The radiosensitivity of rat thymocytes

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γ -Irradiation *in vitro* apparently blocked a plasma-membrane associated, superoxide-producing, NADPH oxidase in rat thymocytes. Differential centrifugation of the mixed thymocytes indicated the smaller lymphocytes (approx. 6 μ m diameter) to be the radiosensitive population. The oxidase system co-isolated in part with thymus nuclei and could be solubilized by detergent treatment [Bellavite, Jones, Cross, Papini & Rossi (1984) *Biochem. J.* 223, 639–648]. Endogenous NADPH was the rate-limiting component for superoxide formation *in vitro*. The level of NADPH was lowered by γ -irradiation, an effect mimicked by GSSG in the presence of 50 μ M-ZnCl₂ to inhibit GSSG reductase. These findings are suggested as the metabolic basis for interphase death of small lymphocytes exposed to ionizing radiation.

INTRODUCTION

The extreme radiosensitivity of some classes of small lymphocytes is well-known; its basis is however still uncertain (Anderson & Standefer, 1983). In the late 1950s, nuclei isolated from various lymphoid tissues were found to contain firmly bound nucleoside phosphates and to generate ATP by an O₂-dependent reaction which was not due to mitochondrial contamination (Osawa *et al.*, 1957). This process was immediately blocked by exposure to 1–2 Gy of ionizing radiation *in vivo* or *in vitro* (Creasey & Stocken, 1959). The biochemistry of the reaction was unclear. Since mitochondrial O₂ uptake and total cellular ATP in lymphoid tissue cells are initially unaffected by low doses of γ -rays, the relevance of the radiosensitivity of nuclear phosphorylation to interphase death of some types of lymphocyte remained controversial, although ATP contents of thymus nuclei from rats exposed to 4 Gy were only 60% of control levels at 1 h after irradiation (Fonagy *et al.*, 1977).

The discovery of an antimycin- and cyanide-insensitive NADPH oxidase in the plasma membrane of various white cell types (Goldstein *et al.*, 1977; Babior *et al.*, 1981; Fantone & Ward, 1982) including rat thymocytes (Kolbuch-Braddon *et al.*, 1984), suggested that the presence of this enzyme system, fortuitously or otherwise, in nuclear preparations might have accounted for the O₂ uptake observed earlier. Consistent with this view, the present paper shows that a plasma-membrane-associated NADPH oxidase in rat thymocytes is inhibited by ionizing radiation at levels of exposure which are lethal for small lymphocytes. The inhibition is indirect, arising from radiochemically-induced oxidation of GSH which diminishes the amount of NADPH available as substrate for the system.

METHODS

Cells and cell fractions

Thymocytes were obtained from male rats of a Wistar strain (100–200 g body wt.) (Kolbuch-Braddon *et al.*, 1984) and suspended in phosphate-buffered saline, pH 7.4 (Cohen *et al.*, 1980), containing 5 mM-glucose. Preliminary experiments showed that Ca²⁺ detectably but not significantly increased O₂ uptake; 0.5 mM-Ca²⁺ was therefore normally present in the buffered saline (cf.

Sparkman *et al.*, 1985). After being allowed to stand for 10 min at 20 °C the cell suspensions were filtered to remove aggregated cells (so-called 'rosettes'; Wroegemann *et al.*, 1980) and counted; 10⁷–3 × 10⁸ cells were used in the various assays. The integrity of the cells was confirmed by Trypan Blue exclusion.

Thymocyte populations were separated either by sedimentation at 1 g at room temperature (10 ml of standard suspension was stood for 0.5 h in a 10 ml cylinder, 1 cm diameter, after which the upper 4–5 ml was removed) or by differential centrifugation over Percoll (ρ = 1.129 g/ml) gradients (Nishimura *et al.*, 1984).

Macrophages were obtained from peritoneal exudates induced in rats by intraperitoneal injections of glycogen (Stuart *et al.*, 1978).

Thymus nuclei used in experiments with detergent were sedimented from iso-osmotic sucrose (0.25 M-sucrose/50 mM-Tris/HCl, pH 7.4, containing 10 mM-MgCl₂) at 800 g for 10 min and resuspended in 50 mM-Tris/HCl, pH 7.4, containing 10 mM-MgCl₂. The suspension was made 0.4% (w/v) in deoxycholate and Lubrol-PX (Bellavite *et al.*, 1984) and the precipitated nucleoprotein was removed by sedimentation or filtration through Nylex.

Unless otherwise stated, cells were irradiated in air at 0 °C or 20 °C with 4 Gy (Cs source; dose rate 0.8 Gy/min) and were examined 0.25–2.0 h after exposure.

