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Monoclonal antibodies recognising the cluster 2 antigen associated with human small cell lung cancer mediate the toxic effects of ricin A chain in an indirect assay of immunotoxin cytotoxicity

E.J. Derbyshire & E.J. Wawrzynczak

Drug Targeting Laboratory, Section of Medicine, Institute of Cancer Research, Sutton, Surrey SM2 5NG, UK.

Summary Monoclonal antibodies (Mabs) submitted to the Second International Workshop on Small Cell Lung Cancer Antigens were screened for their ability to mediate the toxic effects of ricin A chain against the NCI-H69 cell line in an indirect assay of immunotoxin cytotoxicity. Cluster 1 Mabs, recognising the neural cell adhesion molecule, mediated little or no cytotoxic effect in combination with screening agent, ricin A chain linked to an antibody Fab' fragment recognising either mouse or rat Mabs. In contrast, cluster 2 Mabs, recognising an epithelial tumour-associated antigen, generally mediated potent cytotoxic effects with the screening agent, inhibiting the incorporation of ^{3}H-leucine by NCI-H69 cells by between 90% and 99%. Measurements of Mab binding to the NCI-H69 cell line by indirect immunofluorescence and flow cytometry indicated that the cluster 2 Mabs generally bound in higher amounts than the cluster 1 Mabs suggesting that the cluster 1 Mabs were ineffective in the screen because they did not bind to the cells in sufficient amounts. However, Mabs recognising antigens other than cluster 1 bound to NCI-H69 cells in amounts similar to those of the cluster 2 Mabs yet did not mediate potent cytotoxic effects in the indirect assay suggesting that the cluster 2 antigen may be internalised in a fashion favouring the delivery of ricin A chain to the cytosol.

Immunotoxins (ITs) designed for cancer therapy have been made by chemically linking the A chain of the potent toxin ricin to monoclonal antibodies (Mabs) recognising antigens present on the surface of malignant cells. Ricin consists of two polypeptide subunits: the A chain, a ribosome-inactivating enzyme, and the B chain, a galactose-binding lectin. The toxin attaches to galactose-containing glycoproteins and glycolipids present on the surface of mammalian cells via the B chain and is internalised to intracellular compartments that allow the translocation of the A chain into the cytosol. The A chain then rapidly causes the irreversible inhibition of protein synthesis leading to cell death. In comparison with the parent toxin, highly purified ricin A chain is only weakly cytotoxic because it does not associate strongly with the surface of cells and does not gain access to the cytosol as readily.

Ricin A chain ITs are able to exert selective cytotoxic effects because the Mab component allows the IT to bind specifically to cells bearing the target antigen and the A chain can be transported to the cytosol following internalisation of the bound IT. However, different Mabs do not form equally potent ITs with ricin A chain because the cytotoxic activity of an IT depends both upon the amount that binds to the target cell and upon the efficiency with which the target antigen can deliver the IT to the intracellular compartments allowing translocation of the A chain into the cytosol (Blakey *et al.*, 1988). To facilitate the selection of Mabs likely to make ITs with the highest cytotoxic activity, indirect assays allowing the screening of large numbers of unconjugated Mabs were introduced (Weltman *et al.*, 1987; Till *et al.*, 1988). We have also described an indirect assay which accurately predicted the cytotoxic potency of a ricin A chain IT against a human small cell lung cancer (SCLC) cell line in tissue culture (Wawrzynczak *et al.*, 1990).

In this study, Mabs submitted to the Second International Workshop on SCLC Antigens were screened by an indirect assay of IT toxicity against the human SCLC cell line NCI-H69 to identify Mabs in the panel that might form ricin A chain ITs with cytotoxic activity against SCLC. The relative amounts of Mab binding to the cell lines were also measured by indirect immunofluorescence and flow cytometry to determine whether there was a relationship between the level of Mab binding and the cytotoxic effects observed in the indirect assay.

Correspondence: E.J. Wawrzynczak.

