Oxidative product formation in irradiated neutrophils

A flow cytometric analysis

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The effect of irradiation on neutrophil oxidative function was evaluated using a flow cytometric assay of intracellular hydrogen peroxide (H2O2) production. This assay quantitates the H2O2-dependent conversion of the nonfluorescent compound, 2'-7'-dichlorofluorescin (DCFH), into fluorescent 2'-7'-dichlorofluorescein (DCF) on a single-cell basis. Intracellular H2O2 production in response to stimulation with phorbol myristate acetate was not affected by neutrophil irradiation at doses up to 2500 rad. In addition, irradiation of intracellular DCFH and aqueous 2'-7'-dichlorofluorescin diacetate (DCFH-DA) resulted in DCF production, which suggested that oxidative molecules produced by aqueous radiolysis were detected by this assay. This study indicates that radiation doses of 1500 to 2500 rad, which are sufficient to prevent induction of graft-versus-host disease by transfused blood components, are not deleterious to neutrophil oxidative metabolism. **TRANSFUSION** 1987;27:167–170.

TRANSFUSED BLOOD COMPONENTS, unless irradiated, can induce potentially fatal graft-versus-host disease (GVHD) in patients who lack immune competence. Consequently, irradiation of blood products is performed routinely before transfusion into patients with severe cellular immune deficiency.[1-3] The target of blood irradiation is the immunocompetent lymphocyte, which mediates the induction of GVHD.[4] However, other cellular components beneficial to the patient undergoing transfusion should not be affected adversely by irradiation. Therefore, it is desirable to limit the radiation dose to preserve the function of these components.

The effect of irradiation on cellular blood constituents has been the subject of several studies.[5-11] Nevertheless, the most appropriate radiation dose is undetermined, with hypotheses ranging from a minimum mitotic inhibitory dose of 500 rad to a maximum of 5000 rad. The most controversial data have resulted from studies of neutrophil function after irradiation. Defective neutrophil chemotaxis and bactericidal activity do not occur at radiation doses below 10,000 rad.[6,7,9] However, the alteration of neutrophil oxidative metabolism has been reported at varying doses. Specifically, Button et al.[7] reported no significant loss of phorbol ester-stimulated extracellular superoxide production by neutrophils exposed to 5000 rad, whereas Buescher et al.[5] found that neutrophils obtained by continuous-flow centrifugation had highly variable percentages of nitro blue tetrazolium (NBT) reduction positivity after 2500 rad.

The functional significance of the changes seen at less than 5000 rad is unclear. NBT reduction is a semiquantitative method that detects formation of a formazan derivative by superoxide anion (O_2^-) and hydrogen peroxide (H2O2). Therefore, it is a suboptimal technique for establishing the dose-response characteristics of oxidative metabolism inhibition. For this reason we examined the phorbol myristate acetate (PMA; Consolidated Midland, Brewster, NY)-stimulated oxidative response in neutrophils using a sensitive, quantitative flow cytometric assay of intracellular oxidative metabolism.

The assay used in this study was originally described by Keston and Brandt[12,13] and was further characterized and adapted to flow cytometry by Bass et al.[14] It uses the nonfluorescent hydrophobic compound, 2'-7'-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA penetrates the cell membrane due to its hydrophobicity. It is trapped intracellularly by hydrolytic deacetylation to 2'-7'-dichlorofluorescin (DCFH), which is rapidly oxidized by H2O2 in the presence of peroxidase to the highly fluorescent, stable compound, 2'-7'-dichlorofluorescin (DCF). The dose-dependent conversion of substrate DCFH to fluorescent DCF can be detected rapidly on a single-cell basis by flow cytometry, thereby providing a fully quantitative assay of oxidative product formation in neutrophils.

Materials and Methods

Reagents

We used DCFH-DA (Eastman Kodak Co., Rochester, NY); PMA (Consolidated Midland, Brewster, NY); phosphate-buffered saline (PBS; Gibco, Grand Island, NY); ethylene diamine-tetraacetic acid (EDTA; Sigma); DCF; (Sigma Chemical, St. Louis, MO); and ficoll-hypaque (Gallard Schlessinger, Carle Place, NY). The buffer used in the detection of intracellular DCF consisted of PBS with 2 m*M* EDTA, 0.1 percent gelatin, and 5 m*M* glucose, pH 7.4 (PBS-gel).

