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Potentiation of a weakly active ricin A chain immunotoxin recognizing the neural cell adhesion molecule

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SUMMARY

A ricin A chain immunotoxin, SEN36-ricin A chain, directed against the neural cell adhesion molecule (N-CAM) had no selective cytotoxic activity against three different small cell lung cancer (SCLC) cell lines in tissue culture despite expression of the target antigen on more than 98% of cells in each line detected by indirect immunofluorescence. Treatment of the SW2 SCLC cell line with suramin and interferons alpha and gamma increased the level of N-CAM expression only slightly and had no significant effect on the cytotoxic activity of the SEN36 immunotoxin. In the presence of the carboxylic ionophore monensin at a concentration of 0·1 μM, the toxicity of SEN36-ricin A chain to the SW2 cell line was enhanced by 12 000-fold. In contrast, lysosomotropic amines showed little or no potentiation of activity, suggesting that lysosomal degradation was not the major factor limiting the action of the anti-N-CAM immunotoxin. The findings of this study indicate that ricin A chain immunotoxins directed against N-CAM on SCLC are unlikely to have sufficient activity to be useful therapeutic agents in the absence of potentiating agents such as monensin, which can interfere with the normal intracellular pathways of antigen routing.

Keywords ricin A chain immunotoxin monensin neural cell adhesion molecule small cell lung cancer

INTRODUCTION

The cell surface glycoprotein neural cell adhesion molecule (N-CAM) mediates cell–cell interactions via homophilic binding and is implicated in the regulation of tissue pattern formation during embryogenesis [1]. The molecule is expressed with high frequency on a number of human tumours including brain tumours, neuroblastoma, Wilm's tumour, and small cell lung cancer (SCLC) [2,3]. N-CAM has been used as the target for intravenously administered radiolabelled MoAb in neuroblastoma patients [4,5].

In preliminary studies, we examined the potential of N-CAM to serve as an effective target on human SCLC for antibody-toxin conjugates (immunotoxins) made with the ribosome-inactivating protein ricin A chain, by employing an indirect assay of immunotoxin cytotoxicity [6,7]. These studies suggested that N-CAM would not be an effective target for a ricin A chain immunotoxin because the level of MoAb binding to SCLC cells was insufficiently high. Various agents have been reported to modulate the expression of N-CAM at the surface of cells in tissue culture. Ruff *et al.* [8] reported that recombinant human interferon-gamma (IFN-γ) increased the expression of the Leu-7 epitope, which is expressed on a proportion of N-CAM molecules [9], by two-fold or greater in human SCLC cell lines. Guo *et al.* [10] reported that suramin markedly increased the level of N-CAM expression at the surface of a rat glioma cell line and a mouse neuroblastoma cell line.

The aim of the present study was to examine in detail the cytotoxic activity of an immunotoxin prepared by the direct linkage of ricin A chain to the anti-N-CAM MoAb SEN36 in tissue culture and to examine means of enhancing the activity of the immunotoxin. The pharmacological agents which were known to increase cell surface expression of N-CAM or which have previously been shown to enhance the cytotoxic activity of A chain immunotoxins by altering the intracellular fate of internalized immunotoxin were included in this study. Only the carboxylic ionophore monensin was able substantially to potentiate the activity of the SEN36-ricin A chain immunotoxin.

MATERIALS AND METHODS

Preparation of immunotoxins

The mouse IgG1 MoAb SEN36 recognizing N-CAM [11], and the mouse MoAb W3/25, also of the IgG1 isotype, and recognizing the rat homologue of the human CD4 antigen (irrelevant control MoAb) were each linked to ricin A chain via a disulphide bond according to the procedure described by

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Cumber *et al.* [12]. Briefly, the purified MoAb was reacted with N-succinimidyl 3-(2-pyridyldithio) propionate to introduce an average of about two 2-pyridyldisulphide groups into the MoAb. The derivatized MoAb was allowed to react overnight with a 2·5-fold molar excess of freshly reduced ricin A chain and the mixture was applied to a column of Sephacryl S200(HR). Protein fractions of eluate were analysed by SDS–PAGE and fractions corresponding to conjugate molecules consisting predominantly of one ricin A chain molecule linked to one MoAb molecule were pooled to form the final preparation of immunotoxin.

