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Flow-cytometric Determination of Glutathione Alterations in Vital Cells by *o*-Phthaldialdehyde (OPT) Staining

J. TREUMER and G. VALET*

Arbeitsgruppe Krebszellforschung, Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

A flow-cytometric method for the detection of changes of cellular glutathione content in vital cells is described. The reaction is based on formation of a fluorescent product between *o*-phthaldialdehyde (OPT) and reduced glutathione (GSH). OPT is a more GSH-specific dye than other thiol-specific dyes (e.g., bromobimanes), because it forms a cyclic compound with GSH. Changes of GSH induced by oxidation or thiol-blocking agents are visualized in vital cells after a 5-min staining at room temperature.

The protection of the intracellular environment against oxidative damage is of major importance in normal cellular metabolism. The main antioxidative defence is provided by the glutathione redox cycle [1], which is present in practically all cells [2]. Under normal conditions the tripeptide glutathione (glu-cys-gly) is mostly present in its reduced form GSH and only to a small degree in oxidized form GSSG [3]. One can distinguish between the intracellular glutathione content and its functional status which is the ratio of GSH to GSSG.

Determination of intracellular glutathione content and its alteration after exposure to oxidative stress mediated by peroxides or diamide [4] are of particular interest for the biochemical characterization of cells. Information about thiol state, redox state and capability of detoxification of xenobiotics like chemotherapeutics in cancer therapy may be obtained in this way. In some instances, correlations between intracellular glutathione concentration and cellular resistance against alkylating agents are apparent [5–9] and even macrophage-mediated tumor cell cytolysis is modulated by cellular GSH content [10].

The objective of this study was to develop a flow-cytometric method for the measurement of GSH in vital cells. Ortho-phthaldialdehyde (OPT), which is a well established reagent for glutathione determination in tissue homogenates [11–13], was used for this purpose. The OPT method is based on reaction with both glutathione amino- and sulfhydryl groups, yielding a cyclic, highly fluorescent product. Since two molecular groups of GSH are involved in the reaction [14], OPT is a more specific glutathione marker than thiol-specific dyes, e.g. bromobimanes [15].

* To whom offprint requests should be addressed.

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MATERIALS AND METHODS

Cells

All incubations and washing steps were performed in 10 mM HEPES-buffered saline pH 7.4 (HBS). Murine Ehrlich ascites tumor (EA2P8, gift from Dr v. d. Mark, Martinsried, FRG) was obtained by puncture of the peritoneal cavity 10 days after inoculation of 0.4 ml ascites fluid (5×10^8 cells/ml) in C57bl mice. The ascites was diluted between 1/10 and 1/50 with HBS prior to staining to obtain appropriate cell counts (5×10^6 cells/ml). A rat mammary tumor cell line (R323ØAC) was grown in 75 ml polystyrene tissue culture flasks by inoculation of 10^5 cells in RPMI-medium with 10% fetal calf serum (FCS) (Paesel, Frankfurt) at 37°C in air with 5% CO_2 for 7–9 days. The cells were harvested by trypsinization (0.025%), washed twice in HBS by centrifugation (10 min at 200 g) and finally passed through a 40-μm nylon mesh to remove cell aggregates.

Chemicals

100-mM stock solutions of *o*-phthaldialdehyde (OPT, Sigma, FRG), monobromobimane (MB, Calbiochem, FRG) and *N*-ethylmaleimide (NEM, Sigma, Taufkirchen, FRG) were prepared in dimethylformamide (DMF, Baker Chemicals, Deventer, The Netherlands). 100 mM stock solutions of mercurydichloride ($HgCl_2$, Merck, Darmstadt), diamide (Sigma), reduced glutathione (GSH, Sigma) and hydrogen peroxide (HPO, Merck) were prepared in HBS. GSH, diamide and HPO were always freshly prepared to avoid decomposition. A 2 mM solution of *p*-chloromercury-phenylsulfonic acid (PCMS, Sigma) in HBS was used in some experiments instead of HBS. Sodium dodecyl sulfate (SDS) was bought from Serva, Heidelberg.

Spectroscopy

Fluorescence-spectrometer LS-5 (Perkin-Elmer Corp., Norwalk, USA) was mounted on-line to a VAX 11/782 computer. The slit setting was 5 nm (excitation) and 2.5 nm (emission) and quartz cuvettes with 10 mm light pass were used. The spectra were measured in 50 mM Tris/HCl buffer, pH 7.4.

Cell Staining

1×10^6 cells were suspended in 500 μl HBS, 5 μl 0.1 M OPT or 0.1 M MB solution was added. The assay was measured in the flow cytometer after 5 min incubation at room temperature.

