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Role of hydrogen peroxide in the cytotoxicity of the xanthine/xanthine oxidase system

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1. The survival of mammalian epithelial cells exposed *in vitro* to the xanthine/xanthine oxidase system in phosphate-buffered saline (PBS) or serum-containing medium (SCMEM) was investigated. 2. The cytotoxic effect observed depended on the composition of the medium in which the enzymic reaction was carried out; a surviving fraction of 5×10^{-5} was found for cells exposed in PBS and 5.2×10^{-1} for those in SCMEM. 3. The cytotoxic product(s) formed by the xanthine/xanthine oxidase system was relatively stable in PBS; survival of cells incubated after completion of the enzymic reaction was always less than that found for cells exposed during the reaction in the same system. 4. Superoxide dismutase or mannitol present during the enzymic reaction did not inhibit the cytotoxic effect. 5. NaN_3 (a singlet-oxygen quencher and a catalase inhibitor) added to the system in SCMEM caused a reduction in survival to the level observed for cells exposed to the enzymic reaction in PBS. 6. Catalase completely protected cells, but no protection was observed when both catalase and NaN_3 were present in the reaction mixture. 7. A similar cytotoxic effect was produced when cells were treated with H_2O_2 alone. 8. The rate of H_2O_2 decomposition in medium was accelerated by the presence of serum, but this was completely inhibited by NaN_3. 9. It is concluded that H_2O_2 is the major cytotoxic product formed by the xanthine/xanthine oxidase system.

INTRODUCTION

Oxygen radicals are well known for their cytotoxicity and may be implicated in the pathology of some disease states [1–3]. Their toxic effects include membrane damage resulting from peroxidative reactions of polyunsaturated fatty acids (lipid peroxidation) [4–6], as well as attack by reactive oxygen species on proteins [7] and nucleic acids [8–12]. Some anti-cancer agents appear to act via single-electron reduction of O_2 and their cytotoxicity may be explained by redox cycling [5,13].

The superoxide radical anion ($O_2^{\cdot-}$) is a common intermediate of univalent reduction of oxygen. $O_2^{\cdot-}$ itself does not appear to be directly cytotoxic, but is able to initiate radical chain reactions [14]. In contrast, hydrogen peroxide (H_2O_2), which is formed by bivalent reduction of oxygen or dismutation of $O_2^{\cdot-}$, can be directly responsible for cytotoxic effects [15,16].

Xanthine oxidase is one of several enzymes generating $O_2^{\cdot-}$. This enzyme and its mechanism of action have been thoroughly investigated [17–22]. Xanthine oxidase acting aerobically upon xanthine generates $O_2^{\cdot-}$ and H_2O_2 and is used frequently in model systems to investigate cytotoxic effects initiated by $O_2^{\cdot-}$.

Singlet oxygen ($^1O_2$) has been suggested as a toxic agent that can be generated from $O_2^{\cdot-}$ in the xanthine/xanthine oxidase system [23]. Lipid peroxidation of rat liver microsomal preparations exposed to this enzymic system was believed to be promoted by $^1O_2$ [24–26]. However, other authors maintain that $^1O_2$ is not formed as a result of xanthine oxidase activity [27].

Hydroxyl radicals ($HO^{\cdot}$) generated from $O_2^{\cdot-}$ by Haber–Weiss or Fenton-type reactions have also been implicated as toxic products formed by the xanthine/xanthine oxidase system [26,28–31].

The actual mechanism by which cells become damaged while exposed to the xanthine/xanthine oxidase system is still uncertain. However, H_2O_2, as a small, uncharged and relatively stable molecule generated by the system, could cause the cellular injury observed [15,16,32,33]. The investigations described in the present paper indeed show that, in the case of cultured epithelial cells, H_2O_2 is the major cytotoxic species formed by the xanthine/xanthine oxidase system in the reaction mixture.

MATERIALS AND METHODS

Chemicals

Xanthine, xanthine oxidase from buttermilk (grade III), catalase from bovine liver (thymol-free), superoxide dismutase from bovine liver, cytochrome *c* from horse heart (type VI) and NaN_3 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; uric acid and mannitol were from BDH Chemicals, Poole, Dorset, U.K.; H_2O_2 [aqueous 30% (v/v) solution, stabilizer-free] was from Aldrich Chemical Co., Gillingham, Dorset, U.K.; H_2O_2 [aqueous 30% (v/v) solution, stabilizer-free] was from Aldrich Chemical Co., Gillingham, Dorset, U.K.; Eagle's minimal essential medium modified with Earle's salt and foetal bovine serum were from Flow Laboratories, Irvine, Ayrshire, U.K. Serum was stored in small portions at −18 °C and a fresh sample was used for each experiment. Phosphate-buffered saline (PBS)

Abbreviations used: PBS, phosphate-buffered saline; MEM, minimal essential medium; SCMEM, serum-containing minimal essential medium; BSS, balanced salt solution.

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was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 0.132 g of $CaCl_2,2H_2O$, 0.1 g of $MgCl_2,6H_2O$, 1.15 g of $Na_2HPO_4,2H_2O$ and 0.2 g of KH_2PO_4 per litre in deionized water.

Cell line and growth medium

A mammalian epithelial cell line (GPK) was used in all experiments. Cells from passages 18 to 30 were employed in these investigations. The cell cultures were grown in minimal essential medium (MEM) supplemented with 200 mM-L-glutamine (1 ml/100 ml of MEM), 7.5% (w/v) $NaHCO_3$ (0.4 ml/100 ml of MEM), two antibiotics, namely penicillin (10000 i.u./100 ml of MEM) and streptomycin (10 mg/100 ml of MEM), and 10% (v/v) foetal bovine serum.

