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Selective cytotoxicity of an oxygen-radical-generating enzyme conjugated to a monoclonal antibody

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SUMMARY

The monoclonal antibody 8A, which recognizes a human plasma cell-associated antigen, was covalently linked to xanthine oxidase in a conjugate maintaining both immunological and enzymatic properties. A significant degree of target cell lysis was obtained at an enzyme concentration that was ineffective on non-target cells and on myeloid staminal cells (CFU-GM). The cytotoxic activity was abolished by an excess of antibody, by allopurinol and by superoxide dismutase and catalase. A possible use of the conjugate for bone marrow purging in multiple myeloma patients is suggested.

Keywords bone marrow purging immunoconjugates monoclonal antibodies multiple myeloma xanthine oxidase

INTRODUCTION

In the last few years numerous efforts have been devoted to achieving selective killing of cancer cells with conjugates composed of cytotoxic agents linked to antibodies, mainly monoclonal, directed against tumour-associated antigens. Such conjugates, often called 'immunotoxins', have been prepared with conventional antineoplastic drugs or radioisotopes (review by Thorpe, 1985), and, more often, with toxic polypeptides with catalytic (enzymic) activity, either separated from double-chain toxins (ricin and abrin A-chains, diphtheria or *Pseudomonas aeruginosa* A-fragments, review by Pastan *et al.*, 1986), or existing as single-chain ribosome-inactivating proteins (review by Stirpe & Barbieri, 1986). Few conjugates have been prepared with other enzymes, either directly cytotoxic such as phospholipase C (Flickinger & Trost, 1976), or generating toxic free radicals, such as glucose oxidase (Philpott *et al.*, 1974; Akard, Enolist & Gabic, 1986) or, for different therapeutic purposes, with fibrinolytic enzymes (Bode *et al.*, 1985).

We report now the preparation of an antibody-conjugated enzyme consisting of xanthine oxidase linked to a monoclonal antibody. Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine and of the latter to uric acid. In mammalian tissues, the enzyme exists in nature as a NAD^+-dependent dehydrogenase (D form), which can be converted into an oxidase (O form) either irreversibly, by proteolysis (Stirpe & Della Corte, 1969) or reversibly, by oxidation of sulphydryl groups (Della Corte & Stirpe, 1972; Battelli, 1980).

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During the oxidation of hypoxanthine or xanthine catalysed by the xanthine oxidase in its O form, cytotoxic free radicals are produced, which aggravate cell damage when the enzyme is converted from the D into the O form during ischaemic or other pathologic conditions (Engerson *et al.*, 1987).

With present experiments, commercial xanthine oxidase (which is in the irreversible O form) was linked to a monoclonal antibody (MoAb) named 8A recognizing a human plasma cell-associated antigen, present on Raji and U266 cell lines, (Dinota *et al.*, 1987; Tazzari *et al.*, 1987). This conjugate proved significantly more cytotoxic to the target cells than to a cell line not bearing the antigen relevant to the antibody used, and was not toxic to normal human bone marrow precursor cells.

MATERIALS AND METHODS

Materials

Xanthine oxidase purified from buttermilk, catalase from bovine liver, superoxide dismutase from human erythrocytes, xanthine and hypoxanthine (cristalline), ethidium bromide and methyl-cellulose were purchased from Sigma Chemical Co., St Louis, MO, USA.

N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), Sephadex G25 Coarse and Sephacryl S300 Superfine were from Pharmacia S.p.a., Cologno Monzese, MI, Italy.

Iodogen was from Pierce Chemical Co., Rockford, IL, USA; $Na^{125}I$ (specific activity 342 mCi/mmol) was from Amersham International, Amersham, Bucks, UK.

Dimethylformamide (UV spectroscopy grade) was from FLUKA AG, Buchs, CH. Phytohaemagglutinin (PHA) was from Wellcome, Beckenham, England UK. Fluorescein isothio-

cyanate (FITC) conjugated goat-anti-mouse IgG was from Becton Dickinson, Mountain View, USA.