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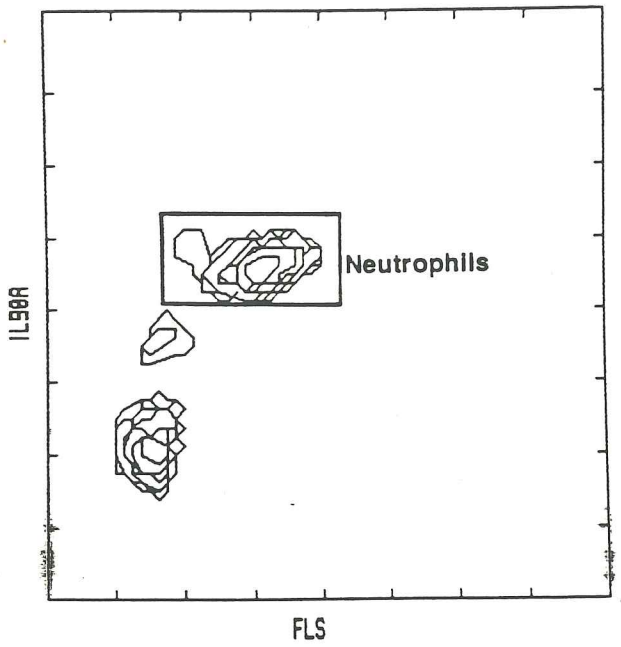

FIG. 1. Bitmap gating of neutrophils. Five thousand leukocytes were examined by light scatter characteristics. FLS: forward light scatter; IL90°–90° light scatter (log scale).

Leukocyte suspension

Heparinized blood was obtained from 10 normal volunteers. Neutrophils were isolated by layering 1 ml of blood over 1 ml ficoll-hypaque. The sedimentation by gravity of agglutinated erythrocytes was allowed to proceed for 30 minutes at room temperature. Platelet- and neutrophil-rich plasma (400 µl) was drawn off of the top layer and resuspended in 3.6 ml PBS-gel, then centrifuged at 100 × *g* for 10 minutes to separate the neutrophils from the platelets. The neutrophil pellet was washed twice and resuspended in 4 ml of PBS-gel, counted (Model ZB, Coulter Electronics, Hialeah, FL), and adjusted to 5×10^6 cells per ml. Neutrophil viability was assessed by trypan blue exclusion; it exceeded 95 percent in all samples.

Cell irradiation

Cell suspensions were incubated with DCFH-DA (final concentration, 5 µ*M*) for 15 minutes at 37°C before irradiation. Irradiation was administered with a Cobalt 60 gamma source at 500, 1500, and 2500 rad. Control cell suspensions were not irradiated.

Measurement of intracellular DCFH oxidation

Cells were separated, incubated with DCFH-DA, irradiated as described above, then stimulated and analyzed immediately after irradiation. The stimulus, PMA (final concentration, 100 ng/ml), was added to a 200-µl aliquot of the cell suspension. Unstimulated and stimulated cells were incubated for an additional 15 minutes at 37°C and examined on a flow cytometer (EPICS-C, EPICS Division, Coulter) equipped with an argon laser set at an excitation wavelength of 488 nm. Green fluorescent emission (510–550 nm) was recorded with a photomultiplier tube. In each experiment, 5000 cells were examined for the following: forward light scatter (FLS), log 90° light scatter (IL90), linear green fluorescence (IGF), and log green fluorescence (ILGF). Neutrophils were separated from the contaminating lymphocytes, monocytes, and platelets by electronic gating on a FLS versus IL90 bitmap (Fig. 1). The intensity of the green fluorescence was collected from neutrophils delineated by the light scatter bitmap (Fig. 2). The high-voltage setting on the photomultiplier tube was calibrated with three distinct fluorescent latex beads (Superbright, Coulter), so that 50, 25, and 12.5 percent of the fluorescent beads fell at channels 180, 77, and 35, respectively (255 total channels). Each preparation was recorded at a single amplification setting of the photomultiplier tube.

Quantative calibration of flow cytometric technique

The quantitative calibration was performed with slight modifications of the method of Bass et al.[20] Neutrophils (5×10^6/ml) were incubated with 0.1, 0.5, 1.0, and 5.0 µM DCFH-DA at 37°C for 15 minutes, washed, and suspended in PBS-gel. The cells were then stimulated with PMA (100 ng/ml). An aliquot was sonicated and the fluorescence of the supernatant was determined by spectrofluorometry. The fluorescence of the sonicates was compared with the fluorescence of solutions of reagent DCF of known concentrations. Parallel nonsonicated cell samples were examined by flow cytometry and the mean fluorescent channel numbers of 10,000 cells were determined. Fluorescent channel numbers were then plotted against the fluorescence of matched sonicated samples (in picomoles DCF/5×10^6 cells). The final expression of DCF production for each mean channel number was expressed in attomoles DCF per cell. Net changes in DCF per cell were analyzed with the *t*-test (unmatched).