Materials and methods

Preparation of immunotoxin screening agents

Ricin was isolated from castor bean cake derived from the seeds of *Ricinus communis* of Sri Lankan origin (Cumber *et al.*, 1985). Ricin A chain was isolated from the toxin by reductive cleavage and further purified using immobilised asialofetuin (Forrester *et al.*, 1984).

An IT screening agent designed for use with mouse Mabs, sheep anti-mouse IgG (SAMIgG) Fab'-ricin A chain, was prepared as previously described (Wawrzynczak *et al.*, 1990). Briefly, SAMIgG F(ab')$_2$ (Sigma) was reduced with 2-mercaptoethanol to yield the Fab' fragment. The free sulphydryl groups of the Fab' preparation were substituted with Ellman's reagent (Aldrich) and the derivative was reacted with a 2.5-fold molar excess of ricin A chain. SAMIgG Fab'-ricin A chain was purified from the reaction mixture by gel permeation chromatography on Sephacryl S200 (HR) (Pharmacia), to remove unconjugated A chain, followed by affinity chromatography on Blue Sepharose CL-6B (Pharmacia) to remove unconjugated antibody fragments. The screening agent retained complete A chain activity when tested in a cell-free assay of protein synthesis using a rabbit reticulocyte lysate (Forrester *et al.*, 1984). The SAMIgG Fab' fragment bound to all subclasses of mouse Ig as determined by a solid-phase ELISA (Wawrzynczak *et al.*, 1990).

An IT screening agent designed for use with rat Mabs, sheep anti-rat Ig (SARIg) Fab'-ricin A chain was prepared by the method described above using SARIg F(ab')$_2$ kindly supplied by Dr C.J. Dean, Institute of Cancer Research, Sutton. SARIg Fab'-ricin A chain retained complete A chain activity and bound to all subclasses of rat Ig.

Preparation of cells and monoclonal antibodies for binding and cytotoxicity experiments

The human SCLC cell line NCI-H69 (Carney *et al.*, 1985), growing as multicellular spheroids in suspension, was routinely maintained in RPMI-1640 medium (Flow) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS)

(Gibco) and 2 mM glutamine (Gibco), and cultured in a humidified atmosphere of 5% (v/v) CO_2 at 37°C. NCI-H69 spheroids were disaggregated mechanically by repeated passage through a 1.1 mm gauge syringe needle to produce a singled cell suspension for use in assays of binding by indirect immunofluorescence and in assays of cytotoxicity by the indirect IT assay.

Stock solutions of Mab (94 samples in coded form) and PBS controls (four samples) were distributed as part of the Second International Workshop on SCLC Antigens. These solutions were either in the form of undiluted hybridoma supernatants or as dilutions of ascites/purified Mab containing approximately the concentration of Mab in the supernatants. The stock solutions also contained 0.1% (w/v) sodium azide. Samples of Mab were prepared for screening in the indirect IT assay by making a 4-fold dilution of each of the stock solutions of Mab with leucine-free RPMI-1640 medium (Gibco) supplemented with 10% (v/v) FCS and 2 mM glutamine (assay medium). Each diluted sample was sterilised by passage through a low protein binding 0.22 μm filtration unit (Millex GV). The same samples were then used to measure the relative amounts of antibody bound to NCI-H69 cells by indirect immunofluorescence and flow cytometry.

Indirect assay of immunotoxin cytotoxicity

Aliquots (0.1 ml) of the diluted, coded Mab samples were distributed into the wells of a sterile 96-well tissue culture plate (Nunclon) and cooled to 4°C. Aliquots (0.1 ml) of a singled cell suspension of NCI-H69 cells at a concentration of 1×10^6 cells ml^{-1} in assay medium were added to each well and the mixtures incubated for 30 min at 4°C. The Mab-treated cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in assay medium (0.2 ml of leucine-free RPMI-1640/10% (v/v) FCS/2 mM glutamine) alone or in assay medium containing the appropriate screening agent at a concentration of 2.5×10^{-8} M. Cultures were incubated for 48 h at 37°C and 1 μCi of L-[4,5-^{3}H] leucine (Amersham) was then added to each well. After incubation for a further 24 h at 37°C, cells were harvested onto filters using a Titertek automated cell harvester. The incorporation of ^{3}H-leucine into cells was determined by liquid scintillation counting of the filters. All assays were performed in triplicate. The incorporation of ^{3}H-leucine by untreated control cultures was >100,000 c.p.m.