RPMI 1640 was from Biochrom, Berlin, West Germany; fetal calf serum (FCS) from Sera Lab, Sussex, UK. Dulbecco's minimal essential medium (MEM) was from Gibco, Paisley, Scotland UK.

Purification and labelling of the antibody

Mouse IgG1 8A monoclonal antibody (8A-MoAb), recognizes an antigen present on the B cell lineage from the CD10$^+$precursors to the last steps of differentiation (Dinota *et al.*, 1987; Tazzari *et al.*, 1987). The hybridoma, grown in complete RPMI 1640 medium, without HAT, was injected in pristane-treated BALB/C mice. Ascitic fluid was collected after 7–10 days, and the antibody was purified by ion-exchange chromatography on DEAE-cellulose (Mason & Williams, 1980).

The 8A MoAb was labelled with ^{125}I using the Iodogen reagent (Fraker & Speck, 1978), in order to monitor antibody content.

Antibody-enzyme conjugation

To a solution of 10 mg of antibody containing a trace of ^{125}I-antibody ($5–10 \times 10^4$ ct/min/mg) in 2 ml of 50 mM borate saline buffer, pH 9·0, 70 μl of a 1 mg/ml solution of SPDP in dimethylformamide, corresponding to a 3-fold molar excess, was added. After vigorous stirring, the reaction mixture was left for 60 min at 28°C and then was applied to a Sephadex G 25 Coarse column (36 cm × 2·0 cm) equilibrated and eluted with 0·14 NaCl containing 5 mM Na-phosphate buffer, pH 7·5 (PBS) to remove the excess of reagent. The antibody derived was collected in a volume of 10–12 ml and a sample was analysed by the method of Carlsson, Drevin & Axen, (1978). An extinction coefficient $A_{280}^{0.1\%} = 1.4$ for 8A MoAb was assumed in order to calculate the number of 2-pyridildithio groups introduced per mol of antibody.

Xanthine oxidase (3·2 U/mg 8A) was reduced for 5 min at 28°C with 20 mM dithiothreitol (DTT) in 50 mM phosphate buffer pH 6·5, and was gel filtered on a Sephadex G 25 Coarse column (36 cm × 2·0 cm) in PBS, directly on the 8A MoAb, with constant stirring. The conjugation was allowed to proceed overnight at 28°C. The reaction mixture was then concentrated to 6 ml by ultra-filtration (Amicon PM 10 membrane) and the conjugate was purified from free enzyme and antibody by gel filtration on a Sephacryl S300 Superfine column (95 cm × 2·6 cm), equilibrated and eluted with PBS buffer, at 4°C. Fractions of 2·4 ml were collected, the xanthine oxidase level was evaluated by its activity and the antibody content was monitored by ^{125}I-radioactivity. The fraction containing the 8A MoAb conjugated to xanthine oxidase was pooled and then concentrated to 6 ml by ultra-filtration (Amicon PM 10 membrane).

The conjugate was filter-sterilized through a 0·22 μm membrane and could be stored in samples under liquid nitrogen without appreciable loss of activity for several weeks.

Xanthine oxidase assay

Xanthine oxidase activity was determined from the uric acid formation measured from the A_{292} as described by Stirpe & Della Corte (1972). The assay was at room temperature in a mixture containing, in a final volume of 3 ml: 0·05 M Tris-HCl buffer, pH 8·1, 60 μM xanthine and the appropriate amount of enzyme. Xanthine oxidase was omitted from reference cuvettes. A unit of enzyme activity is defined as the formation of 1 μmole of uric acid per min.

Reactivity of the conjugate with target cells

The conjugate was assayed on target cells (RAJI and U266 cell lines) by means of a FITC-conjugated goat anti mouse IgG. The binding was scored with a Zeiss fluorescence microscope.

