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Cytotoxic and mutagenic effects of hydrogen peroxide in murine L5178Y sublines

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Hydrogen peroxide belongs to natural (i.e. nonxenobiotic) oxidants that cause cellular stress. It can be considered as a physiological regulatory factor, because it is generated by macrophages and, similarly as other "active oxygen" species, stimulates growth during wound healing, epithelial regeneration and fibrosis [1, 2].

Mammalian cells generate H_2O_2 as a result of dismutation of the superoxide anion radical and in enzymatic (oxidase-catalyzed) reactions. H_2O_2 easily passes the cell membranes and causes DNA breaking [3 - 6]. Genotoxicity of H_2O_2 is due to the product of the Haber-Weiss reaction (proceeding in the presence of transition metal ions), the highly reactive $^{\cdot}OH$ radical [5]. Its reaction with DNA generates single and double strand breaks [3 - 6] and thymidine glycol residues [7].

Ward *et al.* [3] suggest that the cyto- and genotoxic effects of H_2O_2 are due to locally multiply damaged sites. They found that H_2O_2 was much less cytotoxic at 0°C than at higher (24° - 37°C) temperatures [8]; a similar observation has been made by Jonas *et al.* [9].

Effect of H_2O_2 treatment on LY cell proliferation

LY-S[1] cells were found to be clearly more resistant to H_2O_2 treatment than LY-R cells; furthermore, the lethal effect of the treatment was higher at 37°C than at 4°C. Lethally damaged cells usually were scored as non-viable in the treated cell populations during the first and second day after treatment (Fig. 1); delayed appearance of dead cells took place only in LY-S cell culture after treatment with 10^{-5} M H_2O_2 at 37°C (Fig. 1 B). Growth stimulation was observed in LY-S cell cultures in response to 10^{-7} M and 10^{-6} M H_2O_2 treatment at 4°C. It has been suggested that H_2O_2 treatment gives rise to a mitogenic signal [1, 2] directly, through activation of protein kinase C, or indirectly, through a path that involves DNA damage. At lower H_2O_2 concentrations this mitogenic effect is not masked by the lethal effect.

Cytotoxic and mutagenic effects of H_2O_2

Jonas *et al.* [9] described the sparing effect of low (0°C) temperature of H_2O_2 treatment; when the CNCM I-22 cells were pre-incubated with ascorbate, the subsequent exposure to H_2O_2 at low temperature gave effects similar to those at 37°C, whereas on pre-treatment with ascorbate and an iron chelator, desferrioxamine, the previously observed sparing effect became apparent again.

This observation is consistent with the assumption that H_2O_2 toxicity is caused by $^{\cdot}OH$ radicals generated at sites containing reduced metal ions, whereas the temperature effect depends on the availability of the ions. At temperatures that inhibit cellular metabolism, chromatin-bound transition metal ions do not undergo reduction because the reducing equivalents are not produced. Upon oxidation, metal ions do not catalyze H_2O_2 reduction to $^{\cdot}OH$ and thus, DNA lesions are not generated.

The pronounced sparing effect of low temperature in LY-S cells points to an efficient generation of reducing equivalents at 37°C.

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[1] Abbreviations: HGPRT, hypoxanthine:guanine phosphoribosyl transferase; LY-S and LY-R, murine leukaemic lymphoblast strain L5178Y-S and L5178Y-R, respectively

