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An ineffective monoclonal antibody-ricin A chain conjugate is converted to a tumouricidal agent in vivo by subsequent systemic administration of ricin B chain

Suzanne A. Eccles[1], Deirdre P. McIntosh[2], Helen P. Purvies[1], Alan J. Cumber[2], Geoffrey D. Parnell[2], J. Anthony Forrester[2], Jennifer M. Styles[1], and Christopher J. Dean[1]

[1] Institute of Cancer Research, Division of Tumour Immunology, Block X, Clifton Avenue, Belmont, Sutton, Surrey, SM2 5PX, UK.
[2] Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London, SW3 6JB, UK.

Summary. An immunotoxin comprising a tumour-specific monoclonal antibody (11/160) coupled to ricin A chain, although inactive in in vitro cytotoxicity assays against HSN_{tc} sarcoma target cells, was found to be capable of significant tumouricidal activity in syngeneic rats if potentiated by ricin B chain. The 11/160-ricin A, when bound to tumour cells prior to their inoculation, led to a slight inhibition of tumour growth s. c. compared with untreated sarcoma cells or those coated with antibody alone. However, all tumours in these groups developed progressively (69/69), whereas in those rats receiving 15 μ g or 150 μ g ricin B chain i. v. 5 min after tumour cell inoculation, the 'take rate' was reduced to 75% and 30% respectively, and significantly longer latent periods were evident for those tumours which did develop. Ricin B chain similarly inhibited, in a dose-dependent manner, the lung colonisation potential of 11/160-ricin A coated HSN_{tc} cells. No effects were obtained if the B chain treatment followed inoculation of untreated or antibody-coated cells, suggesting that systemically administered B chain is capable of gaining access to and activating antibody-ricin A chain conjugates bound to the surface of syngeneic sarcoma cells in lung or subcutaneous sites. Tumour inhibition was obtained in some instances with intervals of up to 24 h between inoculation of conjugate-coated tumour cells and B chain. Experiments are in progress to determine if such potentiation may be feasible in a therapeutic rather than a prophylactic setting using this syngeneic solid tumour system.

Introduction

Current attempts to develop potent, selective anti-tumour agents include the coupling of plant toxins (or their isolated A chains) to monoclonal antibodies with specificity for epitopes uniquely or preferentially expressed on tumour cells [16]. Such strategies have yielded immunotoxins capable of profound selective cytotoxicity against certain lymphoid and epithelial cell lines in vitro, and of inhibition of tumour growth in vivo in some circumstances [1, 7, 8, 13]. However, not all antibody-toxin A chain conjugates have proved effective [9, 10, 15]. Using a syngeneic monoclonal antibody directed against an apparently tumour-specific antigen McIntosh et al. [11] demonstrated that one such ineffective antibody-ricin A conjugate could be converted to a specific cytotoxic agent in vitro by addition of ricin B chain. The studies described in this paper were designed to determine if similar effects could be obtained in vivo.

Offprint requests to: S. A. Eccles

Materials and methods

Cells. The cell line HSN_{tc} was derived from a benzpyrene-induced fibrosarcoma of CBH/Cbi hooded rats [4], and maintained in tissue culture in DMEM plus 10% fetal calf serum and antibiotics in a humidified atmosphere of 5% CO_2 in air. Cells were used between the 5th and 20th in vitro subculture.

Antibody and ricin preparations. 11/160 is a rat monoclonal antibody of IgG_{2b} subclass obtained by fusion of the rat myeloma Y3 Ag.1.2.3 with spleen cells from a CBH/Cbi rat bearing a syngeneic HSN tumour [12]. An antibody-ricin A chain conjugate (11/160-NH-CO_2CH_2-SS-ricin A) was prepared as already described [14] using antibody purified from ascitic fluid using ion-exchange chromatography and ricin A chain obtained by reduction of the holotoxin. The capacity of the conjugate to bind to target tumour cells, and of the A chain to inhibit ribosomal protein synthesis in a cell-free system were found to be unimpaired [11]. However, 11/160-ricin A tested at 10^{-7}M (a concentration in excess of that required to saturate cell surface antigens) had no detectable cytotoxic effect against HSN_{tc} target cells in vitro. The ricin B chain was freed from A chain and residual holotoxin by chromatofocusing for use as a second stage reagent. This preparation also had no cytotoxic activity when tested separately.