Xanthine/xanthine oxidase systems: determination of $O_2^{\cdot-}$, H_2O_2 and uric acid

Two xanthine/xanthine oxidase systems were compared: (*a*) compartmentalized, in which 1.5 units of xanthine oxidase were contained in a 1 cm-wide dialysis bag immersed in 23 ml of 0.3 mM-xanthine solution; the separation of xanthine oxidase from its substrate enabled the interruption of the reaction at any time; (*b*) uncompartmentalized, in which 150 munits of the enzyme were added directly to 23 ml of 0.3 mM-xanthine solution prepared in PBS. For both systems, in some experiments the xanthine solution was bubbled with O_2 for 1 h before the inclusion of xanthine oxidase.

The 1 h enzymic reaction was carried out at 37 °C in 25 ml conical flasks, the contents of which were slowly stirred with a magnetic stirrer. pH 7.0 ± 0.1 was used for all experiments and was measured with a PW 9410/30 digital pH-meter (Pye–Unicam, Cambridge, U.K.). The linearity error of the instrument was less than ± 0.02 pH full-scale deflection.

All products formed in the investigated systems were determined spectrophotometrically (SP8-400UV/VIS spectrophotometer; Pye–Unicam) with appropriate blank samples. $O_2^{\cdot-}$ was determined by observing the reduction of cytochrome *c* at 550 nm [34] by using an absorption coefficient of 2.1×10^4 $M^{-1} \cdot cm^{-1}$ [35]. Superoxide dismutase was utilized to determine the contribution of $O_2^{\cdot-}$ in the reduction of cytochrome *c* [34]. H_2O_2 was measured by two methods: iodide oxidation and horseradish-peroxidase-coupled oxidation [36]. Uric acid determinations were carried out by observing the absorption at 295 nm [37]. The absorption coefficient of uric acid was 1.36×10^{-4} $M^{-1} \cdot cm^{-1}$.

Cell exposure to the xanthine/xanthine oxidase system

(*a*) Compartmentalized system. A xanthine solution (0.35 mM) was prepared in a serum-free medium (MEM) or in phosphate-buffered saline (PBS) supplemented with L-glutamine, 7.5% $NaHCO_3$ and antibiotics in the proportions described for the growth medium. Serum [10% (v/v) final concentration] was added immediately prior to the experiments to produce serum-containing medium (SCMEM). In the case of xanthine dissolved in PBS, PBS was added instead of serum. The desired pH was adjusted by titration with 1 M-HCl and was measured before and after experiments.

The xanthine solution was filtered through a 0.22 μm-pore-size Millipore filter, and 22 ml portions were distributed into 25 ml conical flasks. Subsequently, a 1 cm-wide dialysis bag containing xanthine oxidase (6 units), or serum-free medium/PBS as control, was placed in the flask. In one set of experiments, 1.6×10^6 cells suspended in 1 ml of growth medium or PBS were added. The flasks were then incubated for 1 h at 37 °C and their contents were slowly stirred with a magnetic stirrer. In another set of experiments, the enzymic system without cells was incubated as above for 1 h, after which the dialysis bag with xanthine oxidase was removed and 1.6×10^6 cells suspended in 1 ml of growth medium or PBS were added and incubated as above.

After incubation the cells were recovered by centrifugation and the concentration of uric acid in the supernatant was determined spectrophotometrically; the concentration was found to be 160 ± 10 μM.

(*b*) Uncompartmentalized system. A xanthine solution (0.35 mM) was prepared in PBS. A further portion of PBS (10% of the final volume) was added to give the same xanthine concentration as in the compartmentalized system. This system was bubbled with O_2 for 1 h before experiments, then filtered through a 0.22 μm-pore-size Millipore filter, and 22 ml portions were distributed into 25 ml conical flasks. Subsequently, 1.6×10^6 cells suspended in 1 ml of PBS and 150 munits of xanthine oxidase were added and the experiments carried out as described in (*a*).

Determination of survival of cells exposed to the xanthine/xanthine oxidase system

The recovered cells were resuspended in growth medium and counted in a Neubauer haemocytometer. The cell suspension was diluted to the desired density, plated into Petri dishes (NUNC Products, Roskilde, Denmark) and incubated for 9 days at 37 °C in an atmosphere of 5% CO_2 in air. The colonies formed were stained with Crystal Violet, and counted to determine their plating efficiency. Final results were expressed as a surviving fraction, calculated as the ratio of the plating efficiency for treated cells to the plating efficiency for control cells.

Cell treatment with H_2O_2

Portions (22 ml) of PBS, of PBS supplemented with either 10% serum or catalase (33 units/ml) or of SCMEM were filtered through a 0.22 μm-pore-size Millipore filter and placed in 25 ml conical flasks. Subsequently, 1.6×10^6 cells suspended in 1 ml of the appropriate medium were added, and 1 ml of H_2O_2 diluted in PBS or MEM (serum-free) to a required concentration, or 1 ml of PBS or MEM as a control, was mixed with this cell suspension. Afterwards the flasks were incubated for 1 h at 37 °C with their contents stirred with a magnetic stirrer. The remaining procedures were the same as those described above.

Determination of the decomposition rate of H_2O_2 in different media

Portions (9.92 ml) of PBS or MEM, with or without 10% serum or with 10% serum and 3.1 mM-NaN_3, were placed in 15 ml glass beakers, and 80 μl of 17.5 mM-H_2O_2 in PBS or MEM respectively was added to each medium giving a final concentration of 140 μM-H_2O_2. Immediately afterwards the beakers were placed in a water bath at 37 °C.

