Biochemical Pharmacology, Vol. 43, No. 4, pp. 705–715, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

ROLE OF LIPID PEROXIDATION AND DNA DAMAGE IN PARAQUAT TOXICITY AND THE INTERACTION OF PARAQUAT WITH IONIZING RADIATION

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(*Received* 17 *June* 1991; *accepted* 7 *November* 1991)

Abstract—Since the introduction of paraquat (PQ) as a herbicide in 1963, there have been many speculations concerning the critical lesion in PQ toxicity. Damage to membrane lipids might be an initial event leading to PQ-induced cell killing. The ability of PQ to induce lipid peroxidation was tested in liver homogenates of the mouse. Lipid peroxidation was indeed induced by PQ and shown to be dose dependent, starting to be significant at 2.5 mM. Subsequently, a possible correlation between lipid peroxidation and PQ-induced cell death was investigated in mouse fibroblasts (LM) and Ehrlich ascites tumour (EAT) cells using a clonogenic assay. It was found that in order to be cytotoxic PQ needs enzymatic activation (incubation at 37°). In both cell lines, PQ-induced cell killing appeared to be dose dependent, starting at a dose of 0.5 mM. Supplementation of LM cells with the antioxidant vitamin E had no effect on PQ-induced cell killing and modification of the membranes of LM cells by incorporation of the polyunsaturated fatty acid 20:4 (arachidonic acid) did not sensitize the cells to PQ toxicity. PQ had no effect on the glutathione (GSH) level in EAT cells and complete GSH depletion by DL-buthionine-(*SR*)-sulfoximine could not sensitize the cells to PQ toxicity. In LM cells PQ-induced cell killing was enhanced after complete GSH depletion by DEM. This sensitization might, however, be attributed to the binding of DEM to proteins. From these results it seems unlikely that lipid peroxidation is the primary cause for PQ-induced cell killing. Another critical target in PQ toxicity is DNA. This possibility was investigated in EAT cells. PQ was found to induce DNA damage (detected by the alkaline unwinding assay) in the same dose range that caused cell death. A good correlation was obtained for cell killing after PQ treatment and DNA damage measured 2 hr after 37° post-incubation. A proposed possible interaction between PQ and X-rays was also investigated. In EAT cells, X-ray-induced cell death was significantly enhanced by pre-incubation with PQ at doses of 0.5 mM and above. At the level of 10% survival an enhancement factor of 1.6 could be observed by treatment with 1 mM PQ when cell killing by PQ is not taken into account. Induction as well as processing of radiation-induced DNA damage seems to be unaffected by pre-incubation with PQ. The mechanism of radiosensitization by PQ is yet unclear.

Paraquat (1,1′-dimethyl, 4,4′-bipyridil; PQ†) is a non-selective contact herbicide which is used in agriculture in more than 100 countries all over the world. Provided the recommendations for use are followed and there is adherence to safe working practices, PQ does not pose a health risk. However, a large number of cases of accidental or suicidal poisoning from PQ has been reported [1]. The most characteristic feature of PQ toxicity is the active accumulation of PQ in the lung resulting in extensive damage of this tissue. Other organs known to be affected are the liver, kidney, thymus and adrenal glands [1–3].

In several studies, the biochemical mechanism of PQ toxicity has been investigated. It has been demonstrated that PQ undergoes a one-electron-reduction by the flavoenzyme NADPH-cytochrome P450 reductase [3–7]. Thereby, the free radical is formed which can react rapidly with molecular oxygen to produce the superoxide anion radical with the regeneration of the PQ dication. Thus, in the presence of sufficient supply of reducing equivalents, repeated cycles of PQ reduction and reoxidation can occur producing large amounts of reactive oxygen species. As a consequence, the balance between oxygen radical generation, normally associated with cellular metabolism, and their dissipation by cellular defense systems (e.g. superoxide dismutase, catalase, peroxidase, glutathione, vitamin E) is disturbed, allowing reactive oxygen species to attack biomolecules. Membrane damage induced by lipid peroxidation, inactivation of proteins or damage to DNA may subsequently lead to cell death.

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† Abbreviations: PQ, paraquat (1,1′-dimethyl,4,4′-bipyridil); EAT, Ehrlich ascites tumour; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; LM, mouse fibroblasts; BSA, bovine serum albumin; BSO, DL-buthionine-(*SR*)-sulfoximine; GSH, glutathione; DEM, diethylmaleate; TBA, thiobarbituric acid; MDA, malondialdehyde; 20:4, arachidonic acid (5,8,11,14-eicosatetraenoic acid).

Despite extensive investigations, the critical target of PQ-induced cell death is still unknown. While it is suggested that the herbicidal action of PQ is most likely due to the peroxidative degeneration of membrane lipids [8], the role of lipid peroxidation in mammalian PQ toxicity is more ambiguous [5, 7, 9–12]. In order to address this question a study was initiated where the ability of PQ to initiate lipid peroxidation was first tested in liver homogenates. Subsequently, a possible correlation between PQ-induced lipid peroxidation and cell death was investigated in two clonogenic cell lines (EAT cells and mouse fibroblasts). Since the direct determination of low amounts of lipid peroxides is impossible with the technique available, it was decided to use indirect approaches. At first, it was tried to sensitize cells to PQ-induced cell killing by supplementation of the membranes with arachidonic acid or depletion of intracellular glutathione. Secondly, a possible protection of the cells by incorporation of the potent antioxidant vitamin E into the membranes was studied.

Alternatively or consequently, DNA damage might be the critical lesion in PQ toxicity. This possibility was studied in the second part of this report. In EAT cells the effect of PQ on DNA damage was determined and a possible correlation with cytotoxicity is discussed.

An agent also capable of generating free radicals of oxygen is ionizing radiation. In the case of low linear energy transfer radiation (X-rays, γ-rays), about two-thirds of the biological damage in cells occurs indirectly by water-derived oxygen radicals (mainly hydroxyl radicals), killing the cells by attacking critical subcellular targets [13]. Since PQ as well as ionizing radiation is capable of initiating the formation of reactive molecules of oxygen, the cellular mechanism(s) leading to PQ- or radiation-induced cell death [14] might have certain similarities. Furthermore, it might be that when PQ and radiation are applied together, some interactive mechanisms may determine the extent of the toxicity. There are two reports in the literature suggesting a synergistic action of PQ and X-rays on mammalian cells [15, 16]. This interaction of PQ and X-rays, together with the active accumulation of PQ into the lung, lead these investigators to suggest that PQ might be useful in the therapy of lung cancer, acting as a radiosensitizer. We further investigated the suggested interaction of PQ with X-rays in EAT cells.