Flow Cytometry

The Fluvo-Metricell flow cytometer [16] was equipped with a Cytomic 12 microprocessor data acquisition and linked to an Interdata 74 computer (Perkin-Elmer) [17]. List mode data were stored on magnetic tape. The hydrodynamically focused sizing orifice was 95 μm in diameter, 100 μm long and was operated at a constant current of 0.23 mA. Fluorescence was excited by a HBO-100 high-pressure mercury arc lamp between 300 and 400 nm. The cell fluorescence was collected by two light channels between 388 and 440 nm (F1) and between 450 and 700 nm (F2). Data evaluation was performed with FORTRAN programs [18].

HPLC

HPLC separations of lysates of OPT-stained cells were made to discriminate between fluorescence associated with high-molecular and low-molecular fractions. A gel sieve column Ultropak TSK-G 3000SW (LKB, Bromma, Sweden), pump 2150 (LKB) and UV-monitor 2238 (LKB) (260 nm) were used in combination with a 25-μl quartz continuous-flow cell (Hellma, Freiburg) for fluorescence measurement. 5×10^6 cells in 0.5 ml HBS were incubated with OPT as described above, washed twice with HBS by 10 min centrifugation at 200 g and lysed by adding 0.5 ml 50 mM Tris-HCl buffer containing 0.5% SDS. Additional disintegration was obtained by sonification (MSE model 7100, 100 W, microtip, 15 sec, Colora, Lorch, FRG). The sonicated cell lysate was centrifuged (10 min, 3000 g), filtered through a 0.45 μm Millipore filter and loaded onto the molecular sieve column, which was equilibrated and eluted with a 50 mM Tris-HCl buffer, pH 7.2, containing 2 mM EDTA and 0.5% (w/v) SDS. The eluate was monitored for protein by UV absorbance at 260 nm. Simultaneous

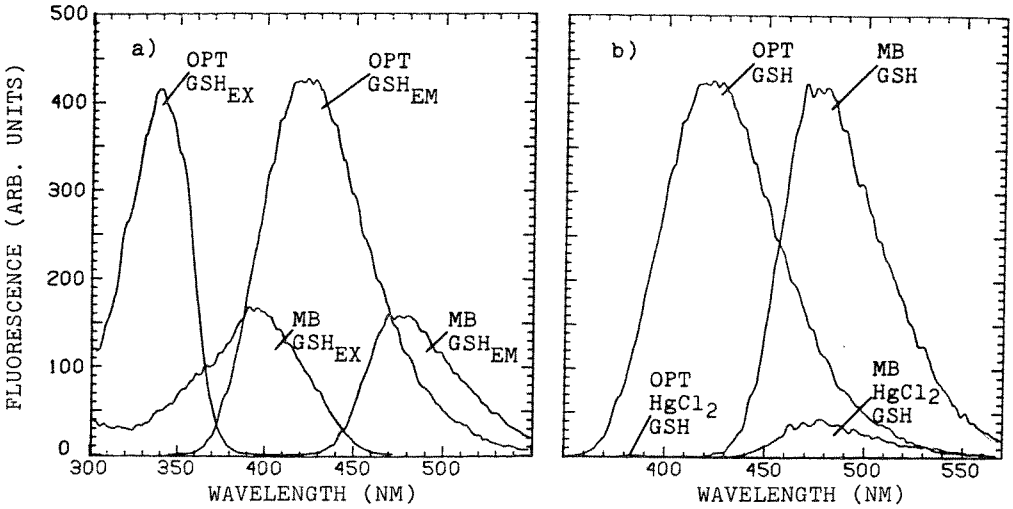

Fig. 1. Excitation- (*EX*) and emission spectra (*EM*) of (*a*) equimolar complexes of glutathione- and thiol-reactive dyes. (*b*) Preincubation with $HgCl_2$ inhibited the reaction completely (*OPT*) or partially (*MB*).

fluorescence detection was performed by the quartz flow cell that was mounted in the fluorescence-spectrometer. The fluorescence was excited at 338 nm and the emission was measured at 420 nm. The fluorimetric data were standardized to the maximum absorbance value of the major protein peak.

RESULTS

Formation of fluorescent adducts between GSH and the thiol-reactive dyes OPT and MB is illustrated in fig. 1 *a*. The fluorescence intensity is significantly greater for OPT, thus providing a sensitive detection of GSH. $HgCl_2$ was a potent inhibitor of this reaction (fig. 1 *b*): A twofold molar excess of $HgCl_2$ over GSH and OPT reduced fluorescence to 0.5% of the initial intensity. MB, despite a tenfold excess of $HgCl_2$, still showed a residual fluorescence. This fluorescence is probably due to hydrolysis of MB in aqueous media, leading to overestimation of thiol concentration. NEM, a potent thiol-blocking agent, also reduced formation of OPT–GSH fluorescent products, but to a lesser degree (data not shown).