Rats. Specific pathogen-free male or female inbred CBH/Cbi rats 10–12 weeks of age, reared and maintained in plastic film isolators were used in all experiments.

Preparation of antibody- or antibody-ricin A coated tumour cells. HSN_{tc} cells were grown to approximately 90% confluency in Nunc 80-cm^2 tissue culture flasks. The growth medium was removed, cell monolayers washed in phosphate buffered saline (PBS) and then incubated with either 3 ml PBS alone (control) or PBS containing saturating concentrations (100 μg/ml) of 11/160 antibody or 11/160-ricin A conjugate for 15 min at room temperature. Cells were removed from the plastic by addition of 27 ml

of PBS-EDTA and incubation for a further 15 min at 37 °C, a procedure previously found to release cells without damaging the trypsin-sensitive 11/160 epitope. Cells were spun for 10 s at 500 *g*, washed twice in PBS-EDTA and resuspended at 5×10^6 viable cells/ml in PBS. The cells were placed on ice to inhibit clumping, and injected at once into syngeneic rats.

Assays for tumour growth and lung colonisation potential. Groups of age- and sex-matched rats were inoculated i. v. (via the jugular vein) or s. c. (left and right flanks) with 10^6 viable HSN_{tc} cells, or tumour cells pre-treated with 11/160 antibody, or 11/160-ricin A conjugate. Ricin B chain was made up in 20 mM lactose and injected i. v. as a second stage reagent at doses of 15 or 150 μg per rat at intervals of 5 min to 24 h following tumour cell inoculation. Once s. c. tumours became palpable, 3 perpendicular diameters were measured 3 times weekly, and on termination of two experiments tumours were dissected out and weighed. Animals receiving tumour cells i. v. were examined daily, and all groups killed when the controls showed signs of respiratory distress. The lungs were removed, weighed, fixed in Bouin's fluid and surface tumour colonies counted the following day.

Results

Growth of tumours inoculated at s. c. sites

Figure 1 represents a typical experiment showing the growth of untreated HSN_{tc} sarcoma cells in comparison with those coated with 11/160 or 11/160-ricin A, and the effects of two different doses of ricin B chain on the latter group. It is apparent that the growth of tumours deriving from conjugate-coated cells was slightly but significantly inhibited compared with the other two control groups. However, there were no differences in the latency period (time to palpable tumours), the slope of the growth curve or the final tumour incidence (100% in all cases). In contrast, the i. v. administration of 15 μ g ricin B chain 5 min following s. c. inoculation of HSN_{tc}-11/160-ricin A cells led to a 3-day delay in tumour appearance and enhanced growth inhibition. In the animals receiving 150 μg B chain the latent period was increased from 7 to 21 days, and the final tumour incidence was reduced to 33%. The tumour-free animals were kept for 60 days but no further tumours developed. Ricin B chain (150 μg) administered 5 min after inoculation of HSN_{tc} cells alone, or antibody-coated tumour cells, had no effect on tumour growth (data omitted for clarity).

This experiment was repeated 4 times with comparable results, and the interval between tumour cell inoculation and B chain administration was increased to 6 or 24 h. The pooled results are shown in Table 1. The data confirm that

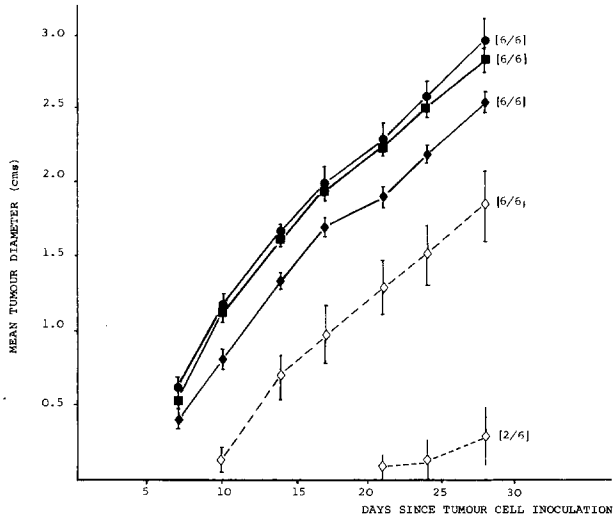

Fig. 1. Subcutaneous growth of HSN_{tc} cells alone ——■——; pre-treated with 11/160 monoclonal antibody ——●——; or pre-treated with 11/160-ricin A conjugate ——◆——. In 2 groups 15 μg ——◇—— or 150 μg -------◇------- ricin B chain was administered i. v. 5 min following 11/160-ricin-A-coated tumour cells. Each point shows the mean diameter (±SD) of 6 tumours, 3 perpendicular diameters of each being measured. Figures in parentheses give the number of inoculated sites at which tumours developed. These results are representative of 5 experiments