MATERIALS AND METHODS

Preparation of a liver homogenate. Mice (male syngeneic C57-Black, 3–5 months old) were killed by cervical dislocation, and the livers were rapidly isolated. From this moment on, all procedures were performed in ice in order to minimize auto lipid peroxidation. After washing in isotone (0.15 M) KCl solution, the livers were weighed and minced. Finally, a 10% (w/v) liver homogenate was prepared in 0.15 M KCl using a Potter–Elvehjem homogenizer.

Cell culturing. EAT cells were grown in suspension culture in RPMI 1640 medium (Flow Laboratories, Irvine, U.K.) supplemented with 10% fetal calf serum (Gibco, Paisley, U.K.), 50 μg/mL streptomycin (Mycopharm, Delft, The Netherlands) and 50 IU/mL penicillin G (Mycopharm), in a shaking incubator at 37°, as described by Jorritsma and Konings [17]. The doubling time of the cells was 12–14 hr. In all experiments exponentially growing cells were used.

Mouse fibroblast LM cells (American Type Collection, CCL 1.2) were adapted to growth in suspension culture in the serum-free, lipid-free and protein-free medium of Higuchi [18] supplemented with 20 mM Hepes buffer pH 7.4, 1 g/L of methyl cellulose, 50 IU/mL penicilline-G, 50 μg/mL streptomycin, 10 μg/mL sodium dextran sulfate as described by Wolters and Konings [19]. Additionally, the following trace elements were added to the culture medium: 0.25 μg/L $CuSO_4 \cdot 5H_2O$, 4.00 μg/L H_2SeO_3, 0.20 μg/L $MnCl_2 \cdot 4H_2O$, 1.00 μg/L $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$, 0.10 μg/L $SnCl_2 \cdot 2H_2O$ and 100.00 μg/L $Na_2SiO_3 \cdot 9H_2O$. The cells were grown at 37° in serum flasks in a shaking incubator. The doubling time of the cells was 24–26 hr.

Modification of the fatty acid composition of cell membranes. The membranes of LM fibroblasts were enriched with the polyunsaturated fatty acid 5,8,11,14-eicosatetraenoic acid (20:4, arachidonic acid, Sigma, Grade I) by the method of Spector and Hoak [20]. Briefly, exponentially growing cells were incubated in complete culture medium supplemented with 100 μM 20:4 complexed to 25 μM fatty acid-free BSA (Sigma) for 24 hr. Because of the risk of peroxidation, 20:4 coated BSA was kept in the dark. The fatty acid composition was assayed by GLC as described by Wolters and Konings [19]. Control cells were grown with 25 μM fatty acid-free BSA present in the culture medium. Doubling time of the cells was 24–26 hr. In all experiments exponentially growing cells were used excluding Trypan blue for more than 95%. Prior to use the cells were centrifuged at 100 *g* for 5 min and resuspended in fresh medium to remove non-incorporated 20:4 or BSA.

Modification of the vitamin E content of cell membranes. LM fibroblasts with an increased vitamin E content were prepared as described by Wolters and Konings [21]. Briefly, cells (5×10^5 cells/mL in a total volume of 50 mL) were grown in complete culture medium supplemented with 230 μM vitamin E (DL-α-tocopherol, Sigma) complexed to 25 μM fatty acid-free BSA for at least 10 doubling times. Because of the risk of peroxidation, vitamin E was kept in the dark. Vitamin E content was assayed by GLC as described by Kormann [22]. Control cells were grown with 25 μM fatty acid-free BSA present in the culture medium. Doubling time of the cells was 24–26 hr. In all experiments exponentially growing cells were used excluding Trypan blue for more than 95%. Prior to use the cells were centrifuged at 100 *g* and resuspended in fresh medium to remove unincorporated vitamin E or BSA.

Depletion of glutathione. In order to eliminate the GSH content of EAT and LM cells non-toxic depleting agents were needed. Konings and Penninga [23] showed that BSO is suitable for the elimination of GSH in EAT cells, whereas in LM cells the concentration of BSO necessary to eliminate GSH completely is toxic. Therefore, in LM cells DEM

has to be used to eliminate GSH. In EAT cells, however, DEM appeared to be toxic.

I. BSO (Sigma) is a competitive inhibitor of glutamylcysteine synthetase, which is a key enzyme in the biosynthesis of glutathione [24]. EAT cells were incubated with BSO for 16 hr at 37° (500 μM BSO; 3×10^5 cells/mL in a total volume of 40 mL). At the end of the incubation period, the cells were washed with fresh culture medium. This BSO treatment resulted in a complete depletion of GSH without any deleterious effect on cell viability.

II. DEM (Sigma) is an α,β-unsaturated electrophil which reacts non-enzymatically (slowly) and enzymatically (fast) by glutathione-*S*-transferase with GSH [24]. The cells were incubated with DEM for 1 hr at 37° (500 nmol DEM; 10^6 cells/mL in a total volume of 50 mL). At the end of the incubation period, the cells were washed with fresh culture medium. This DEM treatment resulted in a complete depletion of GSH without any deleterious effect on cell viability.

DNA labelling of the cells. DNA labelling of the cells was obtained by growing the cells (about 1×10^5 cells/mL) in culture medium containing 2 μM [^{3}H]thymidine (Du Pont, New Products, Boston, MA., U.S.A. sp. act. 20 Ci/mol) and 5 mM unlabelled thymidine, for a period of 44–48 hr (about four doubling times). After centrifugation (5 min, 100 *g*), the cells were resuspended in fresh medium containing 5 mM unlabelled thymidine. The cells were incubated for 60 min (chase), centrifuged and resuspended in normal growth medium at a density of about 1.1×10^6 cells/mL.

Conditions for incubation of the cells with PQ. For each experiment a solution of PQ (methyl viologen hydrate; Janssen Chimica, Beerse, Belgium) was freshly prepared by dissolving PQ in the appropriate medium at a concentration 10 times higher than the final concentration required.

I. PQ solution (1.5 mL) was added to 13.5 mL 10% (w/v) liver homogenate (about 1 mg protein/mL), yielding final PQ concentrations between 0 and 10 mM. The incubation was performed in a shaking waterbath at 37°. For the determination of lipid peroxidation, samples of 800 μL were taken 0, 0.5, 1, 2, 3 and 4 hr after the incubation.

II. PQ solution (100 μL) was added to 900 μL cell suspension (1.1×10^6 cells), yielding final PQ concentrations between 0 and 2 mM. The cells were incubated for 2 hr in a shaking waterbath at 37° or in icewater. Incubation was stopped by washing the cells twice with fresh medium in order to remove extracellular PQ. Thereafter, cells were processed for assessment of lipid peroxidation, DNA damage, intracellular GSH or clonogenic ability.