Microscopic observations revealed that OPT stained the entire cell body blue, sometimes superimposed by a quickly bleaching yellow fluorescence. MB-stained cells displayed a uniform turquoise fluorescence.

Analysis of OPT-stained (1 mM) cell lysates by molecular sieve chromatography showed fluorescence linked to both high-molecular protein and low-molecular GSH-containing fractions (fig. 2 *a*). Greater variations in either fluorescence distribution or ratio between protein and fluorescence were observed, depending on the type of cells and on the growth conditions. Formation of GSH–OPT low-molecular weight fluorescence was quantitatively abolished in the presence of 2 mM $HgCl_2$, whereas high-molecular weight Protein–OPT fluorescence was only slightly affected (fig. 2 *b*). Moreover, GSH–OPT-containing fractions had fluorescence with peak intensity at longer wavelengths than protein–OPT-containing fractions (fig. 3).

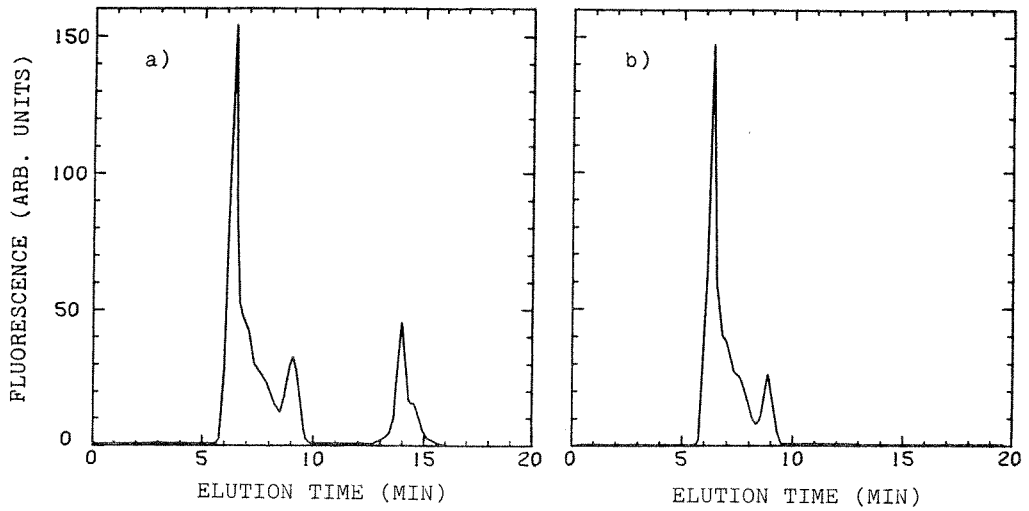

Fig. 2. HPLC molecular sieve separation of OPT-stained cell lysates. (*a*) OPT fluorescence was split into high-molecular protein-containing fractions (6–10 min elution time) and low-molecular GSH-containing fractions (13–15 min elution time). (*b*) Preincubation with 2 mM $HgCl_2$ completely reduced formation of fluorescence in GSH-containing fractions, while protein-containing fractions were significantly less affected.

Analysis of OPT-stained vital cells by flow cytometry is shown in fig. 4*a*. Due to its spectral properties, most of the OPT–GSH fluorescence appears in the green fluorescence channel, whereas the blue fluorescence channel represents mainly protein-bound OPT. Concentration and time-dependent staining experiments recommended incubation of cells in 1 mM OPT for 5 min. Longer staining or higher OPT concentrations gave no increase of blue fluorescence.

Correlation between OPT fluorescence and intracellular glutathione functional status was found, especially after alterations of the cellular free GSH pool:

(*a*) Blocking of the thiol group by incubation of cells in 2 mM $HgCl_2$ prior to OPT staining resulted in significant loss of green fluorescence (fig. 4*b*, table 1).

