Structural damage to lymphocyte nuclei by H₂O₂ or gamma irradiation is dependent on the mechanism of OH⁺ radical production

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Summary Normal human lymphocytes were exposed to OH⁺ radicals produced indirectly by exposure to H₂O₂ or directly by gamma irradiation. Using a flow cytometry technique to measure changes in nucleoid size, it was found that generation of OH⁺ in each system produced a characteristic relaxation in nuclear supercoiling. Exposure of cells to H₂O₂ produced a metal-dependent step-wise relaxation in extracted nucleoids, while gamma irradiation induced a gradual dose-dependent increase in nucleoid size. The site-specific metal-dependent changes produced in lymphocytes incubated in H₂O₂ should also occur in gamma irradiated cells, but the characteristic effects on nuclear supercoiling would not be detected within the background of random DNA damage. The importance of metals in maintaining the supercoiled loop configuration of DNA within the protein matrix suggests that free radical damage at metal locations may be particularly toxic for the cell.

Strand breaks constitute major lesions in cells exposed to gamma irradiation (Nilsson & Johanson, 1981; Hutchinson, 1985). DNA damage of this nature results, in part, from indiscriminate multi-site attack by OH⁺ radicals generated during water radiolysis (Eqs. 1 & 2) (Hutchinson, 1985; Ward, 1985). Similar radical species and DNA damage occur when H₂O₂ interacts with reduced metal ions associated with chromatin (Eq. (3) (Mello-Filho & Meneghini, 1984; Ward et al., 1985; Goldstein & Czapski, 1986).

\[
\text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^+ + e^- \\
\text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{OH}^- + \text{H}_2\text{O}^+ \\
\text{M}^{60+} + \text{H}_2\text{O}_2 \rightarrow \text{M}^{(n+1)+} + \text{OH}^- + \text{OH}^- \quad (3)
\]

Fenton reaction

The relationship between strand break formation and cellular lethality remains uncertain. Ward et al. (1985; 1987), have proposed that cell death induced by H₂O₂ does not relate to strand-break formation, while others maintain that a cell’s attempts to repair this kind of damage leads to a series of metabolic disruptions and substrate depletions which result in cell lysis (Schaufstatter et al., 1985; 1986).

Few studies have addressed the possibility that preferential damage at specific regions of the nucleus may be more detrimental for the cell than random lesions. Observations correlating cell survival with the degree of DNA synthesis inhibition (Cramp et al., 1982; Elkind, 1985), imply that cell death may result from disruption of the processes and structures that direct DNA synthesis and transcription. Thus, analysis of damage to the higher structure of the nucleus, rather than just the DNA, may provide additional insight into the sequence of toxic events that occur when cells are exposed to radiation or H₂O₂.

The higher order chromatin structures that support DNA replication may be examined by extracting nuclei from cells using buffers that remove most stabilising proteins (Cook et al., 1976). Such nuclei, termed nucleoids, comprise DNA in the form of supercoiled loops, each loop representing a complete replication unit (Vogelstein et al., 1980; Lebkowski & Laemmli, 1982; Lewis & Laemmli, 1982). This supercoiled structure can be compacted by the intercalating dye ethidium bromide, or lost altogether when strand breaks are induced in the loops by radiation or chemical treatment (Cook & Brazell, 1976). We have developed an alternative method to velocity sedimentation for analysing changes in nucleoid supercoiling. Relaxation within damaged loops of DNA can be detected as increased light scatter when nucleoids are stained with ethidium bromide and passed through a flow cytometer (Milner et al., 1987).

In this study we have induced DNA strand breaks in human lymphocytes using gamma rays or H₂O₂ and monitored the overall structural consequences using nucleoid flow cytometry.

Materials and methods

Preparation of cells

Peripheral blood mononuclear cells were isolated from the blood of healthy adult volunteers by centrifugation through Lymphoprep separation medium (Gibco Ltd., Paisley, UK). Cells were washed twice in RPMI 1640 (Flow Labs., Irvine, UK) and adjusted to a final concentration of 2 x 10⁶ viable cells ml⁻¹ in RPMI 1640 containing 10% foetal calf serum (FCS) (Flow), 1% glutamine (Flow), 5 U ml⁻¹ streptomyacin sulphate (Evans Medical Labs Ltd., Middlesex, UK) and 5 U ml⁻¹ benzylpenicillamine (Glaxo Labs Ltd., Middlesex, UK). Mononuclear cell preparations were consistently greater than 97% viable and consisted of 10-20% monocytes.

