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A comparison of anti-lymphocyte immunotoxins containing different ribosome-inactivating proteins and antibodies

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SUMMARY

Immunotoxins were prepared with several single-chain ribosome-inactivating proteins (RIPs type 1) and with the A-chain of ricin linked to the $F(ab')_2$ fragment of sheep anti-mouse IgG. The cytotoxic activity of these conjugates was tested on human lymphocytes pretreated with an anti-CD3 murine MoAb. The immunotoxins inhibited DNA synthesis in phytohaemagglutinin (PHA)-stimulated lymphocytes with IC_{50}s (concentrations causing 50% inhibition) ranging from $8{\cdot}9 \times 10^{-13}$ to $5{\cdot}7 \times 10^{-11}$ M (immunotoxins containing dianthin 32, saporin, pokeweed antiviral protein from seeds (PAP-S), bryodin, momordin, momorcochin, and trichokirin), 1×10^{-8} M (immunotoxin containing gelonin) and 5×10^{-9} M (immunotoxin containing ricin A-chain). The immunotoxin containing saporin linked to the anti-mouse IgG $F(ab')_2$ fragment was also highly toxic to human lymphocytes pretreated with anti-CD2, -CD3, -CD5 and -CD45 MoAbs, with IC_{50}s $\leqslant 10^{-11}$ M. Immunotoxins were prepared also with saporin linked to MoAbs against various CD antigens. The immunotoxin prepared with the anti-CD3 antibody had the highest specific cytotoxicity to human lymphocytes.

Keywords immunotoxins lymphocytes MoAbs ribosome-inactivating proteins

INTRODUCTION

A number of selectively toxic conjugates have been prepared by linking toxins to MoAbs directed against a variety of cells to be eliminated [1]. Several toxic moieties have been used to construct these 'immunotoxins': bacterial or plant toxins, active chains of toxins, ricin A-chain being the most widely used, or A-chain-like ribosome-inactivating proteins (RIPs type 1, as opposed to the toxins, RIPs type 2) purified from several plants [2]. The latter proteins seem to offer the advantages of being numerous, stable, and easy and safe to prepare [3].

The properties and activity of immunotoxins may differ, depending on their components. Thus immunotoxins prepared with antibodies directed against distinct antigens on the same cell may have different activity [4], presumably because some are inefficiently internalized or are delivered to intracellular sites where they are inactivated. The nature of the toxic moiety also affects the properties of immunotoxins: an immunotoxin containing gelonin, a type 1 RIP, was toxic to human melanoma cells, whereas immunotoxins prepared with the same antibody and ricin or abrin A-chains were less effective [5]. Similarly, immunotoxins prepared with saporin bound to the anti-CD5 antigen OKT1 and T-101 MoAbs were more active on human lymphocytes than an immunotoxin prepared with the T101 antibody and ricin A-chain [6].

Recently immunotoxins were used in a different way, namely the cells to be killed were treated with a relevant, free antibody, followed by an immunotoxin consisting of a toxin linked to an anti-IgG antibody [7]. With this 'indirect method' it is not necessary to make a new conjugate to test each MoAb. It is possible to use a mixture of different antibodies with one conjugate and, at least in some cases, indirect immunotoxins prepared with whole ricin or abrin showed a higher cytotoxicity as compared with direct ones [8]. For these reasons the use of the indirect method could be advantageous, for instance in the *ex vivo* purging of bone marrow.

