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Oxidative Stress to Human Lymphocytes by Xanthine Oxidoreductase Activity

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The *in vitro* toxicity of the reactive oxygen species generating enzyme xanthine oxidoreductase (XOR) to human peripheral blood lymphocytes was studied after stimulation with phytohaemoagglutinin or anti-CD3/CD28 antibodies. Apoptosis and necrosis were induced by the XOR/hypoxanthine system in a time- and concentration-dependent manner. CD8+ lymphocytes showed a higher sensitivity than CD4+ cells to the XOR/hypoxanthine system. The occurrence of apoptosis was demonstrated by annexin-V binding to injured cell membrane, which was the most precocious alteration observed, followed by the increment of transglutaminase activity, which was significant at the lowest XOR concentration used. Nuclear damage was assessed by the increased hypodiploid nuclei and by DNA migration on gel electrophoresis, which turned to an apoptotic pattern before the occurrence of cell membrane necrotic lesions. Apoptosis was induced by XOR activity proportionally to substrate concentration and was prevented by the competitive enzyme inhibitor, allopurinol. The hydrogen peroxide scavenging enzyme, catalase, gave a higher protection than superoxide dismutase from the toxicity caused by the XOR/hypoxanthine system. Necrosis occurs in a variable percentage indicating that reactive oxygen species may trigger both apoptosis and necrosis in proliferating human lymphocytes, mostly depending on XOR concentration.

Keywords: Apoptosis; Human lymphocytes; Necrosis; Reactive oxygen species; Xanthine oxidoreductase

INTRODUCTION

The xanthine oxidoreductase (XOR) oxidises hypoxanthine to xanthine and the latter to uric acid. The enzyme exists as an NAD-dependent dehydrogenase (EC 1.1.1.204) which can be converted into an oxidase (EC 1.1.3.2.2) either irreversibly, by limited proteolysis,[1] or in a reversible manner by chemical[2] or enzymatic[3] oxidation of thiol groups. The oxygen-dependent

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activity of the enzyme generates superoxide anion and hydrogen peroxide, which may produce hydroxyl radical in the presence of traces of transition metals.[4] These reactive oxygen species (ROS) may have cytotoxic effects,[5] involving DNA damage,[6–8] and are responsible for oxidative stress when XOR activity is increased.

An elevation of the oxidase activity of the enzyme was observed in different pathological conditions as a consequence of (i) the enzyme conversion from dehydrogenase to oxidase,[9] (ii) the increment of total XOR activity,[10] or (iii) the release of the enzyme from injured tissues into plasma[11] where it is completely converted into an oxidase.[12] The consequent increase of ROS production may at least in part contribute to cell injury and aggravate the tissue damage by amplifying the lesion area (reviewed by de Groot and Littauer[13]).

The XOR that leaked out from altered cells into the circulation can bind to sulphated glycosaminoglycans on the cell surface of vascular endothelium[14,15] and cause damage at other sites,[16] e.g. lung injury[17–19] through the production of ROS. These oxidants may also extend tissue damage by participating in the recruitment of inflammatory cells (reviewed by Jaeschke[20]). XOR-derived ROS may, indeed, affect blood cells by activating a neutrophil chemotactic factor,[21] increasing the adhesion of leukocytes[22] and the cytokine production by monocytes,[23] and inducing the accumulation of leukocytes in liver microvasculature after hepatic/splancnic ischaemia/reperfusion.[24]

Oxidative stress has been shown to induce apoptosis of endothelial cells *in vitro*[25] and it has been suggested that an excess of circulating XOR could damage endothelia.[26] A high level of substrate, which is available when there is a high catabolism of nucleic acids such as in leukaemia and in hypoxia, may favour the occurrence of oxidative stress by XOR. The *in vitro* toxicity of this enzyme to human peripheral blood lymphocytes has been reported,[27,29] and DNA single-strand breaks caused to these cells through the oxidative stress produced by the activity of this enzyme were observed.[8,28,30] Moreover, the XOR/hypoxanthine system may induce apoptosis in blood mononuclear cells presumably by generating ROS.[31]

Present work was performed to define the mechanism of XOR cytotoxicity to human peripheral blood lymphocytes and the conditions, which are required to induce apoptosis and/or necrosis. Many variables should be taken into account that may influence the usefulness of one test or another to classify cell death as apoptosis or necrosis, including etiological agent, cell type, conditions of the action, and time of parameter determination. However, none of the alterations reported as typical during apoptosis (reviewed by Majno and Joris[32]) can definitely be considered as the hallmark of this phenomenon.

The toxicity to lymphocytes induced by the XOR/hypoxanthine system-generated ROS was evaluated using different enzyme and substrate concentrations and exposure times, in order to correlate these parameters with the progressive occurrence of cellular alterations that are characteristic of apoptosis or necrosis. The involvement of the enzyme activity and reaction products in such alterations was also studied with the use of the competitive inhibitor allopurinol and utilising the ROS scavenger enzymes superoxide dismutase and catalase.

Freshly isolated human lymphocytes are known to be more resistant to apoptosis than proliferating cells, suggesting that activation of resting cells may increase their sensitivity to apoptotic agents.[33] Resting CD4+ and CD8+ lymphocytes express a different concentration of surface thiols, which is increased in both subset after mitogenic activation in correlation with their differential sensitivity to oxidative stress.[34] Since the mitogenic response of CD4+ lymphocytes to activation by anti-CD3/CD28 antibodies

was shown to be higher than that of CD8+ cells,[35] the differential sensitivity of the two major T lymphocyte subsets to oxidative stress induced by the XOR/hypoxanthine system was investigated.

To better understand the mechanism of lymphocytes killing, we selected various tests to visualise apoptotic changes to cell membrane, cytoplasm and nucleus, which may appear at different times. In particular, we performed: (i) the determination of tissue transglutaminase activity (tTG, EC 2.3.2.13), which catalyses apoptosis-associated protein modifications in the cytoplasm;[36] (ii) the gel electrophoresis of DNA, to distinguish between apoptotic fragmentation and necrotic digestion; (iii) the cytofluorimetric evaluation of apoptotic hypodiploid nuclei by propidium iodide (PI) staining in lysed cells; (iv) the analysis of cell membrane damage by annexin-V binding to apoptotic cells with externalisation of phosphatidylserine[37] and by PI staining of nuclei in non-permeabilised necrotic cells; (v) the microscopic analysis of morphologic alterations.

MATERIALS AND METHODS

Determination of Xanthine Oxidoreductase Activity

An appropriate amount of XOR suspension (from buttermilk, Serva Electrophoresis Gmbh, Frankfurt, Germany), which is in the O_2-dependent form, was centrifuged for 10 min at 16,000*g* at 4°C. The supernatant was discarded, the pellet was dissolved in an equal volume of cold H_2O, diluted with complete medium and sterilised by filtration.