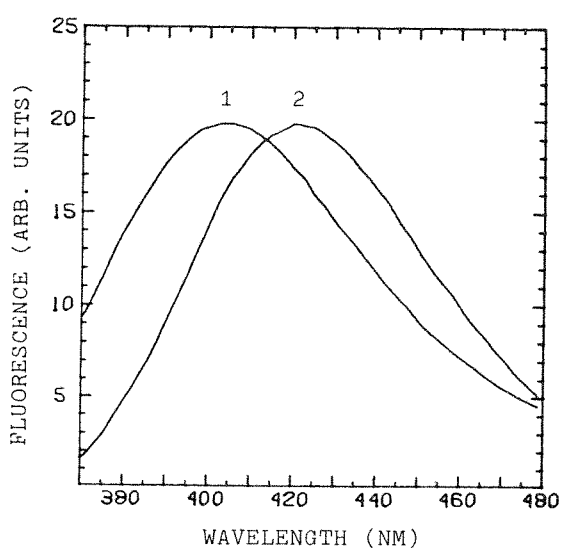

Fig. 3. Emission spectra of *1*, protein-containing; *2*, GSH-containing fractions of OPT-stained Ehrlich ascites cell lysates of fig. 2. Protein-containing fractions exhibited fluorescence at shorter wave fluorescence than the GSH-containing fractions. The spectra were excited at 338 nm.

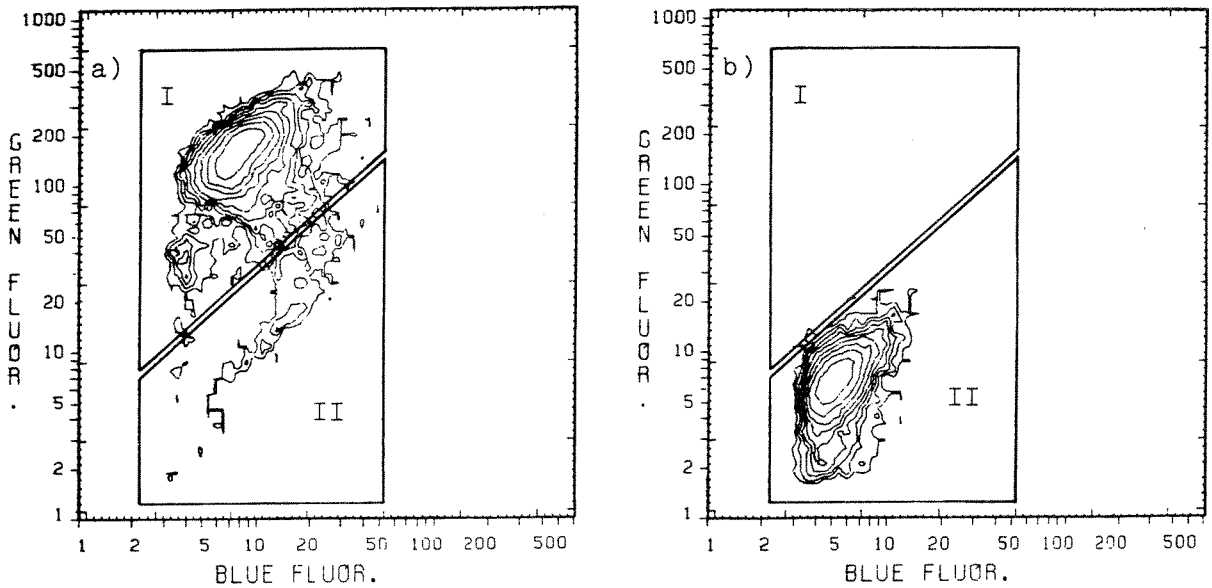

Fig. 4. (*a*) Flow-cytometric analysis of OPT-stained Ehrlich ascites cells. (*b*) Preincubation with 4 mM $HgCl_2$ greatly reduced green fluorescence, while blue protein-fluorescence was only moderately affected.

However, blue protein fluorescence still remained which could not be abolished by exposure to $HgCl_2$ in concentrations up to 20 mM.

(*b*) Incubation with PCMS, a non-permeating thiol-blocking agent, reduced peak intensity of fluorescence in flow-cytometric measurements and HPLC experiments. Only a moderate reduction in attainable fluorescence resulted (table 1), indicating that the OPT fluorescence was located mostly inside the cell.

(*c*) Oxidation of the thiol group by incubation of cells in 2 mM diamide or 2 mM HPO caused a decrease in green GSH fluorescence (table 1).

(*d*) Cellular GSH content was elevated by incubating cells between 0 and 110 min at 37°C in 0.4 mM cysteamine, which leads to an increased glutathione synthesis from precursors. The increase in green OPT fluorescence in vital cells was only moderate (5–10%) (data not shown).

