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Monoclonal Antibody Therapy: "Model" Experiments with Toxin-Conjugated Antibodies in Mice and Rats

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A. Introduction

The advent of cell fusion techniques for producing monoclonal antibodies has stimulated world-wide effort in the search for antibodies with specificity for neoplastic cells. In parallel, a number of laboratories have attempted to devise ways of attaching cytotoxic agents to antibodies with the aim of generating potent anticancer agents from monoclonal antibodies that exhibit sufficient specificity for cancer cells.

The most successful ploy for arming antibody molecules has been to couple them to highly poisonous toxins such as abrin, from the jequirity bean, and ricin, from the castor bean. Abrin and ricin are glycoproteins comprising two polypeptide subunits, A and B, joined by a disulphide bond. The B-chain binds to galactose-containing molecules which are to be found on most cell surfaces and the A-chain is believed to penetrate the plasma membrane or the membrane of an endocytic vesicle and kill the cell by damaging ribosomes (reviewed in [1]).

B. Anti-tumour Effects of Antibody-Abrin and Antibody-Gelonin Conjugates

In the first series of experiments, $Thy_{1.1}$-expressing lymphoma cells growing in mice or in tissue culture were attacked with the $F(ab')_2$ fragment of monoclonal anti-$Thy_{1.1}$ antibody (IgG_{2a}) coupled to abrin. The antibody-toxin conjugate and a control conjugate made with the $F(ab')_2$ fragment of normal murine IgG_{2a} were prepared using a mixed anhydride derivative of chlorambucil as the coupling agent as described previously [2]. The simplest conjugates, consisting of one molecule of $F(ab')_2$ and one of abrin, were purified. No loss in the binding capacity of the anti-$Thy_{1.1}$-abrin to antigens upon AKR-A and BW5147 lymphoma cells was apparent from indirect immunofluorescence analysis in 100 m*M* lactose [3].

In vitro, the anti-$Thy_{1.1}$-abrin conjugate was a very effective and moderately specific cytotoxic agent for AKR-A and BW5147 cells (Fig. 1). Treatment of the cells with the conjugate at 2.5×10^{-11} *M* sufficed to reduce their capacity to incorporate ^{3}H-leucine into protein by 50%. The cytotoxic action of the conjugate upon BW5147 cells was similar in potency to that of unconjugated abrin, whereas with AKR-A cells the native toxin was about tenfold more effective. The cytotoxic effect of the conjugate was specific, as shown by the comparative ineffectiveness of the control conjugate which reduced the leucine incorporation of the BW5147 and AKR-A cells by 50% at 2.5×10^{-9} *M* and 5×10^{-10} *M* respectively.

The therapeutic value of anti-$Thy_{1.1}$-abrin was assessed against AKR-A and BW5147 cells growing in T-cell-deprived CBA mice. The $Thy_{1.1}$ antigen expressed by the lymphoma cells is not found in CBA mice and so it constituted a tumour-specific antigen in this model system. When 10^5 AKR-A cells were injected intraperitoneally, they grew progressively, initially as an ascitic tumour, and killed the mice between 18 and 21 days later. Treatment of the mice with 1.5 pmol anti-$Thy_{1.1}$-