Oxygen uptake and enzyme assays

O₂ uptake was recorded with a Clark-type electrode vessel (Wood, 1978) of 2.1 ml capacity, at 37 °C. Superoxide production was followed in a double-beam spectrophotometer at 25 °C or 30 °C by the reduction of cytochrome *c* (Cohen *et al.*, 1980) in the presence or absence of superoxide dismutase (Sigma). Cyanide was present to block mitochondrial oxidation.

Antimycin was added to assay systems in ethanolic solution (10 μ l in a final volume of 2.0 or 0.5 ml). Additions of the diluent alone did not affect the assays.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glutathione reductase (EC 1.6.4.2) were measured by change of absorption at 340 nm in a double beam spectrophotometer in a final volume of 0.5 ml at 25 °C.

The reactions were started by the addition of glucose 6-phosphate or GSSG (final concentrations 0.5 or 1.0 mM; NADP or NADPH 0.5 mM). When the supernatant from detergent-treated nuclei was assayed, KCN (1 mM) and antimycin (0.8 μ M) were present in both cuvettes.

RESULTS

Oxidase activity in normal and irradiated rat thymocytes

Oxidase is measured in a number of ways: by chemiluminescence (Hume *et al.*, 1981; Kolbuch-Braddon *et al.*, 1984) or superoxide production, or, less sensitively, by antimycin-resistant O_2 uptake (Kolbuch-Braddon *et al.*, 1984). Because of its simplicity, this latter method was used initially. With $(1-2) \times 10^8$ cells in the electrode vessel, 0.2 μ M-antimycin reproducibly diminished O_2 uptake of the mixed population of thymocytes to about 9% of the total uptake (Table 1). With cell numbers outside this range, concentrations of antimycin had to be adjusted. Antimycin-resistant O_2 uptake was 40% lower in cells which had received 4 Gy, with no detectable effect on total O_2 uptake. The response to radiation was however variable (Table 1).

More sensitive and selective estimation of NADPH oxidase is available by measuring superoxide production. Cytochrome *c* is largely impermeant to cells, so restricting the reaction in intact thymocytes to cell membranes. Superoxide production was diminished to an extent similar to that with antimycin-resistant O_2 uptake but the rate of reaction was 20-fold faster [88 nmol of cytochrome *c* reduced/min (Table 2) versus 1.9 nmol of O_2 uptake/min (Table 1)]. The extent of the reaction appeared to be limited by the availability of endogenous substrate, which in control suspensions allowed 130 nmol of cytochrome *c* to be reduced/ 10^8 cells at 25 °C. This was lowered by 30–40% in the irradiated cells.

To enhance the effects of irradiation, glucose metabolism through the glycolytic pathway was restricted by preincubation with iodoacetamide, thus forcing glucose oxidation through the pentose phosphate route. At all concentrations of iodoacetamide tested total O_2 uptake

Table 2. Effects of γ -irradiation on superoxide production in rat thymocytes

Cells (approx. 10^7) were exposed to 4 Gy and assayed 0.5–1.5 h after irradiation. Control activities are expressed as nmol/min per 10^8 cells $\pm$ S.E.M. ($n \leq 3$). Cells were separated and superoxide production measured as described in the Methods section. The Percoll-separated cells were those from the 50%/60% Percoll interface.

Preparation	Superoxide production	
	Control (nmol/min per 10^8 cells)	Irradiated (% of control)
Mixed thymocytes		
30 °C	88 $\pm$ 15	66.5 $\pm$ 2.7
25 °C	71 $\pm$ 12	74.0 $\pm$ 8.0
Separated cells		
Thymocytes sedimented at 1 g (30 °C)		
Top	92	82
Bottom	116	100
Percoll-separated thymocytes (30 °C)	68	20 $\pm$ 16
Macrophages (25 °C)	151	100

was detectably more inhibited in cells exposed to 4 Gy than in unirradiated cells (Fig. 1a). Preincubation in 4 mM-iodoacetamide reduced O_2 uptake in control cells by 40%, but because the pentose phosphate pathway is relatively unaffected by the inhibitor, the proportion of antimycin-resistant O_2 uptake doubled ($\times 2.6$, $\times 2.0$). This enhancement in the proportion of O_2 uptake which was resistant to antimycin was very sensitive to irradiation and was almost completely prevented by 4 Gy (Fig. 1b). The dose-response curve was superimposable on that found for $^{32}P_i$ incorporated into rat thymus DNA and for $^{32}P_i$ uptake into histone H1 after X-irradiation *in vivo* (Ord & Stocken, 1958, 1966).