The amount of H_2O_2 in the medium was determined spectrophotometrically, as indicated above. The rate of

Table 1. Determination of $O_2^{\cdot-}$, H_2O_2 and uric acid formed in the xanthine/xanthine oxidase system

$O_2^{\cdot-}$, H_2O_2 and uric acid were determined spectrophotometrically: $O_2^{\cdot-}$ by observing the reduction of cytochrome *c* at 550 nm, H_2O_2 by measurement of the absorption at 352 nm by using the iodide oxidation method, and uric acid by determination of the absorption at 295 nm. Two xanthine/xanthine oxidase systems were investigated: uncompartmentalized, in which 150 munits of xanthine oxidase were added directly to 23 ml of 0.3 mM-xanthine solution prepared in PBS (175 μM-cytochrome *c* was used), and compartmentalized, in which 1.5 units of xanthine oxidase were placed in a dialysis bag and the enzymic reaction was carried out in 23 ml of 0.3 mM-xanthine solution in PBS (35 μM-cytochrome *c* was used). The results represent the means for at least two experiments.

Products of xanthine/xanthine oxidase reaction	Uncompartmentalized system				Compartmentalized system			
	No additional O_2		Bubbled with O_2		No additional O_2		Bubbled with O_2	
	Absorbance (*A*)	Concentration (μM)	Absorbance (*A*)	Concentration (μM)	Absorbance (*A*)	Concentration (μM)	Absorbance (*A*)	Concentration (μM)
Uric acid(A_{295})	1.93	150	1.95	152	0.24	18.0	0.26	19.0
H_2O_2 (A_{352})	0.95	167	0.84	148	0.10	17.6	0.10	17.6
$O_2^{\cdot-}$ (A_{550})	1.39	66	1.40	67	0.11	5.2	0.08	3.8

decomposition of 140 μM-H_2O_2 solution at 37 °C was determined by measurement of the H_2O_2 concentration remaining in each of the 10 ml samples at 15 min intervals for 1 h. The first measurement was carried out 30 s after the addition of H_2O_2 to the medium.

RESULTS

Determination of $O_2^{\cdot-}$, H_2O_2 and uric acid formed in the xanthine/xanthine oxidase system

The total amounts of uric acid, $O_2^{\cdot-}$ and H_2O_2 formed during the 1 h incubation of the reaction systems are shown in Table 1. In the uncompartmentalized system, in which xanthine oxidase was added directly to the xanthine solution, the generation of 167 μM-H_2O_2 and 66 μM-$O_2^{\cdot-}$ corresponded to the formation of 150 μM-uric acid. The higher concentration of H_2O_2 than of uric acid was due to the sequence of spectrophotometric measurements, in which determination of H_2O_2 followed the uric acid determination. Changing the order of the procedure resulted in a more accurate determination of the ratio of the compounds (see Table 1, uncompartmentalized system bubbled with O_2). There were no such difficulties in the case of the compartmentalized system, in which the enzymic reaction could be interrupted at any time. In this system the concentration of the products measured was approximately one order of magnitude lower than that found for the uncompartmentalized system (Table 1). However, the ratios of uric acid to $O_2^{\cdot-}$ and to H_2O_2 were comparable in the two systems and approximate to about 0.35 and 1.00 respectively.

The concentrations of uric acid, H_2O_2 and $O_2^{\cdot-}$ remained unchanged in both systems when the xanthine solution was bubbled with O_2 for 1 h before the enzymic reaction.

Adding superoxide dismutase (75 units/ml) to the reaction mixture entirely inhibited the $O_2^{\cdot-}$-induced reduction of cytochrome *c*.

Cell survival after 1 h incubation in 0.3 mM-xanthine solution

The surviving fraction of cells incubated for 1 h in a 0.3 mM-xanthine solution was determined. The cell survival varied between 67% and 100%, but was shown to be independent of the solvent (PBS or MEM), the pH of the solution (range 6.5–7.5) or the presence of uric acid (200 μM), mannitol (10 mM), NaN_3 (630 μM), superoxide dismutase (75 units/ml) or catalase (475 units/ml) in the incubation medium (results not shown).

Survival of epithelial cells exposed to the xanthine/xanthine oxidase system without compartmentalization of the enzyme

The survival of cells exposed to the enzymic system in which xanthine oxidase was added directly to the xanthine solution in PBS was 4.8×10^{-4} (Fig. 1). Superoxide dismutase did not protect the cells; the surviving fraction of cells incubated in the xanthine/xanthine oxidase system in the presence of superoxide dismutase was 2.3×10^{-4}, a value comparable with that obtained for cells exposed to the enzymic system alone (Fig. 1). In sharp contrast, the addition of catalase or a combination of catalase and superoxide dismutase abrogated the cytotoxic effect almost completely, increasing the cell survival by more than three orders of magnitude, to 9.2×10^{-1} and 9.4×10^{-1} respectively (Fig. 1).

Cell survival after 1 h incubation in the compartmentalized xanthine/xanthine oxidase system in SCMEM or PBS

The survival of cells exposed to the compartmentalized xanthine/xanthine oxidase system was found to be strongly dependent on the composition of the incubation medium in which the enzymic reaction was carried out (Fig. 2). The surviving fraction for cells exposed to the xanthine/xanthine oxidase system in SCMEM was 5.2×10^{-1}, whereas in PBS it was four orders of magnitude less (5×10^{-5}).