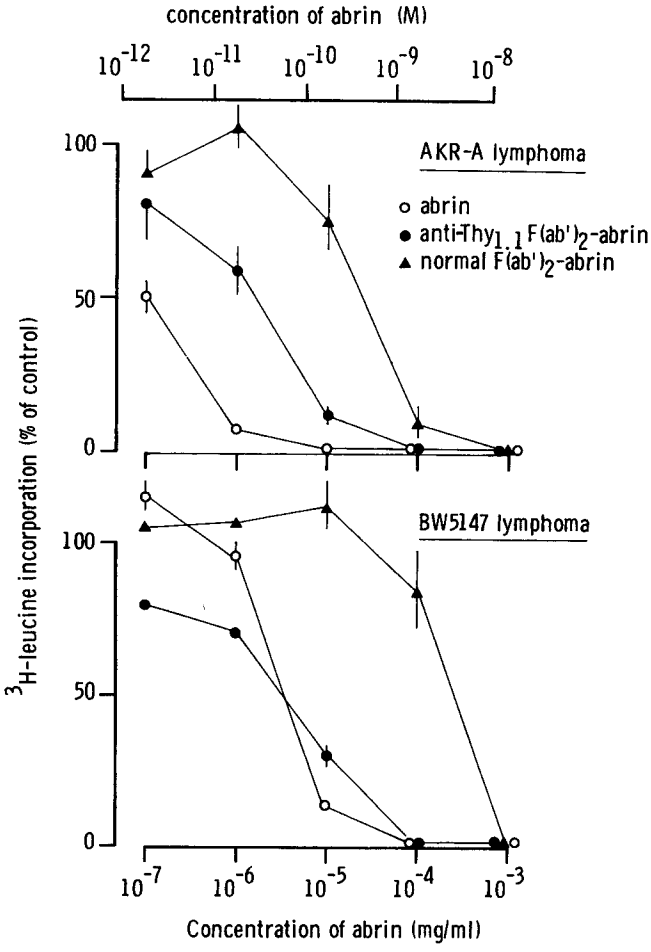

Fig. 1. The cytotoxic effects of abrin alone (○) and of conjugates with the *F(ab')*$_2$ fragments of monoclonal anti-Thy$_{1.1}$ antibody (●) or of normal mouse IgG$_{2a}$ (▲) upon AKR-A and BW5147 cells in tissue culture. The cells were treated for 1 h at 37 °C with abrin or the conjugates and then were washed and 23 h later 1 μCi ^{3}H-leucine was added to the cultures. The ^{3}H-leucine incorporated during a 24 h period is expressed as a percentage of that in untreated cultures. Vertical lines represent one standard deviation on the geometric mean of triplicate determinations. Treatment of the cells with unconjugated anti-Thy$_{1.1}$ *F(ab')*$_2$ at concentrations as high as 10^{-7} *M* did not alter their rate of leucine incorporation

abrin administered intraperitoneally 1 day after the lymphoma cells extended the median survival time of the animals by 5.5 days (Fig. 2). Neither 1.5 pmol anti-Thy$_{1.1}$ *F(ab')*$_2$ alone, nor abrin at a dose corresponding to half the LD_{50}, prolonged the survival time of the animals. Experiments in which graded numbers of untreated AKR-A cells were injected intraperitoneally into T-cell-deprived mice established that an extension in median survival time of 5.5 days was approximately that which resulted from a 100-fold reduction in the number of tumour cells injected, suggesting that the anti-Thy$_{1.1}$-abrin conjugate had eradicated 99% of the lymphoma cells. This deduction was supported by the finding in a further experiment that 40% of animals which received 10^3 tumour cells (equivalent to 100 lethal doses, since about ten cells are needed to kill an animal) were cured by their treatment with the conjugate. However, when the conjugate was administered intravenously rather than intraperitoneally, no anti-tumour action was observed, as was also the case when established subcutaneous tumours were attacked. Thus the therapeutic activity of the conjugate was disappointing and was only apparent when small numbers of tumour cells were attacked with conjugate delivered directly to the site of tumour growth.

The problem with conjugates containing intact toxins is that they are highly poisonous to animals, probably because they bind non-specifically to cells through the galactose-binding site on the B-chain of the toxin moiety. This has the important corollary that conjugates injected intravenously might be expected to form semi-stable complexes with glycoproteins free in the plasma or upon the surface of erythrocytes. This could delay the diffusion of the conjugate out of the bloodstream, a notion consonant with the observation above that intravenously administered conjugate was ineffective. One way around these problems is to link antibodies directly to the isolated toxin A-chain. Alternatively, use could be made of one of the virtually non-poisonous inhibitors distributed widely in the plant kingdom whose action upon eukaryotic ribosomes is apparently identical to that of ricin A-chain (reviewed in [4]). One such inhibitor is gelonin, from the seeds of *Gelonium multifluorum* [5].