Experiments were also performed using fluoride to block glycolysis. Preincubating the cells with fluoride increased their antimycin-insensitive O_2 uptake and

Table 1. Effects of γ -irradiation *in vitro* on O_2 uptake by rat thymocytes

Cells $[(1-2) \times 10^8/\text{assay}]$ were irradiated at ambient temperature with 4 Gy. O_2 uptake was measured at 37 °C 0.5–2.0 h after exposure and is expressed as nmol/min per 10^8 cells in controls. Final concentration of antimycin was 0.2 μ M. Aggregation and sedimentation of the thymocytes are described in the text. Macrophages were obtained from peritoneal exudates induced by injecting glycogen intraperitoneally. Values are means $\pm$ S.E.M. with the numbers of experiments given in parentheses, or actual values of single or duplicate experiments.

Preparation	O_2 uptake			
	Control (nmol/min per 10^8 cells)		Irradiated (% of control)	
	Total	+ Antimycin	Total	+ Antimycin
Mixed thymocytes	21.8 $\pm$ 3.6 (13)	1.9 $\pm$ 0.05 (13)	103 $\pm$ 14 (5)	60 $\pm$ 26 (7)
Thymocytes after aggregation at 37 °C	29.3 $\pm$ 1.3 (3)	2.7 $\pm$ 0.75 (3)	99 $\pm$ 5.4 (3)	100 $\pm$ 0 (3)
Thymocytes sedimented at 1 g				
Top	19.1, 16.2	4.6, 2.0	86, 92	65, 74
Bottom	23.7, 25.3	2.8, 2.5	100, 101	100, 96
Macrophages	65	19.3	98	116

Fig. 1. Effects of irradiation on iodoacetamide-treated rat thymocytes

(a) Basal O₂ uptake (% of that in control cells without iodoacetamide) in cells incubated with increasing concentrations of iodoacetamide. The abscissa shows the concentration of iodoacetamide in which cells were incubated for 5 min before O₂ uptake was measured. (The final concentration during measurement was one-half of this.) ○, Control cells; ●, cells exposed to 4 Gy. Results are the means of two or three experiments with each concentration of iodoacetamide. (b) Iodoacetamide-enhanced antimycin-insensitive O₂ uptake relative to that in unirradiated cells. Cells (1.5×10^8) were incubated for 5 min in 4 mM-iodoacetamide, as in (a). ○, O₂ uptake; ●, enhancement in antimycin-insensitive O₂ uptake after incubation with 4 mM-iodoacetamide (enhancement in unirradiated cells is taken as 100). Antimycin was present at 0.2 μ M.

superoxide production (Table 3). If the suspensions were made 10 μ M in Zn²⁺ to inhibit glutathione reductase (Ord & Stocken, 1963; Eggleston & Krebs, 1974) slight stimulation in oxidase activity was detected, consistent with some utilization of NADPH by glutathione reductase in control cells. The presence of Zn during the irradiation enhanced radiosensitivity with or without fluoride (Table 3).

Glucose utilization *in vitro* by the pentose phosphate pathway

The increased radiosensitivity of the oxidase system as glucose metabolism was forced into the pentose

phosphate pathway by iodoacetamide prompted direct estimation of the effect of 4 Gy *in vitro* on [1-¹⁴C]glucose oxidation in the presence of 0.2 μ M-antimycin. At 37 °C the reaction was linear for at least 1.5 h. Neither ¹⁴CO₂ production nor the associated ³²P_i uptake was affected by exposure, but the rate of ¹⁴CO₂ release (0.12 ± 0.04 nmol/min per 10^8 cells at 37 °C) was less than 10% of the rate of antimycin-resistant O₂ uptake (Table 1). Rates of glucose utilization *in vivo* in the presence of agents possibly promoting its uptake are unknown. The lowered glutathione content of cultured thymocytes compared with freshly prepared cells and the marked rise in GSSG/GSH ratio (Lacombe *et al.*, 1985) indicates a change in their normal metabolism and suggests that NADPH availability *in vitro* is diminished.

Table 3. Effect of fluoride and Zn²⁺ on the radiosensitivity of the superoxide-generating oxidase system in rat thymocytes

Thymocyte suspensions were exposed to 4 Gy in the presence or absence of 10 μ M-ZnCl₂. Antimycin-resistant O₂ uptake (final concentration of antimycin 0.2 μ M) or superoxide production was measured in cells which had or had not been preincubated with NaF (1 or 4* mM) for 5 min. Activity in control cells taken as 100. Control rate of antimycin-resistant O₂ uptake at 37 °C was 1.7 nmol/min per 10^8 cells; that of superoxide production at 25 °C was 87 nmol of cytochrome *c* reduced/min per 10^8 cells.