Conditions for X-irradiation. After PQ incubation, the cells were immediately put into icewater and irradiated with 0–12 Gy in air. X-irradiation was performed using a Philips–Müller MG 300 machine operating at 200 kV and 15 mA. The X-rays were filtered using 0.5 mm Cu and 0.5 mm Al. The dose rate was 6 Gy/min. Subsequently, the cells were washed twice with RPMI 1640 in order to remove extracellular PQ. The cells were then processed for assessment of clonogenic ability and the occurrence of DNA damage.

Lipid peroxidation assay. PQ-induced stimulation of lipid peroxidation was measured in liver homogenates by the TBA assay of Wilbur *et al.* [25], modified as described by Konings *et al.* [26]. The amount of TBA reactive compounds was related to the amount of protein as determined by the method of Lowry *et al.* [27].

GSH assay. Total glutathione, referred to as GSH, was measured using the method of Griffith [28]. The amount of GSH was related to the amount of protein as determined by the method of Lowry *et al.* [27].

Trypan blue exclusion test. Prior to each experiment the dye exclusion capacity of the cells was determined using 0.4% Trypan blue in 0.9% NaCl solution and a hemocytometer counting chamber. Only cell suspensions excluding Trypan blue for more than 95% were used.

Determination of cell survival. Cell survival was determined by testing the colony forming ability on soft agar as described before by Jorritsma and Konings [17]. After treatment, the cells were diluted appropriately to obtain about 100 colonies per plate. For feeder cells (1×10^5 cells per plate), EAT and LM cells, respectively, were used which had been supralethally irradiated with about 180 Gy of X-rays. Cells were plated on Petri dishes of 60 mm (Greiner, Nürtingen, F.R.G.) containing 0.5% agar (Difco, Detroit, MI, U.S.A.) in RPMI 1640, supplemented with 15% new born celf serum (Gibco) plus 50 μg/mL streptomycin and 50 IU/mL penicillin G. The agar plates were incubated at 37° in a humidified CO_2-incubator (5% CO_2, 95% air) for 7 days (EAT cells) or 14 days (LM cells). Colonies containing more than 50 cells were counted. The plating efficiency was 60–80% for LM cells and 60–90% for EAT cells.

Determination of DNA damage. After treatment of the cells with various concentrations of PQ and/or different doses of X-rays, the cells were washed twice and resuspended in 1 mL cold RPMI 1640 medium. DNA damage was determined in samples immediately after treatment (for initial damage) or after post-incubations up to 2 hr at 37° (to allow damage processing). DNA damage was assayed by the method of Ahnström and Erixon [29], as modified by Jorritsma and Konings [17]. By this method, single and double strand breaks as well as alkali labile sites are detected.

Briefly, ice-cooled triplicate samples of 100 μL (about 1×10^5 cells) were treated with an alkaline buffer (pH 12.3) and kept in the dark at 20° for 30 min. After rapid neutralization and addition of sodium dodecyl sulphate, the samples were stored at −20°. Prior to column chromatography, the samples were thawed and sonicated (Branson Sonifier: 30 sec, 50 W). The samples were heated to 60° and 2 mL of each sample was applied to a hydroxylapatite column. All steps of the column chromatography were carried out at 60°. After washing the columns, single stranded DNA was eluted with 2×2 mL 0.15 M phosphate buffer (pH 6.8) and double stranded DNA was eluted with 2×1.5 mL 0.4 M phosphate buffer (pH 6.8). The fractions of single (SS) and double stranded (DS) DNA were collected and counted using cold liquid scintillation. The percentage of DNA that remained

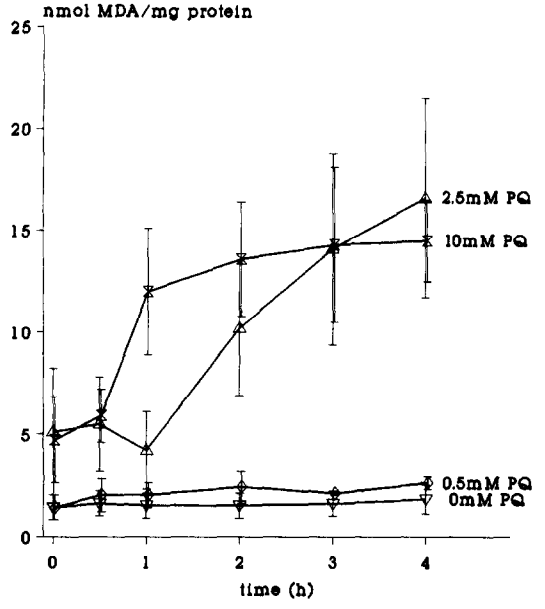

Fig. 1. Lipid peroxidation in liver homogenates as a function of the incubation time at various doses of PQ. Homogenates of the mouse liver were incubated with 0–10 mM PQ at 37° for up to 4 hr. At indicated intervals MDA was measured by the TBA procedure and related to the amount of protein. Each data point represents the mean value ± SEM of three independent experiments.

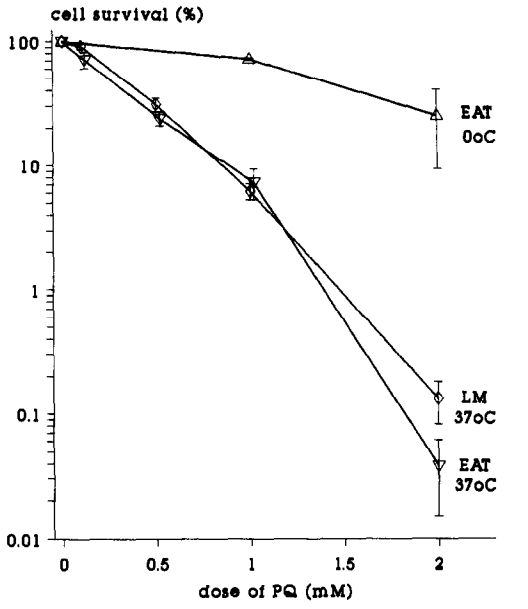

Fig. 2. Survival of EAT and LM cells after a PQ treatment at 37° or 0° (EAT cells). Cell survival is plotted relative to the plating efficiency of untreated controls. Cells were incubated for 2 hr with PQ at indicated concentrations. Each data point represents the mean value ± SEM of at least three independent experiments.

double stranded was calculated by the formula (DS/DS + SS) × 100% and expressed as Gy-equivalents using 0–12 Gy X-ray dose–response curves as calibration standards [17].

