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Cytotoxic properties of a ricin A chain immunotoxin recognising the cluster-5A antigen associated with human small-cell lung cancer

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Summary. The cytotoxic properties of a ricin A chain immunotoxin made with the mouse monoclonal antibody SWA20, recognising a family of sialoglycoprotein antigens selectively expressed by human small-cell lung cancer (SCLC), were examined using a panel of tumour cell lines in tissue culture. SWA20–ricin-A-chain was selectively toxic to the SW2, NCI-H69 and GLC-8 SCLC cell lines, inhibiting the incorporation of [^{3}H]leucine by 50% at a concentration of 0.2–2 nM, but had no selective activity against the NCI-H23 and NCI-H125 lung adenocarcinoma or the control CEM T-lymphoblastoid cell lines. The SWA20 immunotoxin intoxicated the SW2 cell line rapidly, inhibiting [^{3}H]leucine incorporation by 50% within 2 h compared with 0.5 h for ricin. Analysis of the effects of SWA20–ricin-A-chain on the growth of SW2 cells using a limiting-dilution clonogenic assay revealed that the immunotoxin could eliminate 95% of clonogenic malignant cells. Although SWA20–ricin-A-chain was found to be rapidly active against the majority of tumour cells, its action was limited by the presence of insensitive cells expressing low levels of the target antigen.

Key words: Ricin A chain – Immunotoxin – Small-cell lung cancer – Cluster-5A antigen

Introduction

Two international workshops on human small-cell lung cancer (SCLC) antigens have identified a number of groups or clusters of monoclonal antibodies (mAb) that define distinct cell-surface antigens associated with SCLC [7, 8].

SCLC cluster 5 includes mouse mAb of the IgM class that recognise a family of sialoglycoproteins of 90–135 kDa [9]. Cluster-5A mAb of the IgG class identify a different but probably related group of N-linked sialoglycoprotein antigens of 40, 100 and 180 kDa [10, 11]. Both cluster-5 and -5A antigens are strongly expressed on 45%–50% of SCLC tumours and co-ordinately expressed in about 35% [5]. Expression of these antigens is practically absent from other pulmonary and extrapulmonary tumours and occurs only at low levels in bronchial epithelium and other normal tissues [5, 10].

We have been investigating the potential of antigens associated with SCLC to act as targets for immunotoxins containing the ribosome-inactivating protein ricin A chain [12, 13]. A preliminary experiment indicated that a ricin A chain immunotoxin, prepared with the cluster-5A mAb SWA20, had selective toxic activity against the SW2 SCLC cell line in tissue culture [14]. The aim of the present study was to examine in detail the potency and selectivity of the cytotoxic action of SWA20–ricin-A-chain against the SW2 cell line and a panel of SCLC and non-SCLC tumour cell lines.

Materials and methods

Preparation of immunotoxins. Ricin A chain was attached to the mouse mAb SWA20 and 2AL-1, both of the IgG2a isotype, via a disulphide bond according to the procedure described previously [2, 13]. Briefly, 2-pyridyldisulphide groups were introduced into the mAb by reaction with a fivefold molar excess of *N*-succinimidyl 3-(2-pyridyldithio)propionate. The derivatised mAb were reacted overnight with a 2.5-fold molar excess of freshly reduced ricin A chain. The reaction mixture was applied to a column of Sephacryl S200(HR) and fractions of eluate containing predominantly conjugate molecules consisting of one A chain molecule attached to one mAb molecule were pooled.

Tumour cell lines. The panel of SCLC cell lines comprised the variant SW2 cell line and two classic cell lines: NCI-H69 [1], provided by Dr. L. Kelland at the Institute of Cancer Research, Sutton, UK, and GLC-8 [6], provided by Dr. L. de Leij at the University Hospital, Groningen, The Netherlands. The human lung adenocarcinoma cell lines NCI-H23 and NCI-H125 [1] were provided by Dr. V. Macaulay, Institute of Cancer Research. The human T-lymphoblastoid cell line CEM was obtained from the American Type Tissue Culture Collection.

