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# REDOX BUFFERING ABILITY OF LYMPHOID CELLS EVALUATED BY THE OXIDATION OF 2',7'-DICHLOROFLUORESCIN

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The redox buffering activity of several lymphoid cells against endogenous and exogenous $H_2O_2$ has been evaluated using 2',7'-dichlorofluorescin diacetate ($DCFH_2$-DA).

The mechanism of 2',7'-dichlorofluorescin ($DCFH_2$) oxidation has also been investigated. It was found that while the oxidation by external $H_2O_2$ is completely inhibited by azide or cyanide, the oxidation by endogenous species is still present, even under anaerobic conditions. The data herein reported indicate that autoxidation and peroxidation of $DCFH_2$ are distinct reactions. Hence only by addition of increasing concentrations of exogenous hydrogen peroxide, the fluorescence of DCF can be used to evaluate the cellular ability of scavenging $H_2O_2$. By this method we have found that the erythroleukaemia cell line K562 and promyelocytic line HL-60 show a faster rate of $DCFH_2$ oxidation than peripheral blood leukocytes (PBL), mature T-cells (MOLT-3 and MOLT-4) and B-cells (DAUDI). Using this method the balance between antioxidant enzymes activity and the redox state of the cell can be easily assessed by fluorescence both in single cells and in cell populations.

KEY WORDS: 2',7'-dichlorofluorescin diacetate, lymphocytes, oxygen radicals, peroxidase, catalase.

## INTRODUCTION

Aerobic cells must protect themselves from oxygen radicals which are unavoidably formed in variable amounts in the course of oxygen reduction to water. To this purpose they have a complex armoury of antioxidant chemicals and enzymes such as vitamins A, E and C, reduced glutathione, catalase, glutathione peroxidase and superoxide dismutases. In recent years evidence has been growing that many pathological states may indeed originate from an insufficient defence against oxygen radicals (see for reference British Journal of Cancer 55, suppl. VIII, 1987). On the other hand these radicals also have a dramatic effect, appealing for therapeutic purposes, in the killing of invading microorganisms and tumour cells. Indeed tumour cells show a reduced ability to handle oxygen radicals.[1]

For all these reasons several efforts have been made to assess quantitatively the overall antioxidant activity of cells and tissues. Much work has been devoted to the quantitation of each of the above mentioned low and high molecular weight components as well as of the various active oxygen derivatives. Many of these studies have suggested that a balanced proportion of several antioxidant agents rather than the

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absolute concentration of each component is important in cell defence[2], although this issue needs further experimental support. In search for an easy system of assessing the ability of whole cells to deal with active oxygen species, we took advantage of the reaction of 2′,7′-dichlorofluorescin with $H_2O_2$.[3] This reaction has already been used to quantitate $H_2O_2$ in aqueous solutions and in neutrophils during the respiratory burst.[4]

2′,7′-dichlorofluorescin diacetate ($DCFH_2$-DA) is a non-fluorescent analog of fluorescin diacetate which readily diffuses into cells. Here the acetate is hydrolysed by intracellular esterases producing 2′,7′-dichlorofluorescin ($DCFH_2$). $DCFH_2$, still non-fluorescent, is trapped within the cells because of its polarity. When exposed to $H_2O_2$, $DCFH_2$ is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). In neutrophils, preincubated with $DCFH_2$-DA, the stimulation of the respiratory burst, where $H_2O_2$ is produced in large amounts, causes the oxidation of cytosolic $DCFH_2$. The resulting fluorescence is linearly related to the extent of the respiratory burst.[5] In the present study we used $DCFH_2$-DA to evaluate the ability of whole or lysed cells to scavenge exogenous $H_2O_2$, such activity being dependent on the content of the antioxidant enzymes and on the cellular reducing power. Therefore the natural ability of the cell to eliminate exogenous $H_2O_2$, leading to variable intracellular levels of hydrogen peroxide, is inversely related to the rate of fluorescence development. Using this method we were able to demonstrate that normal, mature leukocytes are more resistant to $H_2O_2$ stress than their promyelocytic and erythroleukaemic transformed progenitors. Furthermore we have also investigated the mechanism of $DCFH_2$ oxidation by endogenous oxidants.