RESULTS

Membrane damage as a possible critical lesion in PQ toxicity

During the process of lipid peroxidation, polyunsaturated fatty acids are peroxidized by free radical mechanisms. MDA is a low-molecular weight end product which is formed during the peroxidative degradation of polyunsaturated fatty acids with three or more double bonds. Thus, the amount of MDA produced can be used as a measure for lipid peroxidation [30]. Figure 1 shows the amount of MDA in liver homogenates as a function of the incubation time with and without PQ. In control samples little auto lipid peroxidation was observed during 4 hr. Incubation with 0.5 mM PQ did not result in a significant induction of lipid peroxidation. When 2.5 or 10 mM PQ was added to the liver homogenate non-specific extinctions were detected (timepoint zero). With 2.5 mM PQ, no lipid peroxidation was found during the first hour of incubation. Thereafter, the production of MDA started to increase reaching an amount of about 3–4 times that of control samples 4 hr after the beginning of incubation. With 10 mM PQ, lipid peroxidation increased rapidly between 0.5 and 1 hr. Subsequently, the level of MDA production rose slowly and reached a plateau at about 2 hr. Again, the final MDA concentration was about 3–4 times that of control samples. Thus, in mouse liver homogenates PQ could stimulate lipid peroxidation.

Although PQ may, in principle, cause cell death by lipid peroxidation, no clear relationship has been demonstrated yet. Therefore, experiments with EAT and LM cells were initiated. These cell lines are clonogenic and the membranes of LM cells can easily be supplemented with unsaturated fatty acids or vitamin E.

Figure 2 shows the cell survival of EAT and LM cells as determined by the clonogenic assay after a 2 hr incubation with 0–2 mM PQ. At 37° cytotoxicity appeared to be comparable for the two cell lines. The lowest dose tested, 0.1 mM PQ, caused no significant cell kill. For doses from 0.5 mM PQ and higher, progressive cell killing was observed. When PQ was applied to EAT cells at 0°, to discriminate between direct chemical and enzymatic reactions underlying the PQ toxicity, PQ seemed to cause significant cell killing at a dose of 2 mM. However, as will be discussed later, the observed cytotoxic effect is not induced during the incubation at 0°, but is the result of the presence of residual PQ, causing cell lethality during post-incubation at 37° to allow colony formation.

For the investigation of a possible role of lipid peroxidation in PQ-induced cell death, the membranes of LM fibroblasts were altered. First, LM cells were grown in the presence of the antioxidant vitamin E in order to investigate possible protection against PQ-induced lipid peroxidation

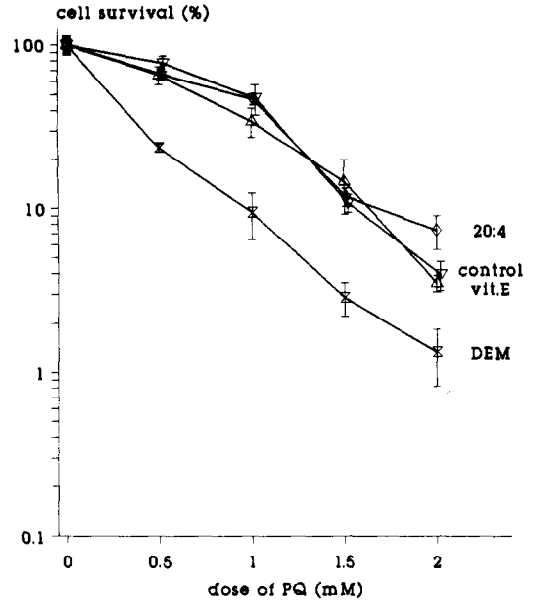

Fig. 3. Survival of normal (control), GSH depleted, 20:4 or vitamin E-supplemented LM cells. Intracellular GSH content was depleted completely by a non-toxic pretreatment with 500 nmol DEM for 1 hr at 37°. Cells were supplemented with 20:4 or vitamin E by culturing them in the presence of these compounds as described in Materials and Methods. Thereafter, cells were incubated with PQ at indicated concentrations for 2 hr at 37°. Cell survival is plotted relative to the plating efficiency of untreated controls. Each data point represents the mean value ± SEM of three independent experiments.

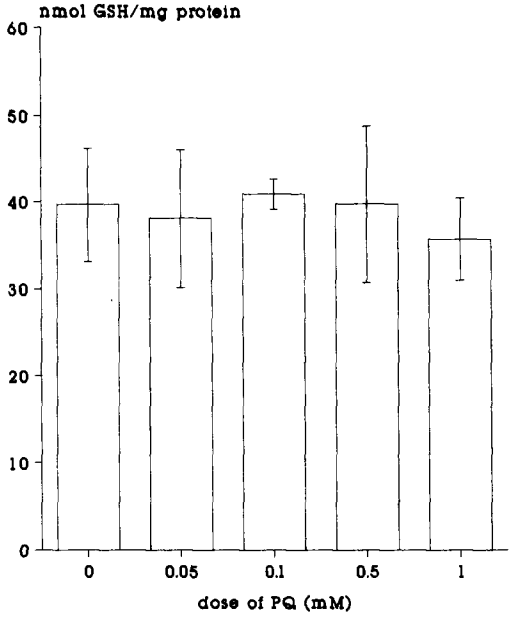

Fig. 4. Effect of PQ treatment on the GSH content of EAT cells. Cells were incubated with PQ at indicated concentrations for 2 hr at 37°. Each data point represents the mean value ± SEM of three independent experiments.

thereby decreasing PQ toxicity. By this treatment, the vitamin E content of the membranes could be enhanced by a factor of about 400, which is in accordance with the previous report of Wolters and Konings [21]. Nevertheless, vitamin E-supplemented cells displayed the same sensitivity to PQ as non-supplemented cells (Fig. 3). Secondly, LM fibroblasts were grown in the presence of the polyunsaturated fatty acid arachidonic acid (20:4) which is highly sensitive to peroxidative damage. Supplementation of LM fibroblasts with 20:4 enhanced polyunsaturated fatty acid content of polar lipid acyl chains from about 7% in the control cells to about 35% in the modified cells which is comparable with the earlier data of Wolters and Konings [19]. By this manipulation, however, also no effect on PQ-induced cell death was found (Fig. 3).