Cells	Antimycin-resistant O ₂ uptake		Superoxide production	
	Control	+ Zn ²⁺	Control	+ Zn ²⁺
Normal	100	108	100	108
+ 4 Gy	80	65	88	76
+ NaF	150	157	153*	238*
+ NaF + 4 Gy	135	120	—	148*

Irradiation of separated populations from thymocytes

Thymocyte preparations contain a mixed population of cells including small numbers of radio-resistant erythrocytes and fibroblasts and a few per cent macrophages. The majority of cells are lymphocytes, between 80–90% of which are located in the cortex. Classical radiobiology established that the highly radiosensitive population was the non-proliferating small cortical lymphocytes, most of which are normally destined to die *in situ* in the thymus (see Anderson & Standefer, 1983).

In preliminary attempts to increase the reproducibility of the irradiation response, some preparations of thymocytes were allowed to aggregate at 37 °C instead of 20 °C, prior to filtering and measuring O₂ uptakes. A prominent gelatinous coagulum formed, but cells in the filtrate were radio-insensitive (Table 1). If the thymocyte preparations were stood at 20 °C for 0.5 h, some separation of radio-sensitive thymocytes occurred into the less rapidly sedimenting population of cells (Tables 1 and 2). These cells had a slightly lower total O₂ uptake

which was further diminished by irradiation (Table 1). Differential centrifugation through Percoll gradients effected a separation of radio-sensitive cells (Table 2) which showed a 3-fold increase in sensitivity. The affected cells at the 50/60% Percoll interface (approx. 70% of those recovered) corresponded to fraction 2 of Nishimura *et al.* (1984), which were positive for Thy 1.2, Ly 2.2 and the peanut agglutinin receptor.

Further observations confirmed selective radio-sensitivity within the mixed thymocyte population. Macrophages relatively free from small lymphocytes were obtained from peritoneal exudates. Antimycin-resistant O_2 uptake and cytochrome *c* reduction in these cells were unaffected by 4 Gy *in vitro* (Tables 1 and 2).

When size distribution in the various populations was examined, the increased radiosensitivity correlated with the presence of an increased proportion of small thymocytes (6–7 μ m diameter).

Fig. 2. Effect of 4 Gy of ionizing irradiation on superoxide production by supernatants from detergent-treated thymus nuclei

Solubilized preparations from thymus nuclei were obtained as described in the Methods section and were kept at 0 °C until assay. Superoxide production at 25 °C was followed spectrophotometrically at 550 nm by the reduction of cytochrome *c* in the presence of KCN (1 mM) and antimycin (0.8 μ M). The final volume in cuvette was 0.5 ml. The reaction was initiated by the addition of cytochrome *c*. After completion of the reaction, NADPH was added to both cuvettes and cytochrome *c* reduction was followed for a further 1–2 min. Arrows show the times at which NADPH was added. (a) Spectrophotometric traces from control (—) and irradiated (4 Gy; ---) preparations (200 μ l sample, equivalent to 1.92×10^8 cells). NADPH was added to 0.5 mM. (b) Spectrophotometric traces from control preparations (—), control preparations that had been made 50 μ M in $ZnCl_2$ (---) and preparations that had received 4 Gy + Zn^{2+} (----). The sample was 200 μ l, equivalent to 0.87×10^8 cells; NADPH was added to 0.1 mM. Traces from the irradiated samples $\pm$ Zn were superimposable.

The substrate for the plasma-membrane-associated oxidase

In neutrophils and macrophages both NADH and NADPH can be utilized by the superoxide-producing system. K_m and other studies suggest (Gabig *et al.*, 1984) that NADPH may be the physiological substrate. The greater amount of antimycin-resistant O_2 uptake in unirradiated fluoride- or iodoacetamide-treated thymocytes would be predicted if NADPH was a substrate.

This was confirmed directly. Thymocyte nuclei were sedimented out of iso-osmotic sucrose medium at 800 g, resuspended and brought to 0.4% (w/v) in deoxycholate and Lubrol (Bellavite *et al.*, 1984); 30% of the superoxide producing activity was released into the supernatant. The activity was unaffected by added glucose or glucose 6-phosphate but NADPH markedly stimulated the reaction (Figs. 2a and 2b); NADH at the same concentration (0.5 mM) produced only 10% of the effect.