Table 1. Growth of HSN_{tc} pre-treated with 11/160 or 11/160-ricin A with or without ricin B chain potentiation. Results are expressed as the mean weights and diameters of tumours at termination of the experiment (28–30 days following tumour cell inoculation). The *P* values are obtained from *t*-test analysis (Students/Cochran) and compare experimental groups with group-1 controls (HSN_{tc} alone) or group-5 (11/160-ricin A pre-treated) where appropriate

Group	Pre-treatment	Ricin B post-treatment	*n*	Tumour weight (g) mean ± SD	*P* vs 1	*P* vs 5	*n*	Tumour diameter (cm) mean ± SD	*P* vs 1	*P* vs 5	Tumour incidence (%)
1	None	None	10	6.42 ± 1.40			22	2.74 ± 0.26			100
2	None	150 μg 5 min	6	6.33 ± 2.48	NS		6	2.97 ± 0.33	NS		100
3	11/160	None	10	8.31 ± 2.47	NS		12	2.85 ± 0.31	NS		100
4	11/160	150 μg 5 min	6	8.02 ± 1.41	NS		6	2.81 ± 0.21	NS		100
5	11/160-ricin A	None	10	4.41 ± 1.56	<0.01		23	2.43 ± 0.27	<0.001		100
6	11/160-ricin A	15 μg 5 min	6	1.43 ± 0.87	<0.001	<0.001	20	1.38 ± 0.98	<0.001	<0.001	75
7	11/160-ricin A	15 μg 6 h	6	3.18 ± 1.18	<0.001	NS	18	2.34 ± 0.25	<0.001	NS	100
8	11/160-ricin A	15 μg 24 h	6	0.67 ± 0.70	<0.001	<0.001	18	1.84 ± 0.69	<0.001	<0.01	94
9	11/160-ricin A	150 μg 5 min	6	0.05 ± 0.10	<0.001	<0.001	10	0.24 ± 0.41	<0.001	<0.01	30
10	11/160-ricin A	150 μg 6 h	6	3.23 ± 0.61	<0.001	NS	6	2.34 ± 0.16	<0.001	NS	100
11	11/160-ricin A	150 μg 24 h	6	3.74 ± 0.38	<0.001	NS	6	2.50 ± 0.09	<0.001	NS	100

NS = not statistically significant ($P > 0.05$). *n* = total number of animals assayed in each group. The data represent up to 5 pooled experiments

Table 2. Lung colonisation potential of HSN_{tc} cells pre-treated with 11/160 or 11/160-ricin A chain with or without potentiation by ricin B chain. 10^6 cells were inoculated via the left jugular vein, and B chain via the right jugular vein at the doses and time intervals shown. Statistical analysis and Key as Table 1. The data represent 5 pooled experiments

Group	Pre-treatment	Ricin B post-treatment	*n*	Tumour colony no. mean ± SD	*P* vs 1	*P* vs 5	[a] Lung weight (g) mean ± SD	*P* vs 1	*P* vs 5	Tumour incidence (%)
1	None	None	18	156 ± 60			5.85 ± 3.75			100
2	None	150 µg 5 min	3	> 200	NS		8.41 ± 3.06	NS		100
3	11/160	None	15	142 ± 62	NS		4.55 ± 2.15	NS		100
4	11/160	150 µg 5 min	3	> 200	NS		6.95 ± 2.08	NS		100
5	11/160-ricin A	None	18	135 ± 66	NS		4.21 ± 2.40	NS		100
6	11/160-ricin A	15 µg 5 min	18	13 ± 10	< 0.001	< 0.001	1.59 ± 0.25	< 0.001	< 0.001	94
7	11/160-ricin A	15 µg 2 h	9	64 ± 78	< 0.001	< 0.001	2.60 ± 0.73	< 0.01	< 0.05	100
8	11/160-ricin A	15 µg 6 h	12	133 ± 81	NS	NS	2.86 ± 1.72	< 0.05	NS	100
9	11/160-ricin A	15 µg 24 h	12	100 ± 79	NS	NS	2.44 ± 0.77	< 0.01	< 0.05	100
10	11/160-ricin A	150 µg 5 min	6	1.7 ± 1.2	< 0.001	< 0.001	1.47 ± 0.10	< 0.001	< 0.001	83
11	11/160-ricin A	150 µg 6 h	6	95 ± 62	NS	NS	1.91 ± 0.27	< 0.01	< 0.01	100
12	11/160-ricin A	150 µg 24 h	6	92 ± 53	NS	NS	1.89 ± 0.26	< 0.01	< 0.01	100

