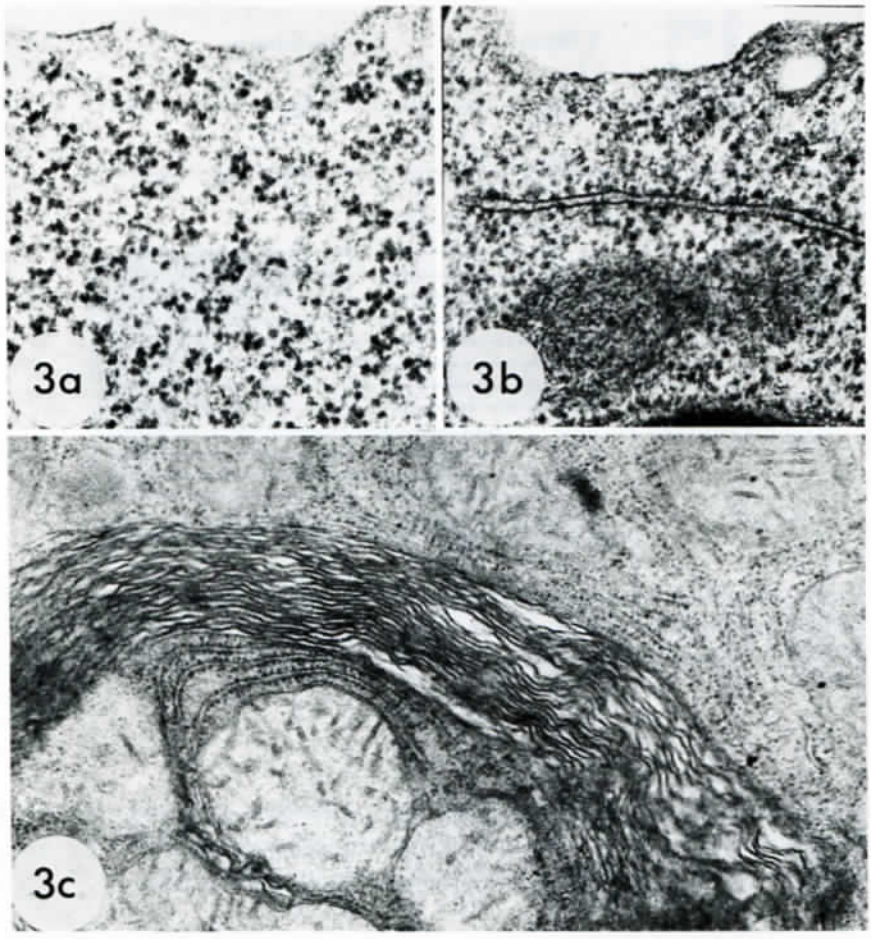

Figure 3. Ultrastructural changes induced by ricin and A-chain.
a, b: Polyribosome configuration in WEHI-7 cells. a) Control, b) After incubation with ricin 1nM for 3 h (x 55,000).
3 c: Lamellar concentric body in a murine hepatocyte 48 h after treatment with 3 mg/kg A-chain of ricin (x 24,000).

B In vivo *properties of ricin and A-chain*

1. *General toxicity*

The LD_{50} of ricin after a single i.p. administration corresponds to 0.32 micrograms/mouse, the one of pure A-chain to 466 micrograms/mouse or 23.3 mg/kg (Table II). Consequently, A-chain which is about 1500 times less toxic than ricin cannot be regarded as a very toxic drug and if it becomes separated from the antibody of an IT *in vivo*, its toxicity will be very limited. In an

TABLE II
Lethal doses of ricin and pure A-chain

		Lethal doses[b]		
		μg/mouse	mg/kg	N[c]
Ricin	LD_{50}	0.32 (0.26-0.38)[a]	0.016	7
	LD_{10}	0.18 (0.14-0.23)[a]	0.009	7
A-chain	LD_{50}	466 (406-534)[a]	23.3	3
	LD_{10}	330 (284-381)[a]	16.5	3

a) 95% confidence interval.
b) 7 days after ricin or A-chain injection into CD_1 mice of 20 g, from Charles Rivers, France.
c) (N) number of experiments with 10 CD_1 mice/group.

experiment of short-term cumulative toxicity over 5 or 10 days, doses corresponding to the LD_{50} were 4–5 times lower than a single injection.

2. *Histopathological alterations*

After a lag period of about 10 h ricin at an intravenous dose corresponding to its LD_{50} provokes lesions mainly in the RES and the vascular system with disseminated intravascular coagulations and changes related to the shock syndrome. Such lesions were particularly apparent on the endothelium, the Kupffer cells of the liver and on the endothelium of myocardial capillaries. The thymus, spleen, lymph nodes and bone marrow presented a severe loss of cell

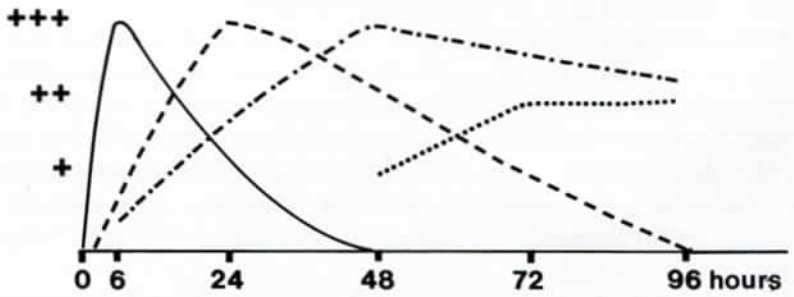

Figure 4. Kinetics of histopathological changes. After a sublethal dose of pure A-chain i.v. lesions appeared in: Intestine (crypts) (—), liver hepatocytes (– – –), thymus and adrenals (•—•) and kidneys (tubules) (. . .).

population and lesions of cytolysis. Parenchymal alterations in liver and heart were delayed and probably a consequence of the vascular lesions.

In comparison, A-chain at a dose corresponding to its LD_{50} led to a quite different distribution of histopathological changes (Fig. 4). Necrotic lesions were rapidly seen in the crypts of Lieberkuhn reaching maximum intensity 2–4 h after A-chain injection. They tended to disappear 1 day later when lesions of the liver parenchyma became dominant. With the electron microscope, concentric lamellar bodies could be observed, derived from the partially degranulated rough endoplasmic reticulum (Fig. 3c). The liver lesions decreased on days 2 and 3 but at the same time tubular lesions of the kidney became apparent reaching a maximum on day 4. The necrotic lesions were paralleled by functional lesions. Leukocytosis was maximal 2–8 h after A-chain administration, and lesions of the adrenal cortex reached a maximum on day 2.

The different localization of ricin and A-chain lesions could be explained by the immediate binding of ricin to every cell with which it is in contact, i.e. the blood cells and the endothelium, while A-chain without a binding site penetrated into organs and mainly attacked tissues with high mitotic index such as the crypts of Lieberkuhn or cells involved in detoxification, i.e. liver parenchymal cells or tubular cells in the kidney (Richer et al. 1981).

III. ANTIBODIES

A toxic effector molecule on a tumor-directed carrier should be attached to a molecule possessing a specific recognition function, i.e. hormones (Oeltman & Heath 1979) or lectins (Chang & Neville 1977) or more selective carriers such as antibodies against tumor-associated antigens (Masuho et al. 1979, Gilliland et al. 1980, Krolick et al. 1980, Casellas et al. 1982).

Antibodies used for the coupling to the A-chain of a toxin should be purified, because a high proportion of non-antibody gamma-globulins, which are contained in fractions of immune sera after salt precipitation of polyclonal antibodies, would diminish the specific activity of IT.

In the beginning of our studies, when monoclonal antibodies were not yet available, we used goat anti-hapten antibodies, because they could be obtained in gram quantities and purified by immunoadsorption (Poncelet 1979). The polyclonal antibodies showed an affinity constant for cell-bound TNP of about $2\times10^{7}M^{-1}$. As soon as the lymphocytic hybridization technique was performed in our laboratory, the polyclonal anti-DNP antibodies were replaced by monoclonal IgG2b antibodies with an affinity constant of $1.7\times10^{8}M^{-1}$ (Pau et al. 1980).

A monoclonal IgM antibody against the antigen Thy 1.2 of mouse T-lymphocytes was purchased from Olac, England. It was purified by precipitation with ammonium sulfate and successive filtrations on Sepharose 6B.

Complement lysis (50%) was obtained at a concentration of 0.9×10^{-10}M.

The monoclonal antibody T101 (Royston et al. 1980) was a gift from Dr Royston. It is an IgG_{2a} directed against a 65,000 dalton antigen on immature and mature human T-lymphocytes and has complement-fixing activity in concentrations of about 10^{-10}M. High purification could be obtained with protein A-Sepharose.