The aim of present work was to ascertain the effect of (i) the toxic moiety, (ii) the antibody, (iii) the linking reagent, and (iv) the manner of delivery on the efficacy of immunotoxins against T lymphocytes and various cell lines. To this effect, ricin A chain and several type 1 RIPs were linked to an $F(ab')_2$ of an anti-mouse IgG antibody, and their effect on human lymphocytes pretreated with anti-CD3 murine MoAb was compared. The immunotoxin prepared with saporin and antibodies against murine IgG was also tested with the indirect method with antibodies against other cell antigens. The same anti-CD

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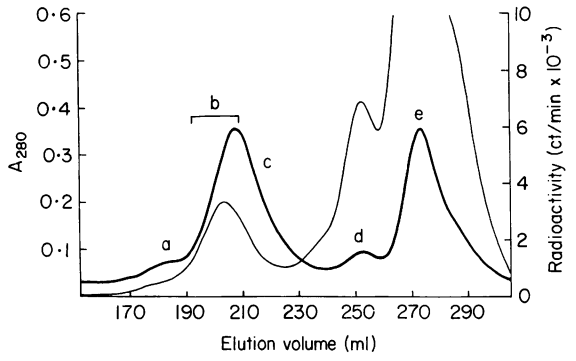

Fig. 1. Separation by gel filtration on Sepharose S200 HR of the saporin-anti-CD3 immunotoxin. The eluate was monitored spectrophotometrically at 280 nm (thick line) and by radioactivity (thin line) derived from ^{125}I-labelled saporin. a, High molecular weight conjugate; b, low molecular weight conjugate; c, free antibody; d, saporin dimer; e, saporin. The fractions collected are indicated by the horizontal bar.

antibodies were directly linked to saporin and the activity of these immunotoxins was tested on human lymphocytes.

MATERIALS AND METHODS

Cells

Human peripheral lymphocytes were obtained from healthy volunteers by Ficoll–Hypaque gradient. Other cells used as targets for the various antibodies were L540, from Reed-Sternberg cells (CD30$^+$, CD45$^+$), CEM, from T cell neoplastic lymphocytes (CD4$^+$), Raji, from a Burkitt lymphoma (positive for 8A and 62B1 antibodies) and U266, from a multiple myeloma (positive for 62B1 antibody). A CTLL murine T cell line (CD3$^-$) was used as an antigen-negative cell with the anti-CD3 MoAb.

Ribosome-inactivating proteins

Deglycosylated ricin A-chain [9] was a generous gift of Dr P. E. Thorpe. The following type 1 RIPs, all from seeds except when otherwise stated, were prepared as described by Barbieri *et al.* [10]: gelonin, from *Gelonium multiflorum*, bryodin, from *Bryonia dioica* roots, saporin, from *Saponaria officinalis*, dianthin 32, from *Dianthus caryophyllus* leaves, pokeweed anti-viral protein from seeds (PAP-S) from *Phytolacca americana*, momordin, from *Momordica charantia*, momorcochin, from *Momordica cochinchinensis*, and trichokirin, from *Trichosanthes kirilowii*. N-succinimidyl-3-(2-pyridyldithiopropionate) (SPDP) was from Pharmacia (Uppsala, Sweden) and 2-iminothiolane was from Sigma Chemical Co. (St Louis, MO). RIPs were labelled with ^{125}I with the method of Fraker & Speck [11] with the Iodogen reagent (Pierce).

Antibodies

The anti-CD antigen MoAbs (hereafter referred to as anti-CD antibodies) used were: anti-CD2, from Dr M. J. Crumpton, UCHT1 (anti-CD3), UCHT4 (anti-CD8) and C11 (anti-CD45) [12], from Prof. P. Beverley and HSR-3 (anti-CD30), from Dr A. Engert (Imperial Cancer Research Fund Laboratories, London, UK). TEC-T4 (anti-CD4) was from Technogenetics (Torino, Italy); 8A and 62B1, against plasma cell-associated antigens [13], were prepared in the Istituto di Ematologia.

F(ab′)$_2$ fragments of anti-mouse IgG polyclonal sheep antibodies (M 1522), affinity purified, were purchased from Sigma.