Mannitol and NaN_3 were used to scavenge HO$^{\cdot}$ radicals and to quench $^1O_2$ respectively. Both compounds were added to the xanthine solution before the initiation of the enzymic reaction. No protection by mannitol was observed when the reaction was carried out in either PBS or SCMEM. The survival of cells exposed to the xanthine/xanthine oxidase system in PBS was 1.6×10^{-5}

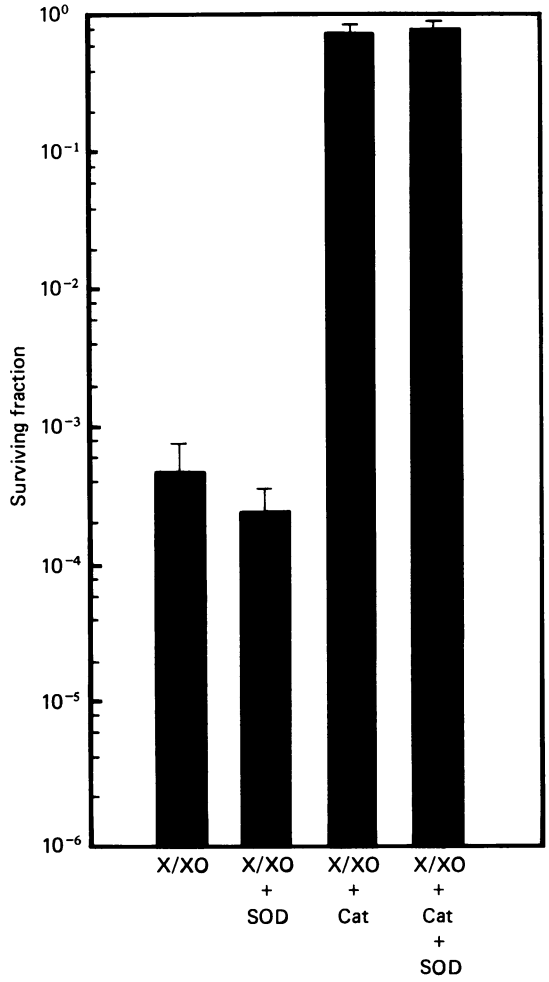

Fig. 1. Survival of epithelial cells after 1 h incubation in the uncompartmentalized xanthine/xanthine oxidase system in PBS

Xanthine oxidase (150 munits) was added directly to 0.3 mM-xanthine solution with/without catalase or superoxide dismutase. Survival of 1.6×10^6 cells exposed to this system for 1 h was determined by clonogenic assay (for details see the Materials and methods section). Key: X, xanthine (23 ml); XO, xanthine oxidase; Cat, catalase (3500 units/ml); SOD, superoxide dismutase (75 units/ml). The data consist of the means for at least three experiments. The error bars show the S.D.

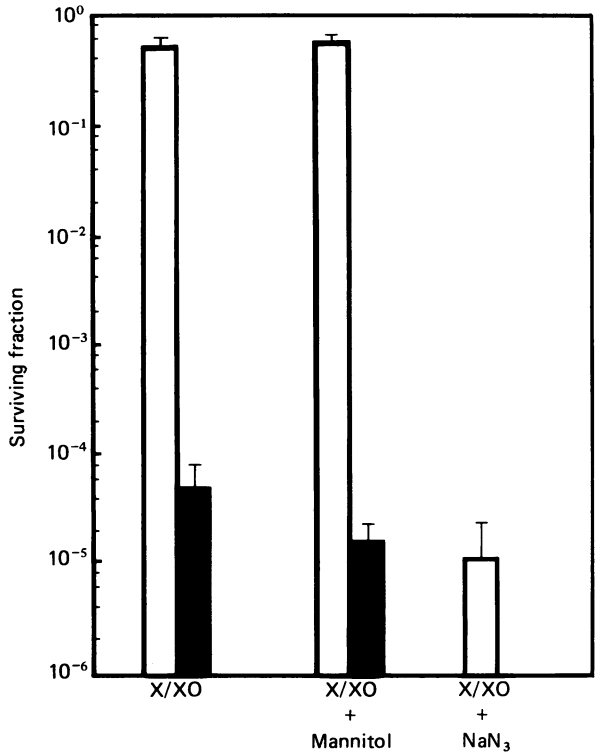

Fig. 2. Survival of epithelial cells after 1 h incubation in the xanthine/xanthine oxidase system in serum-containing medium or PBS

A dialysis bag containing 6 units of xanthine oxidase (XO) was placed in 0.3 mM-xanthine solution (X, 23 ml) with or without 10 mM-mannitol or 540 μM-NaN_3. Cells (1.6×10^6) were exposed to the system in serum-containing medium (□) or PBS (■) for 1 h. Cell survival was determined by clonogenic assay. The data consist of the means for at least three experiments. The error bars show the S.D.

in the presence of mannitol, as compared with 5×10^{-5} without mannitol (Fig. 2). When the enzymic reaction was carried out in SCMEM the respective values of the surviving fraction were 5.9×10^{-1} and 5.2×10^{-1} (Fig. 2).

The addition of NaN_3 to the system in SCMEM was found to increase the cytotoxicity. The survival of cells was 1.1×10^{-5} and comparable with that observed when the reaction was carried out in PBS in the absence of azide (Fig. 2).

Persistence of cytotoxic products generated by the xanthine/xanthine oxidase system

Epithelial cells were incubated in SCMEM or PBS in which the xanthine/xanthine oxidase reaction had been carried out previously to enable the determination of the stability of a toxic product(s) formed in the reaction mixture (for details see the Materials and methods section). The cytotoxicity of this modified system was higher than that observed during the enzymic reaction in SCMEM and PBS respectively (Figs. 3 and 4). In the system in SCMEM a decrease of about 50% in the relative survival was observed when cells were exposed after instead of during the reaction, the cell survival being 1.6×10^{-2} and 3.8×10^{-2} respectively (Fig. 3). The surviving fraction for cells incubated after the reaction was decreased to 3.3×10^{-6} when NaN_3 was present during the enzymic reaction. This was about one order of magnitude less than the corresponding value for cells treated under the same experimental conditions but during the xanthine/xanthine oxidase reaction (Fig. 3).