Accordingly, monoclonal anti-Thy$_{1.1}$ antibody was coupled to gelonin, using the SPDP reagent which introduces a disulphide linkage between the two protein molecules [6]. The conjugate was, however, only weakly cytotoxic to AKR-A or BW5147 cells in tissue culture, a concentration of 10^{-7} *M* being needed to reduce the leucine incorporation of the cells by half. This result contrasts with the extremely potent and

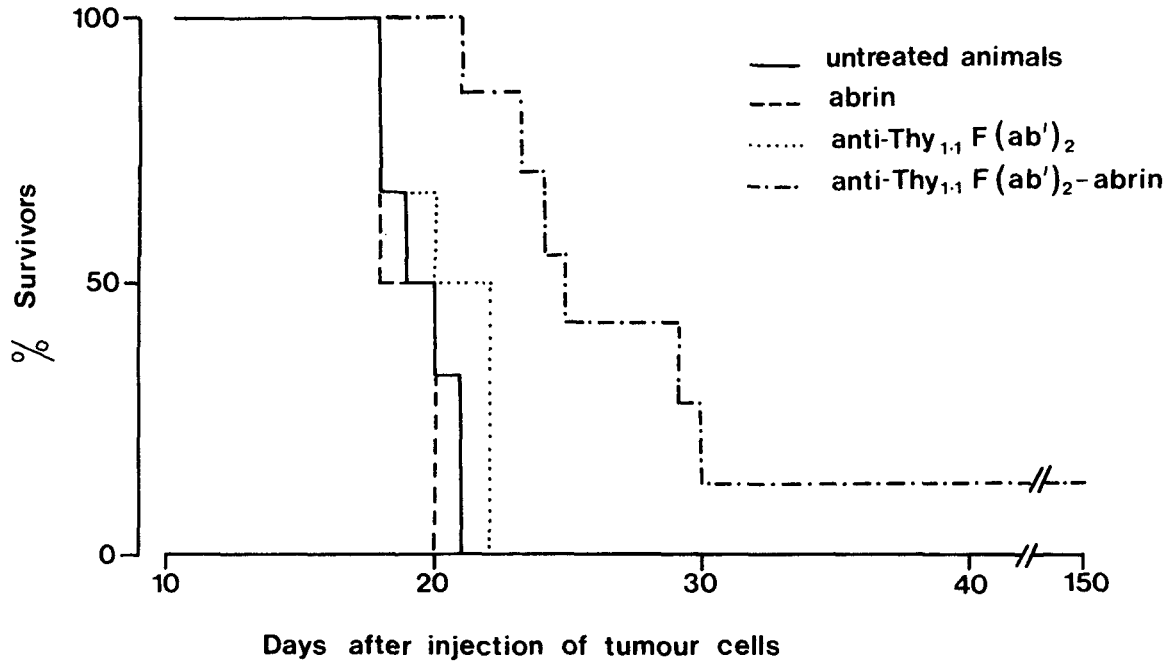

Fig. 2. Prolongation of survival of immunologically deficient CBA mice bearing a Thy$_{1.1}$-expressing lymphoma following the administration of anti-Thy$_{1.1}$ *F(ab')*$_2$-abrin. The mice were injected intraperitoneally with 10^5 AKR-A lymphoma cells and 1 day later received an intraperitoneal injection of 1.5 pmol anti-Thy$_{1.1}$ *F(ab')*$_2$-abrin (–·–·–), 1.5 pmol of unconjugated anti-Thy$_{1.1}$ *F(ab')*$_2$ (·····) or 0.15 pmol of unconjugated abrin (————). The mice were rendered T-cell deficient by a procedure of thymectomy, whole body irradiation and reconstitution with normal CBA bone marrow