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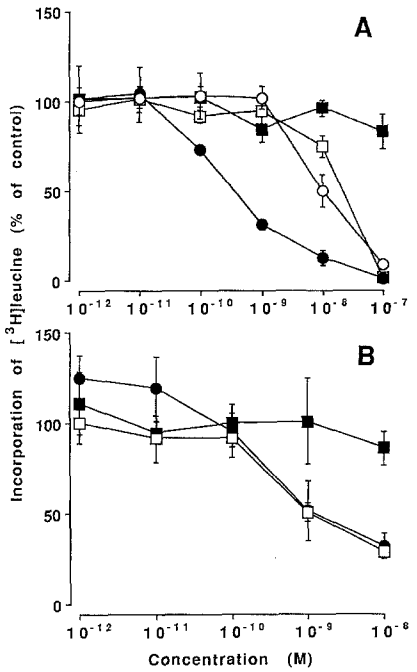

Fig. 1 A, B. Toxic effects of SWA20–ricin-A-chain and other agents against the SW2 cell line in tissue culture. **A** SW2 cells were incubated for 48 h in the presence of SWA20–ricin-A-chain (●), 2AL-1–ricin-A-chain (○), SWA20 (■), or ricin A chain (□) at the concentrations shown, and for a further 4 h in the presence of [^{3}H]leucine. **B** SW2 cells were incubated for 30 min at 0° C with SWA20–ricin-A-chain at the concentrations shown either alone (○), or in combinaton with unconjugated SWA20 (■) or with 2AL-1 (□) at a concentration of 0.1 μM. Cells were then washed and incubated in the presence or absence of the respective mAb at a concentration of 0.1 μM. The results are expressed as the incorporation of [^{3}H]leucine quoted as a percentage of the incorporation by untreated control cultures. The means and standard deviations of quadruplicate (**A**) or triplicate (**B**) determinations are shown

Cell lines were maintained in a humidified atmosphere of 5% CO_2 in air at 37° C. The lines were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/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 the experiments as described previously [13]. The T-lymphoblastoid cell line grew as a suspension of single cells in tissue culture. Cell suspensions for [^{3}H]leucine incorporation cytotoxicity assays were prepared in medium containing leucine-free RPMI-1640 medium (assay medium).

[^{3}H]Leucine incorporation assay. The [^{3}H]leucine incorporation assay was performed essentially as described previously [13]. Briefly, cell suspensions at a final density of 1×10^5 cells/ml were incubated for 48 h in the presence of immunotoxins or other agents. Control cultures were incubated in the presence of assay medium alone. Cultures were incubated for a further 4–24 h in the presence of 1 μCi [^{3}H]leucine. Cells were then harvested and counted for [^{3}H]leucine incorporation. Monolayer cultures of the lung adenocarcinoma cell lines were distributed into 24-well plates in 0.1 ml aliquots containing 5×10^4 cells and incubated with test samples in a final volume of 1 ml for 48 h before being pulsed with [^{3}H]leucine for 4 h.

Kinetics of protein synthesis inhibition. Samples (0.1 ml) of SWA20–ricin-A-chain or ricin at a concentration of 20 nM were mixed with 0.1 ml single-cell suspension of the SW2 cell line and incubated for various times at 37° C before being pulsed with 5 μCi [^{3}H]leucine for 1 h at 37° C, harvested and counted for the incorporation of [^{3}H]leucine.

Limiting-dilution clonogenic assay. The limiting-dilution assay was performed as described by Derbyshire et al. [3]. Single-cell suspensions of the SW2 cell line at a density of 2×10^5 cells/ml in growth medium were mixed with SWA20–ricin-A-chain, ricin A chain or ricin, each at a concentration of 20 nM for 48 h at 37° C. The cells were washed and resuspended in 0.7 ml growth medium to provide cell suspensions for the limiting-dilution assay.

The cell suspensions were serially diluted tenfold in growth medium. From cell suspensions at each cell density, six 0.1-ml samples were added to 0.1 ml growth medium in the wells of a 96-well plate and incubated at 37° C for 14 days. The clonogenic growth of surviving tumour cells was evaluated using inverted phase microscopy to score the number of wells that contained at least one colony. The number of clonogenic units per well in the cell suspensions was calculated by a modification of the Spearman technique [4] according to the formula:

$$\text{clonogenic units/well} = e^{-0.57722 - A}$$

where $A = \ln(5 \times 10^{-1}) + \ln 10/2 - \ln 10s/6$ and s = sum of the wells in which at least one colony was observed.

Results

Cytotoxic effects of SWA20–ricin-A-chain against a panel of human SCLC cell lines

The cytotoxic activity of SWA20–ricin-A-chain was examined against a panel of human SCLC and lung adenocarcinoma cell lines and a control human non-lung tumour cell line in tissue culture using a [^{3}H]leucine incorporation assay. The cytotoxic effects of an isotype-matched control

Table 1. Cytotoxic activity of SWA20–ricin-A-chain, 2AL-1–ricin-A-chain, ricin A chain, and ricin against human tumour cell lines in tissue culture