XOR activity was determined spectrophotometrically by measuring at 292 nm the formation of uric acid from xanthine as described.[1] A unit of enzyme activity is defined as the formation of 1 μmol uric acid per min at 37°C.

Lymphocytes Separation and Treatment

Human mononuclear cells were isolated from buffy coat preparations of peripheral blood from healthy donors by differential sedimentation at 380*g* for 30 min at room temperature on Ficoll Hypaque solution (Pharmacia LKB Biotech., Uppsala, Sweden). Cells were incubated for 3 h in RPMI 1640 medium (Gibco Brl Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS (Gibco) and 1% antibiotics (penicillin/streptomycin, Bio-Wittaker Europe, Verviers, Belgium), to allow adhesion of monocytes. Non-adhering cells were cultured overnight in complete medium before seeding for the time-course experiment with 30 mU/ml XOR. In all other cases, lymphocytes were seeded immediately after separation in 6-well plates (1×10^7 cells/well) in 5 ml complete medium containing 10 μg/ml phytohaemoagglutinin (PHA, DIFCO Lab., Detroit, MI, USA), 100 μM hypoxanthine, or as otherwise specified, and the indicated amount of XOR.

The ROS scavenger enzymes Cu–Zn superoxide dismutase (1 mg/ml, from human erythrocytes, Sigma-Aldrich S.r.l., Milano, Italy) and catalase (1 mg/ml, from bovine liver, Sigma-Aldrich), and the XOR inhibitor allopurinol (1 mM, Sigma-Aldrich) were added in the indicated experiments. The cells were cultured for the indicated time, were then harvested, pelleted by centrifugation for 5 min at 180*g* and washed with 5 mM phosphate buffer, pH 7.5, containing 0.14 M NaCl. After centrifugation as above the cellular pellet was utilised for the following determinations.

The same procedure was followed for γ-irradiated (2000 rad) lymphocytes.

The sensitivity of CD4+ or CD8+ lymphocytes to oxidative stress was tested on cells stimulated with CD3/CD28 monoclonal antibodies (Coulter-Immunotech, Hyaleah, FL, USA) in 24-well trays coated with 1 μg/ml monoclonal antibodies in RPMI 1640 for 24 h at 37°C. Non-adhering mononuclear cells

(2×10^6 cells) were seeded in 1 ml complete medium containing 100 μM hypoxanthine and the indicated amount of XOR for 24 or 48 h.

Determination of Transglutaminase Activity

The cellular pellet was resuspended in 5 mM Tris-HCl buffer pH 8.1 containing 1 mM EDTA and freezed overnight. The samples were thawed and centrifuged for 10 min at 16,000*g* at 4°C and the supernatant was centrifuged for 40 min at 105,000*g* at 4°C. The supernatant from the last centrifugation was dialysed against the same Tris buffer and utilised for tTG activity determination.

Triplicate samples were incubated for 30 min at 37°C in a mixture containing 50 mM Tris-HCl buffer pH 8.1, 3 mM $CaCl_2$, 0.2 mM [1,4(n)-^{3}H] putrescine (6×10^{11} Bq/mol, Amersham Pharmacia Biotech Italia, Milano, Italy) and 0.1 mg N,N-dimethylcasein (Sigma-Aldrich). The reaction was arrested by precipitation with an equal volume of 10% (w/v) trichloroacetic acid containing 2 mM putrescine. The precipitate formed after 24 h at 4°C was collected by centrifugation for 10 min at 16,000*g* at 4°C and was dissolved with 0.1 M NaOH for 30 min at 60°C. The acid precipitation followed by alkaline solubilisation was repeated twice to eliminate unbound putrescine, and the protein-incorporated radioactivity was measured in a β-counter.

A unit of tTG activity is defined as the incorporation of 1 μmol putrescine per min at 37°C. Enzyme activity was referred to protein concentration determined by spectrophotometric absorbance as described.[38]

Flow Cytometry Analysis

Nuclear DNA content was determined by PI staining of permeabilised lymphocytes with the DNA Staining Kit (Coulter-Immunotech), following manufacturer's instructions. PI content was analysed by an EPICS XL flow cytometer (Coulter), with dedicated software to avoid doublets in the G2M area. Apoptotic cells were defined as elements with DNA content <G0/G1 (sub G1).

Apoptotic and necrotic membrane alterations were determined after staining the lymphocytes by annexin-V FITC and PI with the Oncogene Research Products kit (Cambridge, MA, USA) following manufacturer's instructions. The percentage of apoptotic (annexin-V positive/PI negative) and necrotic (both annexin-V and PI positive) cells were calculated from double colour analysis for annexin-V and PI by FACS.

Apoptosis in CD4+ and CD8+ cells was detected by staining the lymphocytes with annexin-V FITC (Oncogene Research Products) and anti-CD4 or anti-CD8 R-phycoerythrin with the Coulter-Immunotech kit following manufacturer's instructions.

Microscopic Analysis

Cells were stained by annexin-V FITC and PI for apoptosis and necrosis detection, respectively, as indicated above and examined by fluorescence microscopy after 1:1 dilution with a 1,4-diazabicyclo[2.2.2] octane solution to delay the fluorescence fading. This solution was prepared by dissolving at 70°C 2 mg of the anti-fading compound in 9 ml glycerol, and was buffered by the addition of 0.2 M Tris-HCl, pH 7.5.

Lymphocytes were cytocentrifuged at 100*g* on a slide, fixed and stained using the May–Grunwald Giemsa technique and examined by light microscopy for apoptotic alterations.

DNA Fragmentation Assessment by Gel Electrophoresis

DNA was extracted from lymphocytes as described.[39] Briefly, the cellular pellet was suspended in Hanks' balanced salt solution and

fixed with 70% ethanol at −20°C for 48–72 h. DNA fragments were selectively extracted from cells by phosphate–citrate buffer for 30 min at room temperature, then treated with RNase A followed by proteinase K. Freshly prepared DNA extracted from each sample well and 1 μg DNA markers (M-Medical Genenco, Firenze, Italy) in phosphate–citrate buffer were diluted with loading buffer following the manufacturer's instructions. Samples and markers were run at 70–85 V for 1.5–2 h on 1.5% agarose gel in 89 mM Tris-borate buffer pH 8.0 containing 2 mM EDTA and 0.5 μg/ml ethidium bromide in a 7×10 cm electrophoresis cell.