Besides vitamin E, GSH is an important antioxidant present in the cell. Therefore, the possible involvement of GSH in PQ toxicity was examined. After the incubation with 0–1 mM PQ intracellular levels of GSH were measured in EAT cells (Fig. 4). Even a 2 hr incubation with 1 mM PQ, killing more than 90% of the cells, resulted in no significant decrease in the level of GSH. Also, a complete depletion of intracellular GSH by BSO did not stimulate PQ-induced cell killing significantly (Fig. 5). Thus, in EAT cells there was no evidence for a protective role of GSH against PQ-induced cell

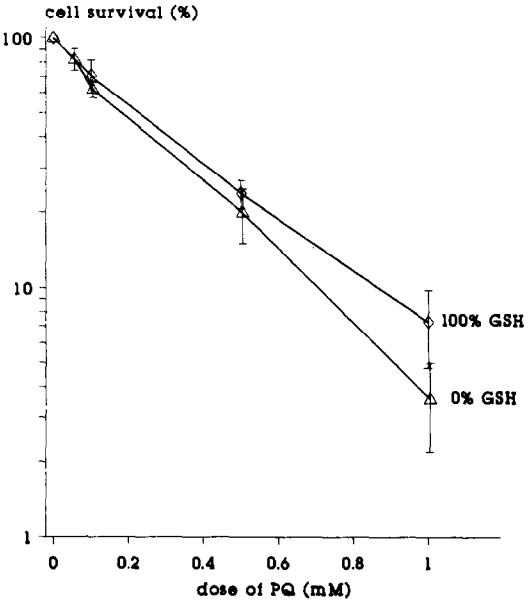

Fig. 5. Effect of GSH depletion on PQ-induced cell killing of EAT cells. Intracellular GSH content was depleted completely by a non-toxic pretreatment with 0.5 mM BSO for 16 hr at 37°. Thereafter, normal and GSH-depleted cells were incubated with PQ at indicated concentrations for 2 hr at 37°. Cell survival is plotted relative to the plating efficiency of untreated controls. Each data point represents the mean value ± SEM of three independent experiments.

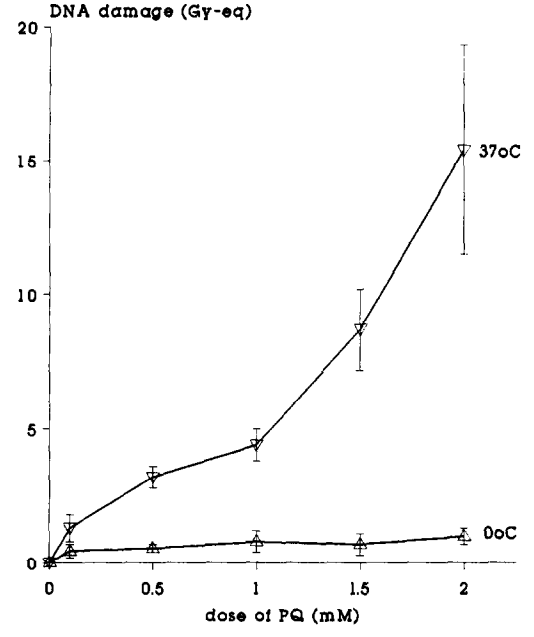

Fig. 6. The induction of DNA damage in EAT cells after a PQ treatment at 37° or 0°. Cells were incubated with PQ at indicated concentrations for 2 hr at 37° or 0°. Immediately after incubation, the amount of DNA damage was assayed by the alkaline unwinding method. The percentage of double stranded DNA was calculated (see Materials and Methods) and expressed as Gy-equivalents using 0–12 Gy X-ray dose–response curves as calibration standards. Each data point represents the mean value ± SEM of at least two independent experiments.

killing. Unfortunately, in LM cells the concentration of BSO necessary to eliminate GSH is toxic [23]. Therefore, DEM had to be used as a non-toxic GSH depletion agent for LM cells. After complete elimination of GSH, PQ-induced cell kill was found to be significantly enhanced (Fig. 3). However, it cannot be excluded that the enhanced PQ toxicity observed after the DEM treatment is a consequence of an effect other than GSH depletion (see Discussion).

DNA damage as the possible critical lesion in PQ toxicity

From the results mentioned above it is unlikely that lipid peroxidation is the cause for PQ-induced cell killing. Therefore, experiments were initiated to investigate the possible role of DNA damage in PQ toxicity. In the same concentration range of PQ as used to examine cytotoxicity (Fig. 2), the induction of DNA damage was measured. A 2 hr incubation with different concentrations of PQ at 37° was found to cause the induction of DNA damage (Fig. 6). This induction appeared to be significant from a dose of 0.5 mM, which is at similar concentrations as found for cell killing. If the PQ incubation was performed at 0°, however, no induction of DNA damage was observed up to 2 hr treatment time.

When the cells were incubated at 37° up to 2 hr after the PQ treatment to measure DNA damage processing, it was found (Fig. 7) that after treatment with 0.5 mM PQ, DNA damage declined during the first 7 min of incubation. Subsequently, the amount of DNA damage increased up to 30 min post-incubation. Thereafter, a decrease of the amount of DNA damage was observed at the timepoints of 1 and 2 hr. Following treatment with 1 mM PQ, DNA damage increased during the first 60 min of post-treatment incubation. At the end of 2 hr post-treatment incubation, the amount of DNA damage was decreased but still significantly higher than the amount of initial DNA damage. For cells treated with PQ at 0° it was observed (Fig. 7) that with the highest concentration (2 mM), causing *no initial* DNA damage, post-incubation at 37° led to the occurrence of a significant amount of DNA damage. This effect must thus be attributed to residual intracellular PQ present in the cells even after thorough washing, and is suggested to be related to the observed "PQ toxicity at 0°" (Fig. 2; see also Discussion). This idea is substantiated by the good correlation between PQ toxicity (for both drug incubations at 37° and 0°) and DNA damage determined 2 hr *after* PQ treatment (Fig. 8).

Interaction of PQ and X-rays

Since it is suggested that PQ acts synergistically with X-rays [15, 16], we also tested the effect of a combined treatment with PQ and X-rays on cell survival and DNA integrity. In Fig. 9, the survival of EAT cells is depicted following a combined treatment with PQ and X-rays. All data are corrected for the cytotoxic effect of PQ alone. In order to compare our data to those from the literature, the combined treatment is presented as if the interaction is due to a true PQ-induced radiosensitization. There is, however, no solid molecular basis for this interpretation so far. By X-rays alone, cell survival was decreased to 10% at a dose of 4 Gy. In the combined treatment with PQ (2 hr, 37°) and X-rays (on ice), cytotoxicity appeared to be more than additive. A pretreatment with 0.5 and 1 mM PQ resulted in dose enhancement factors (DEF*) of 1.3 and 1.6, respectively. It should be kept in mind that in this representation the cytotoxicity of PQ alone is not recorded! Identical results were found when the cells were first treated with X-rays followed by PQ incubation (data not shown). The same doses of PQ applied at 0° did not affect X-ray-induced cell killing (data not shown).

Finally, we determined the induction and post-treatment processing of DNA damage after a combined treatment with PQ and X-rays (Figs 10 and 11). The induction of DNA damage by PQ alone is represented at the 0 Gy point (Fig. 10). No measurable effect of PQ on radiation-induced DNA damage could be detected as implicated by the parallel course of the lines (Fig. 10). Upon 37° post-treatment incubation, the amount of DNA damage induced by X-irradiation (6 Gy) alone (as detected

* $\text{DEF} = \frac{\text{Dose of X-rays in the absence of PQ}}{\text{Dose of X-rays in the presence of PQ}}$ to reduce cell survival to 10%.

