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Review

The response of L5178Y lymphoma sublines to oxidative stress: Antioxidant defence, iron content and nuclear translocation of the p65 subunit of NF-κB★✪

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We examined the response to hydrogen peroxide of two L5178Y (LY) sublines which are inversely cross-sensitive to hydrogen peroxide and X-rays: LY-R cells are radioresistant and hydrogen peroxide-sensitive, whereas LY-S cells are radiosensitive and hydrogen peroxide-resistant. Higher initial DNA breaks and higher iron content (potentially active in the Fenton reaction) were found in the hydrogen peroxide sensitive LY-R cells than in the hydrogen peroxide resistant LY-S cells, whereas the antioxidant defence of LY-R cells was weaker. In particular, catalase activity is twofold higher in LY-S than in LY-R cells. The content of monobromobimane-reactive thiols is 54% higher in LY-S than in LY-R cells. In contrast, the activity of glutathione peroxidase (GPx) is about two times higher in LY-R than in LY-S cells; however, upon induction with selenium the activity increases 15.6-fold in LY-R cells and 50.3-fold in LY-S cells.

Altogether, the sensitivity difference is related to the iron content, the amount of the initial DNA damage, as well as to the efficiency of the antioxidant defence system. Dif-

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Abbreviations: AMT, 3-amino-1,2,4-triazole; Cis-P, *cis*-parinaric acid; FLUO, 2,7-dichlorodihydrofluorescein acetate; GPx, glutathione peroxidase; GSH reduced glutathione, GSSG, oxidised glutathione, MBB, monobromobimane; NAC, *N*-acetylcysteine; PMA, 4α-phorbol-12-myristate-13-acetate; ROS, reactive oxygen species, SOD, superoxide dismutase.

ferential nuclear translocation of p65-NF-κB in LY sublines is due to the more efficient antioxidant defence in LY-S than in LY-R cells.

This paper reviews a several years' work on the response to hydrogen peroxide of two L5178Y (LY) sublines, originally described by Alexander & Mikulski (1961). These sublines are inversely cross-sensitive to hydrogen peroxide and X-rays: LY-R cells are radioresistant and oxidant-sensitive, whereas LY-S cells are radiosensitive and oxidant-resistant (Beer *et al.,* 1983). Since with both damaging agents the main damaging species is the highly reactive hydroxyl radical, this difference in sensitivity presented a highly interesting problem.

Figure 1 shows survival curves after exposure to X-rays and hydrogen peroxide (1 h

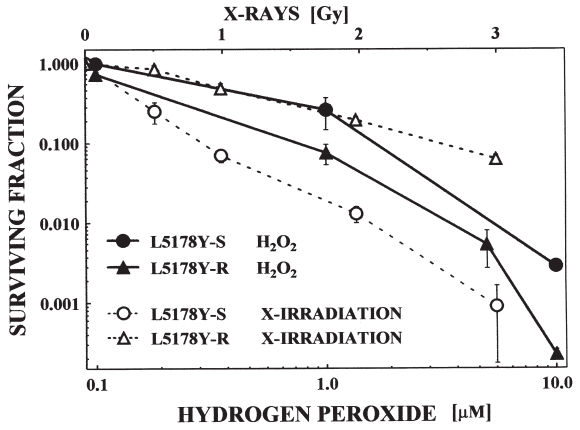

Figure 1. Survival curves for X-irradiated or hydrogen peroxide-treated (1 h, 37°C) LY-R and LY-S cells.

Cloning in soft agar was repeated at least 3 times. Mean results ±S.D. (from M. Kruszewski, PhD Thesis).

treatment at 37°C). The main reason for the high radiation sensitivity of LY-S cells is a deficiency in DNA double strand break (DSB) repair (Wlodek & Hittelman, 1988a; 1988b), apparently caused by impaired non-homologous end-joining (NHEJ) (Kruszewski *et al.,* 1998), whereas homologous recombination repair is functional (Grądzka *et al.,* 1998). Both L5178Y sublines are heterozygous for a Tp53 mutation (TGC → CGC; codon 170) (Storer *et al.,* 1997). Mutation at this site impairs the function of the Tp53 DNA binding domain and thus, indirectly, its transactivating capacity.