Indirect immunofluorescence assays of Mab binding

Initially, the undiluted stock solutions of Mab were used to determine the proportion of NCI-H69 cells that bound each Mab relative to the negative PBS controls supplied as part of the Second International Workshop. The Mabs that reacted with >90% of the cells in this preliminary screen were analysed further to determine the relative amounts of Mab binding to the cells. A total of 1×10^6 NCI-H69 cells suspended in 0.25 ml of ice-cold PBS were incubated with 0.25 ml of each of the diluted Mab solutions for 1 h at 4°C. After a second wash with cold PBS, the Mab-treated cells were resuspended in either 0.5 ml of a solution of anti-mouse Ig, fluorescein-linked whole antibody from sheep (Amersham) or 0.5 ml of a solution of anti-rat Ig, fluorescein-linked $F(ab')_2$ from rabbit (Serotec), diluted 1 in 100 with PBS containing 0.1% (w/v) bovine serum albumin, and incubated for 30 min at 4°C. After a final wash with cold PBS, the treated cells were resuspended in cold PBS (0.5 ml) and 20 μl of a solution of propidium iodide at 100 μg ml^{-1} PBS was added. Flow cytometric measurements were made on an Ortho Cytofluograf 50H equipped with a Lexel 50 mW argon-ion laser tuned to 488 nm and an Ortho 2150 computer system. The parameters measured were forward and orthogonally scattered light, and green (fluorescein, 520 nm) and red (propidium iodide, >630 nm) fluorescence. Red fluorescence was displayed and a gate set to exclude positive (dead) cells. Orthogonal versus forward light scatter was then displayed as a bivariate histogram (cytogram) and an elliptical region set to include single cells and exclude cellular clumps and debris. The green fluorescence of the single cells within this region was displayed and the mean fluorescence intensity (MFI) was recorded.

Results

Indirect immunofluorescence analysis of Mab binding

The ability of all 94 Mabs samples in the panel distributed by the Second International Workshop to bind to the NCI-H69 cell line was analysed by indirect immunofluorescence and flow cytometry. This analysis allowed the selection of 28 Mab samples which bound to greater than 90% of live NCI-H69 cells for further study. The stock solutions of these 28 samples were diluted and sterilised by filtration. Flow cytometry was used to determine the relative MFI of NCI-H69 cells treated with each diluted Mab sample followed by fluorescein-linked anti-Ig. The binding data for these 28 Mab samples are presented in Table I. For ease of comparison, the Mabs have been arranged into groups according to the cluster designations assigned following the cluster analysis of the Second International Workshop and translation of the code.

The majority of the Mab samples binding to greater than 90% of NCI-H69 cells were found to belong to either cluster 1 or cluster 2. The cluster 1 group consisted of 11 samples comprising eight different Mabs. All were mouse Mabs, predominantly of the IgG1 isotype, and bound to 95.9–99.0% of cells. The relative MFI of NCI-H69 cells incubated with these cluster 1 Mabs ranged between 35 and 268. The quadruplicate samples of the Mab NCCLU246 gave relative MFI values similar to one another. The cluster 2 group consisted

Table I Indirect immunofluorescence analysis of NCI-H69 cells treated with workshop Mab samples