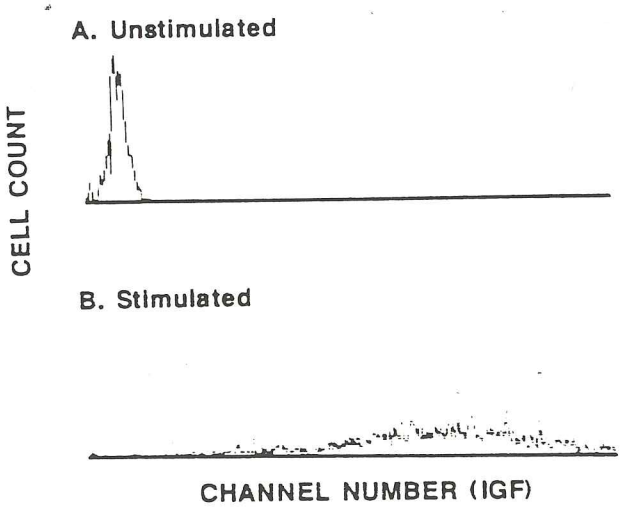

FIG. 2. Distribution of fluorescence in unstimulated and PMA stimulated neutrophils. Neutrophils were incubated for 15 minutes with 5 µ*M* DCFH-DA; PMA, 100 ng per ml final concentration, was added and fluorescence was measured 15 minutes later. The histograms plot the number of cells (ordinate) as a function of fluorescence intensity (abscissa). The mean fluorescence channel of resting and stimulated neutrophil populations was determined in irradiated and control cells.

Results

Fluorescence of unstimulated and stimulated neutrophils incubated with DCFH-DA before irradiation

Unstimulated, unirradiated neutrophils incubated with DCFH-DA fluoresced spontaneously at a low intensity, corresponding to 25.2 ± 3.3 attomoles DCF per cell ($\bar{x}$ ± SEM). Unstimulated neutrophils loaded with DCFH-DA and then irradiated at doses of 500, 1500, and 2500 rad had a dose-related increase in intracellular DCF, reaching a maximum of 59.5 ± 5.0 attomoles DCF per cell at 2500 rad (a 2.4-fold increase).

To determine the mechanism of the increase in fluorescence in unstimulated, irradiated cells, DCFH-DA (5 μM) in PBS-gel alone was irradiated at 500, 1500, and 2500 rad. Spectrofluorometric analysis revealed dose-dependent increases in fluorescence equivalent to 37 picomoles per ml DCF at 0 rad and 96 picomoles per ml DCF at 2500 rad (a 2.6-fold increase). The regression analysis of DCF produced by the irradiation of intracellular and cell-free DCFH-DA revealed a correlation coefficient of $r=0.995$ ($p<0.001$), suggesting a direct oxidative effect of irradiation on the fluorochrome.

PMA stimulation of cells loaded with DCFH-DA and then irradiated at doses up to 2500 rad revealed strong fluorescent responses. The net rise in fluorescence (stimulated minus resting) corresponded to rises in DCF production of 135.1 ± 11.5 attomoles per cell at 0 rad and 123.0 ± 11.8 attomoles per cell at 2500 rad (Fig. 3). The difference between the DCF produced by PMA stimulation in nonirradiated cells and that in cells irradiated to 2500 rad was not significant ($p>0.05$).