specific inhibitory action of the same conjugate upon resting AKR T-lymphocytes in tissue culture [6] and typifies the variable effectiveness of conjugates containing toxin A-chain that has been observed in many laboratories (reviewed in [7, 8]). Nevertheless, when injected intraperitoneally at dose levels of 700 pmol (corresponding to less than 1/50 of the minimal lethal dose for free gelonin) the conjugate prolonged by 1 week the median survival time of CBA mice bearing intraperitoneal AKR-A lymphoma cells.

Several factors could operate in animals to prevent antibody-toxin conjugates from exerting anti-tumour activity of a potency and selectivity predicted by in vitro experiments. Firstly, intact abrin and ricin, ricin A-chain and gelonin are glycoproteins containing mannose and *N*-acetyl glucosamine residues and, by analogy with similar molecules, are expected to be withdrawn from the blood circulation by the reticuloendothelial system which is equipped with receptors for these sugars [9, 10]. Secondly, it could be that the chemical linkage used to form the conjugate breaks down in animals; there is evidence that disulphide linkages are prone to cleavage by reduction or disulphide exchange with thiol-containing molecules [11]. Lastly, it is not known how easily conjugates diffuse out of the bloodstream to their intended site of action. Until the importance of these factors is assessed and countermeasures devised to those which prove problematical, the potential of antibody-toxin conjugates as anticancer agents will remain undetermined.

C. Selective Killing of Malignant Cells in Leukaemic Bone Marrow in Vitro

The treatment of leukaemia patients with high-dose chemotherapy, total body irradiation and allogeneic bone marrow transplantation runs the risk of provoking life-threatening graft-versus-host reactions and rejection of the marrow graft. These problems would not arise if the patient's own bone marrow, extracted before radiochemotherapy, could be treated with an antibody-toxin conjugate to destroy the malignant cells which had infiltrated it and then injected back into the patient on completion of the treatment.

To explore this possibility, we adopted a model system in which a mixture of 10^3 or 10^4 rat T-cell leukaemia cells and 10^7 bone marrow cells was incubated with a conjugate of ricin and the monoclonal antibody, W3/25, washed and injected into 650-rad-irradiated PVG rats [12, 13]. The W3/25 antigen is expressed by the leukaemic cells and by rat T-helper cells, macrophages and thymocytes but is absent from bone marrow stem cells. Toxicity to haematopoietic stem cells was blocked by including lactose in the incubation mixture to antagonise the non-specific binding of the conjugate via its ricin moiety to galactose residues upon their cell surface. The lactose also reduced the quantity of conjugate that bound to erythrocytes and other cells in the inoculum to the level where no signs of toxin-poisoning were seen in the recipient animals.

The bone marrow was acquired from the PVG 1-a strain of rat which is congenic with PVG except that the immunoglobulin light chain genes are derived from the DA strain which expresses the 1-a allotype rather than the 1-b allotype of PVG rats. The survival of haematopoietic stem cells in the leukaemic marrow inoculum treated with the conjugate was measured from their ability to compete with the residual stem cells in the irradiated rat and thus produce B-lymphocytes that expressed the donor (1-a) allotype. As shown in Table 1, there was good, although not complete, preservation of haematopoietic stem cell activity in leukaemic marrow treated with W3/25-ricin at 2.1 μg ricin/ml for 1 h at 37 °C in 100 m*M* lactose. The 1-a chimaerism was between 30% and 46% as compared with 61%–68% in the recipients of untreated marrow cells.