Cell lines

The panel of SCLC cell lines comprised the variant SW2 cell line and the two classic cell lines NCI-H69 [13] provided by Dr L. Kelland (Institute of Cancer Research, Sutton, UK) and GLC-8 [14] provided by Dr L. de Leij (University Hospital, Groningen, The Netherlands) respectively. The human T lymphoblastoid cell line CEM was obtained from the American Type Tissue Culture Collection.

Cell lines were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. The lines were cultured in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (growth medium). SCLC cells growing as aggregates in suspension were disaggregated to predominantly single cells for use in experiments as described previously [15]. The T lymphoblastoid cell line grew as a suspension of single cells in tissue culture. Cell suspensions for cytotoxicity assays were prepared in medium containing leucine-free RPMI-1640.

Indirect immunofluorescence analysis of SEN36 binding

Cells (1×10^5/ml) were incubated in the presence or absence of suramin, human recombinant IFN-α or human recombinant IFN-γ in growth medium for 4 days at 37°C in tissue culture flasks. Cultures were then washed with PBS and a total of 1×10^6 cells were labelled with 200 μl of SEN36 at a concentration of 1×10^{-6} M for 1 h on ice before analysis by indirect immunofluorescence and flow cytometry as described previously [6].

Cytotoxicity assays

Cytotoxicity assays in tissue culture were performed essentially as described previously [15]. Briefly, cell suspensions at a final density of 1×10^5 cells/ml were incubated for 48 h at 37°C in the presence of immunotoxin or other agents. Control cultures were incubated in the absence of immunotoxin. Cultures were incubated for a further 4–24 h in the presence of 1 μCi of ^{3}H-leucine. The cells were then harvested and counted for ^{3}H-leucine incorporation.

Pharmacological agents were stored as sterile aliquots for use in the cytotoxicity assays. Human recombinant IFN-α (Intron A, Kirby-Warwick Pharmaceuticals Ltd.) and human recombinant IFN-γ (IF-RC 1001XXG AMT 2C1B, Boehringer), provided by Dr D. Cunningham and Dr J. Millar, respectively (Institute of Cancer Research, Sutton, UK), were at a concentration of 5×10^6 U/ml in reagent grade water and stored at −70°C. Suramin, provided by Professor A. Fairlame (London School of Hygiene and Tropical Medicine, London, UK), was stored at a concentration of 0·1 M in reagent grade water at −20°C. Potentiating agents were purchased from Sigma Chemical Co. (Poole, UK). Ammonium chloride and methylamine prepared at 1 M, and verapamil and chloroquine at 10 mM in reagent grade water were stored at −20°C. Monensin prepared at 0·1 M and perhexiline at 10 mM in ethanol were stored at −70°C, and were diluted at least 1000-fold in medium in the cytotoxicity assays.

RESULTS

Weak cytotoxic effects of SEN36-ricin A chain

The ability of SEN36-ricin A chain to exert toxic effects against a panel of human SCLC cell lines and a human T lymphoblastoid cell line was tested in tissue culture in parallel with an isotype-matched control immunotoxin of irrelevant specificity, W3/25-ricin A chain, with unconjugated ricin A chain, and with the toxin ricin using a ^{3}H-leucine incorporation assay (Table 1).

