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Short communication

T lymphocyte killing by a xanthine-oxidase-containing immunotoxin

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Summary. We report on the preparation of an immunotoxin consisting of xanthine oxidase, a free-radical-producing enzyme, covalently linked to an anti-CD3 monoclonal antibody. The immunotoxin retained both enzymic and immunological properties and its toxicity to target cells (a) was greater than that of the free enzyme, (b) was proportional to the enzyme concentration, and (c) was reduced either in the absence of hypoxanthine or by an excess of free anti-CD3 monoclonal antibody. The cytotoxicity and selectivity of the hypoxanthine/conjugated xanthine oxidase system were potentiated by the addition of chelated iron and by washing away the unbound immunotoxin prior to the addition of substrate. The same system was not toxic to bone marrow progenitor cells. A possible use of this immunotoxin for the ex vivo purging of organs to be transplanted from T lymphocytes, to avoid the graft-versus-host reaction, is suggested.

Key words: Xanthine oxidase – Anti-CD3 monoclonal antibody – Immunotoxin

Introduction

Immunotoxins are biotechnology products synthesized to achieve selective cell killing by cell targeting of cytotoxic agents. Specific cell recognition is ensured by monoclonal antibodies, to which bacterial or plant toxins or toxin catalytic moieties are linked to obtain cytotoxicity (reviewed in [4, 10]). A few immunotoxins have been prepared with other enzymes, either cytotoxic per se (phospholipase C), or generating toxic products (glucose oxidase, xanthine oxidase) (reviewed in [2, 19]) or converting prodrugs (reviewed in [18]).

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Xanthine oxidase (EC 1.1.3.22) catalyzes the oxidation of hypoxanthine to xanthine and of the latter to uric acid. This enzyme derives from a NAD^+-dependent dehydrogenase (EC 1.1.1.204) either reversibly, by oxidation or blockade of its thiol groups [1, 7], or irreversibly, by limited proteolysis [6]. During the oxidation of substrates xanthine oxidase produces the superoxide anion and H_2O_2 [13], which in the presence of chelated iron are converted into highly reactive hydroxyl radicals by the Haber-Weiss and the Fenton reactions [11]. The conversion of xanthine dehydrogenase into an oxidase may occur in several pathological conditions (reviewed in [5]) and the O_2-dependent enzyme contributes to tissue damage by generating cytotoxic oxygen products [15].

We reported the preparation of immunotoxins containing xanthine oxidase covalently linked to anti-(mouse IgG) polyclonal antibodies or to mouse monoclonal antibodies (8A and 62B1) [2, 8, 23]. The latter antibodies recognize antigens that are expressed on the B cell lineage, including normal and neoplastic plasma cells, essentially sparing haemopoietic stem cells [22]. The xanthine-oxidase-containing immunotoxins ensured a satisfactory purging of bone marrow together with a good sparing of granulocyte/macrophage colony-forming units (CFU-GM), thus appearing suitable for the ex vivo purging of bone marrow prior to autologous transplantation [3].

In this study we describe the preparation and properties of an immunotoxin containing xanthine oxidase covalently linked to an anti-CD3 monoclonal antibody to achieve selective killing of T lymphocytes. A CD3-antigen-targeted saporin-containing immunotoxin proved to be more efficient in killing T lymphocytes than did similar conjugates directed to CD2 or CD5 antigens [24]. Antibody-linked xanthine oxidase could offer the advantage over other conjugates of being virtually devoid of general toxicity, thus eliminating the need of removing it from transplants [8].

The present work was undertaken to study the best conditions for the use of a xanthine-oxidase-containing immunotoxin. In perspective, this conjugate could be useful in the ex vivo purging of T lymphocytes (a) from allo-

geneic transplants, to avoid the graft-versus-host disease, and (b) from bone marrow used in autologous transplantation to avoid relapses of haematological malignancies. This immunotoxin could be also considered for in vivo treatments.

Materials and methods

Materials. Xanthine oxidase (grade III) from buttermilk, catalase from bovine liver, lactoperoxidase from bovine milk, superoxide dismutase from human erythrocytes, bovine serum albumin (BSA), 2-mercaptoethanol and bovine haemin were purchased from Sigma Chemical Co., St. Louis, Mo., USA. Fluorescein-isothiocyanate(FITC)-conjugated goat anti-(mouse-IgG), the monoclonal antibodies anti-CD19, anti-CD11c and anti-CD2, and isotype-matched mouse immunoglobulins were purchased from Becton-Dickinson, Mountain View, Calif., USA. All other chemicals were from the same sources as in previous work [2, 8, 23].