Immunotoxins

Ribosome-inactivating proteins, mixed with a trace of ^{125}I-labelled protein, were linked to antibodies or to F(ab′)$_2$ fragments as described previously, through an artificial disulphide bond introduced with SPDP [14] or with 2-iminothiolane [15]. Antibodies were usually modified with SPDP and RIPs with 2-iminothiolane. The antibody-RIPs conjugates with mol. wt around 200 000, containing 1 mol of antibody, were separated from polymers with higher mol. wt (in excess of 300 000 and containing at least 2 mol of antibody) and from free constituents by gel filtration on a Sephacryl S200 HR column (Fig. 1), and the RIP/antibody ratio was calculated from the radioactivity and the A_{280}. The immunotoxins were analysed by SDS–PAGE as described by Lambert *et al.* [16]. The binding to cells of immunotoxins prepared with anti-CD antibodies was checked by immunofluorescence.

Protein synthesis

Cell-free protein synthesis was measured with a rabbit reticulocyte lysate as described previously [17] with the details given in the legend to Table 1. Conjugates were reduced with 50 mM dithiothreitol before assay.

Cytotoxicity tests

In experiments performed with the indirect method, human peripheral mononuclear cells were incubated with an excess (*ca* 5 μg/ml) of anti-CD antibody for 30 min at 4°C and then washed once with medium. Cells were then seeded in 96-well plates (Sterilin, 10^4 cells/well) and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in 200 μl of RPMI containing 10% AB human serum (deprived of complement) and phytohaemagglutinin (PHA) (10 μg/ml), in the presence or absence of immunotoxins consisting of RIPs linked to anti-mouse IgG polyclonal antibodies (free RIPs in controls). Additional controls were run with anti-mouse IgG immunotoxins alone. After 48 h 1 μCi/well of ^{3}H-thymidine was added and after further 24 h the cells were harvested on glass fibre disks and the insoluble radioactivity determined in a scintillation counter with 5 ml Optiphase scintillation liquid.

In direct experiments cells were incubated for 48 h in the presence or absence of immunotoxins consisting of saporin linked to the various monoclonal anti-CD antibodies.

Other experiments were performed with CEM, L540, and Raji cells, incubated with MoAbs anti-CD4, -CD30, -CD45, 8A and 62B1. After 24 h of incubation in complete RPMI 1640 medium, cells were pulsed with ^{3}H-leucine (2 μCi/well), harvested and analysed as described above.

Cloning inhibition assay

Cells of the Raji and U266 cell lines (10^6 in 1 ml) were incubated in RPMI 1640 in the presence of various dilutions of immunotoxins prepared with 8A and 62B1 antibodies. After a 2-h incubation at 37°C the cells were centrifuged at 600 *g* for 5 min, resuspended with 1 ml of complete RPMI 1640 and 10^3 cells were seeded in Petri dishes containing 850 μl of complete RPMI 1640 medium and 150 μl of human plasma containing 10% (v/v) of 3·8% Na-citrate (Vacutainer system). Clotting of the medium was obtained by addition of 40 μl of a $CaCl_2$ solution (55 mg/ml). Colonies were counted with an inverted microscope at 6–8 days, according to the cell kinetics. Control samples with no additions to cells were run in parallel.

Table 1. Characteristics of the anti-mouse IgG immunotoxins prepared with ribosome-inactivating proteins (RIPs) and $F(ab')_2$ fragments