[a] Normal rat lungs weigh 1.1 – 1.3 g

HSN_{tc} cells treated with conjugate (group-5) produced smaller tumours than those treated with PBS or 11/160 antibody alone. The reduction in tumour burden was maintained in all groups of HSN_{tc}-11/160-ricin A inoculated animals which subsequently received ricin B chain i. v. (groups 6-11). In addition, a statistically significant dose-dependent potentiation of the anti-tumour effect was obtained with ricin B chain administered after 5 min. At later time intervals B chain potentiation could not be reproducibly demonstrated, although in 3/5 experiments animals receiving 15 µg B chain i. v. at 24 h developed smaller tumours than controls. The pooled data also suggested a statistically significant effect of this treatment protocol (group-8). A similar reduction was not demonstrable in the few animals treated with 150 µg B chain at 24 h (group-11). It is possible that smaller numbers of tumour cells would have been more effectively controlled.

Growth of tumours in lung following i. v. inoculation of HSN_{tc}

Table 2 shows that ricin B chain administered i. v. is capable of inhibiting lung colonisation of HSN_{tc} pre-treated with antibody-ricin A conjugate. Significant reductions in tumour weight were obtained with both dose levels of B chain at post-treatment intervals up to 24 h. In these experiments, no statistically significant reduction of tumour colony number or weight was obtained with 11/160-ricin A pre-treatment alone (unlike at s. c. sites), although the mean values were lower. Similarly, the effects of B chain on reducing tumour colony number did not achieve statistical significance at 6 and 24 h. This was due to the large standard deviation of the mean reflecting wide ranges in colony numbers scored. This variation was probably due to the difficulty in obtaining pure single cell suspensions from tumour cell monolayers without using proteolytic enzymes.

Discussion

It has previously been reported that the in vitro activity of cytotoxic antibody-ricin A chain conjugates (e. g. those directed against Thy 1.1 antigens on T cell leukaemia, or against Ig on B cell tumours) could be enhanced by free B chain [19] or by antibody-B chain conjugates recognising either the target antigen [17] or the immunotoxin [18]. Also it has been demonstrated that inoculation into mice of the separated ricin subunits, either simultaneously or separated by time intervals of up to 8 h, resulted in whole animal toxicity [2]. These findings were interpreted as showing that the A and B subunits could re-associate in vivo to form active toxin and encouraged us to test whether B chain potentiation could be achieved with ineffective antibody-ricin A chain conjugates in vivo.

We were able to demonstrate that a syngeneic tumour-specific immunotoxin bound to target cells in the lung or at subcutaneous sites could be rendered tumouricidal by the systemic administration of non-conjugated ricin B chain. This effect was not obtained with tumour cells pre-treated with antibody alone. Interestingly, a slight but significant anti-tumour effect was seen when the growth of 11/160-ricin A pre-treated cells was compared with that of untreated or antibody-coated cells in the absence of B chain. This effect was not observed in vitro [10, 11] and was possibly due to enhanced recognition of the former cells by host macrophages with receptors for mannose residues which are present in both the ricin subunits [6]. This hypothesis could be tested by preparing 11/160-abrin A chain conjugates since this toxin A chain is non-glycosylated.

When ricin B chain was administered soon after the 11/160-ricin A pre-treated tumour cells, complete inhibition of tumour growth was achieved in up to 70% of animals. Since the number of cells inoculated (10^6) was significantly greater than the TD_{50} for this sarcoma (10^2), we consider these results promising. When B chain administration was delayed the results were less predictable, and suggested complex dose-time relationships and possible secondary host effects in the degree of potentiation observed. Growth inhibition of s. c. tumours was obtained in 5/5, 1/5 and 3/5 assays with 15 µg B chain administered after 5 min, 6 h or 24 h, respectively. These data seem to suggest that an interval of 24 h is more advantageous than 6 h. One possibility is that at 6 h acute inflammatory reactions around the tumour site interfere with the extravasation and/or binding of B chain subunits, or alternatively,

at 24 h a waning B chain potentiation could be supplemented by activated host anti-tumour effector mechanisms. The phenomenon was also seen in 2/4 lung colonisation assays, although less evident in the pooled results. Clearly further work is required to analyse in more detail the kinetics of B chain potentiation in vivo and its susceptibility to modulation by host factors.