Monoclonal antibody				
Code no.[a]	*Name*	*Isotype*[b]	*% of positive cells*	*MFI*
Cluster 1 Mabs				
4	RNL1	IgG1	97.9	66
12	MOC1	IgG1	98.9	103
21	MOC191	IgG2a	99.0	104
31	NCCLU246	IgG1	97.8	39
41	NCCLU246	IgG1	96.1	38
58	NCCLU246	IgG1	95.9	39
60	SEN6	IgG1	96.6	91
61	SEN36	IgG1	98.6	68
74	NE150	IgG1	97.7	268
77	NE25	IgG1	96.9	248
82	NCCLU246	IgG1	96.3	35
Cluster 2 Mabs				
11	MOC31	IgG1	99.4	509
14	MOC58	?	99.3	554
16	MOC151	IgG1	97.9	229
20	MOC181	?	99.6	492
53	MOC31	IgG1	99.0	419
75	PE35	IgG1	98.5	316
80	AUA1	IgG1	98.6	423
90	SL2.21	IgG2a	98.0	530
Other Mabs				
18	MOC171	IgG1	98.3	204
27	SM1	IgM	95.3	45
54	NCCST433	IgM	92.7	n.d.
59	SWA11	IgG2a	98.7	324
66	SWA21	IgG3	97.2	n.d.
67	SWA22	IgG3	98.6	n.d.
73	LCA2	rat IgG2b	98.4	585
78	Fib75	IgG2a	98.9	423
96	LCA3	rat IgG2a	96.9	62

[a]Code number assigned by the Second International Workshop on SCLC Antigens. [b]All samples were mouse Mabs of the given isotype, where known, except the two Mabs indicated as rat Mabs. n.d. not determined due to insufficient material.

of eight samples comprising seven different Mabs. All were mouse Mabs, predominantly of the IgG1 isotype, and bound to 97.9–99.6% of cells. The relative MFI of NCI-H69 cells incubated with these cluster 2 Mabs was generally higher than that of cells incubated with the cluster 1 Mabs ranging between 229 and 554. The duplicate samples of the Mab MOC31 both gave high relative MFI values. The third group, consisting of Mabs that were unclustered or that were assigned to clusters other than clusters 1 and 2, comprised seven mouse Mabs and two rat Mabs of various isotypes. The relative MFI values of this group ranged between 62 and 585.

Indirect assay of immunotoxin cytotoxicity

The 28 Mab samples selected for further study on the basis of their binding to a high proportion of NCI-H69 cells were screened for their ability to mediate toxic effects against the cell line by the indirect assay of IT cytotoxicity. Figure 1 shows the results of the indirect assay. The samples have been grouped by cluster as for the binding data in Table I.

NCI-H69 cells treated with cluster 1 Mabs in the absence of the screening agent displayed between 0 and about 25% inhibition of ^{3}H-leucine incorporation relative to untreated cell cultures. Treatment of cells with cluster 1 Mabs followed by the screening agent gave no further significant inhibition of ^{3}H-leucine incorporation in the majority of cases (Figure 1a). The greatest effect, which was less than 30% inhibition relative to antibody treatment alone, was displayed by sample 74 (NE150). This Mab was also the cluster 1 Mab found to have the highest relative MFI (see Table I).

In contrast to the cluster 1 Mabs, all but one of the Mabs assigned to cluster 2 gave powerful cytotoxic effects in the indirect assay, inhibiting the incorporation of ^{3}H-leucine by between 90% and 99% in the presence of the screening agent, compared with between 0 and about 15% in the absence of the screening agent (Figure 1b). The greatest cytotoxic effects were mediated by sample 14 (MOC58) and sample 90 (SL2.21) which bound to the cells in the highest amounts. Sample 16 (MOC151) showed less than 20% inhibition of ^{3}H-leucine incorporation in combination with the screening agent relative to the effect of antibody alone. In the binding studies, MOC151 had a slightly lower relative MFI (229) compared with that (316–554) of the other cluster 2 Mabs examined (Table I).