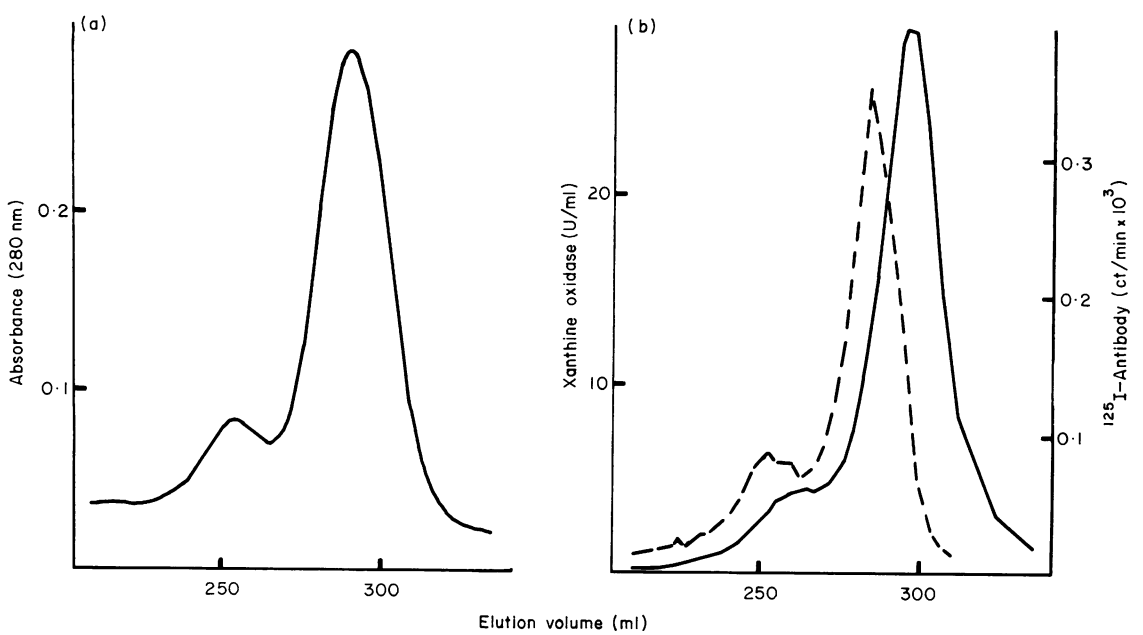

Fig. 1. Purification of the 8A-xanthine oxidase conjugate by gel filtration on Sephacryl S300 superfine. Experimental conditions are described in the text. (a) Protein elution profile. (b) (---) Xanthine oxidase activity. (——) Antibody level.

Cell line assay

Target (Raji, U266) and non-target (NS1) cell lines were maintained in culture flasks, with RPMI 1640 complete medium supplemented by 10% fetal calf serum, glutamine and antibiotics. On the day of the experiment, the cells were harvested, counted, checked for viability with ethidium bromide and adjusted at the concentration of 1×10^6/ml.

Ethidium bromide dye exclusion test

Samples (100 μl) of cell suspension were incubated for 24 h with 100 μl of appropriate dilutions of free or conjugated enzyme, activity ranging from 8·75 to 0·53 mU/ml, in a final volume of 1 ml of complete RPMI 1640, in the presence of 100 μM hypoxanthine (or the appropriate amount for the experiment with different substrate concentration). The cells were washed in RPMI 1640 complete medium and then resuspended in 1 ml of RPMI containing 10 μg of ethidium bromide. Cell death was evaluated with a cytofluorimeter Ortho Spectrum III, analysing forward scatter vs red fluorescence scatter.