Statistical Analysis

The results were evaluated by ANOVA test. Statistically significant differences were compared by Dunnett's and Tukey's tests.[40]

RESULTS

Concentration–response Experiment

The occurrence of apoptosis in proliferating human lymphocytes after 72 h of culture in the presence of a XOR/hypoxanthine system was indicated by the concentration-dependent increase of the tTG activity and of the number of hypodiploid cells, which were significant ($P < 0.05$) by Dunnett's test starting from 10 ($2.29 < t > 4.54$) to 100 ($3.10 < t > 3.23$) mU/ml XOR, respectively (Fig. 1). The annexin-V binding test (Fig. 1, inset) confirmed that the amount of apoptosis increased in an XOR concentration-dependent manner, until a plateau of 54.3% apoptotic cells was obtained with 100 mU/ml enzyme. Some necrosis (17.0%) was also detected at 100 mU/ml XOR, which increased to 33.1% at the higher concentration of the enzyme. A cytofluorimetric profile of DNA

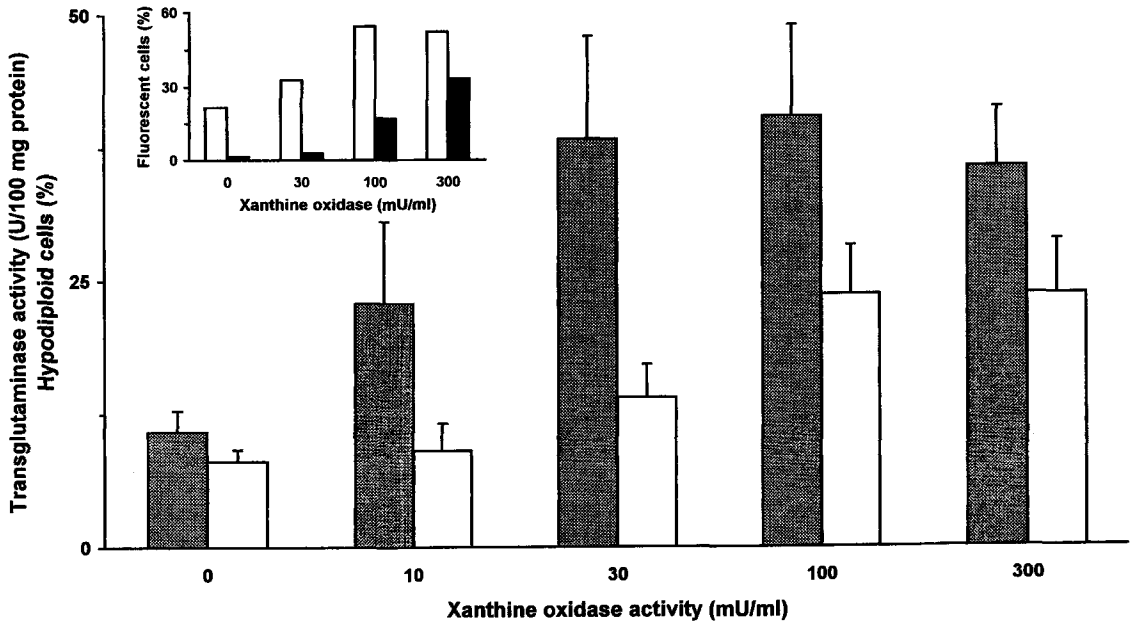

FIGURE 1 Effect to proliferating human lymphocytes of increasing XOR concentration on transglutaminase activity (grey columns) and percentage of hypodiploid cells (white columns) after 72 h in the presence of 100 μM hypoxanthine. Results are means ± S.E. of seven experiments. The insert shows the percentage of annexin-V (white columns) or both annexin-V and propidium iodide (black columns) positive cells from a single experiment in the same experimental conditions. Analysis of variance showed a significant difference between XOR concentrations on transglutaminase activity ($F = 6.97$; d.f. 4/95; $P = 0.0001$) and percentage of hypodiploid cells ($F = 4.76$; d.f. 4/29; $P = 0.0045$).

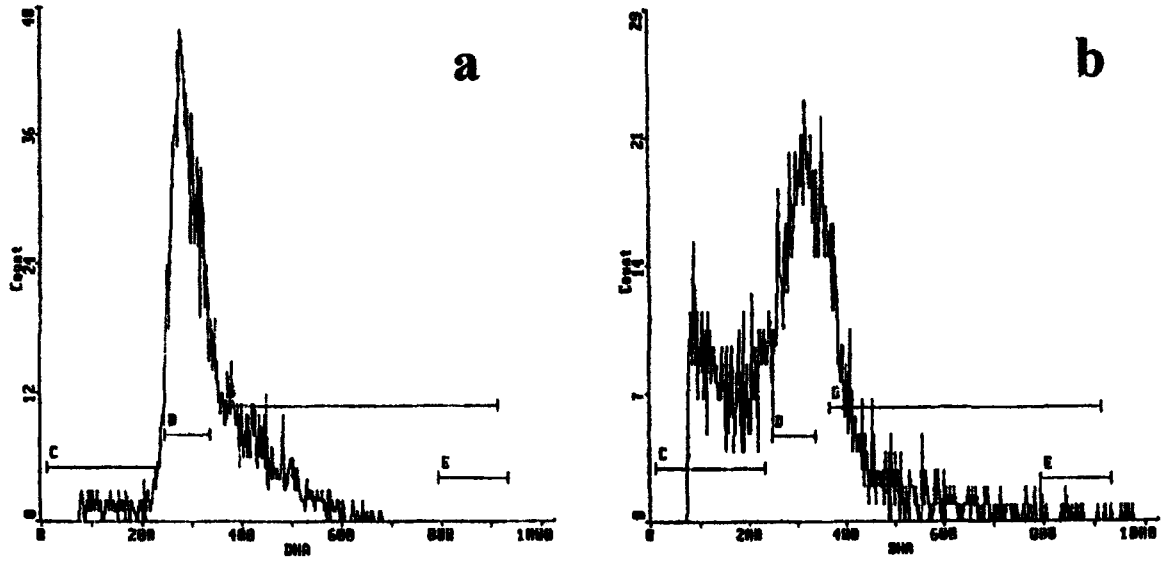

FIGURE 2 Cytofluorimetric determination of DNA content in proliferating human lymphocytes nuclei by propidium iodide staining after 72 h exposure to 100 μM hypoxanthine (a) in the absence (control cells) or (b) in the presence of 100 mU/ml XOR.

content in control and XOR/hypoxanthine-treated lymphocytes is shown in Fig. 2a,b.

Increasing concentration of hypoxanthine caused a proportional increment of tTG activity and percentage of hypodiploid lymphocytes, after 48 h-exposure to 100 mU/ml XOR (Fig. 3). An experiment with different concentrations of XOR showed that the inhibitor allopurinol completely prevented the increment of tTG activity and percentage of hypodiploid cells induced by XOR/hypoxanthine system up to 30 mU/ml enzyme after 48 h of culture (Fig. 4).