The majority of the other Mab samples, which were assigned to other clusters or which were unclustered, showed little or no inhibition of ^{3}H-leucine incorporation with the screening agent compared to antibody alone (Figure 1c). This was the case for sample 59 (SWA11) which had a relatively high MFI (324) similar to that of the cluster 2 Mabs mediating potent cytotoxic effects in the indirect assay. Sample 54 (NCCST433), sample 67 (SWA22) and sample 78 (Fib75) exhibited approximately 50% inhibition of ^{3}H-leucine incorporation. Fib75 also had a high relative MFI (423). The most effective Mab in the group was sample 73 (LCA2) which had a high relative MFI (585) (Table I) and reduced ^{3}H-leucine incorporation by more than 98% in combination with SARIg Fab'-ricin A chain (Figure 1c).

In control experiments, the screening agents, at a final concentration of 2.5×10^{-8} M, had no inhibitory effect upon ^{3}H-leucine incorporation by NCI-H69 cells that were not first exposed to Mab. Moreover, mouse Mabs that mediated cytotoxicity in conjunction with SAMIgG Fab'-ricin A chain, exhibited no cytotoxic effects when incubated instead with unconjugated ricin A chain alone at 2.5×10^{-8} M, or with an unconjugated SAMIgG Fab' fragment alone at 2.5×10^{-8} M, or with a simple mixture of the A chain and Fab' fragment at the same concentrations.

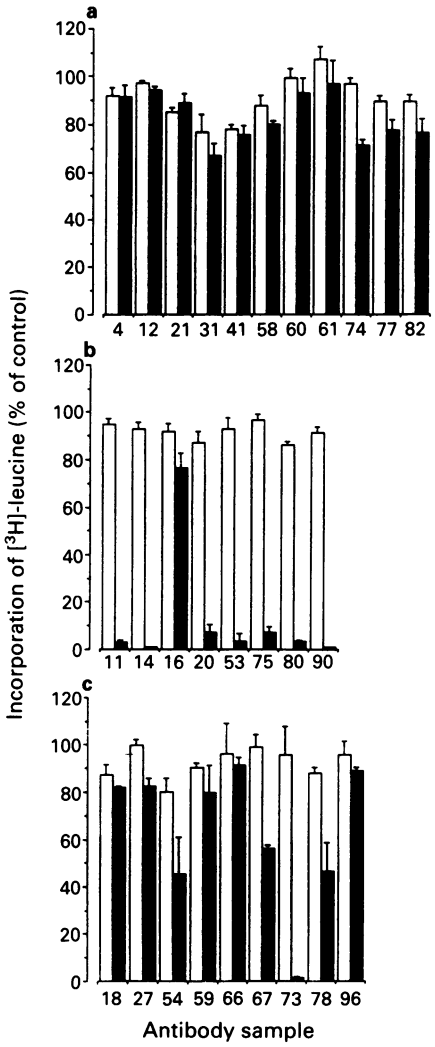

Figure 1 Indirect screen of immunotoxin toxicity to the NCI-H69 cell line in tissue culture. NCI-H69 cells exposed to anti-SCLC Mabs at 4°C were incubated at 37°C in the presence (filled bars) or absence (open bars) of the appropriate screening agent at a concentration of 2.5×10^{-8} M. **a**, cluster 1 Mabs, **b** cluster 2 Mabs, **c** Mabs belonging to other clusters or unclustered Mabs. The results are expressed as the percentage of ^{3}H-leucine incorporated by cell cultures not exposed to Mab or to screening agent. The mean values of triplicate samples are shown. The error bars denote the standard deviations from the mean values unless too small to be discerned.

Discussion

In this study, an indirect assay of IT cytotoxicity was used to screen coded Mab samples distributed by the Second International Workshop on SCLC Antigens for their ability to mediate the toxic effects of ricin A chain against a human SCLC cell line NCI-H69 in tissue culture. Mab samples were first selected for screening on the basis of binding to a high proportion of cells to ensure that large cytotoxic effects could be measured were antigen-positive cells to prove susceptible to the combination of Mab and screening agent. The 28 samples selected were subsequently revealed to contain 24 different Mabs. These Mabs were found to belong predominantly either to cluster 1, recognising the neural cell adhesion molecule (Patel *et al.*, 1989), or to cluster 2, recognising a tumour-associated epithelial membrane glycoprotein (Durbin *et al.*, 1990). The cluster 1 Mabs had little or no effect upon ^{3}H-leucine incorporation in combination with the screening agent in the indirect assay whereas the majority of the cluster 2 Mabs mediated potent cytotoxic effects, inhibiting the incorporation of ^{3}H-leucine by between 90% and 99% in the presence of the screening agent.