In collaboration with Drs I. and K. E. Hellström we were able to use their monoclonal antibody 96.5 directed against a melanoma-associated antigen P 97 (Brown et al. 1980). Complement-dependent lysis was obtained in concentrations of about 3×10^{-10}M (Hellström et al. 1981). Purification was also performed with protein A-Sepharose.

IV. CHEMICAL LINKAGE

Antibody and A-chain have to be chemically linked without decreasing their functions. Considering the natural linkage of A- and B-chains in ricin we thought that such a labile disulfide bridge might be necessary to obtain A-chain activity. We therefore tested first a disulfide bridge and compared it to a stable thioether or a disulfide bridge with longer spacer arm.

1. *Labile disulfide bridge*

The heterobifunctional reagent 3-(2-pyridyldithio)-propionic acid was first reacted with a limiting amount of carbodiimide in order to activate the carboxylic function and then coupled to the antibodies in conditions allowing us to obtain a statistical substitution of two to four activated disulfide groups per antibody molecule. The antibodies were then reacted with an excess of A-chain which binds through its free sulfhydryl group to the activated disulfide groups of the antibody. In general, a final substitution of about 1.5 to 2 A-chains per IgG was obtained. Similarly, in the case of the high molecular weight IgM antibodies, a substitution of 8 A-chains per antibody molecule was attained.

The nature of the heterobifunctional reagent as well as the general chemical conditions chosen avoided the denaturation of antibody and A-chain involved. With all IgG conjugates, A-chain activity in an acellular system was completely recovered after cleavage of the disulfide bridge using β-ME. In the case of IgM conjugates only 3 out of 8 coupled A-chains were found biologically active after cleavage. The anti-DNP IT had similar affinity constants as the unmodified antibody and the anti-human T-cel IT demonstrated binding identical to the unmodified antibody in an indirect immuno-fluorescence test by FACS analysis. Only the anti-melanoma IT showed a slight loss of 25% activity in binding (FACS) and competition assays, and the IgM anti-Thy 1.2 IT had a slight decrease of 30% in a complement-dependent lysis

test. Steric inhibition of the antibody binding site by A-chain may explain the slight decrease of antibody activity, which seems to depend on the nature of the antibody.

2. *Stable thioether bridge*

When the disulfide bridge was replaced by a stable thioether bridge using 6-maleimidocaproic acid as a bifunctional coupling reagent, which introduces a 9 atom spacer, IT retained 30% of its activity in an acellular system. This suggests that A-chain linked to the antibody by a stable bridge, which cannot be cleaved by β-ME, does not need to be separated from the antibody to become active on isolated ribosomes. This decrease of activity was induced by steric inhibition, due to the presence of the antibody. The same conjugate tested on an intact cellular system lost about 99% of activity compared to a disulfide conjugate. With respect to free A-chain it was only twice as active. It can be concluded that the stable bridge does not prevent A-chain being active on ribosomes but its cytoplasmic penetration seems to be severely disturbed. A-chain may therefore need separation from the antibody to reach the ribosomes, while the antibody remains fixed to the membrane antigen.

3. *Long spacer disulfide bridge*

In the hope of increasing IT activity we tested whether a longer disulfide bridge could be more easily broken. This was tested by coupling A-chain and antibody through an 11-atom long spacer arm, using 7-aza 8-oxo 10-(-2-pyridyldithio)decanoic acid as the bifunctional coupling reagent. The longer arm enhanced A-chain activity on an acellular system by about 3 times, if care was taken that no thiols could split the disulfide bond. However, in presence of thiols in this system or on intact cells no difference in activity could be found between a spacer arm 4 or 11 atoms long, so that a longer disulfide bridge is not an advantage for ITs.

V. CELLULAR MODELS

Different cell models were chosen for the study of ITs, from a more simple, artificial model in the beginning, to animal and human tumors in later models.

1. *Artificial antigen*

Before monoclonal antibodies could be produced it was difficult to obtain highly specific and purified antibodies against differentiation antigens. Therefore an artificial antigen, the hapten TNP, was chosen, which could be used as a marker of almost every cell line. Under controlled experimental conditions the

modification does not affect cellular behavior, as verified with different *in vitro* and *in vivo* tests (Jansen et al. 1980). TNP-haptens per cell were in large excess when compared to the maximum quantity of antibodies which could be bound per cell (about 5×10^7 on a TNP-HeLa cell). The estimated area covered by the antibody binding sites corresponded to the entire cell surface. Different cell lines were modified with TNP, especially HeLa cells and WEHI-7 cells.

2. *Mouse differentiation antigen*

When the first monoclonal antibodies against a differentiation antigen became available, we replaced the artificial antigen by the natural cell membrane antigen, Thy 1.2 of mouse T-lymphocytes. This antigen is also present on several mouse leukemia cell lines. We chose the WEHI-7 cell line obtained from the Salk Institute, because it showed high antigen density.

3. *Human differentiation antigen*

Our first approach to human cancer involved the use of the monoclonal antibody T101 (Royston et al. 1980) against the human differentiation antigen T 65, in collaboration with Dr. Royston, La Jolla. As in the mouse system, this antigen is present on human T-leukemia lines, from which we chose the CEM line provided by Royston. The administration of an IT directed against a differentiation antigen on patients carries the danger that it damages tumor cells and normal cells to the same extent. If, however, the differentiation antigen is not present on the precursors of normal cells, a complete recovery of the normal cells could be expected after the end of IT administration.

4. *Human tumor-associated antigen*

We were able to take one more step towards the selectivity of ITs for human tumors in collaboration with Drs I. and K. E. Hellström by using their antibody 96.5 (Brown et al. 1980). The corresponding antigen is associated with human melanoma and present on melanoma cell lines or on fresh biopsy material from patients with melanoma (Woodbury et al. 1980, Brown et al. 1981). Although the antigen is not entirely specific for tumor cells it is expressed in much higher amounts in many melanomas than in normal adult tissues. The SK-MEL 28 cell line derived from a human melanoma was selected as target because of its high antigen density per cell.

VI. IMMUNOTOXIN: *IN VITRO* ACTIVITIES

Immunotoxins definitely acquired several advantages: specificity, efficiency,

the ability to be potentiated, stability and reproducibility. Nevertheless some limitations do remain.

A *Advantages*

1) *Immunological specificity*

Specificity of immunotoxins for their target cells was demonstrated in four models:

a) The heteroclonal anti-DNP IT inhibited 50% of protein synthesis (IC_{50}) on TNP-HeLa target cells with concentrations 500 times lower than free A-chain. The increase of concentration necessary for A-chain to induce nonspecific activity on the same cell line correlates with the specificity of IT and can be estimated by the ratio of free A chain *versus* IT concentration for IC_{50}. This factor is called "specificity factor" (SF) and graphically shown by an arrow, the tail of which represents the A-chain effect (IC_{50}) and the head the specific effect of IT on the same target cells (IC_{50}) (Fig. 5a) (Jansen et al. 1980).

An IT containing the $F(ab')_2$ fragment was as active as the one with whole antibody (Fig. 5a). A monoclonal antibody (Pau et al. 1980) with an affinity constant of $1.7\times10^{8}M^{-1}$ increased the specificity factor three times as compared to a heteroclonal antibody with an average affinity constant of $2\times10^{7}M^{-1}$ on WEHI-7 cells.

b) The IT against the Thy 1.2 differentiation antigen reached a higher specificity factor of about 2,000–6,000 on the mouse leukemia WEHI-7 (Fig. 5b). The specificity increase may be partially due to higher sensitivity of the WEHI-7 compared to HeLa cells, as found with the IT against DNP (Fig. 8). Antibody without complement showed almost no activity and an unrelated anti-dextran IT, also containing an IgM antibody, was as active as free A-chain (Blythman et al. 1981).

c) An IT against the human T-cell antigen with the T 101 antibody led to a specificity factor of 7,000 if incubated for at least 48 h (Fig. 5c). It was found that this IT had rather long kinetics. Antibodies alone had no effect and an anti-DNP IT needed higher concentrations than A-chain alone.

d) A more tumor-selective IT due to the monoclonal antibody against the human melanoma-associated antigen P97 also reached a specificity factor of the same order as the preceding IT, if the adherent melanoma cells were used in suspension (Fig. 5d). After adherence, they are less sensitive (Casellas et al. 1982). As expected, antibodies alone were inactive and an anti-DNP IT on melanoma cells without TNP was less efficient than free A-chain.