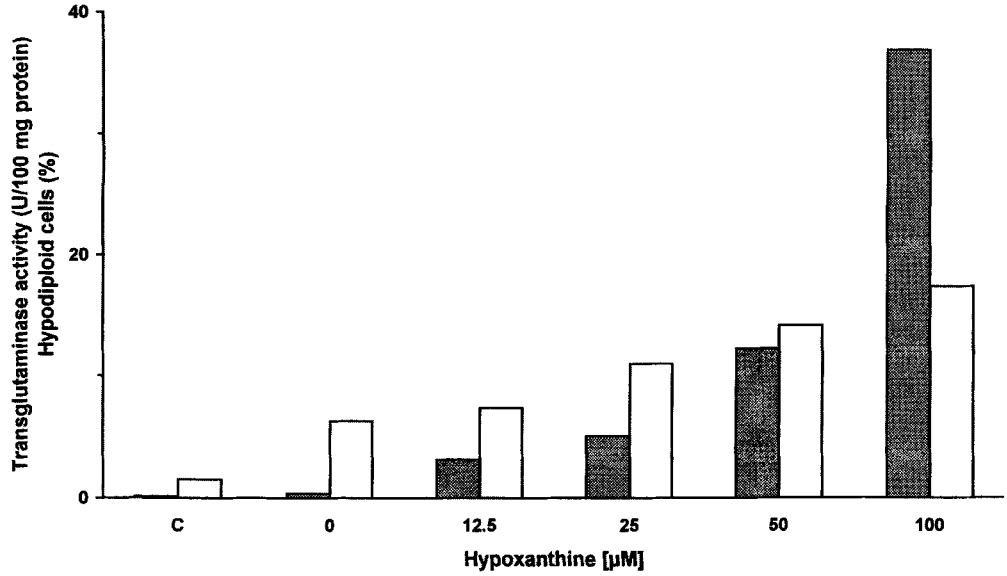

FIGURE 3 Effect to proliferating human lymphocytes of increasing hypoxanthine concentration on transglutaminase activity (grey columns) and percentage of hypodiploid cells (white columns) after 48 h in the presence of 100 mU/ml XOR. Control cells (C) were cultured in the presence of 100 μM hypoxanthine without XOR. The results of a single experiment in triplicate samples are shown.

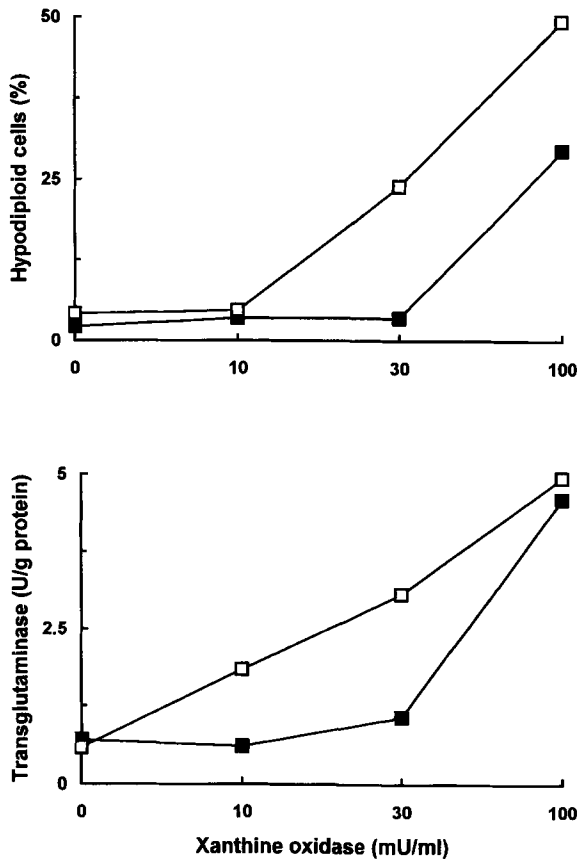

FIGURE 4 Effect of increasing XOR concentrations on the percentage of hypodiploid cells and transglutaminase activity in proliferating human lymphocytes after 48 h in the presence of 100 μM hypoxanthine with (■) or without (□) 1 mM allopurinol. The results of a single experiment are shown.

Time-course Study

A time-course study of the effect of the XOR/hypoxanthine system on lymphocytes was performed. An experiment in the presence of a relatively low concentration of XOR (30 mU/ml) showed a small increase of tTG activity in treated lymphocytes, irrespective of the exposure time, and a three-times increment of annexin-V positive cells starting from 24 h treatment, as compared to control cells (Fig. 5). Necrotic cells were 34.6% and 51.0% after 48 h and 72 h, respectively (Fig. 5, inset). In the same experimental conditions, no increase was observed in the number of hypodiploid nuclei in XOR-treated lymphocytes, as compared to not-treated cells at the same culture time (results not shown). Morphologic alterations characteristic of apoptosis were visible in the same conditions after 24 h (Fig. 6). The annexin-V binding was observed even earlier (after 18 h) by fluorescence microscopy (Fig. 7a,b). A cytofluorimetric dot plot of annexin-V and PI staining in control and XOR/hypoxanthine-treated lymphocytes is shown in Fig. 7c,d.

The time-course study of the effect of the XOR/hypoxanthine system on lymphocytes was repeated with a higher dose of XOR (100 mU/ml). A progressive formation of hypodiploid cells and a parallel increase in the tTG activity were observed. The latter was significantly different from control cells by Dunnett's test ($P < 0.05$) from 24 h ($2.35 < t > 4.83$) onwards (Fig. 8). Apoptosis was already present in 63.9% and necrosis in 20.7% of the cells after 24 h treatment, as assessed by annexin-V and PI staining (Fig. 8, inset).

Gel-electrophoretic Migration of DNA

The gel-electrophoretic migration of DNA fragments extracted from human proliferating lymphocytes is shown in Fig. 9. In a concentration–response experiment, cells treated for 48 h with the XOR/hypoxanthine system showed the formation of a ladder and an accumulation of DNA fragments in the highest base-pair range, which was less marked at the highest XOR concentration (300 mU). A ladder was at least in part visible also in control cells cultured in the absence of the enzyme with or without the substrate and even in lymphocytes not stimulated with PHA (Fig. 9a). In a time-course experiment (Fig. 9b), a progressive DNA accumulation at a similar base-pair range was observed both in cells exposed to 100 mU XOR and in γ-irradiated cells. Almost, no DNA fragmentation was observed in control cells cultured in the presence of hypoxanthine and