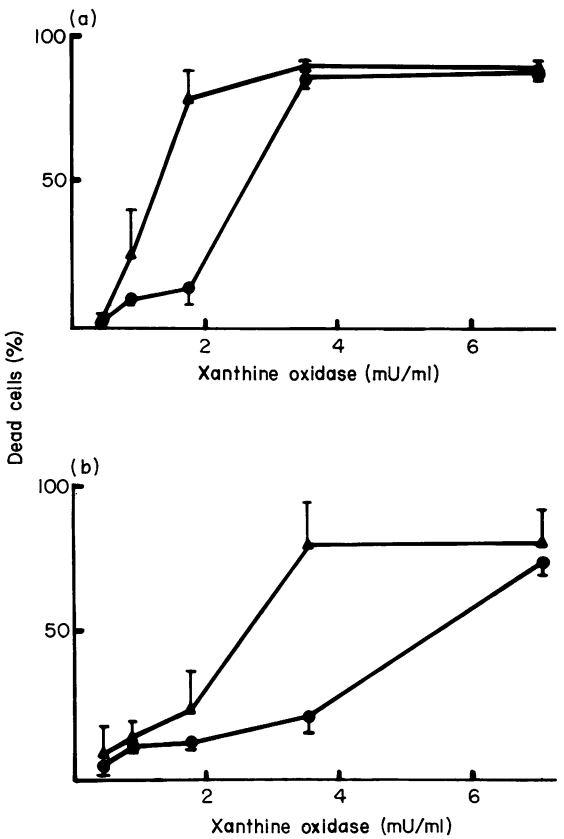

Fig. 2. Cytotoxicity of free and 8A MoAb-conjugated xanthine oxidase on target cells. The evaluation of cell death was made by the ethidium bromide dye exclusion test in the presence of xanthine oxidase free (●) or antibody-conjugated (▲): (a) Raji cell line (b) U266 cell line. Results are mean values ± S.E.M. of four experiments.

Inhibition of colony growth in plasma clot

RAJI cells (1500), incubated in the presence of free or conjugated enzyme and hypoxanthine were washed, resuspended in 1 ml of RPMI complete medium supplemented with citrated human plasma (15%) and plated in 35 × 10 mm Petri dishes. The clotting of the final medium was obtained by adding calcium chloride solution (final concentration 15 mM). After 4 days incubation at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air, aggregates (> 8 cells) were counted with an inverted microscope (Zeiss). U266 cells were not used due to a very low cloning efficiency in this system.

Evaluation of toxicity on CFU-GM

Samples of heparinized bone marrow, obtained from healthy volunteers, were fractionated by Ficoll-Hypaque gradient, the cells were washed twice and incubated with free or conjugated enzyme in the presence of hypoxanthine. After 24 h the cells were washed, checked for viability with ethidium bromide and assayed for CFU-GM (Iscove *et al.*, 1971). Briefly, 2×10^5 cells were resuspended in 2·5 ml of Dulbecco's MEM supplemented with 0·9% of methylcellulose, 20% of FCS, and 10% of supernatant obtained from a 7 day culture of PHA-stimulated human lymphomonocytes (final concentration 1 μg/ml). Every experiment was performed in triplicate.

The cells were plated in 35 × 10 mm Petri dishes, incubated at 37°C in a water-saturated atmosphere of 5% CO_2 and 95% air. CFU-GM scoring was made at days 7 and 14 with a Zeiss inverted microscope, by evaluating clusters (> 20, < 50 cells) and colonies (> 50 cells).

RESULTS

Antibody-enzyme conjugate

Two pyridyl disulphide groups were introduced into the antibody molecule at a mean ratio of 2·5 residues per 8A molecule.

The intrinsic sulphydryl groups of xanthine oxidase, reduced by DTT treatment, reacted with pyridyl-disulphide groups of

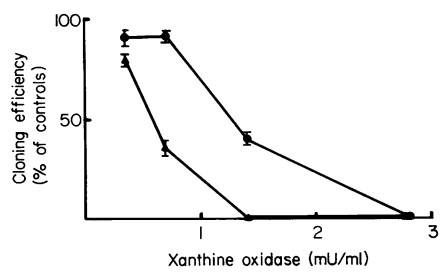

Fig. 3. Inhibition of colony growth. Cloning efficiency of Raji cells was tested in the presence of (●) xanthine oxidase or (▲) 8A-conjugate. Results are means ± S.E.M. of triplicate values of a representative experiment.