CHARACTERISTIC FEATURES OF THE CELLULAR RESPONSE TO HYDROGEN PEROXIDE

The diagram shown in Fig. 2 presents the main features of the cellular response to hydrogen peroxide. Hydrogen peroxide itself is not highly cytotoxic, however, in the presence of iron ions it undergoes the Fenton reaction that generates the highly reactive and damaging hydroxyl radicals. The final effect of hydrogen peroxide treatment depends on the extent of this reaction, the ferrous ions available, and the supply of reducing equivalents from the ongoing metabolism that allow the recycling of iron ions. As shown in the diagram, the enzymes of the antioxidant defence may further modulate the effect. Also, high efficiency of DNA damage repair contributes to the lowering of the cellular lethality following hydrogen peroxide treatment.

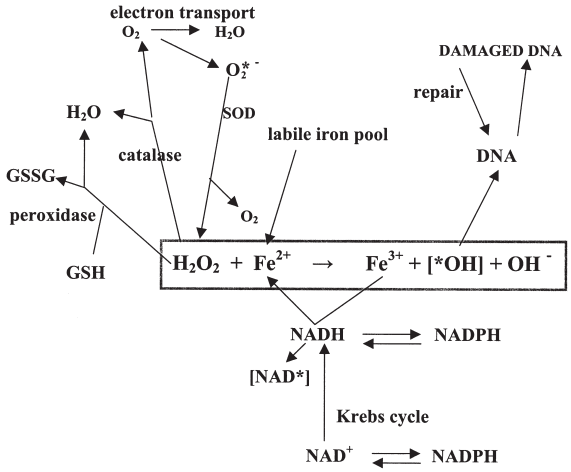

Figure 2. Main features of the response of a mammalian cell to hydrogen peroxide treatment.

Fenton reaction is framed.

The most important features of the cellular response to hydrogen peroxide that were examined in the pair of LY sublines are presented in Table 1. We compared the effects of treatment at 37°C and 0°C, using several end-points: clonogenic ability, DNA damage (estimated by the single cell gel electrophoresis; comet assay) and mutation frequency. (Kruszewski & Szumiel, 1993; 1994; Kruszewski *et al.,* 1994). It can be seen that the temperature effect (in other words, the effect of the metabolic activity) is lower in LY-R (sensitive) than in LY-S (resistant) cells. Even without the supply of reducing equivalents the damage to DNA is very high in LY-R cells. A more detailed examination of DNA lesions (Fig. 3) shows that both single strand breaks (SSB) and base damage is higher in the more sensitive LY-R cells. This may be taken as an indication of a differential availability of iron ions in the vicinity of DNA.

Table 1. Effects of hydrogen peroxide treatment (1 μM, 1 h) of LY cells at 0°C and 37°C

End-point examined	LY-R	LY-S
Survival 0°C	0.25 ± 0.02	0.63 ± 0.05
Survival 37°C	0.07 ± 0.01	0.26 ± 0.08
Initial DNA damage 0°C (tail moment)	14.4 ± 1.4	8.1 ± 0.9
Initial DNA damage 37°C (tail moment)	17.0 ± 1.4	13.3 ± 1.7
Mutation frequency per 10^5 survivors at *hgprt* locus, 0°C	2.4 ± 0.6	1.8 ± 0.1
Mutation frequency per 10^5 survivors at *hgprt* locus, 37°C	2.1 ± 0.3	3.4 ± 0.3
Overall temperature effect	low	high

Data from Kruszewski *et al.,* 1994

DIFFERENCE IN IRON CONTENT IN LY SUBLINES

Iron was estimated in the nuclei of LY cells by atomic absorption spectrometry (Szumiel *et al.,* 1995) and the labile iron pool (available to chelators) with calcein (Lipiński *et al.,* 2000). As shown in Table 2, the expectation concerning the iron content in LY sublines was justified. Consistent with this result was a higher protective effect of iron chelator, desferroxamine, in LY-R than in LY-S cells (Kruszewski *et al.,* 1995).