## MATERIALS AND METHODS

### *Reagents*

Phosphate buffered saline (PBS), foetal calf serum (FCS), Iscove's RPMI-1640 culture media, as well as plastics, L-glutamine, penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and sodium bicarbonate were obtained from Flow Laboratories Ltd. (Irvine, Scotland, UK). Lymphoprep was from Nyeegard & Co. (Oslo, Norway). $DCFH_2$-DA was purchased from Eastman Kodak (Rochester, NY, USA). Sodium cyanide, sodium azide, D-glucose and hydrogen peroxide were obtained from MERCK (FRG). Horse-radish peroxidase (HRP) and catalase were from Sigma (USA). FCS was heat-inactivated at 56° C for 45 minutes. $DCFH_2$-DA was dissolved in anhydrous ethanol (Merck, FRG) and stored at 4° C in the dark for no longer than 3 weeks. Chemically deacetylated $DCFH_2$ was prepared by dissolving 0.5 ml $DCFH_2$-DA (1 mM in ethanol) in 2.0 ml 0.01 M NaOH and incubating for 30 minutes at room temperature. Subsequently 10 ml of 25 mM PBS pH 7.2 were added and the solution stored on ice at 4° C. This was used within two hours. $H_2O_2$ was diluted freshly from stock before each experiment.

### *Cell lines*

The erythroleukaemia cell line K562, B-cell lymphoma line DAUDI and promyelocytic leukaemia line HL-60 were grown in RPMI-1640 culture medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine,

100 U·ml$^{-1}$ penicillin-streptomycin, 1.8 g·l$^{-1}$ Na-bicarbonate and 10 mM HEPES. Flasks were incubated at 37° C in a humidified atmosphere containing 5% (v/v) $CO_2$. The T-cell leukaemia cell lines MOLT-3 and MOLT-4 were grown in similar conditions using Iscove medium and 15% (v/v) FCS.

Peripheral blood leukocytes (PBL) were isolated by the technique of Boyum.[6] For this study 5 healthy volunteer donors were used as controls. Whole blood was layered on lymphoprep (1:1, v/v) and centrifuged at 500 × g for 20 minutes at room temperature. After 3 washes in PBS, the total cells were counted and protein concentration was evaluated using the method of Lowry *et al.*[7]

*Spectrofluorimetric and flow cytofluorimetric analysis*

Static fluorescence was evaluated in a JY-3D Jobin-Yvon spectrofluorimeter linked to an IBM-AT dedicated computer. The sample was excited at 485 nm using a 10 nm slit, and the fluorescent emission was monitored at 530 nm using a 10 nm slit. All measurements were made at room temperature in a 3 ml cuvette using a magnetic stirrer in aerobic conditions.

Single cell fluorescence was determined using a FACS-analyser (Becton-Dickinson, CA, USA) flow cytofluorograph equipped with a HP-2242 (Hewlett-Packard, USA) dedicated computer. Data were analysed using a Consort 30 (Becton-Dickinson, CA, USA) software to produce single and multiparametric correlations as well as Kolmogorov-Smirnov statistical tests. Cells were excited at 480–495 nm and their fluorescence (510–530 nm) for 10,000 events was determined by computer gating using volume (electric impedence) as a triggering signal.

*Evaluation of the peroxidative activity in cells*

Peroxidase activity was assessed spectrofluorimetrically using either whole or sonicated cells at a final protein concentration of 0.2 to 0.3 mg·ml$^{-1}$. DCFH$_2$-DA was added to a 5 $\mu$M concentration and incubated for 3 minutes. This time was adequate for equilibration of intracellular and extracellular DCFH$_2$-DA concentrations in our model; however the incubation time may be extended to 15–20 minutes in cellular models with a reduced esterases activity. Then, various concentrations of hydrogen peroxide, ranging from 10 $\mu$M, were added and the fluorescence recorded for 5 minutes. The tangents of the fluorescence increments (in arbitrary fluorescence units) in time were plotted against the hydrogen peroxide concentration. Higher fluorescence rates are inversely related to the peroxidative ability of the cells. This method may be used to compare the peroxidative activity in different cell lines, in cells treated under different experimental conditions, or to evaluate the ability of new chemical compounds to scavenge/protect cells from a peroxidative stress. The same method may be applied by means of a single cell flow cytofluorograph in order to investigate cell subpopulations haeterogeneity.