Cell line	IC_{50}[a] (M)			
	SWA20–ricin-A-chain	2AL-1–ricin-A-chain	Ricin A	Ricin
SCLC lines				
SW2	$(2.2 \pm 1.4) \times 10^{-10}$	$(1.6 \pm 0.8) \times 10^{-8}$	$(3.2 \pm 0.2) \times 10^{-8}$	$(2.9 \pm 1.7) \times 10^{-13}$
NCI-H69	$(1.7 \pm 1.0) \times 10^{-9}$	$(2.5 \pm 1.5) \times 10^{-8}$	$(2.6 \pm 0.4) \times 10^{-8}$	$(7.9 \pm 3.2) \times 10^{-13}$
GLC-8	$(1.4 \pm 0.5) \times 10^{-9}$	$(4.2 \pm 2.1) \times 10^{-8}$	$(5.8 \pm 2.3) \times 10^{-8}$	$(4.5 \pm 0.1) \times 10^{-12}$
Other lines				
NCI-H23	$>1.0 \times 10^{-8}$	$>1.0 \times 10^{-8}$	$>1.0 \times 10^{-8}$	$(6.3 \pm 1.8) \times 10^{-13}$
NCI-H125	$>1.0 \times 10^{-8}$	$>1.0 \times 10^{-8}$	$>1.0 \times 10^{-8}$	$(5.1 \pm 0.9) \times 10^{-13}$
CEM	$>1.0 \times 10^{-7}$	$>1.0 \times 10^{-7}$	$>1.0 \times 10^{-7}$	$(1.4 \pm 0.2) \times 10^{-12}$

[a] The IC_{50} values given are the means and standard deviations of at least three independent experiments quoted in terms of A chain concentration

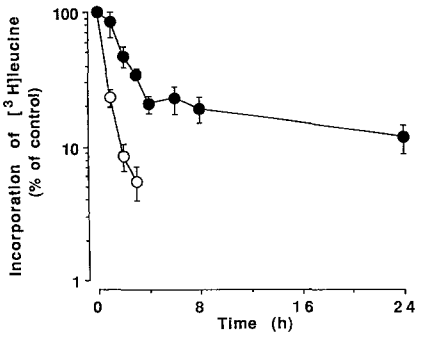

Fig. 2. Kinetics of protein synthesis inhibition by SWA20–ricin-A-chain. SW2 cells were incubated in the presence of the immunotoxin (●) or ricin (○) at a concentration of 10 nM for the stated times and then for a further 1 h in the presence of [^{3}H]leucine. The results shown are the means and standard deviation of quadruplicate determinations of [^{3}H]leucine incorporation expressed as a percentage of values from untreated control cultures

Table 2. Effects of SWA20–ricin-A-chain, ricin A chain and ricin on SW2 colony formation

Dilution	Number of wells containing colonies			
	Control	Ricin	SWA20–ricin-A	Ricin A
5×10^{-1}	6/6	0/6	6/6	6/6
5×10^{-2}	6/6	0/6	6/6	6/6
5×10^{-3}	6/6	0/6	6/6	6/6
5×10^{-4}	6/6	0/6	6/6	6/6
5×10^{-5}	6/6	0/6	1/6	6/6
5×10^{-6}	2/6	0/6	0/6	0/6
5×10^{-7}	0/6	0/6	0/6	0/6
Surviving clonogenic units per well	75567	<1	3296	35340
Inhibition (% of control)	0	>99.999	95.638	54.439

immunotoxin with irrelevant binding specificity, 2AL-1–ricin-A-chain, of unconjugated ricin A chain and ricin toxin were examined in parallel with SWA20–ricin-A-chain (Table 1).

SWA20–ricin-A-chain was selectively toxic to the three SCLC cell lines SW2, NCI-H69 and GLC-8, inhibiting [^{3}H]leucine incorporation by 50% compared to the untreated controls at a concentration (IC_{50}) ranging between 0.22 nM and 1.7 nM. In contrast, the IC_{50} values of 2AL-1–ricin-A-chain and unconjugated ricin A chain were higher than 10 nM against all three SCLC cell lines whereas all the lines were highly sensitive to ricin.

The SWA20 immunotoxin had no selective activity against the two lung adenocarcinoma cell lines NCI-H23 and NCI-H125, which did not express the target antigen at significant levels as judged from indirect-immunofluorescence antibody-binding experiments, nor against the antigen-negative T-lymphoblastoid cell line CEM. All three lines were highly sensitive to ricin, in common with the SCLC cell lines.

Specificity of action of SWA20–ricin-A-chain against the SW2 cell line

The specificity of action of SWA20–ricin-A-chain was examined in detail using the SW2 SCLC cell line in tissue culture. Figure 1A shows a representative concentration/activity curve. SWA20–ricin-A-chain acted in a concentration-dependent fashion, reducing [^{3}H]leucine incorporation by more than 70% at a concentration of 1 nM. In contrast, unconjugated ricin A chain, unconjugated SWA20, and 2AL-1-ricin A chain had no significant effect at equivalent concentrations. SWA20–ricin-A-chain was about 100-fold more potent than unconjugated ricin A chain as judged from IC_{50} values.