When the supernatants from the detergent-treated nuclei were irradiated with 4 Gy, the rate of superoxide production was diminished compared with that by controls (Figs. 2a and 2b); the total production of superoxide was also lowered (Figs. 2a and 2b). Rates with added NADPH were unaffected by exposure (Figs. 2a and 2b), showing that the oxidase itself was not affected by ionizing radiation.

Fig. 3. Thiol/disulphide interactions with NADPH oxidase in supernatants from detergent-treated thymus nuclei

For experimental details see Fig. 2. Arrows show the times at which NADPH was added. (a) Spectrophotometric traces from control preparations (—) and from those in which GSH (0.5 mM) (---) or GSSG (0.5 mM) (----) was present at the start of the reaction (100 μ l sample, equivalent to 0.98×10^8 cells). NADPH was added to 0.5 mM. (b) Spectrophotometric traces from control preparations (—) and from those containing $ZnCl_2$ (50 μ M) (---) or $ZnCl_2$ + GSSG (0.5 mM) (----) (200 μ l sample, equivalent to 1.21×10^8 cells); NADPH was added to 0.1 mM.

Glucose-6-phosphate dehydrogenase and thiol/disulphide involvement in the NADPH oxidase system

The enhancing effect of Zn^{2+} on the radiosensitivity of the oxidase system (Table 3) was provisionally ascribed to the inhibition of GSSG reductase and was seen also with the supernatant from the detergent-treated nuclei (Fig. 2b). GSSG reductase and glucose-6-phosphate dehydrogenase were both active in supernatants from detergent-treated nuclei. GSSG diminished the availability of the endogenous substrate for NADPH oxidase (Fig. 3a) even in the presence of $50 \mu\text{M-Zn}^{2+}$ (Fig. 3b), which inhibits GSSG reductase and substantially increased the activity of the oxidase (Fig. 3b). GSSG has been reported to affect the activity of glucose-6-phosphate dehydrogenase directly and when glutathione reductase is inhibited (Eggleston & Krebs, 1974). Since the presence of Zn^{2+} interferes with spectrophotometric assays of glucose-6-phosphate dehydrogenase, $2 \mu\text{M-HgCl}_2$ was also used to inhibit GSSG reductase. Glucose-6-phosphate dehydrogenase activity in the presence of $2 \mu\text{M-HgCl}_2$ was reduced by 55% in supernatants exposed to 4 Gy, confirming the apparent radiosensitivity of the enzyme (Deakin *et al.*, 1963).

DISCUSSION

The selective radiosensitivity of the oxidase system in small lymphocytes indicates that interference in NADPH availability only occurs in this class of lymphoid cells. Radiochemical oxidation of thiol groups is a general phenomenon (see Ord & Stocken, 1963); in nuclei from thymus, 10 Gy oxidized 0.4 mM of -SH to S-S. Glucose-6-phosphate dehydrogenase activity is also critically dependent on the concentrations of GSSG and the NADP/NADPH ratio and the operation of the glutathione reductase system (Eggleston & Krebs, 1974; Rodriguez-Segade *et al.*, 1985; Levy, 1985). Under conditions when GSSG reductase was thought to be inoperative, $50 \mu\text{M-GSSG}$ lowered the activity of glucose-6-phosphate dehydrogenase by 30% (Eggleston & Krebs, 1974). In the systems used here, availability of NADPH was limiting for superoxide production. Cytoplasmic metabolism in small lymphocytes is low with minimal reductive syntheses. NADPH reoxidation in these cells is therefore likely to be linked to the oxidase system, so that glucose 6-phosphate metabolism by the pentose-phosphate route will be facilitated and lead to antimycin-resistant O_2 uptake and ATP generation.

Barron (1946) was one of the first to suggest radiochemical oxidation of thiol groups as a contributant to the metabolic effects of ionizing irradiation. This may be especially important in interphase death of small lymphocytes. Results reported here suggest the effects of the oxidation are two-fold. Firstly kinetic, consequent upon the utilization of NADPH by glutathione reductase to restore the thiol/disulphide balance. This is seen in the apparent effects of irradiation on superoxide production, which is in fact limited by the availability of NADPH. In the preparations derived from nuclei no glucose or glucose 6-phosphate were added, from which NADPH could be restored. In the thymocyte suspensions the slow uptake of glucose would also prevent maintenance of glucose 6-phosphate levels.

The effects of irradiation on superoxide production were even more apparent when glutathione reductase

was inhibited, and could be mimicked by GSSG in the presence of $50 \mu\text{M-ZnCl}_2$. This suggests that radiochemical oxidation of thiol groups in the system also influenced glucose-6-phosphate dehydrogenase activity directly and further diminished the functioning of the pentose-phosphate pathway in these cells.

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