Ricin B chain converts a non-cytotoxic antibody-ricin A chain conjugate into a potent and specific cytotoxic agent

D.P. McIntosh, D.C. Edwards, A.J. Cumber, G.D. Parnell, C.J. Dean, W.C.J. Ross and J.A. Forrester

Institute of Cancer Research, Royal Cancer Hospital, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, England

Received 27 September 1983

We report the conversion of a non-cytotoxic atibody-ricin A chain conjugate to one displaying specific cytotoxic effects comparable with that of native ricin, by the addition of ricin B chain as a second stage reagent. The results suggest that this conversion is achieved by the association of the added B chain with the A chain of the conjugate, and not through a primary binding of B chain at the cell surface.

Ricin *A chain* *Targeting* *B chain* *Fibrosarcoma*

1. INTRODUCTION

In the pursuit of antibody-directed chemotherapy various expedients have been adopted, one of which has involved conjugation of plant toxins to antibodies [1,2]. Such conjugates generally manifest non-specific cytotoxicity due to the intrinsic binding capacity of the B piece of the toxins. This toxicity may be overcome by the use of antibody-toxin A chain conjugates. Such conjugates can in some cases be effective but in other instances the antibody moiety seems unable to act as a surrogate B chain and permit entry of A chain to the cytosol [3].

2. MATERIALS AND METHODS

The tumour cell line HSNtc was derived from a benzpyrene-induced fibrosarcoma in hooded rats [4] and maintained in tissue culture in DMEM with 5% foetal calf serum and 10% CO_2; 11/160 is a rat IgG2b monoclonal antibody specific for HSNtc that was obtained by fusion of the rat myeloma (Y3 Ag.1.2.3) with spleen cells from a rat bearing the HSNtc tumour [5]. A conjugate of the structure 11/160-NH-CO·CH_2CH_2-SS-ricin A was prepared as in [6].

The capacity of the A chain to inhibit ribosomal protein synthesis after conjugation to the antibody was assayed in a rabbit reticulocyte cell-free system [7] and found to be unimpaired. Binding of the conjugate was assessed by the measurement of ^{125}I-labelled sheep anti-rat γ 2 on the target cells.

The facility of the conjugate to inhibit protein synthesis in the target cell population was assayed by measuring [^{3}H]leucine incorporation into cellular protein after 1-h and 24-h exposures of conjugate to the cells.

3. RESULTS AND DISCUSSION

Fig.1 and table 1 show that these exposures had no effect on the level of protein synthesis and that the conjugate was even less toxic than the unconjugated A chain.

Thus, while the conjugate bound to its receptor on the target cells it seemed that this particular receptor was not permissive for entry of the A chain into the cytoplasm. Ricin B chain was prepared by reduction of the holotoxin and separation of the constituent peptides by chromatofocussing. The preparation was devoid of cytotoxic activity and was investigated as a second stage reagent for potentiation of the A chain conjugate activity.

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

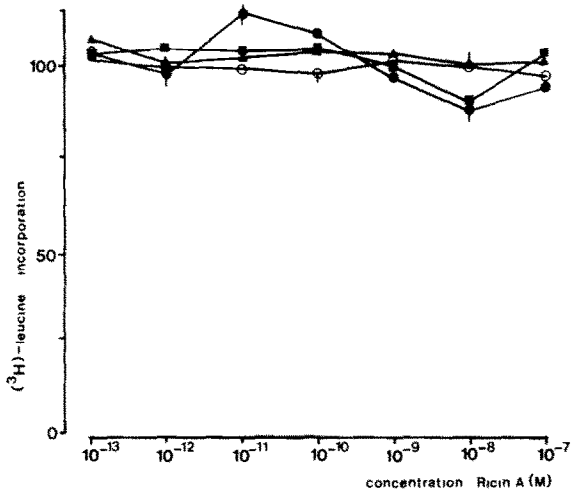

Fig.1. Specific cytotoxic effects of ricin A chain conjugated to monoclonal 11/160 antibody, on HSNtc cells in tissue culture. The cells were plated out at 2×10^5 ml^{-1} in 24 well plates for 24 h prior to incubation for 1 h at 37°C with 11/160-SS-ricin A (■), ricin A (●), 11/160 (▲) and 11/160 + ricin A (○) in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum and antibiotics. Cells were then washed in DMEM (but without toxin A chain and antibody) and incubated at 37°C in fresh culture medium for 27 h. Each well was then pulsed with 1 μCi [^{3}H]leucine for 18 h before harvesting cellular protein and scintillation counting. The [^{3}H]leucine incorporation is expressed as a percentage of that in untreated control cultures which incorporated 43 000 cpm μCi^{-1}. Each data point represents the arithmetic mean of 3 determinations, the standard deviations of which are indicated by vertical lines unless smaller than the points as plotted.