Excess unconjugated SWA20 mAb was able to inhibit the cytotoxic action of SWA20–ricin-A-chain (Fig. 1B). When SWA20 was included in a cytotoxicity assay in which SW2 cells were exposed to the immunotoxin for only 30 min, the cytotoxic activity of the immunotoxin was reduced by more than 50-fold. However, under the same experimental conditions, the isotype-matched control mAb 2AL-1 did not inhibit the cytotoxic activity of the SWA20 immunotoxin.

These results indicated that the selective cytotoxic action of SWA20–ricin-A-chain was dependent upon binding of the immunotoxin to the cell surface via the antigen-binding sites of the mAb component.

Kinetics of acton of SWA20–ricin-A-chain

The kinetics of protein synthesis inhibition by SWA20–ricin-A-chain were determined by incubating SW2 cells in the presence of either the immunotoxin or ricin at a concentration of 10 nM and measuring the effect on [^{3}H]leucine incorporation with time (Fig. 2).

Ricin inhibited protein synthesis extremely rapidly with no appreciable lag phase. [^{3}H]leucine incorporation was inhibited by 50% in just 0.5 h and by one order of magnitude in about 2 h. SWA20–ricin-A-chain also inhibited protein synthesis relatively rapidly. [^{3}H]leucine incorporation was inhibited by 50% in less than 2 h. However after 4 h, inhibition of protein synthesis by the immunotoxin reached a plateau of about 90% compared with more than 95% in the case of ricin. This result was in accord with the 48-h [^{3}H]leucine incorporation assay in which protein synthesis was inhibited by about 90% at an immunotoxin concentration of 10 nM and suggests that a significant proportion of the cells in the population were not immediately susceptible to the action of SWA20–ricin-A-chain.

Effect of SWA20–ricin-A-chain on the clonogenic growth of the SW2 cell line

SW2 cells were exposed to SWA20–ricin-A-chain, ricin A chain, or ricin at a concentration of 10 nM for 48 h under conditions resembling the [^{3}H]leucine incorporation assay.

Estimates of surviving clonogenic units were made by a limiting-dilution assay. Table 2 presents the results of a representative experiment. The cytotoxic activities of SWA20–ricin-A-chain, ricin A chain or ricin were judged from the reduction in the number of colonies evident at different cell densities compared with the untreated control culture. Ricin prevented the development of colonies even at the highest cell densities. SWA20–ricin-A-chain reduced the surviving fraction of clonogenic SW2 cells by more than 95%, whereas unconjugated ricin A chain inhibited the clonogenic growth of SW2 cells by only about 50%.

Discussion

In this study, we demonstrated that the immunotoxin SWA20–ricin-A-chain, directed against the cluster-5A antigen associated with SCLC, exerted toxic effects against a number of SCLC cell lines in tissue culture. Although the cytotoxic effects of the SWA20 immunotoxin were proven to be selective for SCLC, they were of moderate potency and varied over a tenfold range depending upon the target cell line. In the case of the SW2 SCLC cell line, the toxic effects were shown to be selective by comparison with a control immunotoxin of matched isotype but irrelevant specificity, with an antigen-negative control cell line and by competition experiments with unconjugated mAb. SWA20–ricin-A-chain caused a rapid inhibition of protein synthesis in SW2 cells which was only fourfold slower than the rate of intoxication by the highly efficient ricin toxin itself.

The SWA20 immunotoxin eliminated a high proportion of clonogenic SW2 cells in culture but its action was apparently limited even at high concentration because the surviving fraction of clonogenic malignant cells could not be reduced below 5%. The family of sialoglycoprotein antigens on SCLC recognised by cluster-5A mAb is heterogeneously expressed by primary SCLC tumours [5]. This heterogeneity was reflected in the SW2 cell line, which showed a wide variation in surface expression of the cluster-5A antigen within the cell population as judged by indirect immunofluorescence and flow cytometry (unpublished results). Malignant cells expressing little or no target antigen would be unable to bind a lethal dose of immunotoxin and would therefore escape intoxication.

The rapid toxic action of SWA20–ricin-A-chain against cells bearing the cluster-5A antigen and its selectivity for SCLC suggests that immunotoxins against this target antigen might be valuable for therapy of patients with antigen-positive disease. From the findings of this study, the SWA20 immunotoxin could not be expected to be a completely satisfactory antitumour agent in its own right because of the potential for cells expressing low levels of the cluster 5A antigen to escape killing. However, such an immunotoxin could prove useful in conjunction with a second immunotoxin able to recognise an antigen present on cluster-5A-antigen-negative tumour cells or as an adjunct to conventional chemotherapy.

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