The survival of cells incubated after completion of the enzymic reaction in PBS was related to the cell exposure time, decreasing by more than two orders of magnitude over a period of 40 min incubation (cf. Figs. 4*a* and 4*b*). The surviving fraction of cells exposed for 20 min to the final product(s) of the enzymic reaction was 1.3×10^{-3} (Fig. 4*b*), and less than 1×10^{-5} for cells incubated for 1 h (Fig. 4*a*). The survival increased to 9.8×10^{-1} when catalase was added to PBS after the xanthine/xanthine oxidase reaction was terminated and 15 min before cell

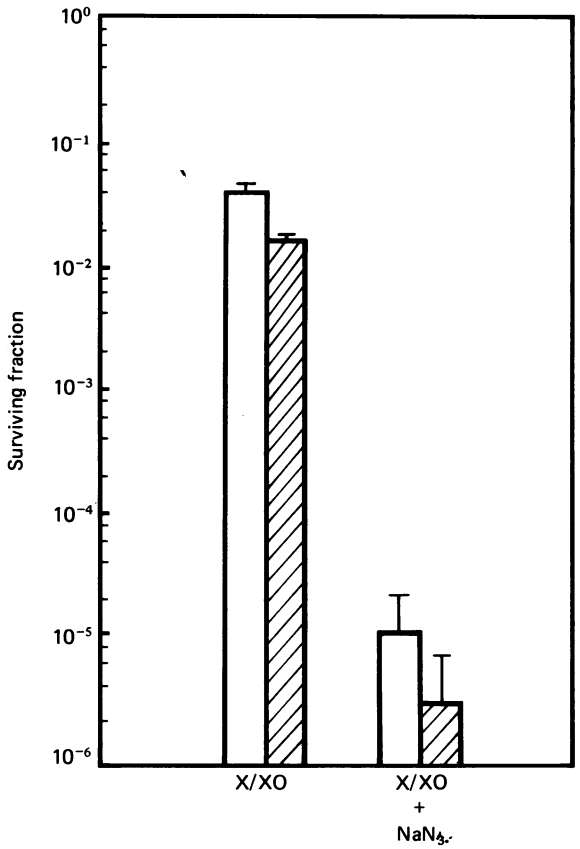

Fig. 3. Comparison of survival of epithelial cells exposed for 1 h to products of the xanthine/xanthine oxidase system in serum-containing medium during or after the enzymic reaction

A dialysis bag containing 6 units of xanthine oxidase (XO) was placed in 0.3 mM-xanthine solution (X) in 23 ml of serum-containing medium with or without 540 μM-NaN_3. Cells (1.6×10^6) were added to the system during the enzymic reaction (□) or after its completion (▨) for 1 h. Cell survival was determined by clonogenic assay. The data consist of the means for two experiments. The error bars show the S.D.

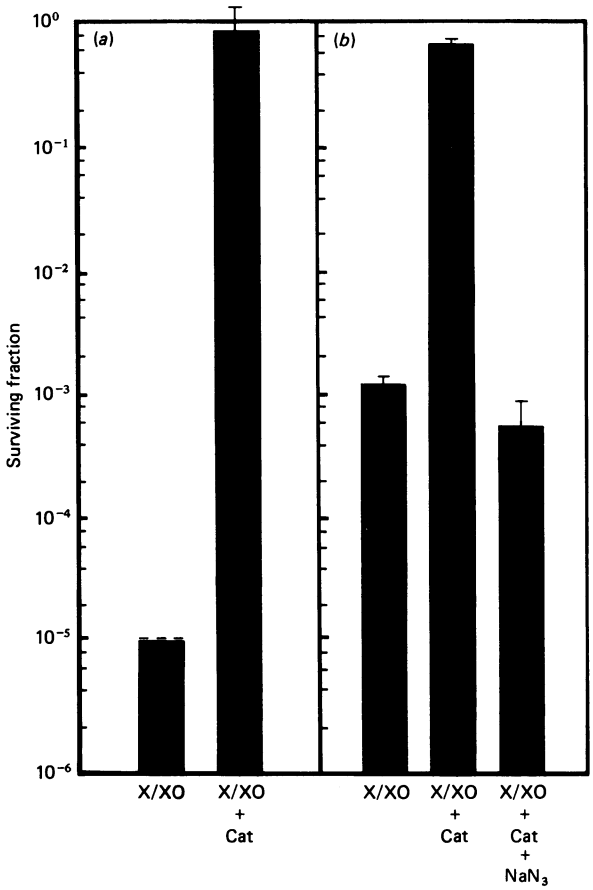

Fig. 4. Effect of catalase and NaN_3 on the survival of epithelial cells exposed to the final reaction products of the xanthine/xanthine oxidase system for 1 h (*a*) or 20 min (*b*)

A dialysis bag containing 6 units of xanthine oxidase (XO) was placed in 0.3 mM-xanthine solution (X) in 23 ml of PBS. Catalase and NaN_3 were added to the system after completion of the enzymic reaction and 15 min before cell exposure. (*a*) Cells (1.6×10^6) were incubated for 1 h after the xanthine/xanthine oxidase reaction was terminated; Cat, catalase (43.3 units/ml). (*b*) Cells (1.6×10^6) were incubated for 20 min after the xanthine/xanthine oxidase reaction was terminated; Cat, catalase (475 units/ml); NaN_3, 310 μM-NaN_3. Cell survival was determined by clonogenic assay. The data consist of the means for two experiments. The error bars show the S.D.

exposure lasting for 1 h (Fig. 4*a*). A similar protective effect with catalase occurred in cells incubated for 20 min after completion of the reaction; the survival was more than three orders of magnitude higher than that for the system that was not supplemented with catalase (Fig. 4*b*). However, adding catalase and NaN_3 after termination of the enzymic reaction and before incubation with cells resulted in a surviving fraction of 5.6×10^{-4}, i.e. similar to that found for the respective control (Fig. 4*b*).

Survival of epithelial cells exposed to H_2O_2

The cytotoxic effect of H_2O_2 was strongly dependent on the H_2O_2 concentration and on the composition of the medium in which the cells were treated (Fig. 5). The survival of epithelial cells exposed to 20 μM-H_2O_2 in PBS was 6.6×10^{-1} and diminished approximately exponentially by about two orders of magnitude with H_2O_2 concentrations increasing up to 98 μM (Fig. 5).