The experiments herein reported were performed using cell lysates or suspensions, incubated aerobically or anaerobically in order to assess the effect on the reaction of azide, cyanide, glucose, catalase and peroxidase. We used the term "peroxidation" to indicate the oxidation of DCFH$_2$ by exogenous $H_2O_2$ and the term "autoxidation" to indicate the intracellular oxidation of DCFH$_2$, where the oxidant species is not known.

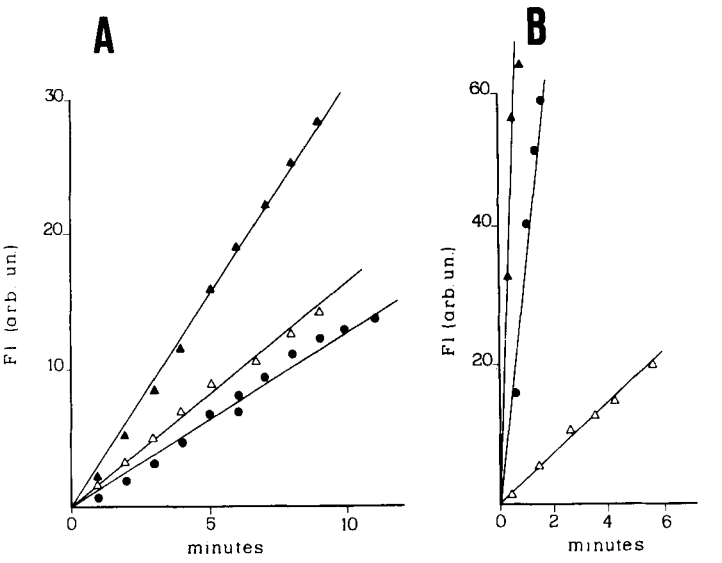


FIGURE 1. Rate of oxidation of chemically deacetylated $DCFH_2$ in PBS (open triangles), lysed K562 extracts (full triangles) and lysed K562 extracts treated for 30 minutes at 90° C (full circles). All experiments were performed in air. A) autoxidation; B) peroxidation in the presence of 1 mM $H_2O_2$.

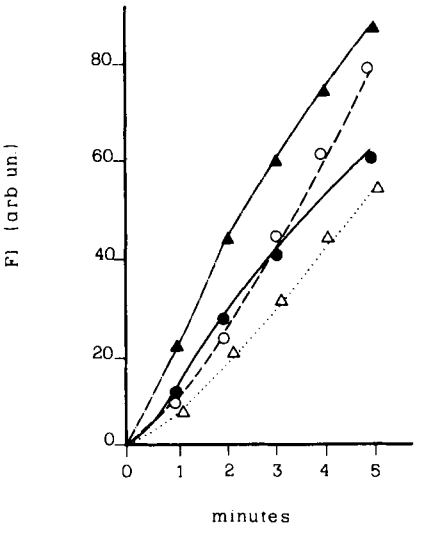


FIGURE 2. Effect of horse radish peroxidase (full triangles = 0.1 $U \cdot ml^{-1}$ HRP) on the rate of $DCFH_2$ peroxidation in lysed K562 cells (full circles = no HRP). The addition of 8 mM (open circles) or 5 mM cyanide (open triangles) to cells with HRP is able to reverse the increased rate of peroxidation (1 mM $H_2O_2$) by HRP. Similar results were obtained using whole cells.