All data are corrected for cell killing by PQ.

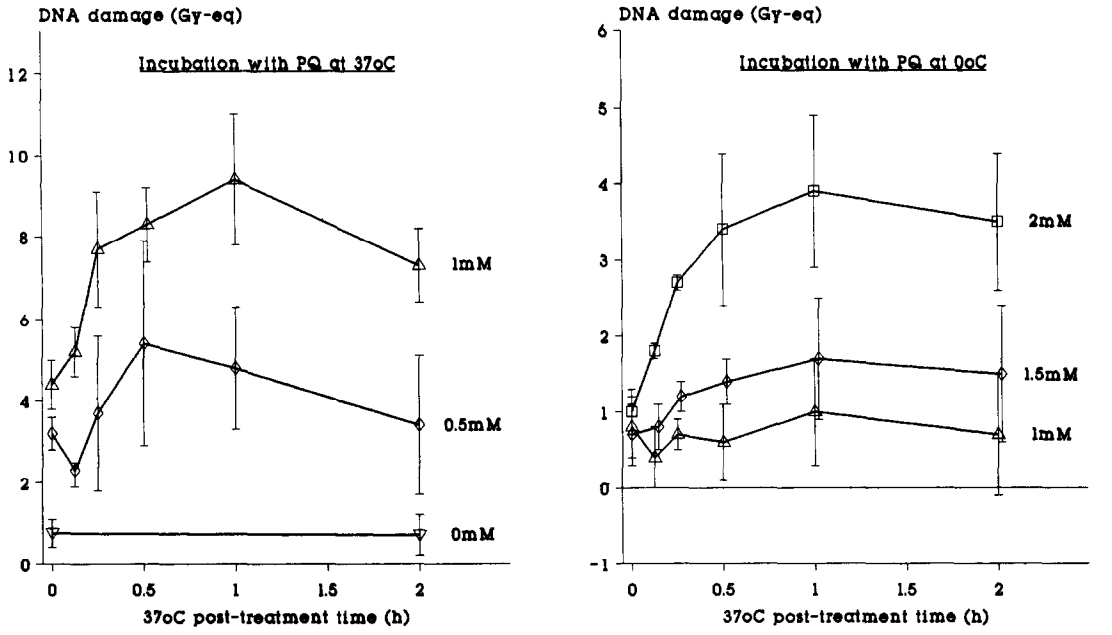

Fig. 7. Processing of PQ-induced DNA damage in EAT cells upon 37° post-treatment incubation. EAT cells were incubated with PQ at indicated concentrations for 2 hr at 37° or 0°. The treatment was stopped by washing the cells twice. After various times of post-treatment incubation at 37°, to allow DNA damage processing, the amount of DNA damage was calculated as Gy-equivalents (see Materials and Methods). This is plotted here versus the various post-incubation times. Each data point represents the mean value ± SEM of at least two independent experiments.

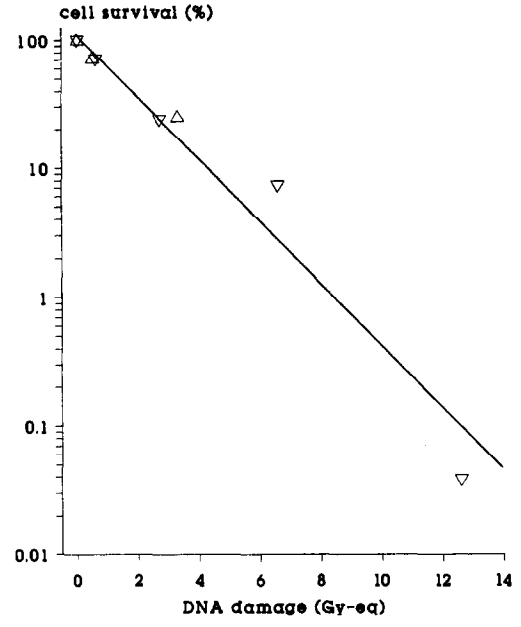

Fig. 8. Cell survival as a function of "residual" DNA damage determined 2 hr after PQ treatment at 37°. Cell survival (Fig. 2) is plotted versus residual DNA damage (Fig. 7) for identical PQ treatments at 37° (▽). Regression analysis of the 37° data yielded a correlation coefficient of $r = 0.9797$. For comparison, the data of the 0° PQ treatment are added to the correlation diagram (△). Regression analysis of the 0° and 37° data yielded a correlation coefficient of $r = 0.9805$.

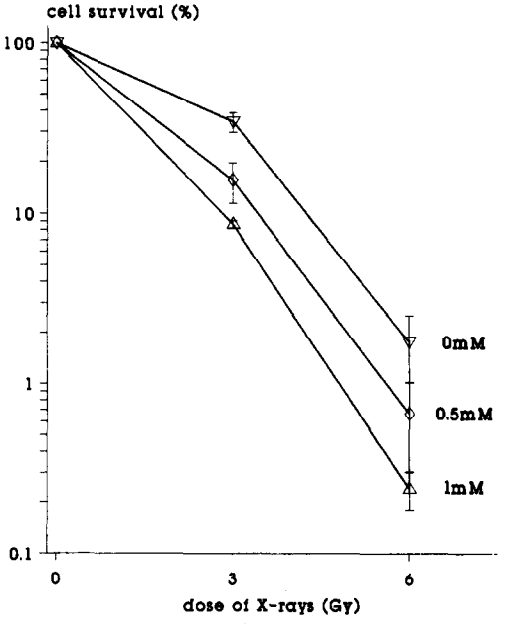

Fig. 9. Survival of EAT cells after a combined treatment with PQ and X-rays. Cell survival is plotted relative to the plating efficiency of untreated controls and corrected for cell killing by PQ alone. EAT cells were treated with PQ at indicated concentrations for 2 hr at 37° followed by X-irradiation at 0° in air. Each data point represents the mean value ± SEM of at least three independent experiments.