Additional controls were performed for each of the models showing that a mixture of free antibodies and free A-chain, unable to combine chemically, behaved as free A-chain. This was demonstrated with the anti-DNP IT (Jansen et al. 1980) the anti-Thy 1.2 IT (Fig. 5e) (Blythman et al. 1981) and the anti-

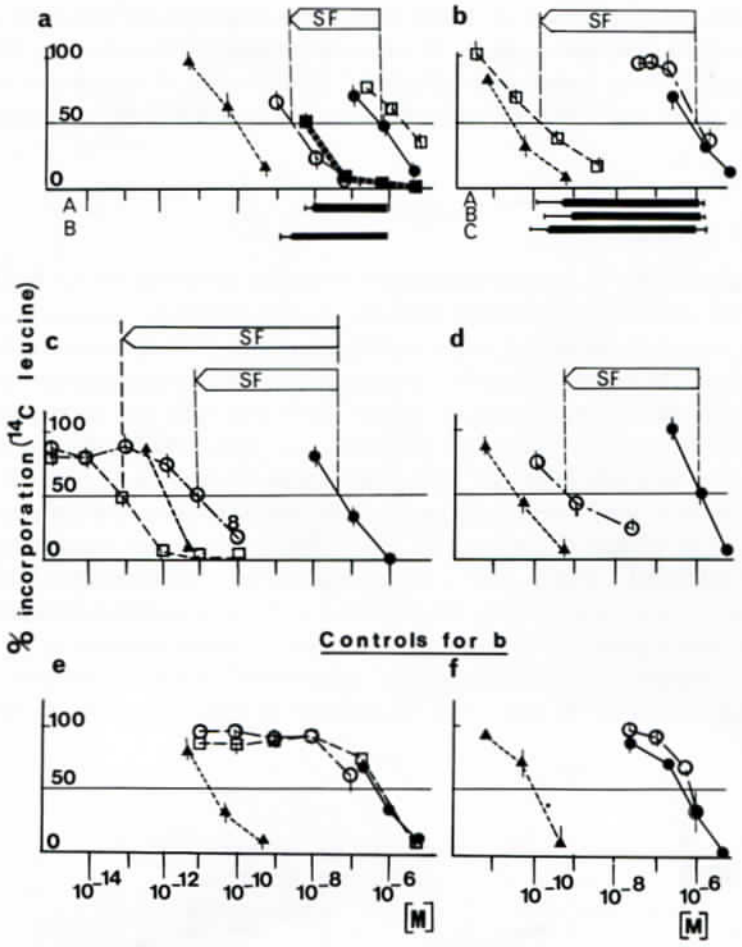

Figure 5. Specific activity of IT on different cell models. Ricin and A-chain are symbolized by (▲—▲) and (●—●), respectively in all cases. SF: specificity factor. (Fig. 5b, e and f reprinted by permission from *Nature* **290**, (5802) pp 145–146. Copyright (c) 1981 Macmillan Journals Limited).

a) artificial model, with TNP-modified HeLa cells. Anti-DNP IT (○—○), anti-DNP IT composed of F(ab')2 fragments (■—■), on TNP-HeLa cells. Anti-DNP IT (□---□), on unlabeled HeLa-cells. A and B are different IT preparations.

b) anti-Thy 1.2 IT (IgM) (□—□) and anti-dextran IT (IgM) (○---○) on WEHI-7 cells. A, B and C are different IT preparations.

c) anti-human T-cell IT on CEM cells with (□---□) and without (○— · ○) NH_4Cl after a 48 h incubation.

d) anti-P97 IT on SK-MEL 28 cells (○—○).

e) specificity controls on Thy 1.2 positive WEHI-7 cells. Anti-Thy 1.2 IgM antibody alone (□---□), a mixture of anti-Thy 1.2 IgM and free A-chain (○—○).

f) specificity controls on Thy 1.2 negative cells (BC-3A). Anti-Thy 1.2 IT (○—○).

melanoma IT (Casellas et al. 1982). On antigen-negative cell lines with similar sensitivity to ricin and A-chain, IT behaved similarly to free A-chain (Fig. 5f), also shown in the mentioned references. Additionally, it was shown that the activity of an anti-DNP IT could be absorbed with DNP-BSA (Jansen et al. 1980).

2. *High efficiency*

The high potency of a toxin should give an IT the ability to kill the last tumor cell. This could not be studied with the protein synthesis inhibition method, which measures an initial toxin interference and is only sensitive at 50% inhibition. The colony formation assay indicates the last consequence of toxin interaction on the proliferation capacity of tumor cells and its sensitivity is sufficient to measure single cell proliferation. About 3000 adherent human melanoma cells (SK-MEL 28) were treated with the specific IT for 24 h. After replacement of the IT with fresh medium the cells were allowed to form colonies during 10 to 15 days (Casellas et al. 1982). There was a linear relation between seeded cells and colonies over a wide range of cell concentrations, although cloning efficiency was variable. Ricin, as well as A-chain and IT, were able to kill the last plated tumor cells, although at different concentrations (Fig. 6). While A-chain needed 10^{-6}M concentrations, ricin and IT killed the last cells with a concentration of 10^{-9}M and 2×10^{-9}M respectively. Here, IT repeatedly revealed

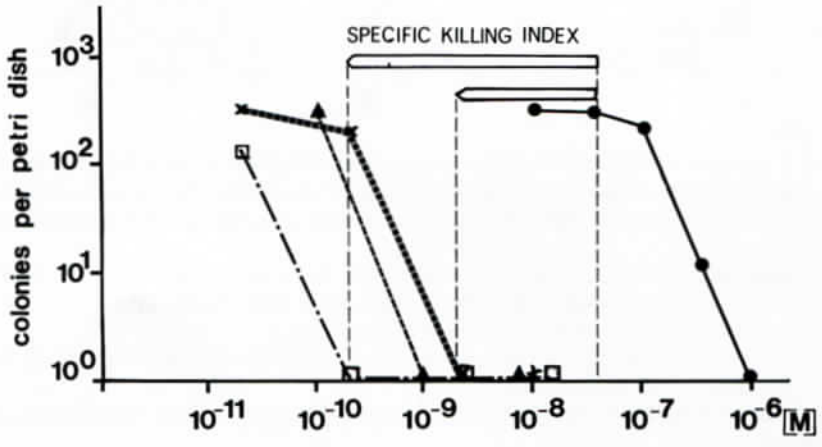

Figure 6. Inhibition of SK-MEL 28 colony formation by anti P97 IT.
Cells were preincubated for 24 h, followed by another 24 h incubation in the presence of the test materials, and then allowed to form colonies for up to 14 days. Ricin (▲—▲), A-chain (●—●), and anti-P 97 IT, with (□—·□), and without (X—X) NH_4Cl.

itself as efficient as ricin. However, the test conditions, especially preincubation time and cloning efficiency, were favorable in these experiments (see C 4).

The colony formation assay allows the estimation of a factor which may be an *in vitro* equivalent of the *in vivo* therapeutic index. It indicates to what extent the concentration at which IT kills the last tumor cells must be increased before free A-chain nonspecifically kills the first control cells. The ratio of ICo of A-chain to IC_{100} of IT of is called "specific killing index". It indicates the concentration range in which IT is 100% efficient without any nonspecific toxicity. Non-specificity is measured by A-chain on the same cell line but other normal cells *in vivo* may have higher A-chain sensitivity and so decrease the "specific killing index". The anti-melanoma IT repeatedly showed a "specific killing index" of 30.

In the colony formation assay also all controls confirmed immunological specificity. The antibody alone had no effect, nor did an anti-DNP IT up to a 10^{-6}M concentration. A mixture of antibody and A-chain behaved similarly to A-chain. On MRC5 cells, which express only low levels of P 97, IT had no specific effect.

3. *Potentiation*

IT induces patch and cap formation as known for bivalent antibodies. With the electron microscope, 1.5 to 3 h after incubation with IT, phagolysosome-like organelles were observed, suggesting that IT might be destroyed in secondary lysosomes (Fig. 7) (Carrière et al. 1980).

We therefore studied different substances known to interfere with lysosomes and found that 10mM ammonium chloride had a highly potentiating effect on IT against natural membrane antigens. Ricin and A-chain activities, however, were not modified. With this potentiation, IT becomes 5 times more active than ricin in the human melanoma model thus reaching a "specific killing index" of 300 in the colony inhibition assay (Fig. 6). There is a considerable increase of activity of the anti-human T-cell IT on the human leukemia CEM by ammonium chloride in the protein synthesis inhibition test (Fig. 5c). IC_{50} is now obtained with 10^{-13}M concentrations, which correspond to less than 1,000 molecules per target cell in the incubation medium.

4) *Stability and reproducibility*

Stability after storage at −20°C in PBS pH 7.4 was followed over 1 year with the anti-DNP IT which always maintained the same specificity factor with slight variations, probably due to the cell line. Reproducibility was studied with different batches of IT (Fig. 5 a, b). Specificity factors were maintained with slightly more variations for specific than for nonspecific activities. In general, IT can be regarded as sufficiently reproducible.