The main proteins that control the cellular level of iron are transferrin receptor (iron uptake) and ferritin (sequestration in the cytoplasm). As summarised in Table 2, the expression level of these proteins in LY sublines differs and this gives an explanation of the differential iron content. Further details on iron content control in LY sublines can be found in Lipiński *et al.* (2000).

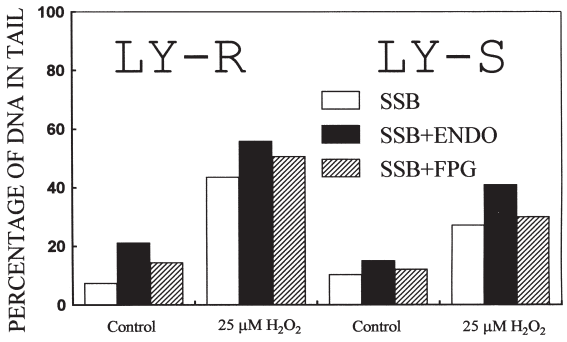

Figure 3. DNA single strand breaks (SSB) estimated directly and after treatment with enzymes that recognise base damage in hydrogen peroxide treated LY cells.

The damage is estimated by the alkaline comet assay without or with enzymes (ENDO, endonuclease III, FPG, formamidopyrimidine glycosylase). The measure of damage is the percentage of DNA in the comet tail.

Table 2. Iron content and iron-controlling proteins in LY cells

	LY-R	LY-S
Iron content in the nucleus (ng/10^6 cells)	7.7 ± 1.8 high	3.1 ± 0.9 low
Labile iron pool [μM]	0.57 ± 0.10 high	0.18 ± 0.05 low
Iron sequestering		
Ferritin L subunit mRNA (densitometry, arbitrary units)	similar 2.0 ± 0.3	similar 3.1 ± 0.6
Ferritin L subunit protein	similar	similar
Ferritin H subunit mRNA (densitometry, arbitrary units)	low 2.1 ± 0.1	high 7.4 ± 0.3
Ferritin H subunit protein (densitometry, arbitrary units)	low 2.3 ± 0.2	high 11.4 ± 3.5
Iron uptake		
Transferrin receptor mRNA (densitometry, arbitrary units)	high 2.3 ± 0.1	low 1.0 ± 0.1

Data from Szumiel *et al.*, 1995, and Lipiński *et al.*, 2000

THE ANTI-OXIDANT DEFENCE AND THE EFFECT OF ITS MODULATION ON DNA DAMAGE

As indicated in the diagram (Fig. 2), the enzymes of the antioxidant defence system, as well as the availability of reduced glutathione can considerably modify the cytotoxic effect of hydrogen peroxide treatment. This feature of LY sublines has been studied (Bouzyk *et al.*, 1997); the main results are shown in Table 3 and discussed below.

The contribution of catalase, glutathione peroxidase (GPx) and glutathione to the defence system differ in LY sublines. Catalase activity is twofold lower in LY-R than in LY-S cells. Accordingly, sensitivity to 3-amino-1,2,4-triazole (AMT), an inhibitor of catalase, is higher in LY-R than in LY-S cells. GPx activity is about two times higher in LY-R than in LY-S cells. After induction with selenium it increases 15.6-fold in LY-R cells and 50.3-fold in LY-S cells. Reduced glutathione (GSH) content (and other monobromobimane (MBB)-reactive thiols) were determined fluorimetrically with MBB and the fluorescence was found 54% higher in LY-S than in LY-R cells.