Discussion

The constant net increase in fluorescence seen in control neutrophils and in neutrophils irradiated to 2500 rad after incubation with DCFH-DA indicates that the membrane-associated enzymatic mechanisms for PMA-stimulated, intracellular H2O2 generation remain fully intact at these doses. The value of DCFH-DA as a determinant of intracellular oxidative metabolism is based on its specificity for H2O2 production.[14,15] Exogenous H2O2 and enzyme systems producing H2O2 (glucose oxidase + glucose and xanthine oxidase + acetaldehyde) have resulted in the oxidation of intracellular DCFH. Furthermore, DCFH oxidation is inhibited by exogenous catalase but not by superoxide dismutase. Also, neutrophils with defective H2O2 metabolism from patients with chronic granulomatous disease do not oxidize DCFH. Cathcart et al.[16] showed that DCFH can be oxidized to DCF by exogenous lipid peroxides. It is unlikely, however, that intracellular lipid peroxide formation would account for a significant fraction of the DCF formation in PMA-stimulated neutrophils. These studies indicate that most intracellular H2O2 production is detected accurately by this assay. Nevertheless, the interpretation of our results with respect to the effect of irradiation on neutrophils in transfused units requires caution. The neutrophils assayed in our study were not obtained by the continuous-flow centrifugation method used in clinical practice. In addition, this assay evaluated in vitro neutrophil oxidative function by the response to PMA. The in vivo function of neutrophils in response to the physiologic stimulants of oxidative metabolism must be inferred from these results.

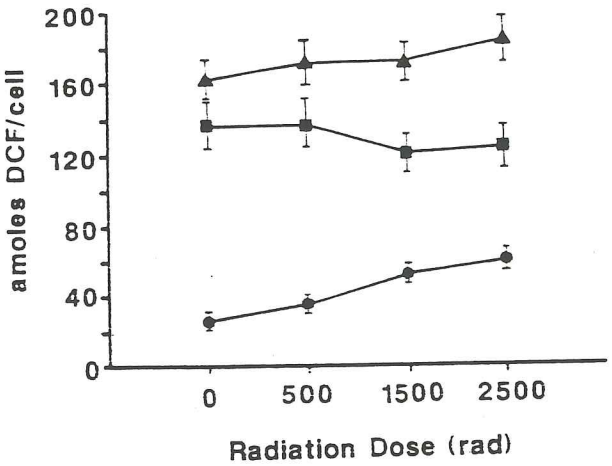

FIG. 3. Effect of radiation on human neutrophil oxidative metabolism. Neutrophils were incubated with DCFH-DA for 15 minutes, irradiated at doses up to 2500 rad, and then stimulated with PMA. Mean intracellular DCF content in attomoles per cell (ordinate) was determined. Radiation dose in rad is plotted on the abscissa. Each point is the mean of at least 5000 individual cell determinations: ●, unstimulated; ▲, stimulated; ■, net increase (stimulated minus unstimulated).

We also demonstrated a dose-dependent increase in the fluorescence of unstimulated, DCFH-DA-loaded and irradiated neutrophils. This rise in fluorescence correlated closely with the radiation-induced fluorescence of DCFH-DA in solution. A possible mechanism for this effect is the radiolytic deacetylation of DCFH-DA and the simultaneous generation of oxidative molecules due to the interaction of ionizing radiation with an aqueous compartment.[17,18] These primary reactive species, including hydroxyl-free radicals, hydrated electrons, and atomic hydrogen ($OH\cdot$, e^-_{aq}, $H\cdot$), are capable of rapidly generating secondary molecules, including H2O2, which may result in the oxidation of DCFH.

Measurements of oxidative metabolism in irradiated neutrophils have been performed by several techniques: quantitation of O2 consumption by respirometry[8]; detection of hexose monophosphate shunt-dependent (HMPS) oxidation of glucose-1-C^{14}; quantitation of myeloperoxidase activity[6]; measurement of superoxide-dismutase-inhibitable reduction of ferric cytochrome C by superoxide anion[7]; and detection of NBT reduction mediated by H2O2 and $O2^-\cdot$.[5,11] Previous studies of HMPS and myeloperoxidase

activity, as well as the analysis of superoxide anion production, support the data presented in this report. These experiments did not show a significant reduction in neutrophil oxidative metabolism at clinically relevant radiation doses below 5000 rad. Conversely, studies of NBT reduction and O2 consumption by respirometry indicated a significant inhibition of intracellular oxidative metabolism at doses of 2500 and 2000 rad, respectively. The reason for this discrepancy is unknown. However, the NBT reduction assay is semiquantitative and limited by subjective interpretation. Furthermore, the consumption of oxygen by living cells, which is measured by respirometry, can proceed along several biochemical pathways and consequently is not specific for "oxidative burst"-related metabolism.

In conclusion, PMA-stimulated production of intracellular H2O2 is unaffected in neutrophils irradiated to doses of 2500 rad. Radiation doses of 1500 to 2500 rad are sufficient to prevent transfusion-related GVHD and are not deleterious to neutrophil oxidative metabolism.

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