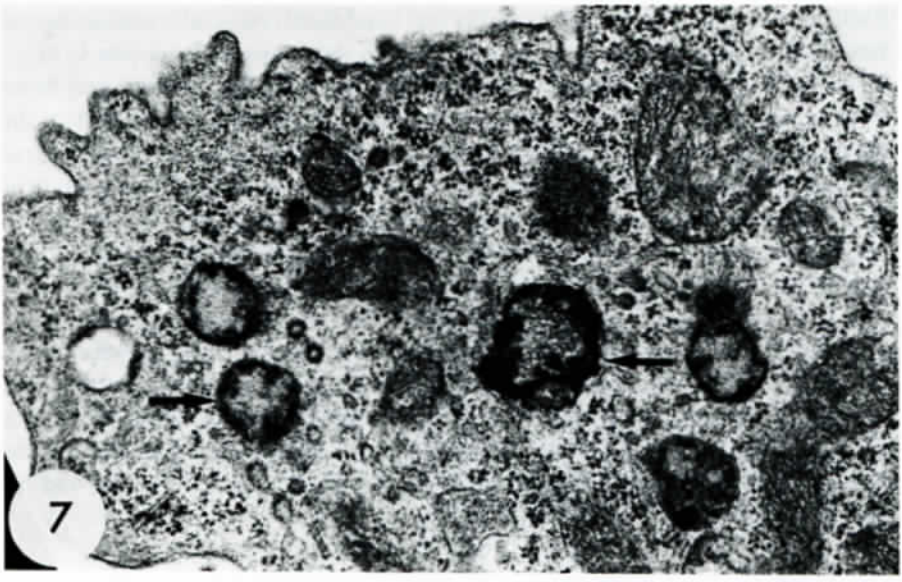

Figure 7. Phagolysosome – like organelles. Arrows indicate such vesicles in a WEHI-7 cell incubated with IT for 3 h (x 32,000).

B *Essential requirements for high IT efficiency*

A slightly more selective cytotoxicity to certain tumor cells as compared to normal cells can sometimes be found with whole toxins or their A-chains (Fig. 2). This is due to different sensitivity of cells to the toxin, i.e. the mouse leukemia cell line L1210 and the fibroblast line 3T3 (Fig. 8). Although such differences are small they were used for therapeutic purposes with ricin (Lin et al. 1970, Fodstad & Pihl 1980).

The essential improvement expected from ITs is a considerable increase of such specificity with the help of the antibody. In order to obtain optimal specificity and potency, several essential requirements can be formulated:

1. *High purification of A-chain*

Since A-chain and B-chain have some affinity for each other, trace amounts of B-chain have to be eliminated since they would otherwise generate nonspecific ricin toxicity. A minimum A-chain toxicity is the best base to obtain specific ITs (Fig. 2). Naturally occurring A-chains such as gelonin (Stirpe et al. 1980) which are not bound to a B-chain, may appear to correspond better to this requirement and have the additional advantage of not being very toxic during the purification process (Thorpe et al. 1981). However, they have a major disadvantage, since

they lack a precisely localized -SH residue. Chemical linkage will therefore lead to heterogeneous products with more or less diminished biological activities.

2. *A nondenaturing labile disulfide bridge*

Hetero-bifunctional disulfide reagents were shown not to denature A-chain or antibody to a great extent. The labile disulfide linkage imitating the natural bond seems to be necessary for activity with ricin A-chain conjugates, although stable bridges with other toxins may show some activity (Thorpe, this issue). Instead of introducing new activated disulfide bridges into antibodies, their natural thiol group in Fab' fragments can be used (Masuho & Hara 1980) which leads to homogeneous conjugates. The yield of this approach, however, should be inferior to artificially introduced activated thiol groups and it is hoped that the coupling of several A-chains per antibody molecule will increase their efficiency. Such studies are in progress.

3. *High affinity of purified antibodies*

If the B-chain of ricin is replaced by an antibody of similar affinity (the anti-DNP antibody) and if the TNP haptens on target cells are present in similar density, distribution and accessibility for antibodies, when compared to the receptors for ricin, such ITs achieve only about 1% of the activity of ricin *in vitro* (Jansen et al. 1981a). This suggests that the B-chain has, besides its binding function, an additional function – to enhance the intracytoplasmic penetration of A-chain. Since an antibody does not replace this function and it only concentrates A-chain on the cell surface, it seems to induce adsorptive endocytosis which is more efficient than fluid endocytosis (Silverstein et al. 1977). The missing "helper" function of the B-chain, however, can be compensated by higher affinity of antibodies, as shown with a monoclonal anti-DNP antibody when compared to antibodies with lower affinity (Pau et al. 1980). The purification of antibodies, which is much easier with monoclonal than with conventional antibodies, avoids a dilution of specific activity by nonantibody gamma-globulins and thus also increases efficiency.

4. *Potentiation of IT activity*

To obtain a maximum increase of IT activity it was thought that its cellular catabolism in the secondary lysosomes could be inhibited. Ammonium chloride is known to increase the pH in lysosomes (Ohkuma & Poole 1978) and proved to enhance IT activity to higher potency than ricin. However, other explanations for ammonium chloride activity are reported in the literature, i.e. it inhibits transglutaminase (Davies et al. 1980) and protects the cell against diphtheria

toxin without interfering with toxin degradation (Dorland et al. 1981). We found that ammonium chloride accelerated IT kinetics considerably, but the precise mechanism is unknown. The potentiation by ammonium chloride can easily be utilized *in vitro* but there are more difficulties in an *in vivo* administration, although a promising result was obtained in a first experiment in the mouse (see VII B 1).

C *Limitations of IT*

1. *Relative cellular resistance*

Differences in the sensitivity to IT cannot easily be studied with IT against natural antigens, because different cell lines will vary in antigen density. An IT against an artificial antigen, the hapten TNP, which can be attached to every cell line, may help in studying sensitivity of IT (Jansen et al. 1981a). A hapten density can be induced on the cell membrane which is largely superior to the amount necessary to completely cover the cells by antibodies. The sensitivity of different mouse and human cell lines to the anti-DNP IT varied to a considerable extent. The concentrations corresponding to IT activity on cell lines of high and low IT sensitivity varied by a factor of about 300 (Fig. 8). TNP as hapten on different membrane proteins does not represent an identical antigen when different cell lines are compared; consequently, the differences in sensitivity may be due, in part, to different antigens modified by TNP or to different antigen densities. It remains highly probable, however, that there are also differences in sensitivity to IT, so that tumors from patients must be tested before treatment for their individual IT sensitivity.

2. *Slow kinetics*

The ITs directed against natural membrane antigens present much slower kinetics than ricin or A-chain (Fig. 9). Kinetic activities of ricin show a short

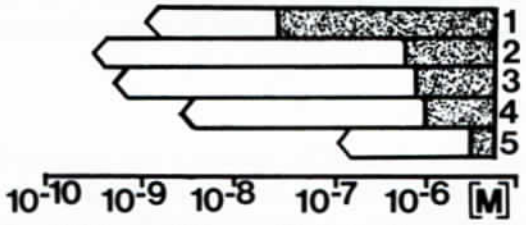

Figure 8. Sensitivity of different cell lines to IT. Each white arrow represents the specificity factor for each cell line, determined with an anti-DNP IT on TNP-labeled cells. 1. L1210; 2. WEHI-7; 3. SK-MEL 28; 4. HeLa; 5. 3T3. The shaded area symbolizes sensitivity of these cells to A-chain.

latent period of about 30 min followed by rapid inactivation of protein synthesis, attaining 50% within 1½ h (Olsnes & Pihl 1976). The kinetics of A-chain are longer. A 50% inhibition is obtained after 3 h, although the A-chain concentration is 5,000 times higher than that of ricin. This is another indication that A-chain follows another entry pathway than ricin. Total inhibition of protein synthesis can only be obtained after 10–12 h with A-chain. This demonstrated the necessity of long incubation periods exceeding 10 h in order to account for A-chain toxicity *in vitro*.

The anti-Thy 1.2 IT needs about 13 h with WEHI-7 cells to kill 50% of them, the anti-human T-cell IT about 20 h on CEM cells (Fig. 9) and the anti-melanoma IT about 30 h on SK-MEL 28 cells (Casellas et al. 1982). The slow kinetics of IT may give the target cells sufficient time to mount defense mechanisms, which reduce the IT efficiency. ITs with slow kinetics need to be continuously present during the whole incubation phase. If they are replaced by culture medium after 1 h, less inhibition is obtained. In clinical application, this means that IT must be maintained at a high blood level for a considerable time in order to achieve maximum effect.