	$F(ab')_2$		RIP				Immunotoxin	
Immunotoxins	Linker SPDP (mol/mol)	Inserted groups (mol/mol)	Activity* IC_{50} (10^{-11} M†)	Linker 2-IT (mM)	SPDP (mol/mol)	Inserted groups (mol/mol)	RIP/$F(ab')_2$	Activity* IC_{50} (10^{-11} M‡)
ITF 1036 $F(ab')_2$-dianthin 32	4·0	2·10	12·0	1·25		1·23	3·41	16·7
ITF 1037 $F(ab')_2$-saporin	2·5	2·52	3·3	1·25		1·01	4·14	8·6
ITF 1038 $F(ab')_2$-PAP-S	3·0	1·67	3·7	1·25		1·13	6·4	41·0
ITF 1039 $F(ab')_2$-gelonin	3·5	2·32	40·0	1·50		0·84	1·79	620·0
ITF 1040 $F(ab')_2$-bryodin	2·5	1·41	12·0		1·85	1·55	2·79	24·7
ITF 1041 $F(ab')_2$-momorcochin	4·0	2·10	12·3	1·25		0·97	5·73	21·0
ITF 1042 $F(ab')_2$-momordin	3·5	2·32	6·0	1·25		0·69	2·36	44·0
ITF 1043 $F(ab')_2$-trichokirin	2·5	1·41	8·7	1·25		0·74	1·94	29·0
$F(ab')_2$-ricin A	3·5	1·34	6·6				2·14	7·8

* Inhibitory activity of cell-free protein synthesis.

† Of the free RIP.

‡ As ribosome-inactivating protein.

Antibodies and RIPs were treated with the linker at the indicated ratio as described in the text. The protein-synthetic inhibitory activity was tested with a rabbit reticulocyte lysate system [17]. Reaction mixtures contained, in a final volume of 62·5 μl: 10 mM Tris/HCl buffer, pH 7·4, 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0·2 mM GTP, 15 mM phosphocreatine, 3 μg of creatine kinase, 0·05 mM amino acids (minus leucine), 89 nCi of L-[^{14}C]-leucine, and 25 μl of a rabbit reticulocyte lysate [18]. Incubation was at 28°C for 5 min. The reaction was arrested with 1 ml of 0·1 M KOH, and the radioactivity incorporated into protein was measured as described [17]. ITF numbers, Italfarmaco code numbers of immunotoxins.

RESULTS

The characteristics of the anti-mouse IgG immunotoxins and their activity on a cell-free protein synthesizing system are given in Table 1. It should be noted that in the case of immunotoxins prepared with saporin, PAP-S and momorcochin the number of RIP moles bound per mol of antibody was higher than the number of groups inserted in the antibody. This was probably due to the linking of covalent polymers of RIPs to a single antibody molecule. The SDS–PAGE of an immunotoxin containing 2·0 mol of saporin per mol of antibody revealed a mixture of conjugates with mol. wts corresponding to a ratio saporin:antibody of 1:1, 1:2, 1:3 and, in smaller amount, 1:4 (Fig. 2).

Only ricin A chain retained full activity after conjugation. In all other cases the inhibitory activity of immunotoxins on cell-free protein synthesis was lower than that of the corresponding free RIPs. This partial inactivation was minimal in the case of saporin, and was greatest (over 90%) in the case of gelonin. The loss of activity of RIPs occurred on modification with the linker and a further loss was observed after the conjugation to the $F(ab')_2$. Preliminary experiments had shown that the inactivation of RIPs was more marked when SPDP was used as a linker (results not shown), and consequently 2-iminothiolane was preferred to modify all RIPs but bryodin. Apparently 2-iminothiolane did not link to this protein, which consequently was modified with SPDP.

In a first series of experiments, anti-mouse IgG immunotoxins containing various RIPs were evaluated with the indirect method with anti-CD3 antibody. The conjugates prepared with deglycosylated ricin A-chain had a cytotoxic activity with IC_{50} 5×10^{-9} M, in the range reported by other investigators [3]. Immunotoxins prepared with type 1 RIPs had IC_{50}s in the 10^{-11}–10^{-12} M range, with the exception of that containing gelonin (IC_{50} 10^{-8} M) (Table 2). In all cases a substantial increase of the specific cytotoxicity was obtained, with an increment of at least two log on the toxicity of a mixture of unlinked antibody and RIP. Differences in the activity of conjugates did not parallel the differences in their IC_{50}s in the cell-free system. An immunotoxin containing saporin added to $CD3^-$ CTLL cells had an IC_{50} above 10^{-8} M (protein synthesis was inhibited by 19% at this concentration, results not shown).