Irradiation of cells

Mononuclear cells were prepared as above and stored under liquid nitrogen in FCS containing 4% dimethylsulphoxide. For each experiment, an aliquot of cells was rapidly thawed, washed twice in fresh RPMI and resuspended to 1 x 10⁶ ml⁻¹ in 100 μl RPMI supplemented with 10% FCS. Viability checks using trypan blue indicated that cells were greater than 90% viable at the start of each experiment. Samples were irradiated using a cobalt-60 gamma ray source at a dose rate of 3 Gy min⁻¹.

Exposure of cells to UV irradiated RPMI 1640

The UV source consisted of two bulbs, wavelengths, 366 and 245 nm (Anderman & Co. Ltd., Surrey, UK). The light source at 366 nm was a Sylvania F8T5/8W bulb with an average light intensity of 17 W cm⁻² at 1 m. The 245 nm source was a G8T5-8W bulb with an average intensity of 10.5 W cm⁻² at 1 m. Briefly, 6 ml aliquots of RPMI 1640 were irradiated in 50 mm petri dishes of 6 cm from the UV source, for various times up to 60 min. Irradiated samples were passed through a 0.2 μm filter (Gelman Sciences Ltd.,

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Nottingham, UK) to ensure sterility and 0.9 ml of each irradiated sample added to 2 × 10⁶ mononuclear cells in 0.1 ml FCS. Control cells received non-irradiated medium. Cells were incubated for 4 or 24 h at 37°C in a humidified 95% CO₂ atmosphere.

Scavenger and chelator studies

Prior to gamma irradiation, cells were incubated with the following free radical scavengers for 30 min: cysteine (50 mM); dimethyl sulphoxide (280 mM); thiourea (500 mM) (all Sigma Chemical Co., Poole, UK). In some experiments mononuclear cells were incubated with the iron chelator desferrioxamine (0.5 mM) (Ciba Labs., Horsham, UK) for 24 h and washed twice in fresh RPMI. Cell aliquots were then exposed to gamma irradiation as above or cultured for 4 or 24 h in UV irradiated medium.

Flow cytometry

Immediately after gamma irradiation or H₂O₂ treatment, cells were lysed in an ice-cold buffer containing 2 m NaCl, 10 mM Tris(hydroxymethyl) and 10 mM EDTA (pH 8). Nucleoids extracted by this procedure were kept on ice for 40 min, then stained with 50 μg ml⁻¹ ethidium bromide and left for 90 s to permit dye intercalation. Analysis was carried out using a Becton Dickinson FACS 440 flow cytometer as described previously (Milner et al., 1987). The intensity of forward light scatter was recorded for each nucleoid and stored as a datum point in a frequency histogram.

Results

A dose-dependent increase in forward light scatter was observed in nucleoids extracted from gamma irradiated lymphocytes. A typical histogram set is shown in Figure 1. The shift to the right in the forward light scatter frequency histogram is quantitated by the increase in median channel numbers; these values are included in Table I. The median value for each frequency histogram assigns a mathematical value to the radiation-induced change, although these values should be interpreted with caution as the shape of the histogram changes with dose. Preincubation of lymphocytes with free radical scavengers had a dose-modifying effect on the light scatter frequency histograms. The median values obtained in these experiments are summarised in Figure 2. Cells incubated with 50 mM cysteine were completely protected, even at the highest irradiation dose of 10 Gy. Dimethyl sulphoxide and thiourea-treated cells irradiated with 10 Gy produced histogram shapes characteristic of a 5 Gy dose and corresponding to a 50% reduction in the median forward light scatter obtained for untreated cells.