Ammonium chloride, which was found to potentiate IT activity, leads to a considerable acceleration of kinetics. Thus anti-Thy 1.2 IT kills 50% of the cells within 1½ h, the anti-human T-cell IT within 3 h (Fig. 9) and the anti-human melanoma IT also in 3 h (Casellas et al. 1982). The slower the kinetics of an IT, the more effective is ammonium chloride. *In vitro* there is no toxicity on control cells after incubation with ammonium chloride at 10mM for up to 4 days. Slow kinetics of IT can therefore be reversed by ammonium chloride.

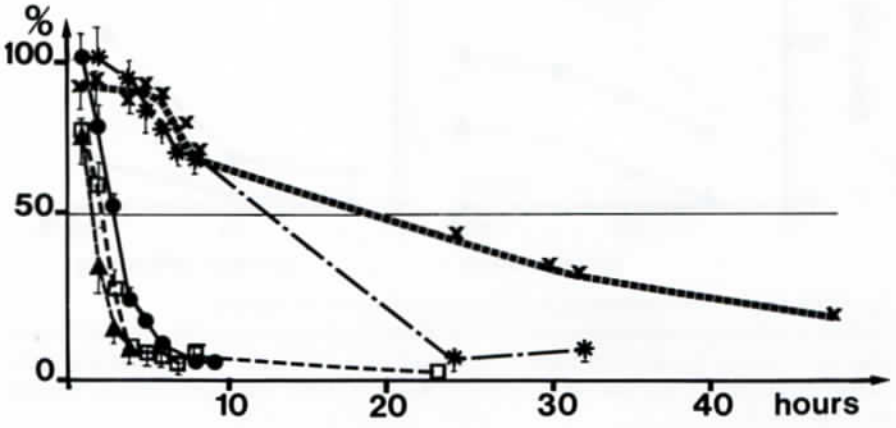

Figure 9. Kinetics of IT *in vitro* activity. Kinetics of ricin (▲—▲), A-chain (●—●) and anti-Thy 1.2 IT (★—★) were tested on WEHI-7 murine lymphoma cells.
Kinetics of anti-human T-cell IT was tested on CEM human leukemic cells, with (□—□) and without (X----X) NH_4Cl.

3. *Comparison with complement-dependent lysis*

The activity of IT was compared with the complement-dependent lysis (50%) of the antibody used in the IT. Two of the four ITs studied seemed to be more potent than the corresponding antibody with complement, the others showed similar potency.

The monoclonal anti-DNP antibody (IgG 2b) in the presence of guinea pig complement needed about 100 times higher doses than the IT (Jansen et al. 1981b). The anti-human T-cell antibody T101 (IgG 2a) induced partial complement lysis in about 10^{-10}M concentration on another cell line (Royston et al. 1980) but did not kill 100% of target cells at higher concentrations. The IT with the same antibody was active at a 10^{-13}M concentration after potentiation with ammonium chloride, and killed 100% of the cells with higher concentrations.

In the two other models, IT was as potent as the antibody with complement. The anti-Thy 1.2 antibody (IgM) with complement was as active as IT at the concentration of 10^{-10}M (Jansen et al. 1981b). The anti-human melanoma

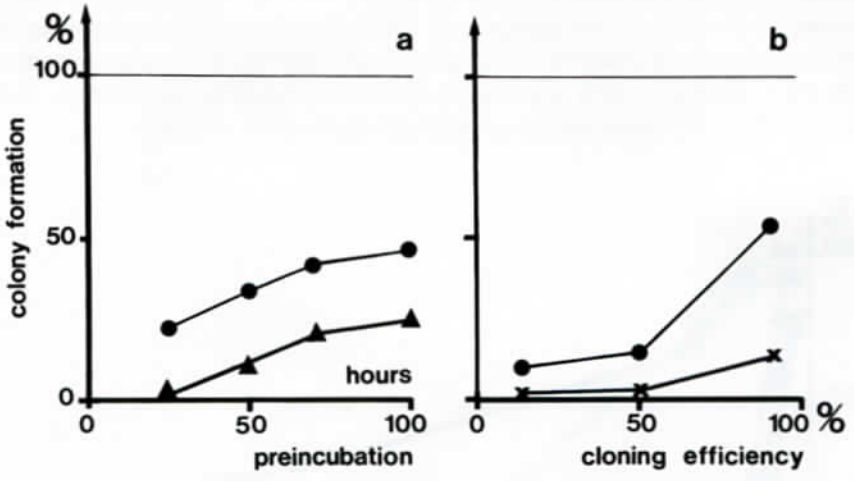

Figure 10. Influence of experimental *in vitro* conditions on IT activity.
a) effect of different preincubation periods. SK-MEL 28 cells were seeded into Petri dishes and allowed to adhere for different times before incubation. Then they were treated with anti-melanoma IT for 24 h with (▲—▲) or without (●—●) NH_4Cl. Preincubation times were 24, 48, 72 and 96 h.
b) effect of cloning efficiency. Anti-melanoma IT was tested on different cell cultures, differing in their cloning efficiency (14, 50, 90%) with (X—X) or without (●—●) NH_4Cl. High cloning efficiency was obtained with cells at the exponential phase of the growth curve, low cloning efficiency resulted from cells taken at confluence.

antibody 96.5 showed about the same activity, 3×10^{-10}M (Hellström et al. 1981) and is also comparable to IT after potentiation (10^{-10}M). However, IT should be more advantageous in an *in vivo* situation, because human complement seems not to be highly active with mouse antibodies (Miller & Levy 1981). The use of Fab fragments (see VI A 1), which are inactive with complement, represents another advantage of ITs, since such ITs should enhance the penetration into organs due to their lower molecular weight of about 80,000.

4. *Dependence on* in vitro *conditions*

When some *in vitro* conditions on IT activity were studied it was found that IT became less active when *in vitro* conditions were more physiological. IT activity on the human melanoma cell line SK-MEL 28 depended to a great extent on the duration of preincubation of target cells, necessary for their adherence before the test, on the incubation time and on the cloning efficiency after the incubation phase. Freshly harvested melanoma cells need some time to adhere to a support. With increasing preincubation time from 1 to 4 days the melanoma cells became more and more resistant to IT although ricin and A-chain activity remained unchanged in a colony formation assay (Fig. 10a). The increase of cell resistance was also seen in the presence of ammonium chloride, but to a lesser extent.

Longer incubation time with IT is favorable for IT efficiency because the slow kinetics require long incubation to reach the maximum effect. With the anti-melanoma IT an incubation up to 4 days increased IT potency and even in the presence of ammonium chloride an incubation of several days was advantageous. On the other hand, a long incubation is necessary to demonstrate A-chain toxicity, which cannot be seen after an incubation of only 1 h. The A-chain toxicity, however, diminishes the specificity factor of IT considerably.

Finally, the cloning efficiency after the incubation phase influences the cellular sensitivity to IT. With high cloning efficiency IT activity decreases. Ammonium chloride only partially compensates the decreased sensitivity to IT (Fig. 10b). Although these test conditions appear to be important with adherent cells, it is not known to date if they are also necessary for nonadherent cells.

VII. IMMUNOTOXIN: *IN VIVO* ACTIVITIES

So far IT has been studied much less *in vivo* than *in vitro* because the production of high amounts of ITs is difficult. The treatment with LD_{10} doses for several days requires large amounts. New methods are under study to produce quantities of more than 10g of A-chain and IT, which will allow more extensive *in vivo* studies in the near future.

A *Physiopathology*

1. *Lethal doses*

A preliminary LD_{50} of IT seems to indicate that it is slightly more toxic *in vivo* than free A-chain. *In vitro,* IT was never more toxic than A-chain, it is therefore likely that the higher toxicity may be due to nonspecific binding of IT by the Fc fragment of the antibodies. This suggests the use of Fab or $F(ab')_2$ fragments of antibodies, which give the same specific *in vitro* activity as IT made with whole antibodies (Fig. 5a).

Treatment of cancer patients with IT containing the A-chain of a plant toxin will be restricted to a short period of a few weeks because of the immunogenicity of the toxin itself and the resulting production of neutralizing and anaphylactic antibodies. In the mouse, a single injection of pure A-chain shows an LD_{10} of 330 micrograms/mouse or 16.5 mg/kg (Table II). Following this, a starting dose in Phase I clinical trials could be estimated on a mg/m^2 basis from one tenth of the LD_{10} in the mouse (Rozencweig et al. 1981) and would correspond to a daily dose of 10.8 mg of antibody-bound A-chain per patient.