Saporin was used in further experiments. The anti-mouse IgG-saporin immunotoxin was tested on PHA-stimulated human lymphocytes pretreated with other antibodies against various CD antigens (Table 3). The immunotoxin was active with all antibodies tested.

Saporin was used also to prepare several immunotoxins with MoAbs against different CD antigens. Consistently with the results obtained with $F(ab')_2$ conjugates, saporin retained

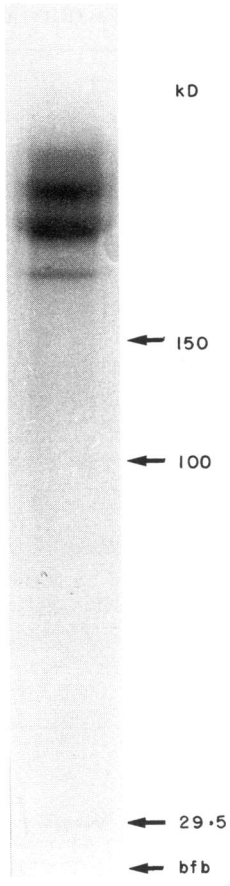

Fig. 2. SDS–PAGE of an anti-CD3-saporin immunotoxin. The immunotoxin was analysed on a 5–10% (w/v) acrylamide slab gel under non-reducing conditions, with sample buffer containing iodoacetamide (10 mg/ml) [16]. Standards of mol. wt were saporin 6 (mol. wt 29 500), an IgG F(ab′)$_2$ (100 000) and an IgG (150 000). bfb, Bromophenol blue front. Gels were stained with 0·1% coomassie blue.

almost full activity on conjugation to various antibodies but anti-CD45, in which case some inactivation was observed.

The saporin-containing immunotoxin prepared with the anti-CD3 antibody was the one showing the highest specific cytotoxicity to PHA-stimulated human lymphocytes (Table 3). All saporin-containing immunotoxins showed less cytotoxicity than with the indirect method, even when the same antibodies were used.

The anti-mouse IgG saporin immunotoxin was tested also with the indirect system on four cell lines, each one pretreated with the relevant MoAb (Table 4). In these experiments the inhibition of protein synthesis or of clonogenic activity was used as an index of toxicity because of the uneven distribution of mitoses among cells of these lines. The immunotoxin was effective in all cases with differences of up to two orders of magnitude between the various cell lines.

DISCUSSION

The results of our experiments confirm that most RIPs can be conjugated to antibodies still retaining significant activity, provided the appropriate linker is used. The only exception was gelonin, which lost a good deal of its inhibitory activity on cell-free protein synthesis, as observed previously [14].

Table 2. Cytotoxicity of immunotoxins prepared with various ribosome-inactivating proteins (RIPs) and assayed with the indirect method

Anti-mouse IgG immunotoxin containing	Anti-CD3 antibody +anti-mouse IgG immunotoxin IC_{50} (10^{-11} M)*
Saporin	0·55
Dianthin 32	1·07
PAP-S	0·26
Bryodin	2·88
Momordin	2·00
Momorcochin	6·76
Trichokirin	3·23
Gelonin	1000
Ricin A chain	500

* As RIP.

Immunotoxins prepared with RIPs linked to anti-mouse IgG F(ab′)$_2$ were added to phytohaemagglutinin (PHA)-stimulated human peripheral lymphocytes pretreated with anti-CD3 MoAb and ^{3}H-thymidine incorporation was measured after 48 h. The RIPs mixed with anti-CD3 antibody and the anti-mouse IgG immunotoxins added without the anti-CD3 antibody had IC_{50}s above 10^{-8} M.