Table I also gives the median light scatter values obtained for nucleoids extracted from lymphocytes incubated in pre-irradiated medium for 4 and 24 h. At 24 h, the increases in the median values for the forward scatter are coincident with the increase in the level of oxidant stress experienced by the whole cells. At 4 h, the median forward scatter value obtained at the highest level of H₂O₂ was lower than at 24 h, although the alteration in the distribution of nucleoid events was similar. The changes in the forward light scatter histograms of nucleoids from lymphocytes incubated in preirradiated medium are shown in Figure 3. A bimodal

Figure 1 A frequency histogram demonstrating the dose-dependent increase in forward light scatter induced by γ-irradiation. Each histogram represents 10,000 separate nucleoid events.

Figure 2 The effect of free radical scavengers on the median forward light scatter histogram. Results are given for non-irradiated (C), γ-irradiated (R) and γ-irradiated cells in the presence of cysteine (CYS), thiourea (THIO) and dimethylsulphoxide (DMSO) as mean ± s.d. in 3 experiments. **P<0.05. ***P<0.001.

### Table 1

| Gamma dose (Gy) | 0 | 2 | 5 | 8 | 10 |
|-----------------|---|---|---|---|----|
| Median channel no. | 52 | 76 | 91 | 113 | 121 |
| Concentration H₂O₂ (μM) | 0 | 25 ± 2 | 78 ± 10 | 170 ± 15 | 204 ± 15 |
| Median channel no. | 25 | 28 | 41 | 70 | 99 |
| % Nucleoid events in larger population | 42.2 | 11.7 | 26.2 | 45.9 | 59.6 |
| Median channel no. | 51 | 63 | 84 | 173 | 179 |
| % Nucleoid events in larger population | 1.1 | 10.0 | 24.6 | 71.9 | 83.7 |
distribution was apparent where there was a dose-dependent decrease in the number of nucleoids in the first peak and an increase in number in the second. To exclude the possibility that analyses were simply detecting dead cells, nucleoids were extracted from permeabilised (>99% dead) cells. The forward scatter profile obtained in this case showed no similarity to that obtained for the oxidant-treated cells. In addition, the red fluorescence profile of nucleoids extracted from oxidant-treated cells did not show the decrease in red fluorescence characteristic of degraded DNA.

The changes within the frequency histograms in Figure 3 are represented in Figure 4 as the percentage of nucleoid events expressing the high scatter profile at each level of oxidant stress. Each value is plotted against the level of H$_2$O$_2$ detected in irradiated medium using the phenol red assay described elsewhere (Allan et al., 1987). Figure 4 also shows that when mononuclear cells were treated with the iron chelator desferrioxamine, prior to incubation in preirradiated medium, the formation of the high scatter population was almost completely prevented. In contrast, desferrioxamine had no significant effect on the radiation-induced changes in light scatter (data not shown).

**Discussion**

Damage to lymphocyte DNA has been examined by a modification of the nucleoid sedimentation technique using a flow cytometer-based laser light scattering system (Milner et al., 1987). The light scattering process from particles in a flow cytometer is a complex function, dependent on both reflection and refraction from the target particle, making it difficult to derive an analytical solution relating light scatter to target size (Hodkinson & Greenleaves, 1963; Loken & Stall, 1982). However, empirically it is possible to show that larger particles of the same type scatter more light than smaller ones. More importantly, the significant advantage of speed and single cell analysis gives the potential for a statistical examination of DNA damage within cell populations.

Nucleoids extracted from lymphocytes exposed to graded doses of gamma rays showed a gradual dose-related increase in the median of the laser scatter histogram. This finding is consistent with gamma radiation producing random DNA strand breaks and other structural alterations, which may inhibit the free rewinding of the DNA supercoils induced by ethidium bromide (Vogelstein et al., 1980). In studies assessing the protective effects of free radical scavengers, cysteine, a potent radioprotector (Sasaki & Matsubara, 1977), completely prevented the radiation-induced increase in light scatter. In the presence of thiouria and dimethyl sulphoxide there was an approximately 50% reduction in radiation-induced nucleoid expansion. Although cysteine, thiouria and dimethyl sulphoxide are not entirely specific for OH$^+$ radicals, the relative protection afforded by each compound is consistent with their rate of reaction with OH$^+$ radicals (Halliwell & Gutteridge, 1985).