Purification and labelling of the antibody. The UCHT1 (anti-CD3) mouse IgG1 monoclonal antibody [16] was a kind gift from Professor P.C.L. Beverley of the Imperial Cancer Research Fund laboratories (London, UK) and was purified from ascitic fluid by protein-A-affinity chromatography with a MAPS II kit (BioRad Chemical Division, Richmond, Calif., USA), following the manufacturers' directions [24]. The antibody was labelled with $Na^{125}I$ (Amersham International, Bucks, UK) by the method of Fraker and Speck [9], using the Iodogen reagent (Pierce UK Ltd., Chester, UK). A specific activity of 22 cpm/pmol protein was obtained.

Preparation and assay of xanthine-oxidase-antibody conjugates. Trace-labelled anti-CD3 monoclonal antibody was linked to xanthine oxidase by *N*-succinimidyl-3-(2-pyridyldithio)propionate reagent (Pharmacia S.p.a., Cologno Monzese, Milan, Italy) and the conjugate was purified from unbound antibody and enzyme by gel filtration as described previously [2, 8, 23]. The antibody and the xanthine oxidase in the conjugate were determined from the radioactivity and from the enzymic activity respectively. The conjugate was divided into aliquots, which were stored in liquid nitrogen and were thawed and assayed for xanthine oxidase activity prior to each experiment.

The binding of the conjugate to target cells was tested by means of indirect immunofluorescence using a FITC-conjugated goat anti-(mouse IgG).

Xanthine oxidase activity was determined from the uric acid formation measured by the change of A_{292} as described [20]. A unit (U) of xanthine oxidase activity is defined as the formation of 1 μmol uric acid/min at 28° C.

Cytotoxicity test. The toxicity to target cells was evaluated by the inhibition of incorporation of [^{3}H]thymidine (5 Ci/mmol, Amersham International, Amersham, Bucks, UK) by lymphocytes stimulated with phytohaemagglutinin (PHA, Wellcome, Beckenham, UK). Peripheral blood lymphocytes were obtained from heparinized venous blood of healty volunteers by gradient separation for 30 min at 400 *g* on Ficoll/Hypaque (Lymphoprep, density 1077 g/ml, Nyegaard, Oslo, Norway). Aliquots of 10^5 cells were distributed in each well of 96-well microtiter flat-bottomed plates in RPMI-1640 medium containing 10% fetal calf serum (FCS, Sera Lab., Sussex, UK).

In the first set of experiments (procedure 1) seven dilutions (0.1–8 mU/ml) of free or anti-CD3-conjugated xanthine oxidase were added. The cells were incubated for 2 h at 37° C in a humidified atmosphere containing 5% CO_2 and 95% air in the presence of 100 μM hypoxanthine, or as otherwise specified. Lymphocytes were then stimulated by the addition of PHA to a final concentration of 10 μg/ml. After 48 h incubation, 2 μCi [^{3}H]thymidine was added to each well. The cells were incubated for 24 h then harvested on paper strips and washed using Skatron equipment and the incorporated radioactivity was measured by a liquid-scintillation counter.

When indicated, an alternative procedure (procedure 2) was followed. Lymphocytes were incubated in sterile tubes with free or conjugated xanthine oxidase (0.5–60 mU/ml) in RPMI-1640 complete medium at 0° C for 30 min to ensure conjugate binding. Cells were washed with the same medium and centrifuged to eliminate unbound conjugate and were distributed in triplicate wells as described above. Lymphocytes were incubated at 37° C for 2 h in the presence of 100 μM hypoxanthine, 25 μM $FeSO_4$ and 25 μM EDTA, or as otherwise specified, and processed as described above.

Immunophenotypic evaluation of peripheral blood mononuclear cells. A panel of monoclonal antibodies including anti-CD19 (pan-B cells), anti-CD11c (monocytes) and anti-CD2 (pan-T cells) was used to evaluate the phenotype of peripheral blood mononuclear cells. Cells were incubated at 0° C for 30 min with the monoclonal antibodies either before or after the cytotoxicity test performed following procedure 2. Isotype-matched mouse Ig served as the control for immunophenotypic evaluation. The cells were washed twice and incubated at 0° C for 30 min with a (*R*)-phycoerythrin-conjugated goat F(ab′)$_2$ fragment of an anti-(mouse IgG) (TAGO, Burlingham, Ala., USA). The percentage of positive cells was determined after three washes by using a fluorescence microscope. At least 300 cells for each sample were scored by two independent investigators.