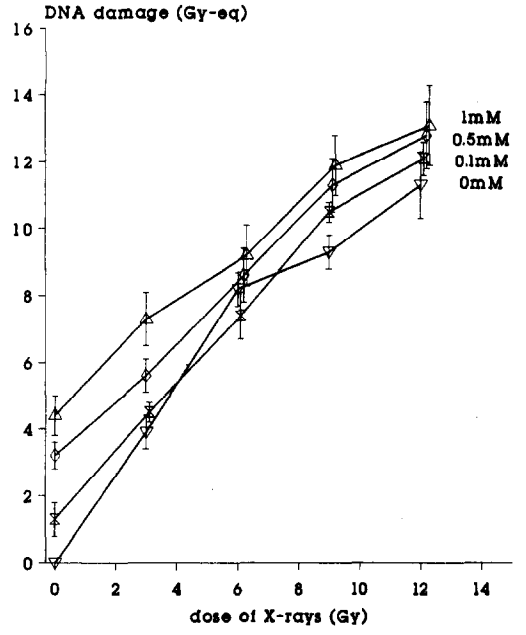

Fig. 10. Induction of initial DNA damage in EAT cells after a combined treatment with PQ and X-rays. EAT cells were treated with PQ at indicated concentrations for 2 hr at 37° followed by X-irradiation at 0° in air. Immediately after the combined treatment the amount of DNA damage was assayed by the alkaline unwinding assay. The percentage of double stranded DNA was calculated (see Materials and Methods) and expressed as Gy-equivalents using 0–12 Gy dose–response curves as calibration standards. Each data point represents the mean value ± SEM of at least two independent experiments.

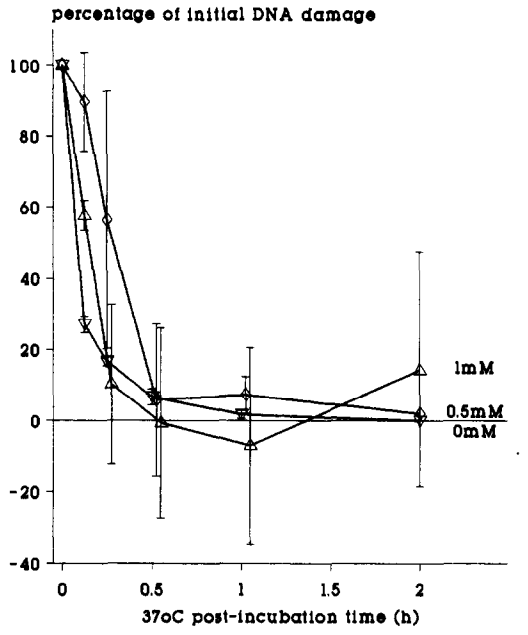

Fig. 11. Processing of DNA damage in EAT cells after a combined treatment with PQ and X-rays. EAT cells were treated with PQ at indicated concentrations for 2 hr at 37° followed by X-irradiation (6 Gy) at 0° in air. After various times of post-treatment incubation at 37°, to allow repair, the amount of DNA damage was calculated as Gy-equivalents (see Materials and Methods), related to the amount of DNA damage initially formed (t: 0 min = 100%), corrected for the processing of DNA damage caused by PQ alone (Fig. 7) and plotted versus the various post-incubation times. Each data point represents the mean value ± SEM of at least two independent experiments.

by the alkaline unwinding assay) was almost completely repaired within 2 hr (Fig. 11). As can be seen in Fig. 11, the rate of repair of X-ray-induced DNA damage seems to be unaffected by PQ pre-treatment. All data are corrected for the processing of DNA damage induced by PQ alone (Fig. 7).

DISCUSSION

Membrane damage as the possible critical lesion in PQ toxicity

In order to address the question whether lipids of cellular membranes might be critical targets in PQ toxicity, the overall capacity of PQ to initiate lipid peroxidation was studied in mouse liver homogenates. Liver homogenates were chosen because of two practical reasons. In homogenates the cellular components can easily interact with PQ and the active oxygen intermediates. Secondly, liver tissue contains a relatively high content of polyunsaturated fatty acids, which are sensitive to peroxidative damage. It was shown (Fig. 1) that PQ in fact, *is* able to cause lipid peroxidation beyond a certain threshold. It is likely that the oxidative stress induced by PQ is defended by cellular antioxidants present in the liver homogenate. Only when the amount of reactive oxygen species produced by PQ exceeds the cellular capacity to dissipate them, peroxidative damage to membrane lipids occurs. In the literature, first evidence for PQ-induced lipid peroxidation in mammalians was found by Bus *et al.* [5]. Using liposomes prepared from rat liver microsomal lipids, an increased degree of peroxidative damage was observed during the incubation with PQ. Similar results were found by Saito *et al.* [31]. Furthermore, Talcott *et al.* [7] demonstrated the induction of lipid peroxidation by PQ in mouse liver microsomes. Whether or not this lipid peroxidation is related to PQ toxicity is yet unclear.

To investigate a possible correlation between lipid peroxidation and PQ-induced cell killing, studies with EAT cells and LM fibroblasts were initiated. As determined by clonogenic ability, the two cell lines showed comparable sensitivity to PQ toxicity (Fig. 2). Since the TBA assay used for liver homogenates was not sensitive enough to detect lipid peroxidation in EAT and LM cells, an indirect approach was used. In irradiation studies from our laboratory [14], it has been shown that radiation-induced loss of membrane integrity of LM cells at high doses of X-rays could be enhanced by supplementation of the membranes with the polyunsaturated fatty acid 20:4 or by depletion of intracellular GSH and supplementation of the membranes with vitamin E resulted in the protection

against irradiation-induced membrane damage (Trypan blue uptake). Therefore, the observed interphase death was suggested to be related to membrane damage induced by radicals of oxygen. Since also in PQ toxicity the involvement of free radicals has been demonstrated [3–7], it was decided to use the same indirect parameters to investigate the role of the membranes in PQ-induced cell kill.

When LM cells were cultured in the presence of 20:4 in order to make the cells more sensitive to peroxidative membrane damage no enhanced PQ toxicity was observed (Fig. 3). Moreover, no protection was found even after a maximal supplementation of the membranes with the antioxidant vitamin E (Fig. 3). This indicates that lipid peroxidation is not the primary target in PQ toxicity. Trush *et al.* [32] could demonstrate an increased induction of lipid peroxidation in isolated lung microsomes derived from rats fed a diet deficient in vitamin E. It is, however, difficult to compare these *in vitro* studies on isolated cell fractions with the reactions that take place in whole cells.

Finally, no protective role of GSH against PQ toxicity was evident in our studies. PQ had no effect on the GSH level in EAT cells (Fig. 4) and complete GSH depletion by BSO could not sensitize the cells to PQ toxicity (Fig. 5). In LM cells, however, PQ-induced cell killing was enhanced after a complete GSH depletion by DEM. In earlier studies from our laboratory [23], it has been found that DEM not only binds to GSH but also to protein thiols. Therefore, the observed sensitization to PQ toxicity by DEM might be attributed to the loss of functional proteins rather than to GSH depletion. In contrast to our findings, there are indications for a protective role of GSH in PQ toxicity. Hagen *et al.* [33] demonstrated in alveolar type II cells a decreased sensitivity to PQ toxicity by the addition of exogenous GSH. Di Monte *et al.* [34] found little PQ toxicity in rat hepatocytes. A dramatic potentiation of PQ toxicity (measured as Trypan blue uptake) was achieved by the inhibition of GSH reductase through non-toxic pretreatment with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). In BCNU-treated cells, PQ caused GSH depletion, lipid peroxidation and a decrease in viability. The correlation between PQ-induced lipid peroxidation and viability was demonstrated by the addition of the antioxidant *N,N'*-diphenyl-*p*-phenylenediamine, which completely blocked PQ-induced lipid peroxidation and prevented PQ cytotoxicity. However, one has to keep in mind that the cellular uptake of a vital dye is the direct result of impairment of the permeability of the membranes whereas a reduced cellular survival as measured by clonogenic ability (as determined in our studies) may be evoked by damage other than to the (plasma) membrane (e.g. DNA). Taking all results together, it has to be concluded that, although PQ seems able to induce lipid peroxidation in liver homogenates (Fig. 1), microsomes [5, 7, 31, 32] and hepatocytes [34], this process is not likely to be the primary cause for PQ-induced cell killing.