A protective effect was observed when SCMEM was used instead of PBS. The survival curve for cells exposed to H_2O_2 in the serum-containing medium was characterized by a shoulder and a slope shallower than that found for cells treated with H_2O_2 in PBS. Raising the H_2O_2 concentration from 142 μM to 280 μM resulted in a decrease in the surviving fraction from 6.8×10^{-1} to 7×10^{-3}, values comparable with the survival observed for cells treated in PBS with H_2O_2 at concentrations ranging from 20 μM to 98 μM (Fig. 5). When catalase (33 units/ml) or 10% serum was added to the PBS, they mimicked the observed protective effect of SCMEM. The survival of epithelial cells exposed to 10 μM-H_2O_2 in PBS was 8.3×10^{-1}. A similar survival was obtained for cells treated with 280 μM-H_2O_2 (i.e. a 28-fold higher dose) in PBS supplemented with either serum or catalase (Table 2).

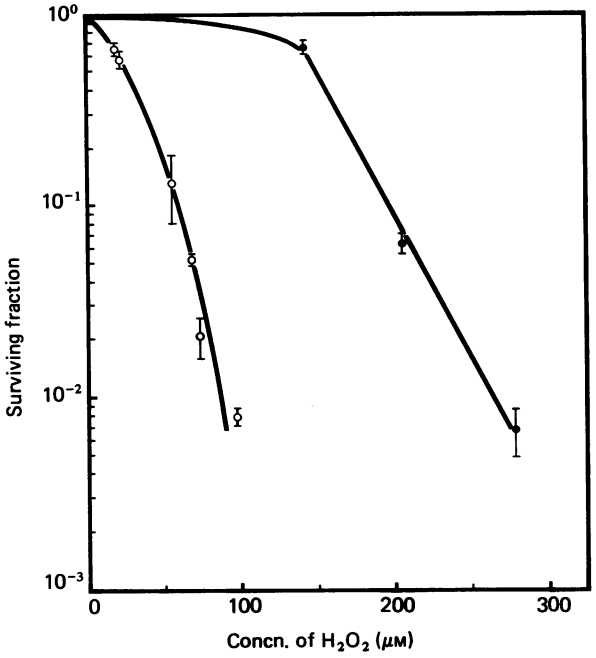

Fig. 5. Survival curves for epithelial cells exposed to H_2O_2 in PBS or serum-containing medium for 1 h

Cells (1.6×10^6) were suspended in 22 ml of PBS (○) or SCMEM (●). A 1 ml portion of H_2O_2 diluted in PBS or serum-free medium to the required concentration was added to the appropriate cell suspension. Cells were incubated for 1 h and their survival was determined by clonogenic assay. The data consist of the means for at least three experiments. The error bars show ± S.D.

Table 2. Protective effect of serum and catalase on epithelial cells exposed to H_2O_2 in PBS

Cells (1.6×10^6) were suspended in 22 ml of PBS with or without 10% serum or catalase (33 units/ml). A 1 ml portion of H_2O_2 diluted in PBS to the required concentration was added to the appropriate cell suspension. Cells were incubated for 1 h, and their survival was determined by clonogenic assay. The results represent the means for three experiments.

Medium	Initial concn. of H_2O_2 added (μM)	Surviving fraction
PBS	10	8.30×10^{-1}
PBS + 10% serum	208	8.36×10^{-1}
PBS + catalase	208	8.22×10^{-1}

Rate of decomposition of H_2O_2 in medium

Measurement of the H_2O_2 concentration remaining in a 10 ml sample of medium kept at 37 °C for 1 h showed that the spontaneous decomposition of H_2O_2 in PBS or MEM amounted to about 7% of the initial concentration, in contrast with the rapid decomposition observed when either PBS or MEM was supplemented with 10% serum (Fig. 6). In the presence of serum, the concentration of H_2O_2 decreased by 30–40% during the first 30 s of incubation, after which the decomposition

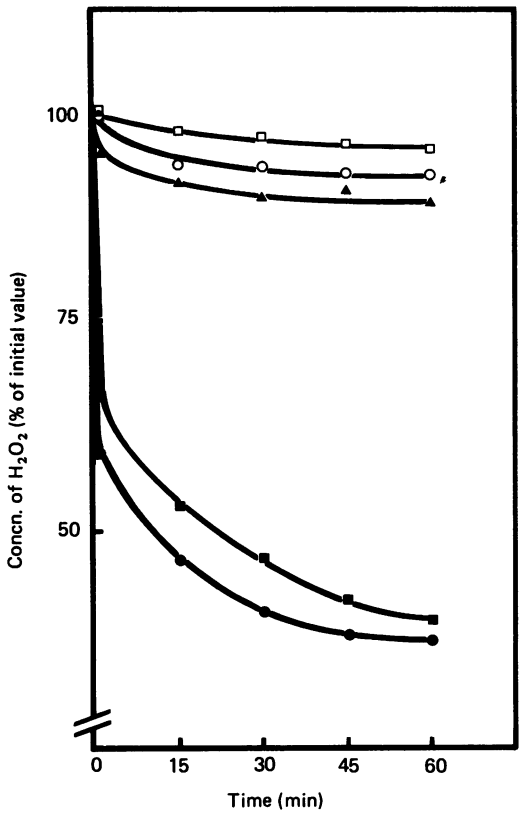

Fig. 6. Rate of H_2O_2 decomposition in different media

Solutions (10 ml) of 140 μM-H_2O_2 were prepared in PBS or MEM with or without 10% serum or with 10% serum and 3.1 mM-NaN_3. The rate of decomposition of the H_2O_2 solution at 37 °C was determined by spectrophotometric measurement of the H_2O_2 concentration remaining in each sample at 15 min intervals for 1 h. The first measurement was carried out 30 s after the addition of H_2O_2 to the medium. ○, PBS; ●, PBS containing 10% serum; □, MEM; ■, SCMEM; ▲, SCMEM containing 3.1 mM-NaN_3. Each point represents the mean for one or two experiments.

became less rapid and the amount of H_2O_2 determined after 1 h was 30% of the initial concentration (Fig. 6). Adding 3.1 mM-NaN_3 to serum-containing medium before the addition of H_2O_2 almost entirely inhibited the acceleration of H_2O_2 decomposition caused by the presence of serum and resulted in a rate of H_2O_2 decomposition comparable with that observed in PBS and serum-free medium (Fig. 6).