DNA damage (estimated by the comet assay) is the same in hydrogen peroxide-treated cells in the presence or absence of AMT; however, after induction of GPx by selenium, DNA damage is considerably lowered: (LY-R cells: about 3.6-fold; LY-S: about 1.7-fold). This sparing effect of selenium is accompanied by decreased growth inhibition in selenium pre-treated, hydrogen peroxide-treated cell cultures, as shown in Fig. 4. Note that thus protected LY-R cells grow better than LY-S cells.

NUCLEAR TRANSLOCATION OF THE P65 SUBUNIT OF NF-κB

One consequence of treatment with oxidants is the activation of members of the Rel/ NF-κB (nuclear factor κB) transcription factor family (reviewed by Baldwin, 1996; May & Gosh, 1997). Therefore, we examined nuclear translocation of this factor in LY sublines, seeking its relation to the inverse cross-sensitivity to X-rays and hydrogen peroxide. NF-κB is present in the cytoplasm as a heterodimer composed of the p50 and p65 subunits and complexed to an inhibitory subunit, IκB. As a consequence of specific stimuli

Table 3. Antioxidant defence in LY sublines

	LY-R	LY-S
Catalase (units/mg protein)	14.08 ± 2.07 low	26.70 ± 2.68 high
Glutathione peroxidase (units × 10^3/mg protein)	5.16 ± 2.28 high	2.49 ± 0.88 low
Glutathione peroxidase after induction with selenium	79.78 ± 15.36 low	126.64 ± 16.37 high
Mn-superoxide dismutase	0	0
Cu, Zn-superoxide dismutase (units/mg protein)	6.85 ± 0.63 high	3.25 ± 0.33 low
MBB-reactive thiols (4 × 10^5 cells, mean fluorescence)	198 ± 29 low	305 ± 49 high

Data from Bouzyk *et al.*, 1991; 1997, and Jaworska *et al.*, 1987

the latter subunit becomes phosphorylated, ubiquitinated and degraded. This is followed by translocation of the heterodimer to the nucleus. Hence, nuclear translocation of the RelA/p65 subunit is often taken as an indication of NF-κB activation. This activation is induced by a variety of agents: their common feature is a direct (exogenous oxidants, ionizing radiation) or metabolic generation of reactive oxygen species (ROS).

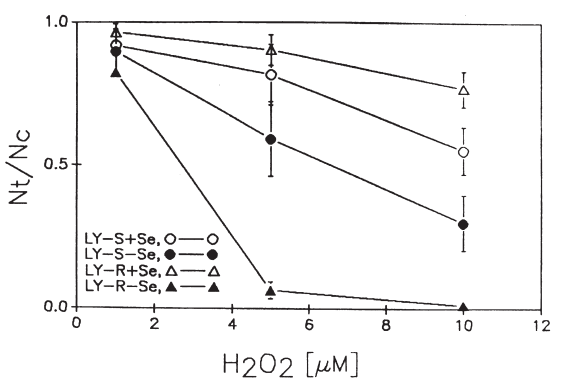

Figure 4. Relative cell numbers in hydrogen peroxide treated LY cell cultures grown in Fischer's medium with or without selenium supplementation before the treatment.

Relative cell number is Nt/Nc; Nt is the number of cells/ml in the treated cell culture, Nc is that in the control, both grown from the same initial cell density for 48 h. Mean results are shown from 2 experiments, standard deviation indicated (reproduced from Bouzyk *et al.*, 1997, by permission of Elsevier).

We have characterized the effect of varying antioxidant status on nuclear translocation of p65-NF-κB in LY cells upon exposure to hydrogen peroxide or X rays. Figure 5 shows some of the pathways to NF-κB activation and the applied ways of their modification. To modify the cellular antioxidant status we treated the cells with the following agents: *N*-acetylcysteine (NAC) as an antioxidant, AMT to inhibit catalase, selenium to induce glutathione peroxidase. Additionally, we examined the effect of a phorbol ester (PMA), a protein kinase