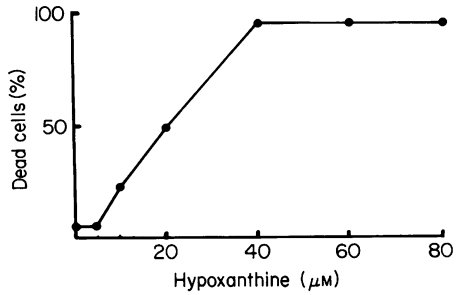

Fig. 4. Substrate concentration curve. The dependence of conjugate cytotoxicity on the hypoxanthine concentration was evaluated with Raji cells in the presence of 3·3 mU/ml of enzymic activity, by the ethidium bromide dye exclusion test.

Table 1. Effect of free and antibody-conjugated xanthine oxidase on cells *in vitro*

	Xanthine oxidase added (mU)					
	0	0·4	0·8	1·6	3·2	6·4
Raji cells						
Experiment 1						
XO	<10*	<10	20	40	90	90
XO-8A		<10	60	90	90	90
XO-8A+allopurinol†		<10	<10	<10	<10	60
XO-8A+8A MoAb‡		<10	<10	15	>90	>90
XO-OxIgG		<10	<10	45	>90	>90
Experiment 2						
XO	<10	10	11	>90	>90	ND
XO-8A		31	>90	>90	>90	ND
XO-8A+SOD§		39	>90	>90	>90	ND
XO-8A+catalase§		10	21	23	66	ND
XO-8A+SOD+catalase		<10	<10	22	25	ND
NS1 cells						
XO	<10	ND	<10	<10	>90	>90
XO-8A		ND	<10	<10	>90	>90

*Values represent the percentage of cell killing, evaluated by means of cytofluorimetry.

†1 mM allopurinol.

‡100 μg/ml 8A MoAb.

§100 mU/ml either superoxide dismutase (SOD) or catalase.

ND, not determined.

the modified antibody. The linked enzyme averaged 1·4 mol per mol of antibody.

The conjugate retained immunological properties and enzymic activity with a yield of 13% for the antibody moiety and 27% for the enzymic activity (Fig. 1).

Cytotoxicity

The conjugate killed target cells with an ID_{50} of 1·7 mU/ml for U266 and 0·8 mU/ml for Raji cell line vs an ID_{50} of 4·7 and 1·7 mU/ml of free xanthine oxidase, respectively. When target cells were incubated in the presence of 1·75 to 3·5 mU/ml of the conjugated enzyme and hypoxanthine, a marked cell lysis, 78 to 80%, was observed with the Raji and U266 cell line respectively. The same concentrations of free xanthine oxidase gave significantly lower values (13 to 21%; $P<0·001$ and $P<0·005$, respectively) (Fig. 2a, b).

A series of cloning assays, performed with Raji cell line, confirmed the values obtained with the ethidium bromide dye exclusion. In particular, at 0·7 mU/ml of conjugated enzyme, cloning efficiency was reduced to 35%, while the same concentration of free enzyme led to 92% of aggregates (Fig. 3). To evaluate the role of both the enzymic and immunological components of the conjugate in the cell killing, experiments were run in parallel with (i) increasing amounts of hypoxanthine, (ii) the xanthine oxidase-competitive inhibitor allopurinol, (iii) an excess of 8A antibody, (iv) an irrelevant conjugate made with bovine IgG, and (v) non target cell line (mouse myeloma-derived NS1).

Target cell killing occurred only in the presence of hypoxanthine as substrate, and was proportional to its concentration up to 40 μM (Fig. 4).

The cytotoxicity of the conjugate was abolished by 1 mM allopurinol and by a 5-fold excess of 8A antibody. No difference in toxicity between the conjugated and the free enzyme was observed when either an aspecific antibody or an irrelevant cell line were used (Table 1).

To ascertain the involvement of oxygen radicals in the conjugate cytotoxicity, superoxide dismutase and catalase were added in some experiments as scavengers. The cytotoxicity of the conjugate was: (i) unaffected by superoxide dismutase (100 mU/ml); (ii) partially decreased by catalase (100 mU/ml); (iii) abolished using both the scavenger enzymes (Table 1).