Table 3. Cytotoxicity of immunotoxins prepared with saporin and different antibodies to phytohaemagglutinin (PHA)-stimulated human peripheral lymphocytes

Antibody	Antibody +anti-mouse IgG immunotoxin IC_{50} (10^{-11} M*)	Anti-CD immunotoxin IC_{50} (10^{-11} M*)
Anti-CD2	<1	1170
Anti-CD3	<1	21
Anti-CD5	9	204
Anti-CD45	<1	69

* As saporin.

Indirect method: the immunotoxin prepared with saporin linked to anti-mouse IgG F(ab′)$_2$ was added to PHA-stimulated human peripheral lymphocytes pretreated with anti-CD MoAbs. Direct method: the immunotoxins obtained with saporin linked to the various anti-CD MoAbs were added directly to PHA-stimulated lymphocytes. In both experiments ^{3}H-thymidine incorporation was measured after 48 h.

The results obtained with the indirect method showed that the most active immunotoxins (IC_{50}s *ca* 10^{-12}–10^{-11} M) are those prepared with RIPs from plant members of the Caryophyllaceae family (saporin, dianthin) and from the taxonomically close Phytolaccacae family (PAP-S). Immunotoxins prepared

Table 4. Cytotoxicity of anti-mouse IgG immunotoxins prepared with saporin and different antibodies to various cell lines

Target cells	Antibody used	Protein synthesis inhibition IC_{50} (10^{-11} M*)	Clonogenic assay IC_{50} (10^{-11} M*)
CEM	Anti-CD4	10	
L540	Anti-CD30	5	
L540	Anti-CD45	<1	
Raji	8A	70	30
U266	8A		80
U266	62B1		10

* As saporin.

Saporin-containing immunotoxins were added to the cells and protein synthesis or clonogenic activity were determined as described in the text.

with RIPs from plants members of the Cucurbitaceae family (momordin, momorcochin and trichokirin) had IC_{50}s above 10^{-11} M. The lower activity of the immunotoxin prepared with gelonin was expected, since this RIP was inactivated already after modification with any linking reagent. Other conjugates prepared with gelonin had IC_{50}s in the range 10^{-7}–10^{-5} [19], 10^{-9}–10^{-8} [20,21], 10^{-10} [22], 10^{-11} [5,16] or 10^{-6}–10^{-12} M [14]. These differences could be due to differences in the preparation of the RIP, in the antibodies, or in the experimental conditions. The potency of ricin A chain immunotoxin was only slightly less than that observed by Martin *et al.* [23] with an anti-CD3 ricin A chain immunotoxin. The cytotoxicity of the immunotoxins was not related to their inhibitory activity on cell-free protein synthesis, as can easily be seen by comparing Tables 1 and 2. This is consistent with previous observations [5,6] indicating that the toxic moiety may affect the entry of immunotoxins into cells. This notion is supported also by the different if not opposite effects of various substances (monensin, chloroquine, NH_4Cl) which enhance the cytotoxicity of immunotoxins prepared with ricin A chain [23,24] but do not modify the cytotoxicity of some RIP-containing immunotoxins [6,25,26].

Different results were obtained with the immunotoxins prepared with saporin linked to different MoAbs of which all but one did not increase the cytotoxicity of saporin to target cells. It should be remembered that efficient immunotoxins were obtained with saporin linked to an anti-CD5 MoAb whereas an anti-CD2 saporin and an anti-CD5 ricin A chain immunotoxin were not effective [4,6].