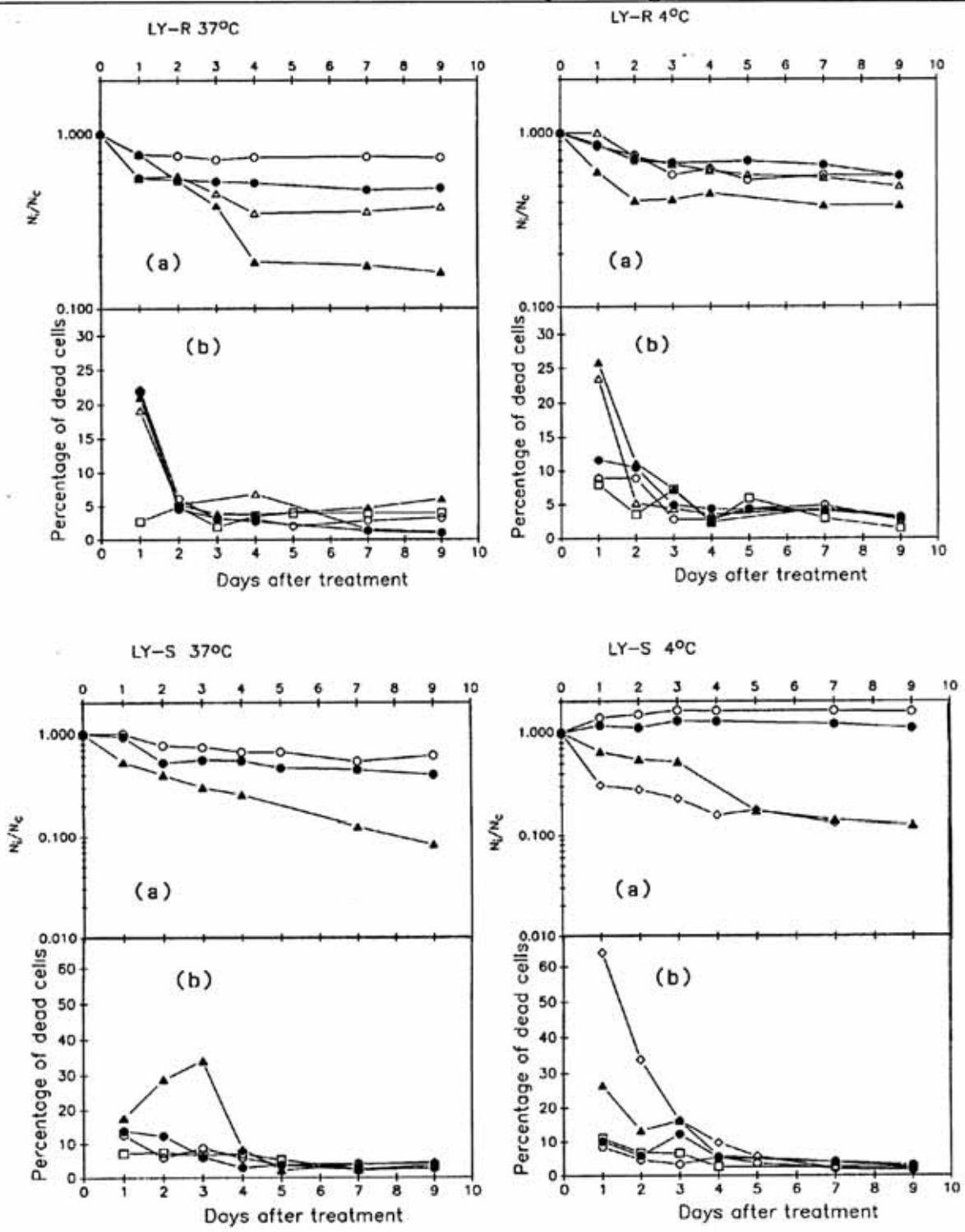

Fig. 1. Growth kinetics (a) and viability (b) observed for the L5178Y-R (A) and L5178Y-S (B) cell strains treated with H_2O_2 at 4°C and 37°C.

Murine leukaemic lymphoblasts LY-R and LY-S were grown in suspension cultures in Fischer's medium supplemented with 8% bovine serum. Cell cultures were diluted every second day to maintain them in the logarithmic growth phase during 9 days after H_2O_2 treatment; cell numbers were counted and % of dead cells estimated from the nigrosine exclusion test [% of dead cells = (number of stained cells/total cell number) × 100]. The results (mean values from 2-3 experiments) were expressed as relative cell numbers, N_t:N_c (N_t – number of living cells in the treated cell population, N_c – number of living cells in the control one, grown from the same initial cell density, 10^5 cells/ml). Concentrations of H_2O_2 were: 10^{-7} M (○), 10^{-6} M (●), 5×10^{-6} M (△), 10^{-5} M (▲), 10^{-4} M (◇). Control (□)

The ratio of concentrations at which under treatment conditions survival of the LY-S cell population is reduced to 37% of the untreated control (survival = 100%), $D_{37}(4^{\circ}C)/D_{37}(37^{\circ}C)$ is equal to 12.5 (see Table 1).

In contrast, for LY-R cells this metabolic aspect of H_2O_2 action seems to be less important: $D_{37}(4^{\circ}C)/D_{37}(37^{\circ}C)$ is equal to 2.33. The higher resistance of LY-S cells upon exposure to H_2O_2 is consistent with the higher activity of catalase in these cells [10].