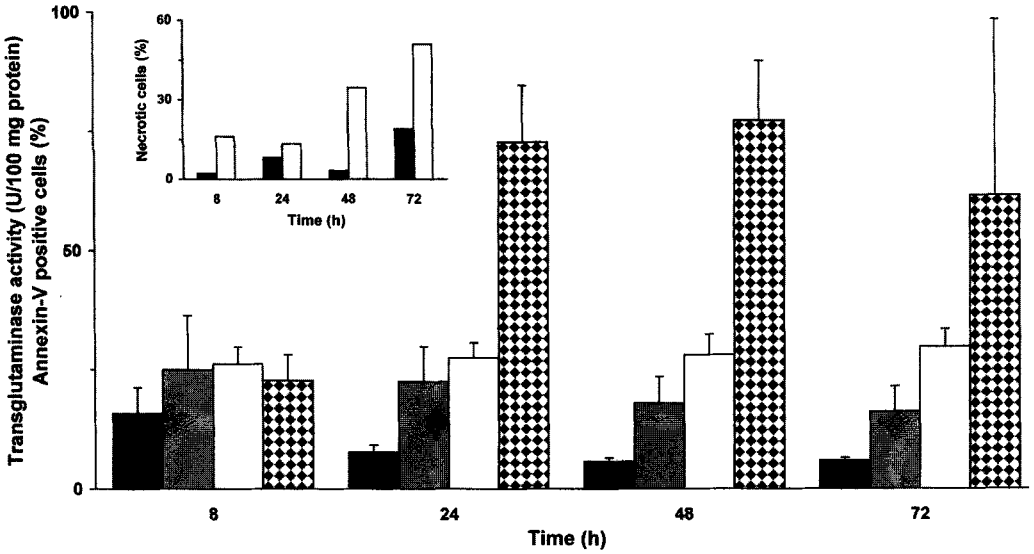

FIGURE 5 Transglutaminase activity (black and grey columns) and percentage of annexin-V positive cells (white and chessboard-like columns) in proliferating human lymphocytes after exposure to 30 mU/ml XOR in the presence of 100 μM hypoxanthine (grey and chessboard-like columns) for increasing times as compared with control cells cultured without XOR (black and white columns). Results are means ± S.E. of three or four experiments. The inset shows the percentage of necrosis in annexin-V and propidium iodide double positive cells in a single experiment with control (black columns) and treated cells (white columns) in the same experimental conditions. Analysis of variance showed a significant difference between control and the XOR/hypoxanthine-treated cells, irrespective of the exposure time, on transglutaminase activity ($F = 30.97$; d.f. 1/62; $P < 0.0001$) and on annexin-V binding ($F = 17.16$; d.f. 1/10; $P = 0.002$).

in the absence of the enzyme, but at the longest time (72 h).

Effects of Scavenger Enzymes

The effects of the scavenger enzymes superoxide dismutase and catalase on the changes induced by the XOR/hypoxanthine system (30 mU/ml XOR for 48 h) on the tTG activity of proliferating human lymphocytes were studied (Fig. 10). The significant increment of tTG activity ($P < 0.05$ by Tukey's test) in XOR-treated cells as compared to that detected in control cells ($t = 7.02$), was reduced when both scavenger enzymes were added to the complete system ($t = 3.98$). This increment was abolished almost completely by allopurinol ($t = 7.79$) and was not observed in the absence of XOR ($t = 10.95$). Although, no significant protection from the effect of the

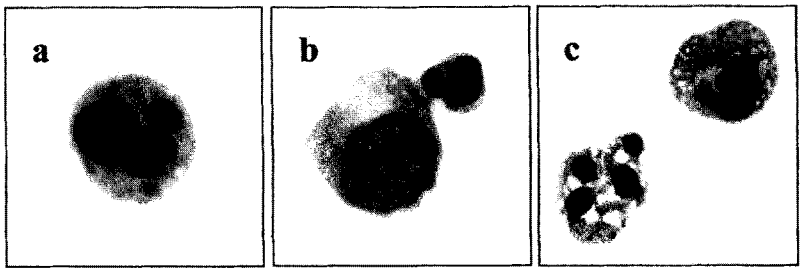

FIGURE 6 Microphotography of proliferating human lymphocytes fixed and stained by May–Grunwald Giemsa after 24 h with 30 mU/ml XOR in the presence of 100 μM hypoxanthine showing: (a) a lymphocyte with apoptotic nuclear alterations, (b) an apoptotic body near a regular lymphoblast and (c) two cells in post-apoptotic necrosis.