Present results are consistent with the higher efficiency of 'indirect' immunotoxins containing whole toxins [8]. In some cases immunotoxins assayed with the indirect method were efficient whereas those prepared with the same RIP linked to the antibody were not. This difference could be due to the linking of more than one immunotoxin molecule to the antibody bound to the cell, ensuring the delivery of a higher amount of toxin to the cell. However, the potency of RIPs is such that a few molecules in the cytoplasm should be sufficient to kill a cell. Thus, it seems more likely that the different load on the antibody linked to the cell may affect its entry into the cytoplasm. Entry of the indirect immunotoxin–antibody complex could be facilitated, for instance if the immunotoxin cross-linked two antibodies bound to the cell, thus facilitating capping. Another possibility is that the anti-mouse antibody complexes and the direct immunotoxins could enter cells through a route different from that followed by direct immunotoxins, and through which could be less exposed to inactivation by cellular enzymes. Whatever the reason, these results weaken considerably the notion that indirect immunotoxins prepared with $F(ab')_2$ or whole antibodies could be used to screen rapidly the efficiency of antibodies as carriers of toxins [7,8,27]. On the other hand, they show that the indirect immunotoxins prepared with $F(ab')_2$ fragments or whole antibodies could be used, at least *in vitro*, with antibodies giving inactive direct immunotoxins, and would allow also the use of unpurified preparations of antibodies, such as ascites liquids or cell culture supernatants.

These results, and the differences observed in the activity of immunotoxins prepared with different antibodies or with different toxic moieties, lead to the conclusion that the efficiency of immunotoxins depends (i) on the antibody, (ii) on the toxic moiety, and (iii) on whether the toxin is linked directly to the antibody or is used in the indirect way. The also show that indirect immunotoxins could be used for the purging of undesired cell populations from bone marrow, possibly with a higher efficiency than direct immunotoxins and with a single immunotoxin allowing the use of a mixture of different antibodies. For this purpose, the toxic moiety to be used to prepare immunotoxins should be chosen taking into account not only the activity, but also availability, stability and toxicity.

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REFERENCES

1 Magerstädt M. Antibody conjugates and malignant disease. Boca Raton: CRC Press, 1991:151–94.

2 Stirpe F, Barbieri L. Ribosome-inactivating proteins up to date. FEBS Lett 1986; **195**:1–8.

3 Lambert JM, Blättler WA, McIntyre GD, Goldmacher VS, Scott CF Jr. Immunotoxins containing single chain ribosome-inactivating proteins. In: Frankel AE, ed. Immunotoxins. Boston: Kluwer Academic Publishers, 1988:175–209.

4 Siena S, Bregni M, Formosa A, Martineau D, Lappi DA, Bonadonna G, Gianni AM. Evaluation of antihuman lymphocyte saporin immunotoxins potentially useful in human transplantation. Transplantation 1988; **46**:747–53.

5 Sivam G, Pearson JW, Bohn W, Oldham RK, Sadoff JC, Morgan AC Jr. Immunotoxins to a human melanoma-associated antigen: comparison of gelonin with ricin and other A chain conjugates. Cancer Res 1987; **47**:3169–73.

6 Siena S, Lappi DA, Bregni M *et al.* Synthesis and characterization of an antihuman T-lymphocyte saporin immunotoxin (OKT1-SAP) with *in vivo* stability into nonhuman primates. Blood 1988; **72**:756–65.

7 Weltman JK, Pedroso P, Johnson S-A, Davignon D, Fast LD, Leone LA. Rapid screening with indirect immunotoxin for monoclonal antibodies against human small cell lung cancer. Cancer Res 1987; **47**:5552–6.