Fig.2 shows the potent cytotoxic effect obtained when binding of 11/160-SS-ricin A to HSNtc cells for 1 h was followed by a 1-h treatment with ricin

Table 1

The effects of ricin, its isolated A and B chains and ricin A chain conjugates on HSNtc cells

Treatment 1	Treatment 2	ID_{50}(M ricin A)
Ricin 1 h	–	1.1×10^{-12}
Ricin + lactose 1 h	–	2.4×10^{-10}
Ricin 1 h	6 × lactose wash	1.1×10^{-11}
Ricin B 1 h	–	$>10^{-7}$
Ricin A 1 h	–	$>10^{-7}$
Ricin A 24 h	–	5×10^{-8}
11/160-ricin-A 1 h	–	no effect at 10^{-7}
11/160-ricin-A 24 h	–	no effect at 10^{-7}
11/160-ricin-A 1 h	ricin B chain 1 h	3.2×10^{-12}
11/160-ricin-A 1 h	ricin B chain + lactose 1 h	5.3×10^{-12}
M10/76-ricin A	ricin B chain 1 h	$>10^{-7}$
Ricin A 1 h	ricin B 1 h	1×10^{-7}
Ricin A 1 h	ricin B + lactose 1 h	$>1 \times 10^{-7}$
Ricin B 1 h	ricin A 1 h	2.6×10^{-11}
Ricin B 1 h	ricin A +lactose 1 h	1.5×10^{-9}
Ricin B + lactose 1 h	ricin A chain	4.5×10^{-9}
Ricin B	M10/76-ricin-A 1 h	2.5×10^{-10}
Ricin B	11/160-ricin-A 1 h	1×10^{-13}

The ID_{50} is the dose required to inhibit the incorporation of [^{3}H]leucine into protein by 50%. HSNtc cells were plated out at 2×10^5 ml^{-1} for 24 h prior to a 1-h incubation at 37°C with materials listed in treatment 1. Cells were washed 3-times in DMEM with 10% foetal calf serum and incubated for 1 h at 37°C with materials listed in treatment 2. The cells were then washed 3-times and incubated for 27 h in DMEM and pulsed with [^{3}H]leucine as described (fig.1). All determinations were made in triplicate

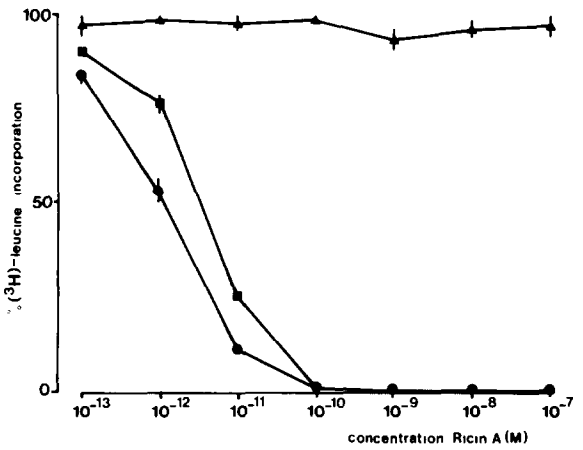

Fig.2. Specific cytotoxic effects of ricin on HSNtc cells and of 11/160-SS-ricin A and M10/76-SS-ricin A followed by ricin B chain at 10^{-7} M. Cells were plated out as described (fig.1) and incubated for 1 h at 37°C with ricin (●), 11/160-SS-ricin A (■) or M10/76-SS-ricin A (▲) in DMEM with 10% foetal calf serum. The cells were washed in medium as before (fig.1) and the ricin-treated cells were incubated and pulsed as described. Cells treated with ricin A chain conjugates were then incubated for 1 h at 37°C with ricin B chain at 10^{-7} M after which they were washed 3-times in medium and incubated, pulsed and harvested as described (fig.1).

B chain at 10^{-7} M. This is compared (fig.2) with the toxicity of whole ricin and a comparable treatment with an A chain conjugate with monoclonal antibody M10/76 [5] which does not react with HSNtc cells. Treatment of another rat fibrosarcoma cell line, MC24 with 11/160-SS-ricin A followed by B chain resulted in no cytotoxic effect. Cytotoxic effects, expressed as ID_{50}'s are shown in table 1. The results obtained after preincubating HSNtc cells with B chain for 1 h prior to the addition of the specific and non-specific conjugates are also shown (table 1).

There seemed two possible mechanisms whereby the inactivated 11/160 conjugate could be converted to a potent cytotoxin by the addition of B chain. It has been shown [8] that, following reduction of the disulphide bond, the A and B subunits of ricin appear to be held together by weak, non-covalent interactions. Therefore, following binding of 11/160-SS-ricin-A to the cell surface, the addition of B chain could result in an annealing of the ricin B chain with the A chain of the conjugate. This would lead to the formation of a quasi-holotoxin conjugate at the cell surface. The second possibility was that the B chain might bind to galactose residues at the cell surface and that this binding would act as a signal for the cell to internalise its membrane-bound ligands.