DISCUSSION

The results presented in this paper demonstrate that the survival of epithelial cells exposed to the xanthine/xanthine oxidase system is significantly diminished. The conditions under which products of the enzymic reaction formed in the medium are toxic to the cells suggest that H_2O_2 is responsible for the cytotoxic effect observed, and this is confirmed by the results obtained from the comparative studies with cells exposed to H_2O_2.

The xanthine/xanthine oxidase system is known to generate $O_2^{\cdot -}$ and H_2O_2. There is some evidence that $O_2^{\cdot -}$ alone formed extracellularly does not cause cell

death [14]. However, its contribution to a metal-ion-catalysed Haber–Weiss reaction, by reducing transition-metal ions and thereby accelerating the cycle of a Fenton-type reaction in which the reduced ions are oxidized by H_2O_2, generating HO$^\cdot$ radicals, is well established [14].

Elimination of $O_2^{\cdot-}$ from the reaction mixture by adding superoxide dismutase to the xanthine/xanthine oxidase system did not diminish the cytotoxic effect (see Fig. 1); on the contrary, cell survival was slightly decreased in comparison with the survival of cells exposed to the xanthine/xanthine oxidase system alone. The results show that $O_2^{\cdot-}$ generated extracellularly is neither directly nor indirectly responsible for the cell damage observed.

Catalase completely protected the cells when they were exposed to the products of the xanthine/xanthine oxidase system, either during or after completion of the enzymic reaction (see Figs. 1 and 4). It is evident, therefore, that H_2O_2 was toxic to the cells either by itself or as a precursor of other toxic species such as HO$^\cdot$, formed via a Fenton-type reaction in the presence of appropriate transition-metal ions. The lack of influence of mannitol on the survival of epithelial cells exposed to the xanthine/xanthine oxidase system in both PBS and SCMEM eliminates the possibility that HO$^\cdot$ radicals formed in the medium contribute to the cellular injury observed (Fig. 2). This is in agreement with the findings reported by Zingler *et al.* [38], which show that HO$^\cdot$ radicals are not produced by the hypoxanthine/xanthine oxidase system in balanced salt solution (BSS) if chelated iron ions are not added to the reaction mixture. BSS, which has a composition very similar to that of PBS, does not contain sufficient metal ions to support significant extracellular conversion of H_2O_2 into HO$^\cdot$. In our system serum is the only source of such metal ions, but medium supplemented with serum also contains several HO$^\cdot$ scavengers, such as amino acids and proteins, with which HO$^\cdot$ is known to react avidly [7,39,40]. Moreover, it is unlikely that HO$^\cdot$ radicals formed extracellularly can damage cells, since it has been estimated that these radicals diffuse approx. 6 nm from a site of their production to a reaction site [41]. Indeed, the amount of HO$^\cdot$ generated in the hypoxanthine/xanthine oxidase system in BSS increases significantly in the presence of Fe–EDTA. However, the cytotoxic effect of such a modified system diminishes considerably and mannitol does not exhibit any protective action [38].

The possibility that the toxicity is due to H_2O_2-mediated formation of O_2-derived radicals in the reaction mixture is excluded both by the stability of the cytotoxic product(s) and by the stability of the H_2O_2 concentration. The stability of the toxic agent(s) is inferred from the survival of epithelial cells incubated after completion of the enzymic reaction, which was always less than that for cells exposed during the reaction in the same system (see Figs. 3 and 4*a*). The extent of the toxic effect was related to the duration of cell exposure, with the cell survival decreasing by more than two orders of magnitude over a period of 40 min. These results are consistent with the accumulation of a stable toxic product(s) during the enzymic reaction, so that the concentration of the noxious species upon completion of the reaction is higher than the average concentration during the period of the reaction. The stability of the toxic agent titrated with the cells excludes any species of relatively short life-span, including those derived from O_2 as the primary agents responsible for the cell injury.

The H_2O_2 concentration in the xanthine/xanthine oxidase system remained essentially unchanged for at least 3 h after termination of the enzymic reaction (less than 1% decrease at 4 °C). Thus H_2O_2-mediated reactions leading to the formation of a cytotoxic product(s) in the reaction mixture can be excluded, since if such reactions occurred a decrease in concentration of H_2O_2 would be observed. Moreover, the abolition of the cytotoxic effect by catalase added after termination of the xanthine/xanthine oxidase reaction points to the involvement of H_2O_2 in the cell damage and suggests that H_2O_2, which is a stable product in the system investigated, is responsible for the reproductive death of cells.