2. In vivo *half-life*

The half-life of IT was measured by the disappearance of IT activity in the serum and not by the half-life of radiolabeled IT molecules. After intravenous injection into mice of an anti-DNP IT which should not bind to target cells, blood samples were obtained by heart puncture and analyzed for *in vitro* activity on TNP-WEHI-7 cells. In preliminary experiments a half-life of 30 min for IT activity was found with an IT dose corresponding to the LD_{50} for a single A-chain injection.

3. *Nonspecific toxicity*

The histopathological changes induced in mice following an injection of an anti-DNP IT, which does not recognize a target in the mouse, were similar to those of free A-chain (see II B 2). Lesions appeared with slightly slower kinetics in the intestinal epithelium (crypts of Lieberkuhn), in liver parenchyma and tubules of the kidney. However, the short half-life of *in vivo* IT activity suggests that A-chain is rapidly liberated from the antibody. Therefore the observed lesions may be attributed to a great extent to free A-chain and not to intact IT.

4. *Immunogenicity*

Immunogenicity of IT has not yet been studied. Some evidence was obtained that anaphylactic reactions could be obtained when treatment with A-chain was

interrupted for several days and then followed by a new administration. Daily treatment with A-chain over 12 days did not lead to anaphylactic reactions. Therefore it can be expected that a short IT treatment over 10 days in patients who are under immunosuppressive treatment may not lead to anaphylactic reactions. The appearance of neutralizing antibodies or immune complexes are other possible complications of IT administration which have not yet been studied.

B *Tumor growth inhibition*

Only a few experiments have been performed until now to obtain an initial idea of *in vivo* IT activity. The experimental conditions were chosen for maximum sensitivity for IT activity, i.e. intraperitoneal administration of tumor cells in limited quantities and i.p. treatment with IT within 1 h. Such conditions are not comparable to a clinical situation.

1. *Artificial antigen*

About 2×10^6 HeLa cells modified with TNP were injected i.p. into groups of 15 nu/nu BALB/C mice. Experimental groups received about 210 micrograms IT

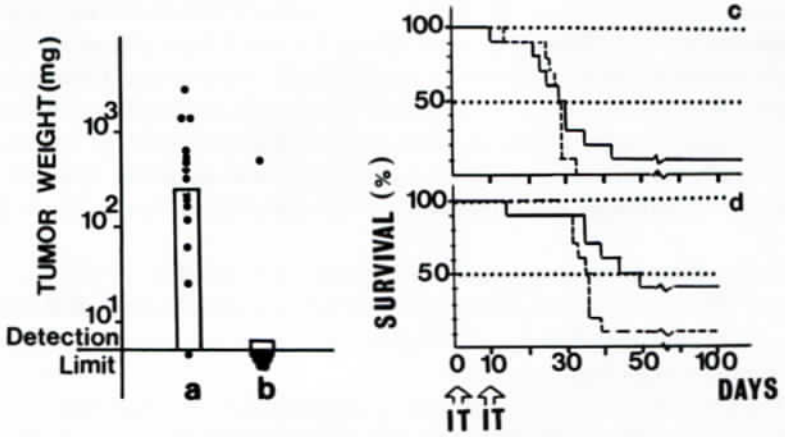

Figure 11. In vivo effect of immunotoxins. (Fig 11 c and d reprinted by permission from *Nature* **290**, (5802) pp. 145–146. Copyright (c) 1981 Macmillan Journals Limited).
a, b) the effect of anti-DNP IT on the appearance and growth of HeLa tumors in nu/nu mice. Tumor weight 25 days after i.p. inoculation of 2×10^6 TNP-HeLa cells into groups of 15 nu/nu mice: a) without injection of immunotoxin, b) after i.p. injection of 210 micrograms IT/mouse within 1 h of tumor cell inoculation.
c, d) *in vivo* test for anti-Thy 1.2 IT activity. Groups of 10 Balb/c mice (Bomholtgaard) were injected (i.p.) with c) 2.4×10^6 WEHI-7 cells or d) 6×10^5 WEHI-7 cells. Within 1 h and 7 days later, 300 micrograms of IT/mouse were injected i.p.. Survival was followed for 100 days.

i.p. per mouse, which corresponded to about 1/10 of the LD_{50} of A-chain. All mice were sacrificed 25 days later and tumors excised and weighed. In the control group, 14 of 15 animals showed tumors up to several grams, while only one animal of 15 treated mice had a visible tumor (Fig. 11 a, b).

With the mouse leukemia model L1210 used to evaluate anticancer drugs, IT may be compared to other drugs. Since there was no monoclonal antibody against L1210 available, we were obliged to utilize the anti-DNP IT on TNP-modified L1210 cells. Such cells have the major inconvenience that the hapten density per cell is diluted by subsequent cell proliferation, therefore only a short treatment can be envisaged. After i.v. injection of 1,000 tumor cells followed by i.p. treatment with 600 μg of monoclonal anti-DNP IT an increased life-span (ILS) of 29% and one definitely surviving mouse were obtained. According to the NCI protocols (Geran et al. 1972) anti-tumor activity of IT is significant because an ILS of more than 25% was reached. However, if an *in vivo* potentiation of IT with three successive injections of ammonium chloride (7 mg/mouse every 15 min) was added, 64% ILS was achieved and three animals were definitely surviving after 30 days. Therefore IT shows activity on the L1210 leukemia *in vivo* and can be potentiated by ammonium chloride.

2. *Mouse differentiation antigen*

Two doses (2.4×10^6 and 6×10^5) of Thy 1.2 positive WEHI-7 cells were injected i.p. into groups of 10 BALB/C mice. About 1 h and 7 days later experimental animals received 300 micrograms of the (IgM) IT. Survival was followed over 100 days. With the highest dose of 2.4×10^6 tumor cells, no significant difference could be found as compared to controls. In the lower dose of 6×10^5 tumor cells a statistically significant prolongation of survival was achieved; however, the percentage of definitely surviving animals was not statistically significant (Fig. 11 c, d).

Different factors may explain these unsatisfactory results:

1. The Thy 1.2 antigen is also present on normal lymphocytes of BALB/C mice, and IT may therefore be adsorbed by them, since they are about 100 times more frequent than leukemia cells.

2. The short half-life of IT reduced its extracellular concentration.

3. The slow kinetics of the anti Thy 1.2 IT combined with the decrease of active IT in the extracellular environment should have considerably diminished its activity.

4. Two IT injections with a 7-day-interval are insufficient for treatment. With noncrossreacting mouse models and an intensive treatment over 10 days with IT it is hoped that better results will be obtained.

VIII. COMPARISON OF IT FROM DIFFERENT ORIGINS

It was clearly shown that the activity of an identical IT can vary to a high degree depending on the conditions of the *in vitro* test systems. ITs of different origin are therefore difficult to compare if they are not tested under the same conditions.

1. *Methods*

The choice of the test conditions concerning, for instance, the preincubation, incubation or postincubation phases of adherent cell lines determine high or low activity of IT. Some experimental conditions favor IT activity considerably and others diminish its potency. Standardization of methods would therefore be necessary in order to proceed to meaningful comparison of ITs. Any well described method could be accepted for standardization; however, it seems that methods including more physiological conditions for target cells represent harder but more realistic test conditions for IT. *In vitro* methods could then, perhaps, give more precise predictions for *in vivo* efficiency.

2. *Interpretations*

Three points are essential for the characterization of ITs: a) their composition, if they are assembled with A-chain, which may be called a ITs, or with whole toxin, then called ab-ITs, b) the lowest concentration at which they are active, and c) how much this concentration may be raised before IT damages cells nonspecifically. If IT is active at very low doses, it should be able to kill tumor cells, which present only a low density of target antigens. If specific tumor antigens exist, they will probably be in very low density. On the other hand it is necessary for IT to be highly specific, which could be indicated by a specificity factor or a specific killing index. High specificity is the essential aim of IT, by which they should be distinguished from classical effector molecules, i.e. anticancer agents. Standardization of methods and interpretations concerning ITs should take these considerations into account.

IX. POSSIBLE APPLICATIONS OF IT TODAY

The application of IT seems to be possible in the near future by *in vitro* "cleaning up" of autologous bone marrow of leukemia patients from tumor cells. After aspiration of autologous bone marrow, leukemia patients may be treated with supralethal doses of chemotherapy and X-ray irradiation. They are then transplanted with their autologous marrow, which has been "cleaned up" of any

tumor cell by IT *in vitro*. The high *in vitro* potency of IT makes it probable that the last tumor cells could be eliminated without damaging bone marrow stem cells.