Lactose at 100 mM has been shown to compete with cellular galactose residues for the binding site of ricin B chain [9] and has been used to abrogate B chain binding of holotoxin conjugates to cellular carbohydrate receptors in in vitro bone marrow cleansing studies [10]. Fig.3 shows that the ID_{50} of ricin on HSNtc cells is increased 100-fold by the presence of 100 mM lactose in the incubation medium. The ID_{50} of 11/160-SS-ricin-A when B chain is added in the presence of 100 mM lactose is unaffected. This result argues against the binding of B chain to cellular galactose residues as the potentiating mechanism, and supports the notion of B chain annealment to the A chain component of the conjugate. This interpretation is in agreement with the findings in [11].

To test this hypothesis further, the interactions of isolated A and B chains as inhibitors of protein synthesis in the presence and absence of lactose have been investigated (table 1). Prior treatment of cells with 10^{-7} M ricin B chain followed by a 1 h incubation with ricin A chain resulted in the formation of a cytotoxic product at the cell surface which is likely to be quasi-holotoxin since neither 1 h treatments with A or B chains result in any cytotoxic effect. The presence of 100 mM lactose inhibited the cytotoxic effect if the sugar was added: (i) during treatment with B chain by competing with the cell surface glycoproteins for the B chain binding sites; (ii) subsequently during addition of the A chain by the elution of a proportion of the surface-bound B chain. Lactose had no effect on the binding of 11/160-SS-ricin-A to HSNtc cells and did not inhibit the subsequent binding of B chain to this conjugate. We conclude from these results that by the addition of ricin B chain we have succeeded in eliciting a specific cytotoxic effect with a formerly inactive ricin A chain conjugate.

The use of toxin B chains as potentiating agents may have immediate application for the in vitro eradication of tumour cells from bone marrow and it has been reported in [12] that the addition of ricin B chain can increase the efficiency of entry of A chain conjugates. The application of the two

treatments of this type in vivo, both of which are non-cytotoxic and which culminate in a cytotoxic event specified by the binding of the first stage monoclonal-antibody conjugate, has exciting implications for the treatment of tumours and their metastatic deposits. However, one might expect that the systemic introduction of ricin B via the circulation will be difficult to achieve due to its propensity to bind galactose residues on plasma glycoproteins, blood cells and vascular endothelium. Blockade of the galactose binding site, which the present experiments suggest is not important to the effectiveness of B chain as a second stage reagent, might be achievable by chemical modification of the peptide, the use of photoactivatable affinity labels or antibodies directed to binding site epitopes. Some of these possibilities are being investigated in vitro and in rats bearing the target tumour.

ACKNOWLEDGEMENTS

We thank Raymond Henry for technical assistance. This work was supported by grants to the Institute of Cancer Research Campaign and the Medical Research Council. D.C.E. is an external member of the staff of the Wellcome Foundation Ltd.

REFERENCES

[1] Youle, R.J. and Neville Jr, D.M. (1980) Proc. Natl. Acad. Sci. USA 77, 5483–5486.
[2] Edwards, D.C. and Thorpe, P.E. (1981) Trends Biochem. Sci., 313–316.
[3] Martinez, O., Kimura, J., Gottfried, T.D., Zeicher, M. and Wofsy, L. (1982) Cancer Surv. 1, 373–388.
[4] Currie, G.A. and Gage, J.O. (1973) Br. J. Cancer 28, 136–146.
[5] North, S.M., Styles, J.M., Hobbs, S.M. and Dean, C.J. (1982) Immunology 47, 397–405.
[6] Thorpe, P.E. and Ross, W.C.J. (1982) Immunol. Rev. 62, 119–158.
[7] Clemens, M.J., Henshaw, E.C., Rahamimoff, H. and London, I.M. (1974) Proc. Natl. Acad. Sci. USA 71, 2946–2950.
[8] Lappi, D.A., Kapmeyer, W., Beglau, J.M. and Kaplan, N.O. (1978) Proc. Natl. Acad. Sci. USA 75, 1096–1100.
[9] Olsnes, S., Saltvedt, E. and Pihl, A. (1974) J. Biol. Chem. 249, 803–810.
[10] Thorpe, P.E., Mason, D.W., Brown, A.N.F., Simmonds, S.J., Ross, W.C.J., Cumber, A.J. and Forrester, J.A. (1982) Nature 297, 594–596.
[11] Housten, L.L. (1982) J. Biol. Chem. 257, 1532–1539.
[12] Youle, R.J. and Neville jr, D.M. (1982) J. Biol. Chem. 257, 1598–1601.