H_2O_2 alone is cytotoxic to a similar degree when epithelial cells are exposed to it under experimental conditions employed in the xanthine/xanthine oxidase system (see Fig. 5). Its toxic effect is strongly dependent on the composition of the medium, with lower cell survival in PBS than in SCMEM. The protective effect of SCMEM appears to be due to the presence of catalase in serum, since the same survival was obtained when the cells were exposed to H_2O_2 in PBS supplemented with the equivalent amount of serum (cf. Fig. 5 and Table 2), and this result was mimicked when catalase was added instead of serum in both systems under investigation (see Fig. 4 and Table 2). Moreover, the presence of NaN_3, known to be a powerful inhibitor of catalase [42], in the xanthine/xanthine oxidase system in either SCMEM or PBS with catalase depressed cell survival to the level found for cells exposed to the enzymic reaction in the absence of both serum and catalase (cf. Figs. 2 and 4*b*). In addition, it was found that the H_2O_2 concentration diminished very slowly in PBS and in serum-free medium (about 7% of the initial concentration in 1 h at 37 °C), but decomposition increased rapidly when both media (i.e. PBS and MEM) were supplemented with 10% serum. This acceleration of H_2O_2 decomposition caused by serum was abolished completely when NaN_3 was present in the reaction mixture (Fig. 6). However, serum contains iron-binding proteins such as ferritin and transferrin. Ferritin may mediate the metal-ion-catalysed Haber–Weiss reaction in the presence of reducing agents ($O_2^{\cdot-}$ among others) [43]. Thus, as apo-transferrin and partially iron-loaded transferrin appear to prevent HO$^\cdot$ generation, presumably by binding the free iron ions or iron released from ferritin [44], the protective effect of serum might be interpreted on this basis. Conversely, since H_2O_2 decomposition is accelerated in the presence of serum and as HO$^\cdot$ radicals formed extracellularly do not contribute to the observed cell damage (see Fig. 2 and ref. [38]), it might be argued that the addition of serum with a high iron content could protect cells by diminishing the H_2O_2 concentration in the xanthine/xanthine oxidase system, where the formation of $O_2^{\cdot-}$ could act as a reductant for the iron. Our data appear to exclude either of these mechanisms since: (1) serum protects equally cells exposed to the xanthine/xanthine oxidase system and to H_2O_2 (i.e. the protection is independent of the presence of $O_2^{\cdot-}$); (2) serum accelerates the rate of decomposition of H_2O_2 in the absence of $O_2^{\cdot-}$; (3) the protection by serum is simulated by catalase; (4) NaN_3 abolishes both the accelerated H_2O_2 decomposition and the protective effect of serum and catalase. We infer, therefore, that the protective

effect exhibited by SCMEM is due to the presence of catalase in the serum.

In summary, the exclusion of $O_2^{\cdot-}$ and HO$^{\cdot}$ formed extracellularly from involvement in the cell damage observed, the stability of the cytotoxic product(s) generated in the xanthine/xanthine oxidase reaction, the constant concentration of H_2O_2 formed in the system, the quantitative similarity in survival of cells exposed to H_2O_2 instead of the products of the enzymic reaction and the involvement of catalase in cell protection by serum-containing medium for both systems lead us to the conclusion that H_2O_2 is the major toxic product of the xanthine/xanthine oxidase system.

Nevertheless, there remains a possibility in the compartmentalized system that the $O_2^{\cdot-}$ formed within the dialysis bag by xanthine oxidase was spontaneously converted into H_2O_2 before diffusion from the bag to the cell suspension. To eliminate this possibility the efficiency of $O_2^{\cdot-}$ and H_2O_2 production was carefully examined. Reduction of cytochrome *c* added to the xanthine solution and the complete inhibition of this reduction by superoxide dismutase present outside the dialysis bag indicate that the cytochrome *c* was reduced by $O_2^{\cdot-}$ diffusing out of the dialysis bag, and additionally that the enzymic activity of superoxide dismutase was not prevented by H_2O_2 formed in the system (Table 1). The amounts of uric acid, $O_2^{\cdot-}$ and H_2O_2 generated in the uncompartmentalized system were higher in comparison with the system in which the enzyme was placed in a dialysis bag. However, the ratios of uric acid to $O_2^{\cdot-}$ and to H_2O_2 remained the same in both systems (see Table 1). This suggests that the semi-permeable membrane limited the rate of xanthine diffusion into the dialysis bag containing the enzyme but did not affect the proportions of $O_2^{\cdot-}$ and H_2O_2 formed. This ratio, as well as the total amount of $O_2^{\cdot-}$ and H_2O_2 in both systems, was unaffected by bubbling the xanthine solution with O_2. This excludes the possibility that, under the experimental conditions employed, O_2 diffusion into the reaction mixture was a limiting factor that could influence the yield of the primary enzymic products.

Our findings are in agreement with the results of previous studies concerning H_2O_2 as the major cytotoxic product formed in the reaction mixture by xanthine oxidase systems [38,45,46]. However, it should be emphasized that the data presented do not attempt to elucidate either the intracellular mechanism(s) by which H_2O_2 damages epithelial cells or the supposed target(s) responsible, directly or indirectly, for the cell death. H_2O_2 toxicity is well documented [9,10,15,16,32,47], but the mechanism(s) of cell injury is still under investigation [12,16,47–50]. It is possible that DNA is the most significant molecular target damaged by H_2O_2 [9,10,12,47]. However, recent data have demonstrated that the lower colony-forming ability of cells exposed to H_2O_2 is not always related to the number of single-strand and double-strand breaks [51], and, therefore, other cellular targets should be considered. In addition, there are a number of characteristic cytoplasmic changes associated with H_2O_2-generated cell injuries that may be directly caused or might be secondary to damage to other targets including DNA [16]. The toxic effect of H_2O_2 on DNA is enhanced in the presence of transition-metal ions [32], and certain metal-ion chelators, as well as HO$^{\cdot}$ scavengers, which can diffuse through a plasma membrane, protect cells from intracellular damage induced by H_2O_2 [32,47,52]. This suggests that intracellular metal-ion-catalysed production of HO$^{\cdot}$ radicals by the Fenton reaction may be responsible for H_2O_2 cytotoxicity. Further work is required to clarify the mechanism of H_2O_2 cytotoxicity and its relevance to conditions *in vivo*.

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