## RESULTS AND DISCUSSION

### *"Autoxidation" and "peroxidation" of DCFH$_2$ are distinct reactions*

The oxidation of 2′,7′-DCFH$_2$-DA aqueous solutions in air is very slow.[3,8,9] However, after deacetylation, the resulting DCFH$_2$ autoxidises at a significant rate in the presence of air. We studied the aerobic oxidation of chemically deacetylated DCFH$_2$ catalysed by K562 lysates (Figure 1A). The data show that cellular lysate significantly enhances the oxidation rate of chemically decacetylated DCFH$_2$ over that observed in PBS alone. This activity is abolished by heat-treatment of cell lysates, indicating that an enzyme is involved in the oxidation of DCFH$_2$. Instead the peroxidation of chemically deacetylated DCFH$_2$ in the presence of 1 mM $H_2O_2$, is catalysed—though at a lower extent—even by a heat-inactivated cell lysate (Figure 1B). This is in keeping with the finding that low molecular weight, non-protein components, such as haematin, may catalyse the peroxidation of DCFH$_2$.[9] Molecular oxygen would not seem to be the primary oxidising agent of the autoxidation of DCFH$_2$-DA by K562 cells, since the reaction takes place at essentially the same rate under aerobic and anaerobic conditions (data not shown). As a matter of fact, it has been reported that organic peroxides are also able to oxidise DCFH$_2$.[9]

Autoxidation and peroxidation of DCFH$_2$ seems therefore to be distinct reactions. The addition of HRP to a K562 lysate increases the rate of peroxidation of DCFH$_2$ (Figure 2). This increase is completely abolished by adding $N_3^-$ or $CN^-$, which always strongly affect the peroxidation of DCFH$_2$ (Figure 3B), while they even increase the rate of autoxidation (Figure 3A). The addition of catalase does not affect the rate of DCFH$_2$ autoxidation observed, but prevents the peroxidation in the presence of 1 mM $H_2O_2$ (data not shown). Taken together these data indicate that DCFH$_2$-DA is metabolised by cells in a complex way. In particular, the distinct behaviour of DCFH$_2$ in autoxidation and peroxidation demonstrate that DCFH$_2$-DA can not be used as such to evaluate the peroxidative activity of cells. Hence we modified this method by adding increasing concentrations of exogenous $H_2O_2$, to be sure that a true peroxidation is taking place.

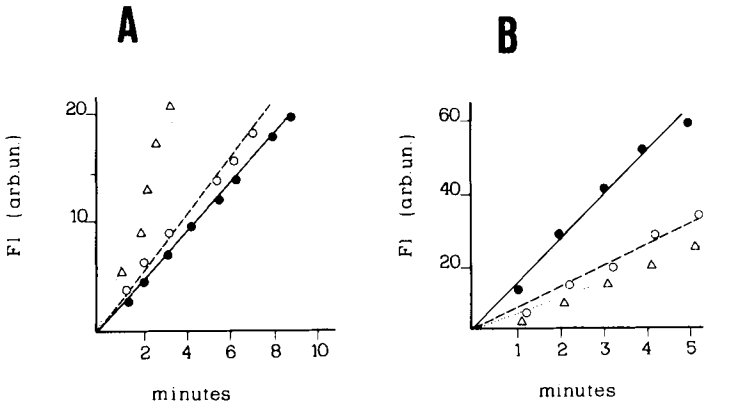


FIGURE 3. Effect of 8 mM azide (open circles) and 5 mM cyanide (open triangles) on DCFH$_2$-DA autoxidation (A) and peroxidation (B) in lused K562 cells (full circles = control).

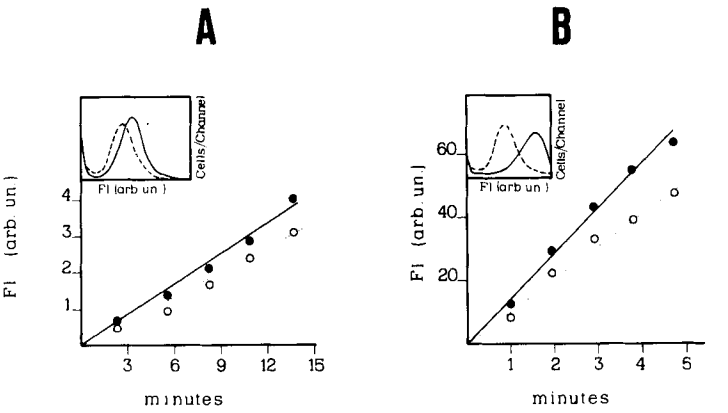


FIGURE 4. Effect of 10 mM glucose (open circles) on $DCFH_2$-DA autoxidation (A) and peroxidation (B) on K562 lysed cells (full circles = control). The inserts show the corresponding flow cytometry data (solid lines = control; broken lines = 10 mM glucose). Cells were incubated at 37° C for two hours in the presence or absence of glucose, before the fluorimetric analysis.