None of the animals that received inocula of 10^3 leukaemic cells incubated with the conjugate developed leukaemia and, of three recipients of 10^4 leukaemic cells, only one did so. Since about ten cells are needed to induce leukamia, it can be calculated that the conjugate had destroyed 99.9% of the malignant cells in the marrow inoculum. This conclusion was supported by other experiments in which animals injected with 10^6 conjugate-treated leukaemic cells were found to develop disease 8–10 days later than recipients of 10^6 untreated cells and at the same time as recipients of 10^3 untreated cells. Neither treatment of leukaemic cells in 100 m*M* lactose with W3/25 antibody alone at 20 μg/ml nor with a control conjugate made with an irrelevant monoclonal antibody, MRC OX8, delayed the appearance of leukaemia in the recipients.

It is concluded that antibodies linked to intact toxins or, as used by Krolick and his colleagues [14], to ricin A-chain, potentially could be used to destroy malignant cells in autologous bone marrow grafts in man.

Table 1. In mixtures of leukaemic cells and bone marrow cells the malignant cells are specifically killed by the antibody-ricin conjugate

Cells injected	Incubation conditions	No. of recipients	Day of appearance of leukaemia	% B-Cell chimaerism
10^3 Leuk. + 10^7 BM	Med.	3	16, 17, 18	–
	Med. + conj.	3	>67, >67, >67	34.0, 37.5, 38.7
10^4 Leuk. + 10^7 BM	Med.	3	16, 16, 16,	–
	Med. + conj.	3	36, >67, >67	–30.6, 46.5
10^7 BM only	Med.	3	–	61.4, 66.2, 67.8

Med., Dulbecco's phosphate buffered saline containing $CaCl_2$, $MgCl_2$ and supplemented with 100 m*M* lactose; conj., W3/25-ricin (M_r 350,000) at a concentration of 2.1 μg ricin/ml and 10 μg IgG/ml. Day of appearance of leukaemia: number of days which elapse after injecting the leukaemic marrow cells before the animal's WBC reached 2×10^4 mm^{-1}. Other details are given by Thorpe [12] and Mason [13].

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References

1. Olsnes S, Pihl A (1976) In: Cuatrecasas P (ed) Receptors and recognition series B: the specificity and action of animal, bacterial and plant toxins. Chapman & Hall, London, pp 129–173
2. Thorpe PE, Ross WCJ (1982) Immunol Rev 62:119–158
3. Ross WCJ, Thorpe PE, Cumber AJ, Edwards DC, Hinson CA, Davies AJ (1980) Eur J Biochem 104:381–390
4. Barbieri L, Stirpe F (to be published) Cancer Surveys 1 (3)
5. Stirpe F, Olsnes S, Pihl A (1980) J Biol Chem 255:6947–6953
6. Thorpe PE, Brown ANF, Ross WCJ, Cumber AJ, Detre SI, Edwards DC, Davies AJS, Stirpe F (1981) Eur J Biochem 116:447–454
7. Olsnes S, Pihl A (1982) In: Drew J, Dorner F (eds) Pharmac. Ther. Vol 15. Pergamon Press, London, pp 355–381
8. Thorpe PE, Edwards DC, Ross WCJ, Davies AJS (1982) In: Fabre J, McMichael A (eds) Monoclonal antibodies in clinical medicine. Academic Press, London, pp 167–201
9. Skilliter DN, Paine AJ, Stirpe F (1981) Biochim Biophys Acta 677:495–500
10. Neufeld EF, Ashwell G (1980) In: Lennarz WJ (ed) The biochemistry of glycoproteins and proteoglycans. Plenum, pp 241–266
11. Edwards DC, Ross WCJ, Cumber AJ, McIntosh D, Smith A, Thorpe PE, Brown A, Williams RH, Davies AJ (1982) Biochim Biophys Acta 717:272–277
12. Thorpe PE, Mason DW, Brown ANF, Simmonds SJ, Ross WCJ, Cumber AJ, Forrester JA (1982) Nature 297:594–596
13. Mason DW, Thorpe PE, Ross WCJ (to be published) Cancer Surveys 1 (3)
14. Krolick KA, Uhr JW, Vitetta ES (1982) Nature 295:604–605