Cluster 1 Mabs generally bound to the cells in lesser amounts than the cluster 2 Mabs which mediated potent cytotoxic effects. It is possible that a higher level of binding of the cluster 1 Mabs to the cells would have resulted in more potent cytotoxic effects. However, similarly weak cyto-

toxic effects were observed in indirect assays of IT cytotoxicity performed with the cluster 1 Mabs SEN6 and SEN36 and in cytotoxicity assays of a covalent conjugate of SEN36 and ricin A chain against another human SCLC cell line SW2 in tissue culture (Wawrzynczak *et al.*, 1990*b*). These findings suggest that the cluster 1 antigen may not be expressed on SCLC cell lines at levels sufficient to bind the cluster 1 Mabs or ITs in the amounts required to give potent cytotoxic effects.

The relatively high levels of binding by the majority of cluster 2 Mabs can account only in part for the potent cytotoxic effects observed because other Mabs binding to the NCI-H69 cells at similar levels did not show such potent effects: SWA11, recognising the cluster w4 antigen, and Fib75, which was unclustered, showed no significant inhibition and about 50% inhibition of ^{3}H-leucine incorporation respectively. Thus, the nature of the target antigen, as well as the amount of Mab bound to the cell, appears to have influenced the potency of action in the indirect assay. It is interesting to note that LCA2, which gave highly potent cytotoxic effects in the indirect assay but which was not assigned to cluster 2 by the Second International Workshop, had been previously assigned to this cluster by the First International Workshop. MOC151 was the only cluster 2 Mab to bind to the NCI-H69 cell line in an amount similar to that of the highest binding cluster 1 Mabs. Thus, the weak cytotoxic effects mediated by this Mab in the indirect assay could have been due to an insufficient level of Mab bound to the cells. An alternative explanation is that MOC151 might recognise an epitope of the cluster 2 antigen distinct from the epitope(s) recognised by the other cluster 2 Mabs; binding of MOC151 to the antigen might not induce internalisation of the antigen in the same fashion as the binding of the other cluster 2 Mabs.

The Mabs previously found to belong to cluster w4 by the First International Workshop, i.e. SWA11, SWA21 and SWA22 (Souhami *et al.*, 1988; Stahel *et al.*, 1988) gave between 0% and about 50% inhibition of protein synthesis in the indirect assay. Interestingly, SWA11 was ineffective in this screen whereas previous studies had indicated that SWA11 mediated the toxic effects of the screening agent against the human SCLC cell line HC12 and that an SWA11-ricin A chain conjugate exerted selective cytotoxic effects against the cell lines HC12 and NCI-H69 (Wawrzynczak *et al.*, 1990). The simplest explanation of this apparent discrepancy is that higher levels of the cluster w4 Mabs must bind to NCI-H69 cells compared with the binding of the cluster 2 Mabs in order to give comparable cytotoxic effects.

In conclusion, this study has shown that Mabs recognising the cluster 2 antigen associated with human SCLC effectively mediated the toxic effects of ricin A chain in an indirect assay of IT cytotoxicity against a human SCLC cell line in tissue culture. The cluster 2 antigen may be a useful target for the action of ricin A chain ITs directed against SCLC because the antigen appears to be internalised in a fashion that favours the transfer of the A chain to the cytosol. Further studies will be necessary to determine the cytotoxic potency of an IT made by the direct attachment of ricin A chain to a cluster 2 Mab and to correlate the potency of such a cluster 2 ricin A chain IT with the levels of target antigen expression on different SCLC cell lines.

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