Effect on bone marrow progenitor cells. Bone marrow cells, aspirated from a healthy donor, were diluted in Iscove modified Dulbecco's medium (IMDM) supplemented with 10% FCS and mononuclear cells were collected after Ficoll/Hypaque sedimentation. After two washes with IMDM, bone marrow cells (5×10^6) were incubated for 30 min at 37° C, prior to a 30-min incubation at 37° C in the presence of 0.001% H_2O_2 in order to exhaust peroxidases activity. The cells were then kept at 0° C for 30 min with free or conjugated xanthine oxidase (50 mU/ml), washed to remove unbound enzyme and then incubated at 37° C for 2 h in the presence of 100 μM hypoxanthine, 25 μM $FeSO_4$ and 25 μM EDTA. Bone marrow cells were then washed and the recovery of CFU-GM and burst-forming units (erythroid) (BFU-E) was evaluated by the clonogenic assay as previously described [14]. Briefly, the cells were resuspended in IMDM supplemented with 24% FCS, 0.8% BSA, 0.1 mM 2-mercaptoethanol, 1 unit partially purified erythropoietin (Toyobo Inc. New York, N.Y., USA), 0.2 mM bovine haemin, 1.32% methylcellulose and 10% leucocyte-conditioned medium obtained from 7-day culture of human peripheral blood mononuclear cells stimulated with 10 μg/ml PHA. Triplicate cultures were incubated at 37° C in a fully humidified atmosphere with 5% CO_2 and colonies were scored after 14 days of incubation.

Statistical analysis. Results are given as means ±SE. Student's *t*-test was used for statistical comparisons. For $P < 0.05$ the differences were considered statistically significant. The xanthine oxidase concentration giving 50% inhibition of [^{3}H]thymidine incorporation (IC_{50}) was calculated by linear regression analysis.

Results

2-Pyridyl disulphide groups were introduced into the monoclonal antibody molecule at a mean ratio of 1.6 residues/anti-CD3 molecule. The anti-CD3–xanthine-oxidase conjugate contained 0.16 U enzyme activity/nmol monoclonal antibody and retained the capacity for binding to T lymphocytes and the catalytic activity of its immunological and enzymic moieties respectively.

The toxicity to PHA-stimulated peripheral lymphocytes of the immunotoxin was significantly greater than that of free xanthine oxidase and was proportional to the concentration of the enzyme (procedure 1, Fig. 1 a).

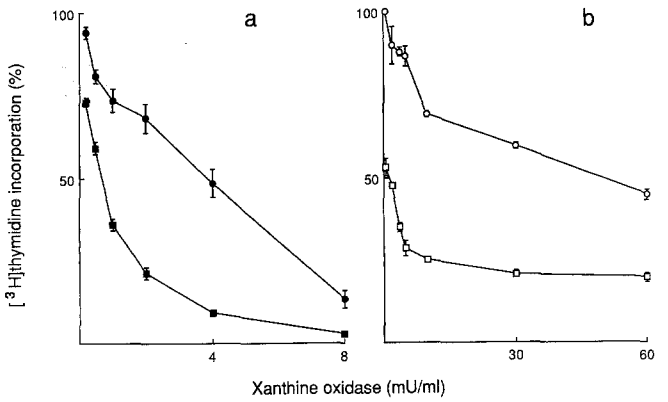

Fig. 1 a, b. Inhibition of [^{3}H]thymidine incorporation by lymphocytes. **a** Lymphocytes were incubated in the presence of free (●) or anti-CD3-conjugated (■) xanthine oxidase, and of hypoxanthine as described in Materials and methods. Results are means ± SE of triplicate values of three experiments. [^{3}H]Thymidine incorporation of control lymphocytes, incubated in the absence of the enzyme, was 112849±2583 dpm. **b** Lymphocytes were treated with free (○) or conjugated (□) xanthine oxidase and the enzyme not bound to cells was washed away prior to the incubation in the presence of hypoxanthine and chelated iron as described in Materials and methods. Results are means ± SE of triplicate values of three experiments. [^{3}H]Thymidine incorporation of control lymphocytes, incubated in the absence of the enzyme, was 61163±4105 dpm

Table 1. Toxicity to lymphocytes of free and anti-CD3-conjugated xanthine oxidase