Ricin was potently toxic to the three SCLC cell lines SW2, NCI-H69 and GLC-8, inhibiting ^{3}H-leucine incorporation by 50% of untreated controls at a concentration (IC_{50}) ranging between $2{\cdot}9 \times 10^{-13}$ M and $4{\cdot}5 \times 10^{-12}$ M. Although the SEN36 MoAb bound to 98% or more of cells from each of the SCLC cell lines (not shown), SEN36-ricin A chain was only slightly more cytotoxic than unconjugated ricin A chain and W3/25-ricin A chain with an IC_{50} higher than 1×10^{-8} M. Indeed, SEN36-ricin A chain was no more toxic to the SCLC cell lines than to the T lymphoblastoid cell line CEM to which significant binding of SEN36 could not be detected (not shown).

Table 1. Cytotoxic activity of SEN36-ricin A chain, W3/25-ricin A chain, ricin A chain, and ricin in tissue culture against small cell lung cancer (SCLC) cell lines in tissue culture

	IC_{50} (M)		
Agent	SW2	NCI-H69	GLC-8
SEN36-ricin A chain	$2{\cdot}4 \pm 1{\cdot}0 \times 10^{-8}$	$2{\cdot}3 \pm 1{\cdot}1 \times 10^{-8}$	$> 1{\cdot}0 \times 10^{-8}$
W3/25-ricin A chain	$3{\cdot}5 \pm 0{\cdot}6 \times 10^{-8}$	$> 1{\cdot}0 \times 10^{-7}$	$> 1{\cdot}0 \times 10^{-7}$
Ricin A chain	$3{\cdot}2 \pm 0{\cdot}2 \times 10^{-8}$	$2{\cdot}6 \pm 0{\cdot}4 \times 10^{-8}$	$5{\cdot}8 \pm 2{\cdot}3 \times 10^{-8}$
Ricin	$2{\cdot}9 \pm 1{\cdot}7 \times 10^{-13}$	$7{\cdot}9 \pm 3{\cdot}2 \times 10^{-13}$	$4{\cdot}5 \pm 0{\cdot}1 \times 10^{-12}$

The IC_{50}s given are the mean values ±s.d. derived from at least three independent experiments quoted in terms of A chain concentration.

Influence of suramin and IFNs upon N-CAM expression and the cytotoxic activity of SEN36-ricin A chain

Suramin and human recombinant IFN-α and IFN-γ were tested for their ability to increase the level of N-CAM expression at the surface of SW2 cells and to enhance the cytotoxic activity of SEN36-ricin A chain.

The levels of SEN36 binding to SW2 cells exposed to suramin and IFNs for 4 days were assessed by indirect immunofluorescence and flow cytometry. Suramin increased the amount of SEN36 that bound to the surface of SW2 cells, though only slightly (Table 2a). Even when cells were exposed to suramin at the highest non-toxic concentration (1×10^{-4} M) the relative mean fluorescence intensity (MFI) of cells increased from 334 to only 383. In a separate experiment, IFN-α and IFN-γ also augmented the binding of SEN36 to the SW2 cell surface only slightly (Table 2b). Concentrations of the IFNs as high as 5×10^4 U/ml increased the relative MFI of SW2 cells from 231 to only 283 and 276 respectively.

In cytotoxicity assays, SW2 cells were incubated in the presence or absence of suramin at a concentration of 1×10^{-4} M or the IFNs at concentrations of 5×10^4 U/ml for 4 days before exposure to SEN36-ricin A chain at a concentration of 2×10^{-8} M (Fig. 1a). Pretreatment with suramin at the highest non-toxic concentration did not significantly affect the susceptibility of SW2 cells to the toxic action of SEN36-ricin A chain. IFN-α and IFN-γ pretreatment appeared to render cells slightly more susceptible to the immunotoxin. However, when SW2 cells were incubated with the IFNs at a range of concentrations and then exposed to SEN36-ricin A chain at a non-toxic concentration of 1×10^{-10} M, ^{3}H-leucine incorporation was reduced by a maximum of only 25% (Fig. 1b), indicating that the potentiating effect of IFN pretreatment was very small.