A limited number of experiments were performed with 150 μg B chain which, when administered 5 min after conjugate-coated tumour cells, consistently inhibited their growth at both s. c. and pulmonary sites more effectively than did 15 μg B chain. The failure to demonstrate potentiation in the s. c. tumour assay with 150 μ g B chain given after 24 h was surprising (since 15 μg was effective) and if confirmed in a larger series may imply that the optimal dose of B chain varies with time of administration. However, in the lung colonisation assay, tumour growth was reproducibly inhibited to a greater degree by the higher dose of B chain at all time points tested, suggesting that the s. c. site may not predict the response of tumours growing in internal organs representing potential sites of metastasis.

Most in vivo studies with antibody-ricin A conjugates have been performed with lymphoid tumours, with a few exceptions. Seto et al. [13] showed that a monoclonal antibody-ricin A conjugate bound to syngeneic mouse mammary carcinoma cells prior to their inoculation s. c. or i. p. was growth inhibitory. However, in this test system the monoclonal antibody alone (which recognises an inappropriately expressed alloantigen) is strongly cytolytic in vitro in the presence of complement, and has in vivo antitumour activity. Our results encourage the view that other non-lymphoid tumours, even those refractory to antibody-A chain conjugates, may be responsive in vivo to the 2-stage regime described.

The effectiveness of the B chain could be due to its ability to potentiate translocation into the cytoplasm of A chain from complexes bound to the cell surface [10]: this may be consequent upon a promotion of internalisation of the complexes to an endocytic compartment. Although the mechanism is unknown it does not appear to depend primarily on binding of the B chain to galactose residues at the cell surface, since the effect in vitro is manifest in the presence of 100 mM lactose [10, 11]. It seems more probable that the B chain is prone to re-association with the conjugated A chain effectively generating a holotoxin-antibody complex. The fact that B chain is able to activate antibody-A chain bound to tumour cells remote from the site of injection supports this mechanism, since following inoculation the B chains will form complexes with galactose-containing plasma glycoproteins via their sugar recognition sites, thus inhibiting interaction with cell surfaces except by exchange reactions. Previously it has been suggested that the galactose-binding property of the B chain may need to be modified in order for it to potentiate specifically the action of antibody-A chain in vivo [17, 18]. In the system we describe this does not appear to be necessary.

We know that the epitope recognised by 11/160 antibody is a relatively stable cell surface component, and that bound antibody is not readily internalised [3]. This feature may be responsible for the fact that ricin B chain, even when administered up to 24 h after conjugate-coated tumour cells, was still able to potentiate anti-tumour effects in vivo in some circumstances. Also, it has been demonstrated that HSN_{tc} cells treated with 11/160-A chain conjugates in vitro retain their susceptibility to B chain potentiation for at least 48 h (D. McIntosh unpublished observations). Clearly, the nature of the target antigen, and in particular its susceptibility to modulation and/or internalisation in response to antibody binding, will determine the kinetics and efficiency of B chain potentiation. A possible problem, which we did not observe, is that recombined ricin A and B chains, if attached to an antibody which can bind to certain normal cells, or if released intact, may lead to toxic side effects in vivo. For these reasons, experiments of the kind described should take into account the nature, distribution and stability of target antigens, the characteristics of the antibody employed, and the interactions between the two.

The advantages of the system described are that using a syngeneic antibody and tumour model in normal immunocompetent hosts the potential of therapeutic single or split dose regimes can be tested. The tumour is capable of metastasis in immune deprived hosts [5] allowing an evaluation of the effectiveness of immunotoxins against disseminated disease, and of the role of various host effectors (e. g. T cells, macrophages). Antibodies with the same specificity as 11/160, but of different isotypes (IgG_{2a}, IgM, IgA, IgE) are available, and can be prepared as $F(ab)'_2$ fragments, which is not possible with certain subclasses of mouse monoclonal antibody. The next step will be to determine if antibody-ricin A chain conjugates can be targeted to HSN_{tc} cells growing in vivo, and whether B chain potentiation can be achieved in therapeutic protocols.

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