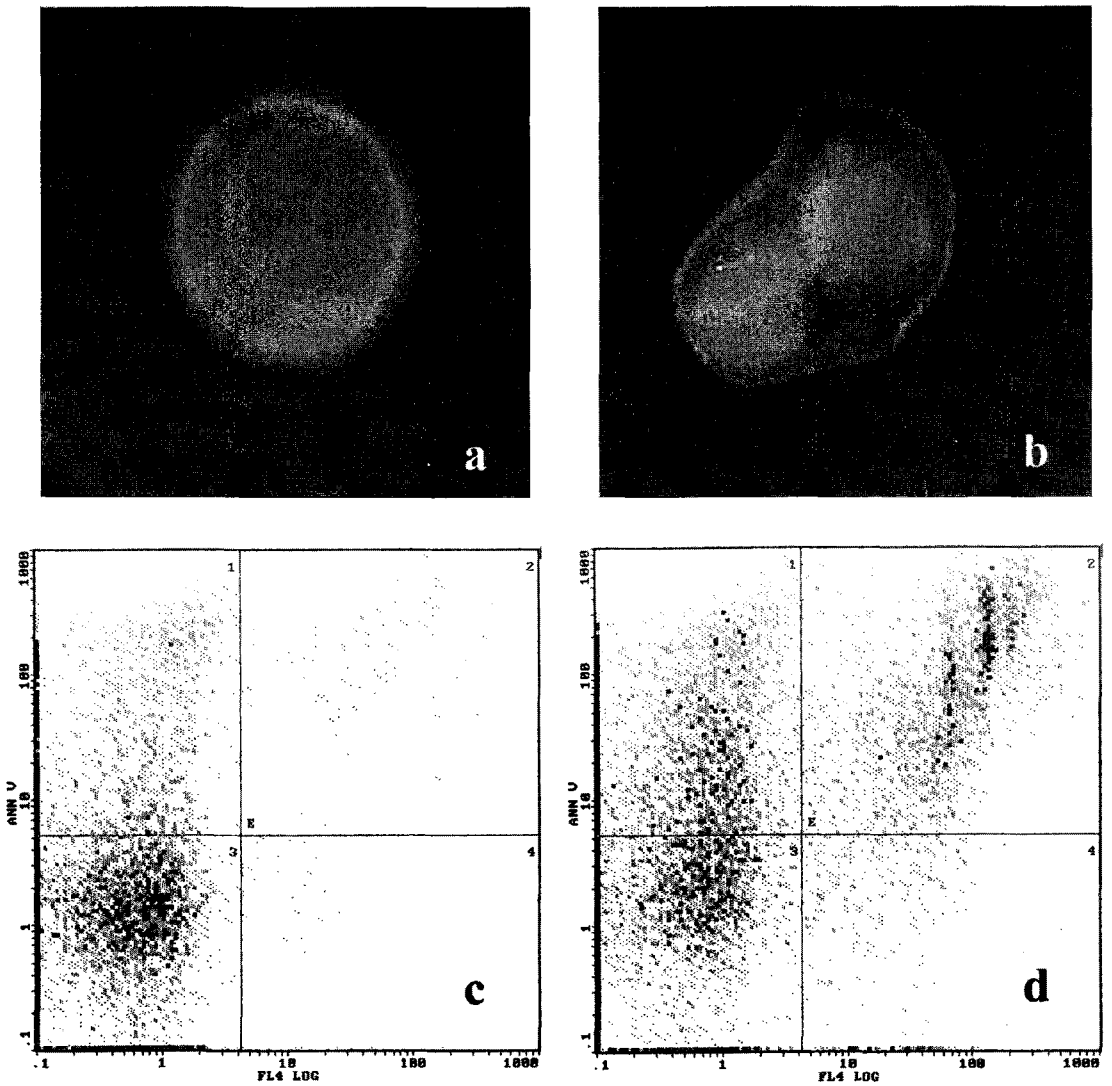

FIGURE 7 Fluorescence microscopy of annexin-V FITC (green) (a) and/or propidium iodide (red) positive cells (b) after 18 h treatment with 30 mU/ml XOR in the presence of 100 μM hypoxanthine. Dot plot of annexin-V FITC/propidium iodide two-parameter cytofluorimetry of proliferating human lymphocytes cultured for 18 h with 100 μM hypoxanthine (c) in the absence (control cells), or (d) in the presence of 30 mU/ml XOR. Lower left quadrant: double negative viable cells; upper left quadrant: annexin-V positive apoptotic cells; upper right quadrant: double positive cells in post-apoptotic necrosis.

XOR/ hypoxanthine system to lymphocytes was given by superoxide dismutase or catalase alone, a partial but significant prevention ($t = 4.50$) of the tTG activity increment was observed in the presence of both scavenger enzymes, as compared to the treatment with superoxide dismutase alone. The low tTG activity level detected in control cells was not significantly different from that observed when hypoxanthine was omitted or allopurinol was added to the complete system.

In one experiment performed in the same conditions, the annexin-V and PI staining test (Fig. 10, inset) confirmed that the apoptosis induced by the complete XOR/hypoxanthine system (75.9% of the cells) did not occur in the

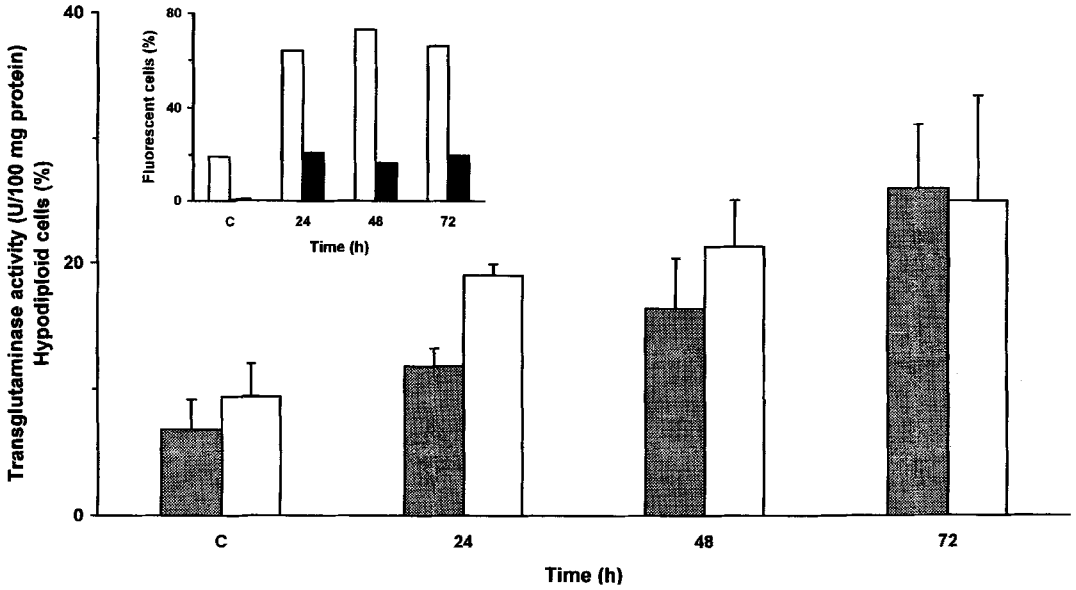

FIGURE 8 Transglutaminase activity (grey columns) and percentage of hypodiploid cells (white columns) in proliferating human lymphocytes after exposure to 100 mU/ml XOR in the presence of 100 μM hypoxanthine for increasing times as compared with control cells (C) cultured for 72 h without XOR. Results are means ± S.E. of three or four experiments. The inset shows the percentage of annexin-V (white columns) or both annexin-V and propidium iodide (black columns) positive cells from a single experiment in the same experimental conditions. Analysis of variance showed a significant difference between control and XOR/hypoxanthine-treated cells after various time on transglutaminase activity ($F = 7.93$; d.f. 3/30; $P = 0.0005$).

absence of XOR (15.5%) or hypoxanthine (15.6%), was prevented by allopurinol (13.4%) and was considerably reduced in the presence of catalase alone (33.8%) or both scavenger enzymes (34.7%). The percentage of necrotic lymphocytes observed after treatment with complete system (15.2%) was increased by the addition of superoxide dismutase to the cultures (25.6%), although this enzyme partially prevented the binding of annexin-V (45.5%).

Sensitivity of CD4+ and CD8+ Cells to Oxidative Stress

CD3/CD28-stimulated lymphocytes showed (Fig. 11) a marked difference in sensitivity to the XOR/hypoxanthine system between CD4+ and CD8+ cells. The annexin-V binding was observed in 21% of the latter already after 24 h treatment, either with 30 or 100 mU/ml enzyme and reached the value of 27% after 48 h with the higher XOR concentration (Fig. 11, inset). No apoptosis was observed in CD4+ lymphocytes cultured in the same conditions.

DISCUSSION

The *in vitro* toxicity of ROS produced by the XOR/hypoxanthine system to proliferating human peripheral blood lymphocytes was studied. All the tests performed to ascertain the mechanism of lymphocytes killing showed both apoptosis and necrosis with a variable percentage depending on the experimental conditions.