Potentiation of SEN36-ricin A chain cytotoxicity by monensin

A panel of established immunotoxin potentiating agents were tested for their ability to enhance the cytotoxic activity of SEN36-ricin A chain against the SW2 cell line in tissue culture as determined by a ^{3}H-leucine incorporation assay. The concentra-

Table 2. Relative levels of SEN36 binding to SW2 cells following (a) suramin and (b) IFN treatment

(a) Concentration of suramin (M)	Relative MFI
0	334
1×10^{-5}	342
1×10^{-4}	383

(b) Concentration of IFN (U/ml)	Relative MFI	
	IFN-α	IFN-γ
0	231	231
5	241	265
5×10^2	248	263
5×10^4	283	276

MFI, Mean fluorescence intensity.

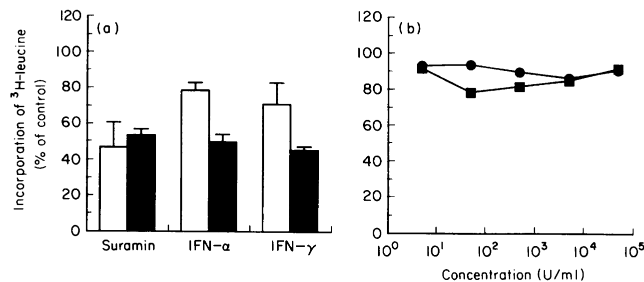

Fig. 1. Influence of suramin and interferons on the cytotoxic activity of SEN36-ricin A chain. (a) SW2 cells pretreated with suramin at a concentration of 1×10^{-4} M, IFN-α or IFN-γ at concentrations of 5×10^4 U/ml (■) or untreated cells (□), were incubated in the presence of SEN36-ricin A chain at a concentration of 2×10^{-8} M for 48 h at 37°C and then for a further 4 h in the presence of ^{3}H-leucine. The mean values ±s.d. of triplicate determinations are shown. (b) SW2 cells pretreated with IFN-α (■) or IFN-γ (●) at the concentrations shown were incubated in the presence of SEN36-ricin A chain at a concentration of 1×10^{-10} M for 48 h at 37°C and then for a further 4 h in the presence of ^{3}H-leucine. The results are expressed as the incorporation of ^{3}H-leucine as a percentage of control cultures incubated in the absence of immunotoxin. The mean values of duplicate determinations are shown.

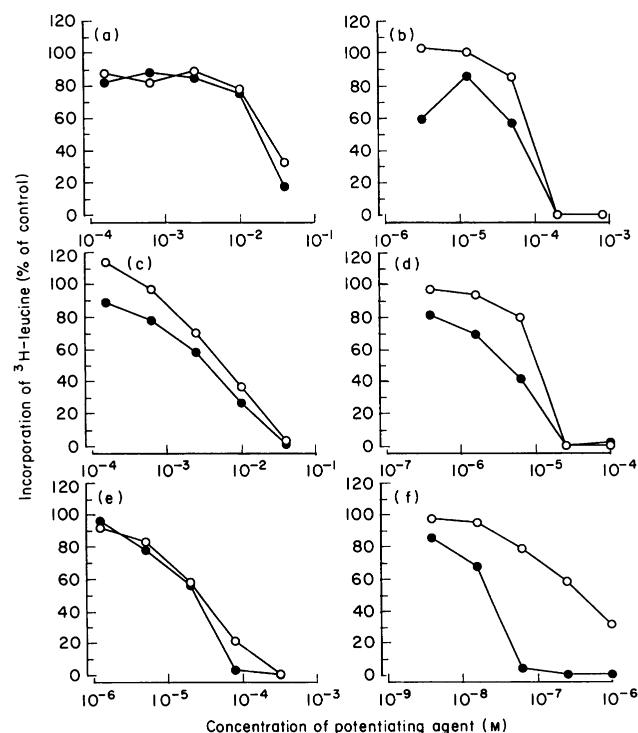

Fig. 2. Toxic effects of SEN36-ricin A chain in the presence of potentiating agents. SW2 cells were incubated for 48 h in the presence of the potentiators shown either alone (○) or in combination with SEN36-ricin A chain at a concentration of 1×10^{-10} M (●), and then for a further 4 h in the presence of ^{3}H-leucine. The results are expressed as the incorporation of ^{3}H-leucine as a percentage of untreated control cultures. The mean values of duplicate determinations are shown. (a) Ammonium chloride. (b) Verapamil. (c) Methylamine. (d) Perhexiline. (e) Chloroquine. (f) Monensin.