If peroxidation of membrane lipids is not the critical toxicological consequence of redox cycling and the production of active oxygen, the question remains as to what the true nature of the toxic lesion might be. Because damaged DNA might severely threaten the cells viability, it was decided to investigate the relation between PQ-induced DNA damage and cell killing.

DNA damage as the possible critical lesion in PQ toxicity

In the same dose range of PQ that caused cell death (Fig. 2), the induction of DNA damage was observed (Fig. 6). After washing the cells twice, in order to remove PQ, post-incubation at 37° generally led to further enhancement of DNA damage (Fig. 7). The increment of DNA damage may be a net result of the balance between DNA repair and further induction of DNA damage by PQ that could not be removed from the cells. A good correlation was obtained for cell killing after PQ treatment at 37° and DNA damage measured 2 hr *after* the PQ treatment (Fig. 8, ∇), indicating that PQ-induced DNA damage indeed relates to PQ toxicity. A relationship between PQ-induced cell killing and DNA damage was also reported by Ross *et al.* [35]. After exposure to 0–5 mM PQ (37°), mouse leukaemia L1210 cells showed a dose-dependent decrease of cell survival paralleled by an increase of PQ-induced DNA single strand breaks as determined by the alkaline elution technique. In a study using *Escherichia coli* bacteria with different repair capacities, Yonei *et al.* [36] demonstrated that PQ cytotoxicity was greater in DNA repair-deficient than in DNA repair-proficient strains. Also, a mutagenic effect of PQ was reported, again indicating DNA damage by PQ. Our data cannot specify the exact type of DNA lesions involved, since the alkaline unwinding assay is a relative aspecific assay, detecting double and single strand breaks as well as base damage and some apurinic–apyrimidinic sites. Thus, to find the type of PQ-induced DNA damage related to cell killing more specific assays are needed. Although the exact lesion(s) causing PQ-induced cell killing remain to be elucidated, it seems likely that PQ toxicity is related to damage at the level of the DNA [this study, 35, 36].

The marginal toxicity seen for PQ treatment at 0° (Fig. 2) might be caused partially by an inhibited uptake of PQ into the cells. From the literature it is known that PQ accumulates in at least some tissues (e.g. the lung) by an energy-dependent process using the endogenous polyamine transport system [37, 38]. This uptake is inhibited drastically by lowered temperatures [38]. For EAT cells it is not known whether PQ is accumulated by an active or a passive process. In any case, PQ had entered the cells during the 0° incubation and remained in the cells even after thorough washing. This has caused DNA lesions upon 37° post-incubation. The final amount of DNA damage corresponds with DNA damage measured after the treatment with 0.5 mM PQ at 37°. Also, the survival of EAT cells (Fig. 2) is the same after the two treatments (i.e. incubation with 0.5 mM PQ at 37° or incubation with 2 mM PQ at 0°). As shown in Fig. 8, the correlation for "residual" (2 hr post-treatment) DNA damage and survival after PQ treatment holds for PQ applied at both 0° and 37°. The data obtained from PQ treatment at 0° indicate that in order to become cytotoxic PQ needs

enzymatic activation. Similar results were found by Sandy *et al.* [11] with rat hepatocytes and diquat, another bipyridil herbicide. When hepatocytes were incubated for 2 hr at 37° with 1.5 mM diquat clear cytotoxicity was observed, whereas no significant cell killing occurred at 4°.

Interaction of PQ and X-rays

Combined PQ and X-ray treatments were found to have a more than additive effect on cell killing [this report, 15, 16]. In the literature [15, 16] the interaction of PQ and X-rays was interpreted as a PQ-induced radiosensitization. However, no evidence has been provided as yet to exclude the reversed situation, being a radiation-induced PQ sensitization. Also here, we cannot discriminate between the two possibilities. Nevertheless, the data were presented as a PQ-effect on radiosensitivity to be able to compare our data to those in literature. In our experiments we obtained dose enhancement factors of 1.3 and 1.6 for (toxic) PQ treatments with 0.5 and 1mM, respectively. Similar data were obtained by Miller *et al.* [16]. The latter authors therefore suggested that PQ might be a suitable radiosensitizer in the clinic, especially in the treatment of lung cancers. This conclusion, however, must be severely criticized since only relative low levels of radiosensitization were seen at high toxic doses of PQ. Furthermore, there is no reason to assume that the drug will specifically accumulate in (lung) tumours. It might even accumulate in healthy lung tissue and thus cause extensive normal tissue damage by both direct toxicity and potentiation of a radiation treatment. Thus, PQ must considered to be unsuitable in radiotherapeutic practice.

Since cell killing by X-irradiation is accepted to be due to the induction of DNA damage [14] and there is now evidence [this report, 35, 36] that the same might be true for PQ toxicity. It is therefore interesting to investigate whether the super-additive effect of PQ and X-rays seen at the level of cell survival is reflected at the level of DNA damage. Using the alkaline unwinding assay, however, the combined treatment only caused an additive effect for the induction of (initial) DNA damage (Fig. 10). Furthermore, PQ did not affect the *rate* of repair of X-ray-induced DNA lesions (Fig. 11). So, within the limits of this assay, the super-additive interaction of PQ and X-rays seen at the level of cell survival cannot be explained at the DNA level. However, the alkaline unwinding assay detects more than one type of DNA damage, making it impossible to distinguish between different types of damage. Specific types of lesions not identified in this assay (e.g. DNA double strand breaks) might be responsible for the super-additive effect seen at the level of cell survival. If, however, these critical DNA lesions have a low induction frequency, it will be very difficult to detect an increase in the bulk of damaged DNA. Therefore, approaches looking at specific types of individual DNA lesions (e.g. DNA double strand break analysis by CHEF electrophoresis [39]) are needed to obtain a better insight in the mechanism underlying the observed effects.

Acknowledgements—This work was supported by a grant (GUKC-8801) from the Netherlands Cancer Foundation.

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