8 Fodstad Ø, Kvalheim G, Pihl A, Godal A, Funderud S. New indirect approach to the therapeutic use of immunotoxins. J Natl Cancer Inst 1988; **80**:439–43.
9 Foxwell BMJ, Donovan TA, Thorpe PE, Wilson F. The removal of carbohydrates from ricin with endoglycosidases H, F and D and α-mannosidases. Biochim Biophys Acta 1985; **840**:193–203.
10 Barbieri L, Stoppa C, Bolognesi A. Large scale chromatographic purification of ribosome-inactivating proteins. J Chromatogr 1987; **408**:235–43.
11 Fraker PJ, Speck JC Jr. Protein and cell membrane iodinations with a sparingly soluble chloroamide,1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem Biophys Res Commun 1978; **80**:849–57.
12 McMichael AJ. Leucocyte typing III. Oxford: Oxford University Press, 1987.
13 Tazzari PL, Gobbi M, Dinota A *et al.* Plasma cell membrane phenotype. Study with new monoclonal antibodies. Clin Exp Immunol 1987; **70**:192–200.
14 Thorpe PE, Brown ANF, Ross WCJ *et al.* Cytotoxicity acquired by conjugation of an anti-$Thy_{1.1}$ monoclonal antibody and the ribosome-inactivating protein, gelonin. Eur J Biochem 1981; **116**:447–54.
15 Dinota A, Barbieri L, Gobbi M *et al.* An immunotoxin containing momordin suitable for bone marrow purging in multiple myeloma patients. Brit J Cancer 1989; **60**:315–9.
16 Lambert JM, Senter PD, Yau-Young A, Blättler WA, Goldmacher VS. Purified immunotoxins that are reactive with human lymphoid cells. Monoclonal antibodies conjugated to the ribosome-inactivating proteins gelonin and the pokeweed antiviral proteins. J Biol Chem 1985; **260**:12035–41.
17 Stirpe F, Gasperi-Campani A, Barbieri L, Falasca A, Abbondanza A, Stevens WA. Ribosome-inactivating proteins from the seeds of *Saponaria officinalis* L. (soapwort), of *Agrostemma githago* L. (corn cockle) and of *Asparagus officinalis* L. (asparagus) and from the latex of *Hura crepitans* L. (sandbox tree). Biochem J 1983; **216**:617–25.
18 Allen EH, Schweet RS. Synthesis of haemoglobin in a cell-free system. J Biol Chem 1962; **237**:760–7.
19 Brust F, Filipp G, Hofmann U *et al.* Antigen-gelonin conjugates: preparation and application in experimental myasthenia gravis. Biol Chem Hoppe-Seyler 1987; **368**:991–9.
20 Wiels J, Junqua S, Dujardin P, Le Pecq J-B, Tursz T. Properties of immunotoxins against a glycolipid antigen associated with Burkitt's lymphoma. Cancer Res 1984; **44**:129–33.
21 Singh V, Sairam MR, Bhargavi GN, Akhras RG. Hormonotoxins. Preparation and characterization of ovine luteinizing hormone-gelonin conjugate. J Biol Chem 1989; **264**:3089–95.
22 Ozawa S, Ueda M, Ando M, Abe O, Minoshima S, Shimizu N. Selective killing of squamous carcinoma cells by an immunotoxin that recognizes the EGF receptor. Int J Cancer 1989; **43**:152–7.
23 Martin PJ, Hansen JA, Vitetta ES. A ricin A chain-containing immunotoxin that kills human T lymphocytes *in vitro*. Blood 1985; **66**:908–12.
24 Casellas P, Bourrie BJP, Gros P, Jansen FK. Kinetics of cytotoxicity induced by immunotoxins. Enhancement by lysosomotropic amines and carboxylic ionophores. J Biol Chem 1984; **259**:9359–64.
25 Casellas P, Dussossoy D, Falasca AI *et al.* Trichokirin, a ribosome-inactivating protein from the seeds of *Trichosanthes kirilowii* Maximowicz. Purification, partial characterization and use for preparation of immunotoxins. Eur J Biochem 1988; **176**:581–8.
26 Goldmacher VS, Blätter WA, Lambert JM, McIntyre G, Stewart J. Cytotoxicity of gelonin conjugated to targeting molecules: effects of weak amines, monensin, adenovirus, and adenoviral capsid proteins penton, hexon, and fiber. Mol Pharmacol 1989; **36**:818–22.
27 Till M, May RD, Uhr JW, Thorpe PE, Vitetta ES. An assay that predicts the ability of monoclonal antibodies to form potent ricin-A chain-containing immunotoxins. Cancer Res 1989; **48**:1119–23.