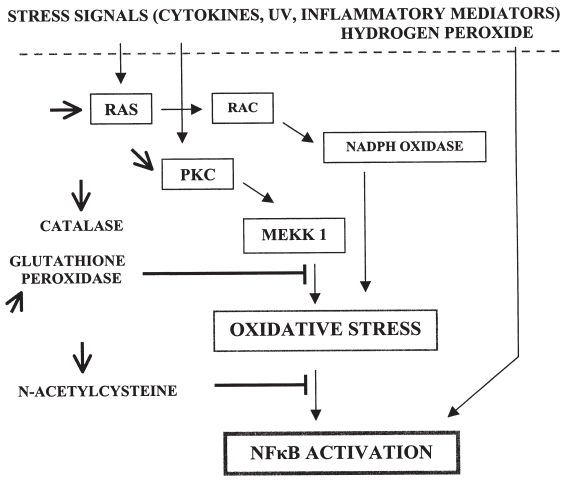

Figure 5. Some of the pathways leading to NF-κB activation and the applied ways of its modification.

Short arrows indicate the modified stages of activation (see text for explanations).

C activator, also activating NF-κB (Mohan & Meltz, 1994) and lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase and hence, of p21ras farnesylation (Sinensky *et al.,* 1990); both compounds are known to induce endogenous generation of ROS (Schreck & Bauerle, 1991; Santillo *et al.,* 1996).

Hydrogen peroxide, phorbol ester and X-rays cause a marked translocation of p65-NF-κB in LY-R cells and a weak translocation in LY-S cells. By manipulating the antioxidant defence status we obtained an alteration in the p65-NF-κB translocation induction in LY cells. As mentioned above, LY cells kept in selenium-supplemented medium for 4–6 days before hydrogen peroxide treatment showed an increase in GPx activity and a diminished DNA damage. This increase in antioxidant defence was accompanied by diminished nuclear translocation of p65-NF-κB in hydrogen peroxide treated cells: it was markedly lower than in selenium untreated, hydrogen peroxide treated cells. The decrease was more evident in LY-R cells.

A differential effect in the pair of LY sublines also was achieved with lovastatin pretreatment (25 μM, 24 h, 37°C). Lovastatin inhibits farnesylation of the Ras protein, and hence, prevents its anchorage in the plasma membrane and its signalling function. The results of lovastatin treatment of LY cells are summarized in Table 4. Treatment with lovastatin followed by hydrogen peroxide caused an increase in p65-NF-κB translocation only in LY-R cells.

From a comparison of the induction of p65-NF-κB nuclear translocation in LY-R and LY-S cells with the lethal effect of the inducing agent we concluded that the induction corresponded to the antioxidant status of the cell but was not consistently related to the sensitivity to the inducing agent. So, the induction was stronger in LY-R than in LY-S cells both after treatment with hydrogen peroxide and X-irradiation, in the former case accompanied by a higher lethal effect, in the latter – by a lower one.

Table 4. Effects of lovastatin in LY cells

End-point examined	LY-R	LY-S
Thiols (MBB-reactive)	↑↑	↓
Lipid peroxides (estimated with cis-P)	slight decrease	
Reactive oxygen species (estimated with FLUO)	↑↑	↑
NF-κB activation in hydrogen peroxide treated cells	↑↑	↓
Apoptosis induction in hydrogen peroxide treated cells	no change	
Growth inhibition		

Data from Sochanowicz *et al.,* 1999

CONCLUDING REMARKS

The presented data on the cellular response of LY sublines to hydrogen peroxide treatment allow us to answer the question posed at the beginning: what is the reason of the differential sensitivity of the closely related cell sublines to hydrogen peroxide? Our present answer is that the difference depends primarily on differential iron homeostasis control and hence, iron content, especially that in the labile iron pool, which is potentially active in the Fenton reaction. As a consequence – there is a substantial difference in the initial DNA damage and the lethal effect of hydrogen peroxide treatment (at micromolar concentrations). The status of the antioxidant defence: thiol content and the activity of catalase and

glutathione peroxidase contribute to the difference, whereas the activation of NF-κB in the pair of LY sublines corresponds with the antioxidant status.

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