A clinical application of IT may be possible in conjunction with classical cancer therapy. After maximum reduction of the tumor load and its metastasis by all classical therapeutic methods, IT may help, by its different therapeutic approach, to kill the last escaping tumor cells. Although IT has some limitations in its actual form, its utilization may be promising, i.e. in the remission phase of leukemia when the residual tumor cells are at a minimum, or in the early stage of melanoma, when metastases are very small but already have spread to different organs, or after surgical excision of a primary tumor in order to inhibit tumor cell escape. However, more selective monoclonal antibodies, i.e. for the leukemia cells, will be needed.

Higher potency of IT *in vivo* will be expected if more knowledge is obtained on the *in vivo* distribution, the rapidity and intensity of IT penetration to tumor tissues, the binding and internalization into the target cell, as well as the metabolism and elimination from the organism. The rapid progress of the last 2 years in the assembly of ITs makes it probable that important progress will be made in the near future.

X. SUMMARY AND CONCLUSIONS

With the aim of constructing antibody-toxin conjugates in which the toxin part should be inactive during the transport and only become active after the binding of the antibody to its target, we coupled the toxic A-chain of ricin to different antibodies. A-chain without a binding site seemed to fulfill these requirements. The A-chain alone has only a low *in vitro* and *in vivo* toxicity. Only a dose corresponding to the LD_{50} (about 460 micrograms per mouse) affects the crypts of Lieberkuhn, liver parenchyma and the tubules of the kidney.

Conjugates between A-chain and antibodies which may be called A-chain immunotoxins (a-IT) became highly specific and efficient for their target tumor cells if the following conditions were met:

1. extreme purification of A-chain to eliminate trace amounts of B-chain contamination,
2. a labile disulfide bridge and a method which couples A-chain to antibody without denaturation,
3. purified antibodies of high affinity,
4. potentiation of IT activity by ammonium chloride.

Such IT have several advantages *in vitro*, since they are: a) immunologically specific, b) highly efficient, c) able to kill the last plated tumor cells, d) highly reproducible, and stable at $-20°C$.

Their limitations are a) a relative cellular resistance to IT, depending on the cell line, b) a potency which is only sometimes higher than complement-dependent lysis, c) slow kinetics, d) a decrease of potency under more physiological *in vitro* conditions.

In vivo IT, similar to A-chain, (LD_{50}, 23.3 mg/kg) is not very toxic and has a half-life of about 30 min in the mouse. Tumor growth of TNP-HeLa cells could be significantly inhibited with an IT against DNP. The life-span of mice injected with TNP-L1210 cells was also significantly prolonged with a similar IT. An anti-Thy 1.2 IT, however, produced only slight tumor inhibition.

A clinical administration of IT may be envisaged, after more intensive animal experiments, in patients with only a few residual tumor cells, i.e. in the remission phase in order to kill the last tumor cells by this different approach. An immediate use of IT, however, could be an *in vitro* destruction of tumor cells present in the autologous bone marrow of leukemia patients. After a supralethal treatment of these patients with drugs or X-ray irradiation they could then be retransplanted with their own bone marrow, which should now be free of tumor cells.

ACKNOWLEDGMENTS

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REFERENCES

Bernstein, I. D., Nowinski, R. C., Tam, M. R., McMaster, B., Houston, L. L. & Clark, E. A. (1980a) Monoclonal antibody therapy of mouse leukemia. In *Monoclonal Antibodies. Hybridomas: A New Dimension In Biological Analyses*, eds. Kennett R. H., Mc Kearn T. J. & Bechtol K. B., p. 275. Plenum Press, New York.

Bernstein, I. D., Tam, M. R. & Nowinski, R. C. (1980b) Mouse leukemia: Therapy with monoclonal antibodies against a thymus differentiation antigen. *Science* **207**, 68.

Blythman, H. E., Casellas, P., Gros, O., Gros, P., Jansen, F. K., Paolucci, F., Pau, B. & Vidal, H. (1981) Immunotoxins: Hybrid molecules of monoclonal antibodies and a toxin subunit specifically kill tumour cells. *Nature* **290**, 145.

Brown, J. P., Woodbury, R. G., Hart, C. E., Hellström, I. & Hellström, K. E. (1981) Quantitative analysis of melanoma-associated antigen p97 in normal and neoplastic tissues. *Proc. natl. Acad. Sci. USA* **78**, 539.

Brown, J. P., Wright, P. W., Hart, C. E., Woodbury, R. G., Hellström, K. E. & Hellström, I. (1980) Protein antigens of normal and malignant human cells identified by immunoprecipitation with monoclonal antibodies. *J. Biol. Chem.* **255**, 4980.

Carrière, D., Casellas, P. & Richer, G. (1980) Immunotoxin: Ultrastructural cytotoxic effect of ricin and immunotoxin on lymphoma WEHI-7 cells. Antibodies as carriers of anticancer drugs or toxins: Quo Vadis (Satellite Symposium of the IV International Congress of Immunology, Paris) Montpellier, France.

Casellas, P., Brown, J. P., Gros, O., Gros, P., Hellström, I., Jansen, F. K., Poncelet, P., Roncucci, R., Vidal, H. & Hellström, K. E. (1982) Immunotoxin potentiated by ammonium chloride specifically and efficiently kills human melanoma cells. (submitted for publication).

Chang, T. M. & Neville, D. M. (1977) Artificial hybrid protein containing a toxic protein fragment and a cell membrane receptor-binding moiety in a disulfide conjugate I. Synthesis of diphtheria toxin fragment A-S-S-Human placental lactogen with methyl-5-bromovalerimidate. *J. Biol. Chem.* **252**, 1505.

Crathorn, A. R. & Roberts, J. J. (1966) Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from deoxyribonucleic acid. *Nature* **211**, 150.

Davies, P. J. A., Davies, D. R., Levitzki, A., Maxfield, F. R., Milhaud, P., Willingham, M. C. & Pastan, I. H. (1980) Transglutaminase is essential in receptor-mediated endocytosis of α 2-macroglobulin and polypeptide hormones. *Nature* **283**, 162.

Dillman, R. O., Sobol, R. E., Green, M. R., Collins, H. A., Griffiths, J. C. & Royston, I. (1981) T101 monoclonal antibody serotherapy in a patient with chronic lymphocytic leukemia (CLL). Proceedings of the A.A.C.R. and the A.S.C.O. C–168, 375.

Dorland, R. B., Middlebrook, J. L. & Leppla, S. H. (1981) Effect of ammonium chloride on receptor-mediated uptake of diphtheria toxin by Vero cells. *Exp. Cell Res.* **134**, 319.

Fodstad, O. & Pihl, A. (1980) Synergic effect of adriamycin and ricin on L1210 leukemic cells in mice. *Cancer Res.* **40**, 3735.

Geran, R, I., Greenberg, N. H., MacDonald, M. M., Schumacher, A. M. & Abbott, B. J. (1972) Protocols for screening chemical agents and natural products against animal tumors and other biological systems (Third Edition). *Cancer Chem. Reports* **3**, 47.

Ghose, T. & Blair, A. H. (1978) Antibody-linked cytotoxic agents in the treatment of cancer: Current status and future prospects. *J. natl. Cancer Inst.* **61**, 657.

Ghose, T., Tai, J., Guclu, A., Norwell, S. T., Bodurtha, A., Aquino, J. & MacDonald, A. S. (1976) Antibodies as carriers of radionuclides and cytotoxic drugs in the treatment and diagnosis of cancer. *Ann. NY. Acad. Sci.* **277**, 671.

Gilliland, D. G., Steplewski, Z., Collier, R. J., Mitchell, K. F., Chang, T. H. & Koprowski, H. (1980) Antibody-directed cytotoxic agents: Use of monoclonal antibody to direct the action of toxin A-chains to colorectal carcinoma cells. *Proc. natl. Acad. Sci. USA.* **77**, 4539.

Gorer, P. A. & Amos, D. B. (1956) Passive immunity in mice against C57 black leukosis E. L. 4 by means of iso immune serum. *Cancer Res.* **16**, 338.

Hellström, I., Brown, J. P. & Hellström, K. E. (1981) Monoclonal antibodies to two determinants of melanoma-antigen p97 act synergistically in complement-dependent cytotoxicity. *J. Immunol.* **127**, 157.

Jansen, F. K., Blythman, H. E., Carrière, D., Casellas, P., Diaz, J., Gros, P., Hennequin, J. R., Paolucci, F., Pau, B., Poncelet, P., Richer, G., Salhi, S. L., Vidal, H. & Voisin, G. A. (1980) High specific cytotoxicity of antibody-toxin hybrid molecules (Immunotoxins) for target cells. *Immunol. Letters* **2**, 97.