Procedure	IC^a_{50} of xanthine oxidase (mU/ml)	
	Free	Conjugated
Procedure 1[b]		
Complete system	4.9	0.6
Minus hypoxanthine	>8.0	1.7
Allopurinol added	>8.0	1.0
Free anti-CD3 added	6.8	2.1
$FeSO_4$ and EDTA added	1.0	0.3
Procedure 2[c]		
Complete system	49.0	1.7
Minus $FeSO_4$ and EDTA	>50	6.0
Allopurinol added	>50	3.3
Free anti-CD3 added	>50	49.0
Superoxide dismutase and catalase added	47.9	2.8
Lactoperoxidase and KI added	>50	3.0

[a] Enzyme concentration inhibiting by 50% the [^{3}H]thymidine incorporation

[b] Lymphocytes were incubated with 0.2–8 mU/ml free or conjugated xanthine oxidase in the presence of hypoxanthine, unless otherwise indicated, and of 1 mM allopurinol, 0.1 μM free anti-CD3, 25 μM $FeSO_4$ and 25 μM EDTA where indicated. [^{3}H]Thymidine incorporation of control lymphocytes, incubated in the absence of the enzyme, was 101213±1263 dpm

[c] Lymphocytes were treated with 0.5–60 mU/ml free or conjugated xanthine oxidase and the enzyme not bound to cells was washed away prior of the incubation in the presence of hypoxanthine, of chelated iron (unless otherwise indicated), and of 1 mM allopurinol, 0.1 μM free anti-CD3, 1 U/ml both superoxide dismutase and catalase, 4 U/ml lactoperoxidase and 0.1 mM KI where indicated. [^{3}H]Thymidine incorporation of control lymphocytes, incubated in the absence of the enzyme, was 70860±1601 dpm

Table 2. Immunophenotypic analysis of peripheral blood mononuclear cells treated with free and anti-CD3-conjugated xanthine oxidase[a]

Treatment	Antigen-bearing cells (%)		
	CD19	CD11c	CD2
Before treatment	7.9	10.6	63.1
Controls	0.6	3.0	81.0
Free xanthine oxidase	0.3	7.0	88.0
Conjugated xanthine oxidase	4.0	10.0	10.0

[a] Peripheral blood mononuclear cells were tested for positivity to CD19, CD11C and CD2 antigens before and after the treatment in the absence (controls) or in the presence of 50 mU/ml either free or conjugated xanthine oxidase. The cells were grown for 3 days, at a concentration of 1×10^6/ml, in RPMI-1640 complete medium supplemented with 10 μg/ml of phytohaemagglutinin. The results are expressed as the percentage of antigen-bearing cells in each sample

Table 3. Effect on normal bone marrow progenitor cells of free and anti-CD3-conjugated xanthine oxidase[a]

Treatment	Colonies/10^5 cells plated	
	CFU-GM	BFU-E
Controls	107	214
Free xanthine oxidase	118	168
Conjugated xanthine oxidase	103	228

[a] Bone marrow cells were incubated in the absence (controls) or in the presence of 50 mU/ml either free or conjugated xanthine oxidase. The results are expressed as the mean of triplicate cultures and represent the number of colonies per 10^5 cells plated. SD was always lower than 10% CFU-GM, granulocyte/macrophage-colony-forming units; BFU-E, burst-forming units (erythroid)

In the absence of substrate hypoxanthine, the cytotoxicity of xanthine oxidase either free or conjugated was lowered (Table 1, procedure 1). An excess of free antibody reduced the toxicity of the immunotoxin. This effect was strongly enhanced when the unbound conjugate was washed away (Table 1, procedure 2). The presence of 1 mM allopurinol reduced by 40%–50% the cytotoxicity of conjugated xanthine oxidase, whereas that of the free enzyme was almost completely abolished. The cytotoxicity of xanthine oxidase either free or anti-CD3-conjugated was potentiated by the addition of chelated iron (Table 1, procedures 1 and 2).

By washing away the unbound xanthine oxidase prior to the addition of substrate and by adding chelated iron during the incubation, the cytotoxicity of the conjugated enzyme was enhanced over that of the free enzyme (procedure 2, Fig. 1 b), with a 30-fold difference in the IC_{50}. Because of the washing, this procedure required a 3-fold higher concentration of xanthine oxidase to have the same effectiveness, but it increased 3.5-fold the selectivity of the T cell killing, as compared to procedure 1 without the washing and without chelated iron. The addition of both superoxide dismutase and catalase or of lactoperoxidase in the presence of KI scarcely affected the toxicity of the hypoxanthine/xanthine oxidase system (Table 1, procedure 2).