Mutation frequencies, induced at the concentration of H_2O_2 corresponding to D_{37} survival level were the same in LY-R cells at either temperature of treatment and similar to that in LY-S cells H_2O_2-treated at 37°C (Table 1). In contrast, mutation frequencies were higher in LY-S cells treated at 4°C, indicating a different relation between pre-mutational and lethal lesions generated at this temperature. Since DNA double strand breaks induced by H_2O_2 treatment seem to constitute the critical DNA lesions [4], the possible explanation of this observation should take into account the impairment of double strand break repair in LY-S cells [12].

Evans *et al.* [13] characterized mutagenic effects of UV and X radiations, as well as alkylating agents in various LY strains at hypoxanthine:guanine phosphoribosyl transferase (HGPRT), thymidine kinase and Na^+/K^+ ATPase loci. LY-S are more sensitive to X rays and alkylating agents than LY-R cells; on the contrary, they are less sensitive to UVC radiation and hydrogen peroxide in comparison to LY-R cells. Notwithstanding the relative sensitivity, LY-S cells were found to be uniformly less mutable than LY-R cells, as previously found for the original LY strains [14]. Mutants scored per 10^5 survivors at 37% survival were the following: LY-R cells, 60 (UVC radiation) and 3 (X rays); LY-S cells, 0.45 (UVC radiation) and 0.61 (X rays) [14].

Explanation of the hypomutability of LY-S cells, supported by the results of Evans *et al.* [13], is that, due to DNA repair deficiency, multilocus lesions are formed in the DNA of LY-S cells upon X-irradiation or treatment with alkylating agents; these lesions lead to low recovery of viable mutants when the examined *locus* is situated in the vicinity of essential genes, as in the case of HGPRT. The described by Evans *et al.* [13] pattern of mutagenic effects of DNA damaging agents in LY-R and LY-S cells is in contrast with the results obtained upon treatment with H_2O_2 under conditions that reduce survival to 37%. At 4°C LY-S mutants were about twice more frequent than LY-R mutants at the same survival level (37%), whereas at 37°C the frequency was the same as in LY-R cells. Reversing the reasoning of Evans *et al.* [13] we can conclude that the applied H_2O_2 treatment induced relatively fewer lethal mutations because multilocus lesions were less numerous; thus, non-lethal mutations could be expressed at higher frequency.

There is no reason to assume that DNA double strand breaks differ qualitatively in X-irradiated or H_2O_2 treated cells; in both cases $^{\bullet}OH$ radical is the most important damaging species. However, a difference in damage localization can be assumed. Radiolysis of ubiquitous water would generate random damage, whereas Fenton reaction would be site-specific:

Table 1

Mutation frequencies in L5178Y cells treated at 4°C or 37°C with H_2O_2 at concentrations corresponding to 0.37 surviving fractions (D_{37})

Mutation frequency in HGPRT locus was determined according to Knaap & Simons [10]. After 7-day expression period cells were plated in soft agar medium supplemented with 5 μg/ml 6-thioguanine as the selecting factor. According to the expected mutation frequencies of LY-R and LY-S cells, 2×10^6 and 3×10^6 cells per Petri dish (10 cm diameter) were plated, respectively; in parallel, the same cells were plated in non-selecting medium on 5 cm diameter Petri dishes, in order to determine plating efficiency. Spontaneous mutation frequencies were < 0.9 and < 0.04 per 10^5 survivors for LY-R and LY-S cell strains, respectively

	L5178Y-R		L5178Y-S	
	H_2O_2 (μM)	Mutation frequency per 10^5 survivors	H_2O_2 (μM)	Mutation frequency per 10^5 survivors
4°C	0.7	2.4 ± 0.4	5.0	4.1 ± 0.6
37°C	0.3	2.2 ± 0.5	0.4	2.6 ± 0.5

metal ions, at least copper, are involved in DNA-nuclear matrix interactions. Such DNA double breaks situated near the nuclear matrix could be preferentially repaired [15]. Hence, a lower proportion of lethal mutations, possibly multilocus deletions or multilocus mutations, can be expected as a result of the treatment at the applied H_2O_2 concentration, in contrast to mutations induced in these cells by X rays, UV radiation and alkylating agents [13, 14].

In summary, the relative sensitivity pattern of LY sublines is dependent on differences in "protecting enzymes", extent of the Fenton reaction and in repair abilities. The relative contributions of these factors remain to be established and confirmed by analysis of the initial DNA damage and repair and determination of iron ions.

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