The redox buffering ability of cells may indirectly be altered by their metabolic state; thus we investigated whether the extent of $DCFH_2$ peroxidation might be an indicator of the cell reducing power. The presence of 10 mM glucose reduces both the autoxidation (Figure 4A) and the peroxidation (Figure 4B) rates in lysed as well as in intact (inserts) cells. This finding may be related to the greater supply of reduced cofactors.

Whole cells and their lysates have a very strong ability of scavenging $H_2O_2$. In fact the complete peroxidation of 5 $\mu$M $DCFH_2$-DA was not achieved even after adding

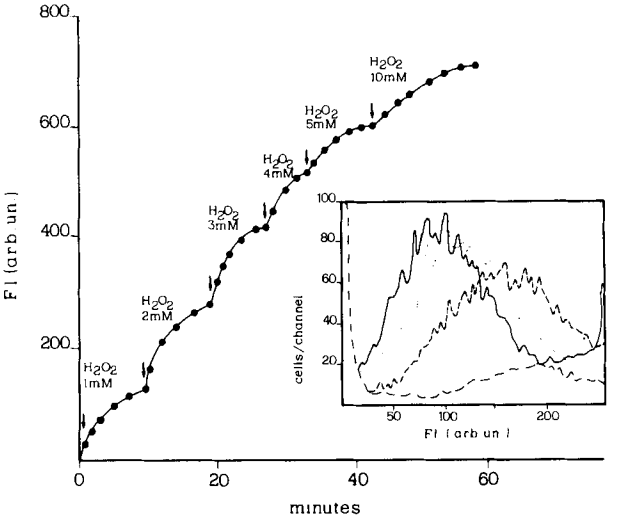


FIGURE 5. Saturation of 5 $\mu$M $DCFH_2$DA with $H_2O_2$ in lysed K562 cells. The insert shows the corresponding flow cytometry data (solid line = control; dotted line = $10^{-5}$M $H_2O_2$; broken line = $10^{-4}$M $H_2O_2$; dashed-dotted line = $10^{-3}$M $H_2O_2$).

10 $\mu$M $H_2O_2$ (Figure 5) or by 24 hours incubation in air (data not shown). For this reason the final concentration of 5 $\mu$M $DCFH_2$-DA seems non toxic and sufficient to evaluate the ability of scavenging high concentrations of hydrogen peroxide.

Interestingly enough all K562 cells behave as a homogeneous population toward $DCFH_2$ oxidation. The insert in Figure 5 shows that in the case of K562 cell line, all cells migrate as a single cluster to yield higher values of DCF fluorescence when observed in a flow cytofluorimeter. The overall antioxidant defences of the cell can be easily and quickly evaluated by $DCFH_2$-DA, regardless of the balance of its individual components.

*Peroxidation of $DCFH_2$ in different lymphoid cells*

In order to assess the ability of this method to discriminate the redox buffering ability of various lymphoid cell lines, first we used $DCFH_2$-DA to compare K562 cells and PBL in view of the activities of their peroxidative enzymes. Then we performed a fast screening of various lymphoid cell lines at different stages of phenotypic maturation.