The immunophenotypic analysis of peripheral mononuclear blood cells (Table 2) showed that 14% of the cells after the treatment with anti-CD3-conjugated xanthine oxidase were B cells and monocytes. In the same sample, only 10% of the cells were CD2-positive, whereas more than 80% of CD2-positive cells were found either in the control or in the free xanthine-oxidase-treated sample, and 76% of peripheral mononuclear blood cells were not stained after the treatment with anti-CD3-conjugated xanthine oxidase. This figure is very similar to the percentage inhibition of [^{3}H]thymidine incorporation observed in procedure 2 of the cytotoxicity test (Fig. 1 b).

It is noteworthy that the immunotoxin was not toxic to CFU-GM and BFU-E (Table 3).

Discussion

Xanthine-oxidase–anti-CD3 immunotoxin retained both the enzymic and immunological properties essential for the selectivity of cell killing.

The absence of the substrate hypoxanthine induced only a moderate reduction of the toxicity of the immunotoxin, possibly because of the xanthine and hypoxanthine present in the serum-containing incubation medium or produced by the cells.

As observed previously [8], chelated iron enhanced the cytotoxicity of xanthine oxidase, thus confirming that the action of this enzyme is mediated by superoxide radicals and H_2O_2 generated during the oxidation of hypoxanthine.

The competitive inhibitor allopurinol effectively inhibited the cytotoxicity of free xanthine oxidase, whereas it scarcely affected that of the anti-CD3-bound enzyme. This suggests that the immunotoxin after its binding to CD3 antigen is internalized by the cell. Consistently, both the scavenger enzymes, superoxide dismutase and catalase, did not afford protection against the cytotoxicity of conjugated xanthine oxidase. Similarly, an internalizable glucose-oxidase-containing immunotoxin was cytotoxic to target cells even in the presence of serum with elevated H_2O_2 scavenger activity, since H_2O_2 generated intracellularly became inaccessible to extracellular scavengers [17].

The association of a hydrogen-peroxide-producing enzyme with a halide peroxidase system has been used to enhance the cytotoxicity of a glucose oxidase immunotoxin [12]. The rationale of this association consists in the sequential production of H_2O_2 and of I_2 and its halogenated derivatives. The addition of lactoperoxidase and KI to our system did not affect the toxicity to lymphocytes of conjugated xanthine oxidase. This lack of catalytic amplification of cytotoxicity is consistent with the possible internalization of the immunotoxin [17].

The addition of free anti-CD3 monoclonal antibody reduced the toxicity of anti-CD3-conjugated xanthine oxidase, presumably by competing with the immunotoxin for the binding to CD3 antigen on the T lymphocyte membrane. This effect was enhanced when unbound conjugate was washed away prior of the incubation with the substrate.

The immunotoxin effectively killed T lymphocytes even in the absence of chelated iron (Fig. 1 a). When the unbound conjugate was washed away and chelated iron was added, the selectivity and cytotoxicity of the immunotoxin were greatly enhanced, but some [^{3}H]thymidine incorporation was still present at the highest concentrations of xanthine oxidase tested (Fig. 1 b). The escape of T lymphocytes from binding to anti-CD3 conjugate is not likely, since an immunotoxin containing the same monoclonal antibody and the ribosome-inactivating protein saporin 6 was able to eliminate CD3$^+$ cells completely [24]. The phenotypic analysis of peripheral blood mononuclear cells showed that a significant percentage of the cells surviving the treatment with anti-CD3-conjugated xanthine oxidase were B lymphocytes and monocytes. This may account, in part, for the residual [^{3}H]thymidine incorporation (Fig. 1 b). In addition, the latter could be due to DNA repair in damaged T lymphocytes. It must be also taken into account that free radicals at low concentrations are per se mitogens and the synthesis of DNA can be the result of two opposite effects: the toxicity and, at a lower concentration, the cell division stimulation [21].

We reported previously that xanthine-oxidase-containing immunotoxins remove most malignant cells from bone marrow [3]. If the present model could be perfected, the use of xanthine-oxidase–anti-CD3 immunotoxin for the ex vivo purging of CD3$^+$ cells from bone marrow for autologous and allogeneic transplants could be considered, with the immense advantage that this immunotoxin is not toxic to normal bone marrow progenitor cells.

The present results show that xanthine-oxidase-containing immunotoxins, like glucose-oxidase–antibody conjugates [17], are effective in the presence of scavenger enzymes for oxygen radicals. Thus they suggest that xanthine-oxidase–antibody conjugates should also be effective in vivo, provided they are targeted to internalizable antigens.

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