Table 2. CFU-GM rescue after exposure to free or 8A MoAb-conjugated xanthine oxidase

XO added (mU)	XO		XO-8A	
	7th day	14th day	7th day	14th day
1·6	78±14*	101±3	76±15	108±28
3·2	73±3	94±15	82±11	106±21
6·4	85±20	121±20	89±15	103±13

*Mean values±s.e.m. of two experiments. Clusters+colonies are expressed as percentage of controls, which were 783 and 308 for the first healthy bone marrow donor, and 308 and 135 for the second one, at the 7th and 14th day respectively. Experimental conditions are described in the text.

CFU-GM rescue

Weak or no toxicity was observed on CFU-GM scoring, both with conjugated and free xanthine oxidase at the same concentrations causing a significant degree of Raji and U266 cell lysis.

In particular, concentrations ranging from 1·6 mU/ml to 6·4 mU/ml had only a limited effect on the growth of 7th and 14th day CFU-GM, with a rescue ranging from 73% to 121% of the control values (Table 2).

DISCUSSION

We prepared an antibody-conjugated enzyme by covalently linking xanthine oxidase in its O form to a monoclonal antibody against a plasma cell associated-antigen. The conjugate could be well separated by gel filtration, from both the excess of free xanthine oxidase and from the unreacted antibody.

In the presence of hypoxanthine, the antibody-conjugated enzyme was twice as toxic to target cells as the free enzyme. The higher cytotoxicity to target cells was due to the specificity conferred by the antibody carrying the enzyme to the target cells, since (i) it was not observed on cells irrelevant to the antibody, (ii) was abolished in the presence of an excess of free 8A antibody, and (iii) was not observed when xanthine oxidase was linked to an IgG irrelevant to plasma cells. The cytotoxic effect was due to the radicals produced by the xanthine oxidase-catalysed reaction, since it was substrate-dependent and could be abolished by the xanthine oxidase inhibitor allopurinol and by catalase and superoxide dismutase.

Thus our results demonstrate that xanthine oxidase in its O form can be made selectively cytotoxic when linked to an antibody. The selectivity, i.e. the increase of specific toxicity conferred upon linkage to the antibody, is still relatively low, as compared with conjugates containing toxins (Frankel, Houston & Issel, 1986). Presumably this is because the cytotoxicity of the conjugated xanthine oxidase is due to free radicals, which are produced on the surface of, but outside, the target cells, and which are freely diffusible and capable of reaching other cells. Hopefully, this obvious disadvantage could be circumvented by changing experimental conditions, since in some cases it could be useful that the cytotoxic agent may act from outside the target cells. This is because it was observed that immunotoxins prepared with certain antibodies were ineffective, due to poor delivery of the toxic moiety inside the cytoplasm. Furthermore, a conjugate of this kind can be used against microrganisms, that are killed by free radicals producing systems (Rosen & Klebanoff, 1979). The abrogation of the specific toxicity in the presence of superoxide dismutase and catalase shows that the mechanism of action is *via* oxygen radicals.

On the other hand, the use of antibody-linked xanthine oxidase could offer some advantages over other conjugates, for experimental if not for therapeutic purposes, in that (i) it is virtually devoid of general toxicity, (ii) its action can be arrested at will with the use of allopurinol, without the need of removing the conjugate, (iii) as compared with other free radical-generating enzymes, xanthine oxidase seems more efficient, since it generates two radicals for each mole of hypoxanthine used as substrate; finally, (iv) xanthine oxidase is present in human tissues, being particularly abundant in the liver and in the intestine (Parks & Granger, 1986), and can be prepared from the dead and eventually by recombinant DNA technique, thus avoiding immunological reactions in administration to patients.

If the present model could be perfected, oxidase-antibody conjugates would appear particularly suitable for the purging of bone marrow of patients with plasmocytoma (and presumably with other malignancies), due to lack of toxicity to myeloid bone marrow precursors.

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