We have shown previously (Allan et al., 1987), that the lymphotoxic effects of preirradiated culture medium over 24 hours are attributable to events involving H$_2$O$_2$, as addition of catalase, an enzyme which specifically degrades H$_2$O$_2$, almost completely prevented cell killing in vitro. Medium supplemented with reagent H$_2$O$_2$ produced identical changes in lymphocyte nuclei to those reported for cells incubated in preirradiated medium. Substantial experimental evidence suggests that H$_2$O$_2$ induces DNA strand breaks by interacting with DNA-bound metals to generate OH$^+$ radicals (Eq. 3) (Meneghini et al., 1984; Ward et al., 1985; 1987; Goldstein & Czapski, 1986). Nucleoids extracted from lymphocytes incubated in preirradiated medium exhibited a stepwise shift from the control scatter profile to a discrete population with increased light scatter. Nucleoids from lymphocytes pretreated with the iron chelator desferrioxamine, did not show these changes, suggesting that the size increase related to metal-dependent reactions involving H$_2$O$_2$.

These studies have shown that OH$^+$ radicals, induced directly in cells by gamma irradiation or indirectly from reactions involving H$_2$O$_2$, generate different patterns of supercoiled relaxation. These differences could reflect structural alterations of the higher order DNA structure. Lebkowski & Laemmli (1982) and others (Dijkwel & Wenink, 1986), have demonstrated the importance of metal ions, notably copper, in stabilising the association between DNA supercoils and the non-histone nuclear matrix proteins. The removal of these ions by metal chelators results in a stepwise expansion of the original nucleoid structure (Dijkwel & Wenink, 1986). The analogous changes in light scatter induced by H$_2$O$_2$ suggest that 'site-specific' OH$^+$ attack could occur at the same metal locations involved in maintaining the overall supercoiled structure.

If the metals within the matrix represent potential interaction sites for H$_2$O$_2$, the data obtained in this study may provide an alternative view of the role of strand breaks in the induction of cell killing by H$_2$O$_2$ or gamma irradiation. In the case of H$_2$O$_2$, single strand break formation continuously competes with the processes of repair (Evans et al., 1986). Ward et al. (1987) have suggested that when the rate of repair is slower than the rate of single strand break formation, irreparable DNA interstrand breaks, such as coincident breaks in both strands of the DNA occur (Ward et al., 1987). We believe that damage to the metal-protein interactions maintaining the DNA on the matrix may constitute an additional form of lethal lesion(s) in cells exposed to H$_2$O$_2$.

The concentration of H$_2$O$_2$ required to induce the extent of nuclear relaxation characteristic of the high scatter peak may be different for different mononuclear cell populations, for example, lymphocytes are far more sensitive to H$_2$O$_2$.

![Figure 3](image-url) A frequency histogram demonstrating the dose-dependent increase in forward light scatter of nucleoids extracted from lymphocytes incubated in preirradiated medium for 4 and 24 h. Each histogram represents 10,000 separate nucleoid events.

![Figure 4](image-url) Effect of desferrioxamine (DFX) on the percentage of nucleoid events occurring in the high light scatter population (see text).
than are mononocytes (Sagone et al., 1984). This could explain the light scatter profiles of nucleoids from 4 h and 24 h incubations. At 4 h, when cells were as viable as controls, extracted nucleoids displayed a range of relaxed nuclear conformations. This may reflect incomplete expression of OH•-mediated events within the nucleus. At 24 hours, the dose-dependent decrease in viable cells correlated with a progressive increase in the number of nucleoids exhibiting a more uniform relaxed conformation. It could be that more susceptible cells, unable to sustain damage within the nucleus, never attain the relaxed nuclear form characteristic of the high scatter peak, but still undergo similar changes in DNA conformation. This process may constitute a lethal lesion for the cells.

The initiation and continuation of the Fenton reaction (3) requires the presence of molecules able to generate metal ions to the reduced catalytic state. Treating cells at 4°C to 24 h more than changes in activity may

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