Jansen, F. K., Blythman, H. E., Carrière, D., Casellas, P., Gros, O., Gros, P., Paolucci, F.,

Pau, B., Poncelet, P., Richer, G., Vidal, H. & Voisin, G. A. (1981b) Assembly and activity of conjugates between monoclonal antibodies and the toxic subunit of ricin (Immunotoxins). In *Monoclonal antibodies and T-cell hybridomas,* eds. Hammerling, G. J., Hammerling, U. & Kearney, J. F., Elsevier/North Holland, Amsterdam (in press).

Jansen, F. K., Blythman, H. E., Carrière, D., Casellas, P., Gros, P., Paolucci, F., Pau, B., Poncelet, P., Richer, G. & Vidal, H. (1981a) Replacement of the B chain of ricin with specific conventional or monoclonal antibodies. In *Receptor-mediated binding and internalization of toxins and hormones,* eds. Middlebrook, J. L. & Kohn, L. D., p. 351. Academic Press, New-York.

Kirch, M. E. & Hammerling, U. (1981) Immunotherapy of murine leukemias by monoclonal antibody I. Effect of passively administered antibody on growth of transplanted tumor cells. *J. Immunol.* **127**, 805.

Krolick, K. A., Villemez, C., Isakson, P., Uhr, J. W. & Vitetta, E. S. (1980) Selective killing of normal or neoplastic B-cells by antibodies coupled to the A-chain of ricin. *Proc. natl. Acad. Sci.* **77**, 5419.

Lin, J. Y., Tserng, K. Y., Chen, C. C., Lin, L. T. & Tung, T. C. (1970) Abrin and Ricin: New anti-tumour substances. *Nature* **227**, 292.

Masuho, Y. & Hara, T. (1980) Target-cell cytoxocity of a hybrid of Fab' of immunoglobulin and A-chain of ricin. *Gann* **71**, 759.

Masuho, Y., Hara, T. & Noguchi, T. (1979) Preparation of a hybrid of fragment Fab' of antibody and fragment A of diphtheria toxin and its cytotoxicity. *Biochem. Biophys. Res. Comm.* **90**, 320.

Mathé, G., Loc, T. B. & Bernard, J. (1958) Effet sur la leucémie L1210 de la souris d'une combinaison par diazotation d'A-méthoptérine et de gamma-globulines de hamsters porteurs de cette leucémie par hétérogreffe. *C. R. Acad. Sci.* (Paris) **246**, 1626.

Miller, R. A. & Levy, R. (1981) Response of cutaneous T cell lymphoma to therapy with hybridoma monoclonal antibody. *Lancet* **II**, 226.

Miller, R. A., Maloney, D. G., McKillop, J. & Levy, R. (1981) In vivo effects of murine hybridoma monoclonal antibody in a patient with T-cell leukemia. *Blood* **58**, 78.

Miyazaki, H., Beppu, M., Terao, T. & Osawa, T. (1980) Preparation of antibody (IgG)-ricin A-chain conjugate and its biologic activity. *Gann* **71**, 766.

Moolten, F. L. & Cooperband, S. R. (1970) Selective destruction of target cells by diphtheria toxin conjugated to antibody directed against antigens on the cells. *Science* **169**, 68.

Moolten, F. L., Zajdel, S. & Cooperband, S. R. (1976) Immunotherapy of experimental animal tumors with antitumor antibodies conjugated to diphtheria toxin or ricin. *Ann. N.Y. Acad. Sci.* **277**, 690.

Nicolson, G. & Blaustein, J. (1972) The interaction of ricinus communis agglutinin with normal and tumor cell surfaces. *Biochim. Biophys. Acta* **266**, 543.

Oeltman, T. N. & Heath, E. C. (1979) A hybrid protein containing the toxic subunit of ricin and the cell specific subunit of chorionic gonadotropin II. Biologic properties. *J. Biol. Chem.* **254**, 1028.

Ohkuma, S. & Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. natl. Acad. Sci. USA* **75**, 3327.

Olsnes, S. & Pihl, A. (1973) Different biological properties of the two constituent peptide chains of ricin, a toxic protein inhibiting protein synthesis. *Biochemistry* **12**, 3121.

Olsnes, S. & Pihl, A. (1976) Abrin, ricin and their associated agglutinins. In *The Specificity and Action of Animal, Bacterial and Plant Toxins* (Receptors and Recognition, series B, volume 1), ed. Cuatrecasas P., p. 129. Chapman and Hall, London.

Olsnes, S. & Pihl, A. (1981) Chimaeric toxins. In *Pharmacology of Bacterial Toxins,* eds.

Drews, J. & Dorner, F. Pergamon Press. (in press).

Olsnes, S., Refsnes, K. & Pihl, A. (1974) Mechanism of action of the toxic lectins abrin and ricin. *Nature* **249**, 627.

Pau, B., Blythman, H. E., Casellas, P., Gros, O., Gros, P., Jansen, F. K., Paolucci, F., Vidal, H. & Voisin, G. A. (1980) Conjugates between a toxin subunit and monoclonal antibodies (immunotoxins) with high specific cytotoxicity. In *Protides of the biological fluids*, 28th Colloquium, ed. Peeters H., p. 497. Pergamon Press, Oxford.

Poncelet, P. (1979) Influence du rapport anticorps/antigène sur la récupération, la pureté et l'affinité des anticorps isolés par immunoadsorption. D. E. A. Université Scientifique d'Aix-Marseille (II), France.

Raso, V. & Griffin, T. (1980) Specific cytotoxicity of a human immunoglobulin-directed F(ab)'-ricin A-chain conjugate. *J. Immunol.* **125**, 2610.

Richer, G., Carrière, D., Blythman, H. E. & Vidal, H. (1981) Histopathological changes induced in mice by the plant toxin ricin and its highly purified subunits A-chain and B-chain. In *Lectins, biology, biochemistry, clinical biochemistry*, ed. Bog-Hansen, T. C., Vol. II. Walter de Gruyter, Berlin. (in press).

Ritz, J., Pesando, J. M., Sallan, S. E., Clavell, L. A., Notis-McConarty, J., Rosenthal, P. & Schlossman, S. F. (1981) Serotherapy of acute lymphoblastic leukemia with monoclonal antibody. *Blood* **58**, 141.

Royston, I., Majda, J. A., Baird, S. M., Meserve, B. L. & Griffiths, J. C. (1980) Human T cell antigens defined by monoclonal antibodies: the 65,000-dalton antigen of T cells (T 65) is also found on chronic lymphocytic leukemia cells bearing surface immunoglobulin. *J. Immunol.* **125**, 725.

Rozencweig, M., Von Hoff, D. D., Staquet, M. J., Schein, P. S., Penta, J. S., Goldin, A., Muggia, F. M., Freireich, E. J. & DeVita, V. T. (1981) Animal toxicology for early clinical trials with anticancer agents. *Cancer Clin. Trials* **4**, 21.

Silverstein, S. C., Steinman, R. M. & Cohn, Z. A. (1977) Endocytosis. *Ann. Rev. Biochem.* **46**, 669.

Stirpe, F., Olsnes, S. & Pihl, A. (1980) Gelonin, a new inhibitor of protein synthesis, non toxic to intact cells: isolation, characterisation and preparation of cytotoxic complexes with concanavalin A. *J. Biol. Chem.* **255**, 6947.

Thorpe, P. E., Brown, A. N. F., Ross, W. C. J., Cumber, A. J., Detre, S. I., Edwards, D. C., Davies, A. J. S. & Stirpe, F. (1981) Cytotoxicity acquired by conjugation of an anti-Thy 1.1 monoclonal antibody and the ribosome-inactivating protein, Gelonin. *Eur. J. Biochem.* **116**, 447.

Thorpe, P. E., Ross, N. C. J., Cumber, A. J., Hinson, C. A., Edwards, D. C. & Davies, A. J. S. (1978) Toxicity of diphtheria toxin for lymphoblastoid cells is increased by conjugation to antilymphocytic globulin. *Nature* **271**, 752.

Tomita, M., Kurokawa, T., Onozaki, K., Ichiki, N., Osawa, T. & Ukita, T. (1972) Purification of galactose-binding phytoagglutinins and phytotoxin by affinity column chromatography using sepharose. *Experientia* **28**, 84.

Vidal, H. (1981) Purification et propriétés de la chaine A de la ricine, proteine toxique extraite des graines de ricin. Thèse doctorat 3eme cycle, Université des Sciences et Techniques du Languedoc, Montpellier, France.

Woodbury, R. G., Brown, J. P., Yeh, M. Y., Hellström, I. & Hellström, K. E. (1980) Identification of cell surface protein p97, in human melanomas and certain other neoplasms. *Proc. natl. Acad. Sci. USA* **77**, 2183.

Youle, R. J. & Neville, D. M. (1980) Anti-Thy 1.2 monoclonal antibody linked to ricin is a potent cell-type-specific toxin. *Proc. Natl. Acad. Sci. USA* **77**, 5483.