The binding of annexin-V to the membrane of apoptotic cells was early detectable by fluorescence microscopy, even at a relatively low XOR concentration, and increased in the first 24 h of treatment, afterwards reached a plateau. The necrotic damage of cell membrane was measurable by cytofluorimetry only with a higher enzyme concentration, or at a longer exposure time. These results are in agreement with

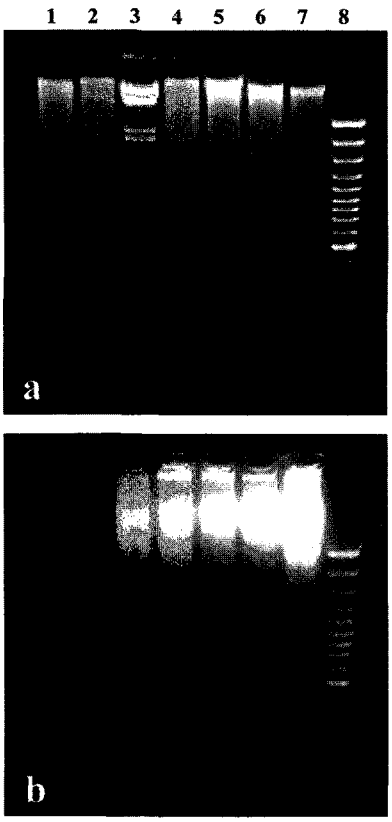

FIGURE 9 DNA fragmentation by gel electrophoresis. (a) Proliferating human lymphocytes were exposed to 100 μM hypoxanthine for 48 h in the presence of 30 (lane 5), 100 (lane 6) or 300 (lane 7) mU/ml XOR. Non-proliferating (lane 1) or proliferating (lanes 2, 4) control cells were cultured without the enzyme in the absence (lanes 1, 2) or in the presence of the substrate (lane 4). Lambda DNA/*Hin*dIII (23.130–564 base-pairs) and 100 bp DNA Ladder Plus markers were run in lanes 3 and 8, respectively. (b) Proliferating human lymphocytes were exposed to 100 μM hypoxanthine in the absence of XOR for 24 (lane 1), 48 (lane 2) or 72 (lane 3) h or in the presence of 100 mU/ml XOR for 24 (lane 4), 48 (lane 5) or 72 (lane 6) h. DNA extracted from γ-irradiated cells and 100 bp DNA Ladder Plus marker were run in lanes 7 and 8, respectively.

previous observations that cell death may take the form of either apoptosis or necrosis depending on the intensity of a variety of etiological agents, apoptosis occurring at low and necrosis at high level of these agents.[41] It has been also described that increasing prooxidant levels may either stimulate growth, trigger apoptosis, or produce necrosis of cells depending on the amount of oxidative stress and duration of exposure.[42]

The pattern of appearance of apoptosis and necrosis and the relative percentages indicate that the XOR/hypoxanthine system precociously induced apoptotic alterations to the cell membrane. It also suggests that, at least in part, necrosis of lymphocytes was a post-apoptotic event, in particular at the highest exposure times and enzyme concentrations. Indeed, a double staining test with annexin-V and PI was able to detect secondary necrosis in lymphatic cells after spontaneous apoptosis induced by a three-day culture.[43]

The apoptosis of proliferating human lymphocytes was dependent on the activity of XOR as shown by the increase of tTG activity and percentage of hypodiploid cells that was proportional to the concentration of XOR and hypoxanthine and to the exposure time. This result was confirmed by the protection exerted by the XOR competitive inhibitor allopurinol, which fully prevented the increment of tTG activity and percentage of hypodiploid cells induced by the XOR/hypoxanthine system up to 30 mU/ml enzyme.

Apoptosis-associated protein modifications in the cytoplasm were investigated by determining the tTG activity. This test showed a high sensitivity, since even the lowest XOR concentration induced a statistically significant increment of tTG activity, which reached a plateau at 30 mU/ml XOR. The protein cross-linking activity of tTG may increase both by inducing the expression of enzyme molecule or by switching the constitutive protein from GPTase activity (reviewed by Chen and Mehta[44]). Taking into account the low level of tTG protein normally detectable by immunostaining in lymphocytes,[45] the increment of its activity after progressive exposure to the XOR/hypoxanthine system suggests an increased tTG expression. However, both mechanisms may be involved in this cell type, because the availability of free Ca^{2+} in stimulated lymphocytes is compatible with tTG activation.[45] The small and constant increment of tTG activity in the

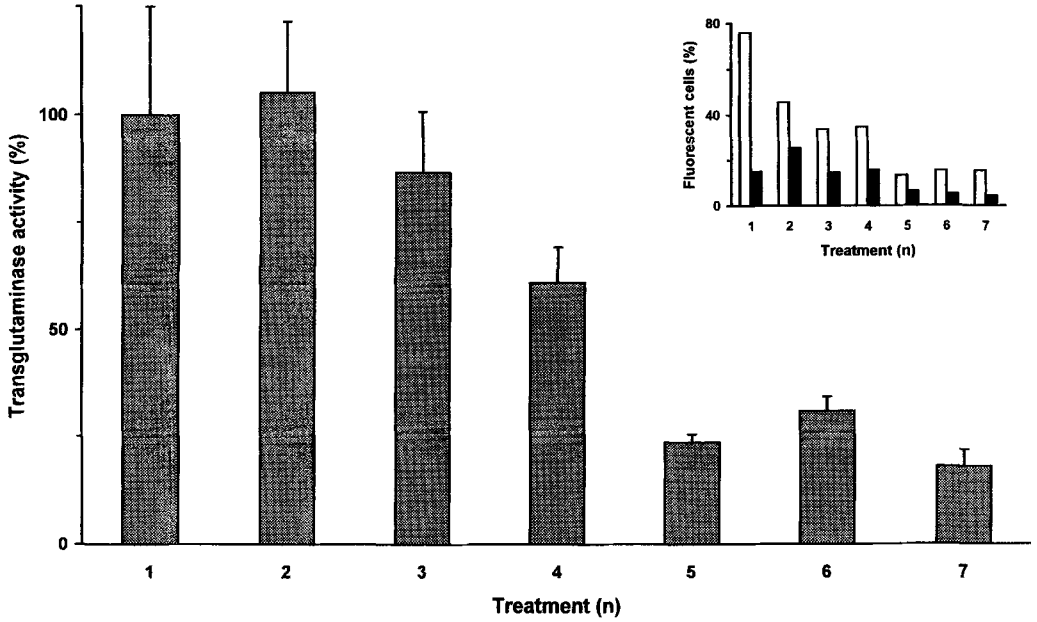

FIGURE 10 Transglutaminase activity in proliferating human lymphocytes after 48 h exposure to 30 mU/ml XOR in the presence of 100 μM hypoxanthine: (1) complete system; with (2) superoxide dismutase (2 U/sample); (3) catalase (2 U/sample); (4) both scavenger enzymes; or (5) 1 mM allopurinol; or without (6) hypoxanthine; or (7) XOR (control cells). Enzyme activity is expressed as the percentage of the complete system value (7.01 ± 1.40 U/g proteins). Results are means ± S.E. of three experiments. The inset shows the percentage of annexin-V positive apoptotic cells (white columns) or both annexin-V and propidium iodide positive necrotic cells (black columns) from a single experiment in the same experimental conditions. Analysis of variance showed a significant difference between the above treatments on transglutaminase activity ($F = 47.05$; d.f. 6/100; $P < 0.0001$).