Table 3. Cytotoxic activity of SEN36-ricin A chain, W3/25-ricin A chain, ricin A chain, and ricin in combination with monensin at a concentration of 1×10^{-7} M against the SW2 cell line in tissue culture

Treatment	IC_{50} (M)	Enhancement factor*
SEN36-ricin A chain	$2{\cdot}0 \pm 2{\cdot}4 \times 10^{-12}$	12 000
W3/25-ricin A chain	$3{\cdot}4 \pm 0{\cdot}9 \times 10^{-9}$	10
Ricin A chain	$2{\cdot}0 \pm 0{\cdot}5 \times 10^{-9}$	16
Ricin	$1{\cdot}6 \pm 0{\cdot}3 \times 10^{-14}$	18

The IC_{50}s given are the mean values ± s.d. of three independent experiments quoted in terms of A chain concentration.

* IC_{50} in the absence of monensin divided by the IC_{50} in the presence of monensin.

tions of potentiators used were chosen to span a range giving inhibition of ^{3}H-leucine incorporation in the assay between approximately 0% and 100%.

SW2 cells were incubated with each potentiator at a range of concentrations either in the presence or absence of SEN36-ricin A chain at a final concentration of 1×10^{-10} M, the highest concentration of immunotoxin which had no inhibitory effect upon ^{3}H-leucine incorporation. The lysosomotropic amines–ammonium chloride, methylamine and chloroquine–and the calcium antagonists—verapamil and perhexiline—had little or no potentiating effect on SEN36-ricin A chain activity (Fig. 2). These results were in contrast to the effects of the carboxylic ionophore monensin. In combination with monensin at a concentration of 1×10^{-7} M, which was only weakly toxic to cells, SEN36-ricin A chain inhibited ^{3}H-leucine incorporation by greater than 95%.

In the ^{3}H-leucine incorporation assay, monensin at a concentration of 1×10^{-7} M enhanced the activity of SEN36-ricin A chain 12 000-fold against the SW2 cell line, reducing the IC_{50} to 2×10^{-12} M (Table 3). The potentiating effect of monensin on SEN36-ricin A chain activity was not entirely selective because the cytotoxic activities of W3/25-ricin A chain, ricin and ricin A chain were each enhanced by between 10- and 18-fold. In addition, there was no enhancement in the cytotoxic activity of SEN36-ricin A chain against the control target antigen-negative CEM cell line (not shown).

DISCUSSION

The major findings of the study were as follows. First, the anti-N-CAM immunotoxin SEN36-ricin A chain had no selective toxic activity against three different target antigen-positive SCLC cell lines in tissue culture. Second, there was no evidence to suggest that suramin or interferons could markedly increase the expression of N-CAM at the surface of the SW2 cell line or the sensitivity of the cell line to the action of SEN36-ricin A chain. Third, the carboxylic ionophore monensin enhanced the cytotoxic activity of SEN36-ricin A chain selectively and substantially, whereas the other established immunotoxin potentiators were only marginally effective.

Although the three SCLC cell lines examined in this study bound the SEN36 MoAb and were highly susceptible to the toxic effects of ricin, they were only weakly sensitive to the SEN36 immunotoxin. This finding was in agreement with the predictions of an indirect assay of immunotoxin cytotoxicity in which SEN36 and seven other anti-N-CAM MoAbs displayed only weak toxic effects in combination with an immunotoxin screening agent [6]. These results demonstrate that ricin A chain immunotoxins directed against N-CAM are unlikely to have any useful activity against human SCLC when used as single agents. However, the ability to potentiate the activity of such an immunotoxin selectively and substantially would clearly be advantageous.