Figure 6 compares the peroxidation rates of $DCFH_2$ induced by externally added $H_2O_2$ in K562 cells and in PBL. The former cells show a faster rate of oxidation than PBL. Even though differences in esterase activity and substrate uptake and release should be taken into account, the data suggest that there is a relationship between the

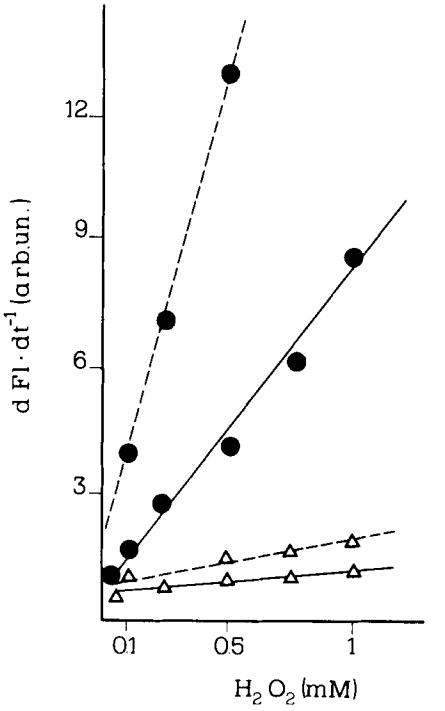


FIGURE 6. Rate of $DCFH_2$ peroxidation by $H_2O_2$ in K562 cells (full circles) and PBL (open triangles), evaluated in whole cells (solid lines) or in lysed cells (broken lines). $DCFH_2$-DA was added at a final concentration of 5 $\mu$M for 3 minutes before $H_2O_2$. The tangents were taken over the first minute kinetics.

TABLE I
Redox-buffering ability of lymphoid cells

| cells | origin | Tg Fl |
|---|---|---|
| K562 | erythroleukaemia | 58.0 |
| HL-60 | promyelocytic leukaemia | 60.0 |
| DAUDI | B-cell lymphoma | 3.1 |
| MOLT-3 | T-cell leukaemia | 3.0 |
| MOLT-4 | T-cell leukaemia | 3.0 |
| PBL-1 | normal leukocytes | 9.0 |
| PBL-2 | normal leukocytes | 10.5 |
| PBL-3 | normal leukocytes | 7.8 |
| PBL-4 | normal leukocytes | 11.0 |
| PBL-5 | normal leukocytes | 9.1 |

The data were collected after 3 minutes incubation with 5 $\mu$M $DCFH_2$-DA followed by the addition of 1 mM exogenous $H_2O_2$. Tg Fl was evaluated measuring the geometric tangent of the fluorescence increment in time, expressed in arbitrary units.

antioxidant enzyme activity and the rate of $DCFH_2$ oxidation. This hypothesis is in keeping with the reported activities of the hydrogen peroxide metabolising enzymes in these cells: Cu,Zn-SOD 1.16 vs 0.65$\mu$g·prot $mg^{-1}$, GSH-Px 2.5 vs 86.3 U·prot $mg^{-1}$, catalase 40 vs 81 U·prot $mg^{-1}$ in K562 cells and PBL respectively.[1]

Lysates from both cell types were also studied in order to take into account the influence of the $H_2O_2$ diffusion rate that may be a source of difference between the two kinds of cells. Figure 6 shows a greater increment of the $DCFH_2$ oxidation rate after disruption of the larger K562 cells. After lysis, therefore, the difference between the two cell lines becomes even more evident.

In order to evaluate the peroxidative activity as a function of cell maturation, we compared the results obtained with K562 cells and PBL with those observed in several other lymphoid cell lines, like HL-60, DAUDI, MOLT-3 and MOLT-4 (Table I). The two most undifferentiated cell lines, K562 and HL-60, showed the fastest rate of $DCFH_2$ oxidation. On the other hand the mature cell lines showed a slow oxidation rate. PBL had intermediate values, probably because of the presence of co-purified monocytes and polymorphonuclear cells, which have high oxidative activity.

## CONCLUSION

This report describes a fluorimetric assay to evaluate the peroxidative activity of whole or lysed cells. $DCFH_2$-DA appears to be a suitable detector of exogenous oxidative stress like $H_2O_2$ as well as intracellularly formed oxidants. This method allows the quantitation of the redox buffering ability of cells, a reaction that is dependent on the overall balance between the activity of the antioxidant enzymes (GSH-Px, catalase), the reducing power of the cells (NADH, ascorbate, glutathione, etc.) and the metabolic precursors (e.g. glucose). $DCFH_2$DA is a simple, fast, sensitive and reproducible marker also for single cell analysis, allowing the study of oxygen metabolism in cell subpopulations.

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