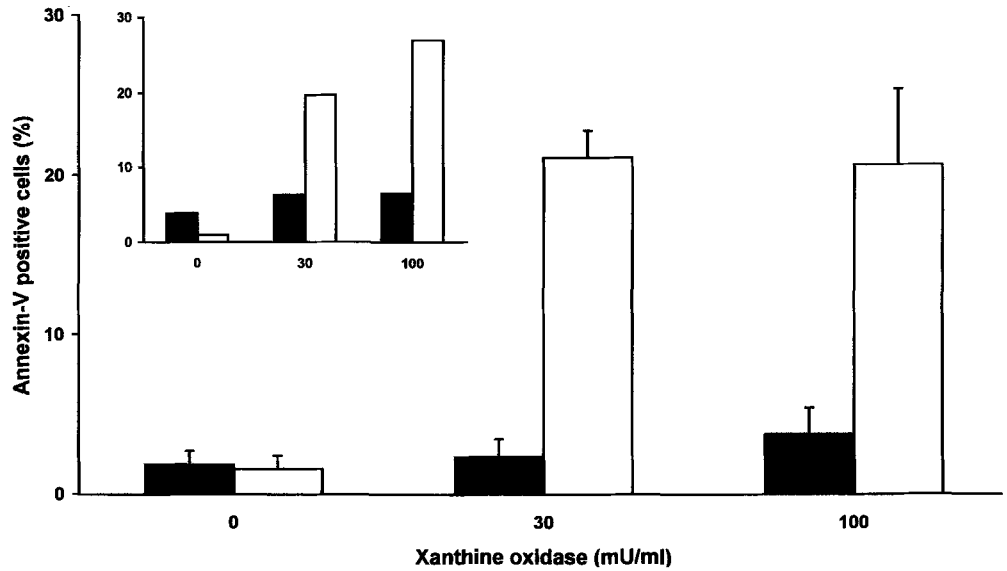

FIGURE 11 Annexin-V FITC positive CD4+ (black columns) and CD8+ (white columns) cells, stimulated by immobilised anti-CD3/CD28 antibodies and identified by phycoerithrin-conjugated monoclonal antibodies. Cells were cultured with 100 μM hypoxanthine and the indicated concentration of XOR for 24 h. Results are means ± S.E. of two experiments. The insert shows the percentage of annexin-V positive CD4+ (black columns) and CD8+ (white columns) cells from a single experiment after 48 h exposure to the same enzyme and substrate concentrations.

time-course experiment with 30 mU/ml XOR may be due to the different experimental conditions, i.e. the retarded stimulation of lymphocytes with PHA.

An apoptotic pattern similar to that exhibited by γ-irradiated cells was observed by electrophoretic migration of DNA extracted from XOR/hypoxanthine-treated lymphocytes. Consistent with a previous report,[43] this analysis of DNA showed some apoptotic ladder in control lymphocytes after 48–72 h culture, both in PHA-stimulated and in non-proliferating cells. The accumulation of DNA fragments in the highest base-pair range indicated a higher amount of apoptosis in XOR-treated samples than that shown by control cells. This accumulation was less marked at the highest XOR concentration, in agreement with a more extensive DNA digestion and the generation of very small fragments, which may be lost during the preparation of the sample. The early occurrence of high molecular weight DNA fragments was considered essential for apoptotic cell death[46] and was described also in the apoptosis of lymphocytes induced by hydrogen peroxide.[47]

DNA fragmentation was already detectable by gel electrophoresis after 24 h treatment with an XOR/hypoxanthine system at a relatively high XOR concentration, or after 48 h in the presence of a lower level of XOR. PI staining of apoptotic hypodiploid nuclei showed that the DNA decrease in XOR-treated lymphocytes reached a plateau level later than annexin-V binding and at a higher XOR concentration than the increment of tTG activity. All together these findings suggest that the extracellular ROS-production damages the membrane before inducing alterations of other cellular compartments.

The appearance of cell permeability to PI required higher XOR concentrations or longer exposure times than the emergence of DNA fragmentation. Thus, necrotic cell membrane alterations induced by the XOR/hypoxanthine system followed DNA damage, in agreement with a previous report.[8]

The relationship between the apoptotic effect of the XOR/hypoxanthine system and the production of ROS by XOR activity was investigated through the use of the scavenger enzymes superoxide dismutase and catalase. The results obtained by tTG activity determination and annexin-V and PI staining tests showed a partial protection exerted by scavenger enzymes, in particular by catalase, consistently with previous observations that hydrogen peroxide is the major cytotoxic product formed during the XOR activity.[7] However, the limited effects of the addition of superoxide dismutase or catalase alone suggest that a good deal of the damage is caused by the combined effects of both hydrogen peroxide and of superoxide and/or of other free radicals derived from the latter.

The differential sensitivity of CD4+ and CD8+ lymphocytes to the XOR/hypoxanthine system was tested after stimulating cells with anti-CD3/CD28 antibodies. Apoptosis was induced in CD8+ cells, as indicated by annexin-V test, whereas CD4+ lymphocytes showed the same level of binding as control cells. Since CD8+ lymphocytes were more resistant than CD4+ cells to Fas-dependent apoptosis of activated T cell,[48] our results suggest that the ROS-induced apoptosis was not mediated by Fas.

The responsiveness of the two T cells subsets to activation with anti-CD3/CD28 was reported to be different, being the mitogenic response of CD4+ lymphocytes higher than that of CD8+ cells, which showed an increased rate of apoptosis after some days in culture.[35] These results parallel the pattern of the higher sensitivity of CD8+ cells to oxidative stress we observed, as compared to CD4+ cells, after stimulation with the same antibodies.

The apoptotic membrane damage of CD8+ cells activated with anti-CD3/CD28 antibodies was completed after 24 h, afterwards reaching a plateau irrespective of XOR concentration. The pattern of annexin-V binding was similar to that obtained after induction of mitosis with PHA,

although in the latter case the percentage of apoptotic cells was higher than in the former.

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