In previous studies, cells expressing high levels of surface target antigens have tended to be more susceptible to immunotoxin intoxication than cells expressing a lower level [16–18]. In the present study, suramin and interferons were examined with the intention of increasing the expression of N-CAM and the amount of SEN36 that bound to the SW2 cell surface, thereby to increase the cytotoxic potency of the SEN36 immunotoxin. Guo *et al.* [10] reported that suramin at concentrations of 7×10^{-6} M and 7×10^{-5} M markedly increased the level of N-CAM expression at the surface of a rat glioma cell line and a mouse neuroblastoma cell line following exposure to the drug for 2 days. However, in the present study suramin had little effect upon the amount of SEN36 that bound to the surface of SW2 cells and did not alter the potency of SEN36-ricin A chain.

Ruff *et al.* [8] found that recombinant human IFN-γ at a concentration of 500 U/ml increased the expression of the Leu-7 epitope at the surface of four SCLC cell lines, including the NCI-H69 cell line, by two-fold or greater following exposure to the agent for 4 days. In the present study, both IFN-α and IFN-γ had little effect upon the amount of SEN36 that bound to the surface of SW2 and NCI-H69 cells. The Leu-7 epitope is known to be expressed on only a proportion of N-CAM molecules [9] and the findings in the present study suggest that the two-fold increase in the anti-Leu-7 MoAb binding to the NCI-H69 cell line observed by Ruff *et al.* [8] may not have been matched by a two-fold increase in the level of N-CAM expression. Indeed, Carbone *et al.* [3] recently showed that the Leu-7 epitope was distinguishable from N-CAM on SCLC.

The failure in this study to markedly increase antigen expression and immunotoxin sensitivity stands in stark contrast to the significant enhancement by IFN-γ of the activity of a ricin A chain immunotoxin directed against HLA-DR on a myeloid cell line which was mediated by a 10-fold increase in the density of the target antigen [19]. An alternative mechanism of IFN action could increase the susceptibility of SCLC to immunotoxin cytotoxicity in a manner independent of antigen upregulation [20]. The lack of a significant enhancement in the potency of SEN36-ricin A chain following IFN treatment suggests this phenomenon did not occur in the SCLC system.

Monensin was the most effective of several established potentiating agents in enhancing the activity of SEN36-ricin A chain. This was consistent with the findings of previous studies in which different agents have been compared for their ability to potentiate immunotoxins [21–23]. Monensin is believed to potentiate immunotoxin cytotoxicity by altering the intracellular transport of the macromolecule to compartments that favour A chain translocation to the cytosol rather than by altering binding or the rate of antigen internalization [24,25]. The ability of monensin to potentiate SEN36-ricin A chain cytotoxicity indicates that at least a proportion of the immunotoxin was internalized by the target cell, albeit in a fashion not conducive to efficient A chain translocation to the cytosol. In the

presence of monensin, on the other hand, the efficacy of A chain delivery to the cytosol increased greatly. The relative lack of potentiation by lysosomotropic amines in the present study suggests that lysosomal degradation of SEN36-ricin A chain was not the major factor limiting its cytotoxic activity.

In conclusion, the intracellular fate of the ricin A chain immunotoxin directed against N-CAM was the major factor limiting its cytotoxic potency against SCLC. Agents capable of modulating the expression of the target antigen had no potentiating effect on cytotoxic activity whereas the carboxylic ionophore monensin, which affects intracellular processing, greatly enhanced the potency of the immunotoxin. Methods of enhancing immunotoxin activity with monensin *in vivo* [26-28] might prove useful for increasing the potency of ricin A chain immunotoxins directed against N-CAM.

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