Cells stained with OPT or MB showed similar changes of fluorescence intensity after exposure to $HgCl_2$ or NEM. Treatment with diamide or HPO, however, lead to marked differences: While preincubation with HPO significantly reduced OPT green-fluorescence to levels below those obtained by diamide, MB-stained

Table 1. *Distribution of 1 mM OTP-stained Ehrlich ascites cells between area I (fig. 4) of glutathione-containing cells and area II of glutathione-devoid cells after preincubation of the cells with thiol blocking or oxidizing agents*

Treatment	None	2 mM $HgCl_2$	2 mM HPO	2 mM PCMS
Area I (%)	97.3	0.1	14.0	77.3
Area II (%)	2.7	99.9	86.0	22.7

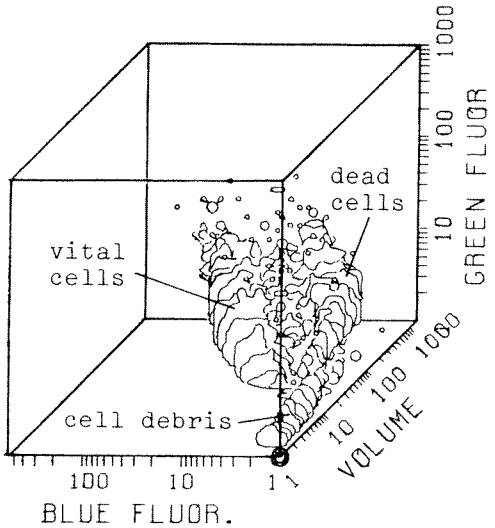

Fig. 5. Cloud display [18] of simultaneous three-parameter measurement of Ehrlich ascites cells stained with 1 mM OPT and 60 μM propidium iodide. Due to their DNA staining with propidium iodide, dead cells become red fluorescent, thus appearing in the green channel. The logarithmic contents of the individual channels of the three-parameter histograms were standardized for this display to the channel with the maximum logarithmic particle content as 100%. Contour lines were plotted at the 10% level of this value to visualize cell- and particle-containing areas. The histogram contains a total of 24 680 particles at a maximum channel content of 184 particles. The contour lines show the positions of all measured cells and particles.

cells showed only a slight decrease in fluorescence. Furthermore, cells stained with 0.5–1 mM MB increased in cell volume. An increasing cell volume was observed with OPT only after pretreatment of cells with $HgCl_2$ or NEM, but not by OPT staining alone.

OPT-stained cells were vital cells, as is shown by simultaneous counterstaining by propidium iodide which stains dead cells (fig. 5).

DISCUSSION

Specific staining of intracellular GSH sulfhydryl groups by thiol-group reactive, fluorescent dyes cannot be accomplished with conventional dyes (e.g., bromobimanes). A widely accepted GSH-staining procedure for tissue homogenates [11–13] was therefore modified for application in flow cytometry. OPT reacts with reduced glutathione yielding a cyclic, highly fluorescent product. OPT is more specific than thiol dyes such as MB because of its simultaneous requirement of amino- and thiol groups for the formation of a fluorescence product [14]. GSH–OPT has a fluorescence emission at higher wavelengths than protein–OPT reaction products (fig. 3), which permits distinction between GSH- and protein fluorescence by flow cytometry (fig. 4*a, b*). Staining of protein SH-groups by OPT is a major source of error in fluorimetric cuvette assays with single wavelength fluorescence detection.

Further advantages of the OPT reaction are: no intrinsic fluorescence of free OPT, and stability of OPT in aqueous solutions. Bromobimanes, in contrast, undergo hydrolysis, thereby forming fluorescent products, which causes overestimation of thiol groups. OPT is of low cellular toxicity because no changes in cell volume are observed.

Different information is obtained by bromobimane staining as compared with OPT staining: Bromobimane is reactive towards every thiol group [15], indicating the whole cellular thiol content, whereas OPT stains preferentially GSH. This

was confirmed by experiments influencing the cellular GSH level, e.g. by thiol blocking with $HgCl_2$ (fig. 4*b*, table 1) or NEM, by oxidation with peroxides (table 1) and diamide, or by increased synthesis in the presence of cysteamine. OPT-green-fluorescence in all experiments reflected the occurrence of the expected GSH alterations. Furthermore, the flow-cytometric analysis of OPT-stained cells was in accordance with data obtained by cell homogenization and the standard colorimetric GSH assay [19] on the cell lysates. Especially changes in fluorescence intensity after oxidative stress by HPO reflected highly specific alterations of cellular GSH. HPO lowers GSH enzymatically via GSH peroxidase [20], which is indicated by OPT staining. The blue OPT–protein fluorescence is not affected.

The potential of this new flow-cytometric method for GSH determination is that metabolic changes of GSH and protein thiol groups can be simultaneously observed in different subpopulations of heterogeneous vital cell preparations shortly after removal from the organism.

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