The role of oxidative stress in the in vitro induction of micronuclei by pesticides in mouse lung fibroblasts

Rosadele Cicchetti and Gabriella Argentin

Department of Public Health and Cell Biology, University of Rome 'Tor Vergata', Via Montpellier 1, I-00133 Rome, Italy

The involvement of the antioxidant enzymes catalase and glutathione peroxidase (both at 0.1 mg/ml) in defence against the genotoxicity of phosphamidon (80 µg/ml) and dieldrin (25 µM) was investigated in order to demonstrate that the two pesticides damage DNA through the generation of reactive oxygen species and therefore of oxidative stress. The pesticide genotoxicity was determined by the cytokinesis-block micronucleus test performed on primary mouse lung fibroblast cultures. Also, 3-aminotriazole (40 mM) and mercaptopussuccinate (0.5 mM), inhibitors of catalase and glutathione peroxidase, respectively, were added to the cultures. Data indicate that catalase causes a decrease only in the damage induced by phosphamidon, while glutathione peroxidase protects against damage induced by both phosphamidon and dieldrin. Simultaneous treatment with antioxidant inhibitors and pesticides results in a decrease in micronucleus frequency and cell number, due to apoptotic death. Our results indicate that clastogenic DNA damage produced by the two pesticides is modulated by antioxidant enzymes and their inhibitors and thus could be due to oxidative stress induction.

Introduction

A range of aberrant phenotypes, including DNA damage, may be induced in mammals exposed to toxic chemicals. Among these chemicals, pesticides represent a potentially serious hazard for human health (Lang, 1993); among the insecticides commonly used are the organophosphates and organochlorines. Organic insecticide poisoning continuously presents to be a major health problem not only in the developing communities but also in Western populations (Burmeister, 1981; Peter and Cherian, 2000; Satoh and Hosokawa, 2000; Jorgenson, 2001; Pilkinson et al., 2001). Patients with organic insecticide poisoning present a wide spectrum of adverse health effects ranging from gastrointestinal symptoms to cardiac, immunological or neurological diseases (Ladics et al., 1994; Le Couteur et al., 1999; Peter and Cherian, 2000). Certain pesticides are also associated with carcinogenesis. Since DNA damage has been correlated with cancer development (Hammar et al., 1994), genotoxicity studies have been carried out on specific pesticides (De Ferrari et al., 1991; Rupa et al., 1991; Dolora et al., 1994).

Cytogenetic studies on phosphamidon (PHO), an organophosphate pesticide, indicate that it can induce chromosome aberrations and micronuclei (MN) in man and mice (Van Bao et al., 1974; Behera and Bhunya, 1980; Usha-Rani et al., 1987; Cicchetti et al., 1999). Recently, dieldrin (DED), an organochlorine pesticide still used in several developing countries and found to accumulate in food (Doody et al., 2000; Romero et al., 2000; Jorgenson, 2001), has also been demonstrated to be genotoxic (Cicchetti et al., 1999); in the same study, MN induced by both pesticides were demonstrated to originate from chromosome breakage and not from loss of whole chromosomes due to malsegregation at mitotic anaphase.

Although the genotoxic activity of PHO and DED in mammals has been well established, the mechanisms underlying their damaging effects on DNA are still unclear. Nevertheless, it is well known that different classes of pesticides induce oxidative stress which may contribute to the toxicity of these xenobiotics (Banerjee et al., 2001).

In this context, the induction of oxidative stress has been suggested as the mechanism by which DED induces its hepatic toxic effect in mice (Klauning et al., 1995, 1998; Bachowski et al., 1997); in addition, some organophosphates have been found to induce several effects on antioxidant enzymes and other oxidative parameters (Hai et al., 1995). PHO in particular has been reported to induce oxidative stress, as shown by inhibition of superoxide dismutase activity, a decrease in glutathione peroxidase activity and lactate dehydrogenase leakage, last this as a general measure of cell membrane damage (Naqvi and Hasan, 1992; Yamano and Morita, 1992).

Although good evidence supports the hypothesis that oxidative stress may be involved in pesticide-induced cell injury, a relationship between genotoxicity and oxidative stress for DED and PHO has not been reported.

The oxidative stress that ensues when the normal balance between the production of reactive oxygen species (ROS) and the antioxidant ability of the target cell is upset has been implicated in many diseases and ROS may interact with various critical cellular macromolecules, including DNA, to produce damage. Several different pathways by which oxidative DNA damage occurs have been proposed, including chemical modification of nucleotides.

Fortunately, mammalian cells possess an efficient biological system to protect themselves from the damaging effects of ROS, which can be produced both endogenously and exogenously. Defence against xenobiotic toxicity, comprised of many kinds of antioxidants, is well characterized in mammals (Noguchi et al., 2000). These are classified by function into four categories: preventive, that suppress the formation of free radicals; radical scavenging, suppressing chain initiation and/or chain propagation reactions; repair; adaptation, with formation and transport of the appropriate antioxidant to the right site.

We report here studies on the role of oxidative damage (and its modulation) in genotoxic events evoked by PHO and DED, analysing the involvement of two preventive antioxidant enzymes, and their inhibitors, in the modulation of the genotoxicity. The antioxidant enzymes tested were catalase (CAT) and glutathione peroxidase (GPOx): both enzymes detoxify H2O2, reducing it to water and oxygen. GPOx utilises the reducing power of glutathione (GSH) and two molecules of glutathione peroxidase activity and lactate dehydrogenase leak-
GSH are oxidised to form the sulfide-bonded compound GSG, reducing a molecule of peroxide; CAT uses H$_2$O$_2$ itself.

Slides were incubated at 37°C in an atmosphere of 5% CO$_2$ in air. The cell culture medium was Ham’s F12, supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin. Culture slides were plated into sterile Slide Flasks (Nunc) containing 3 ml of complete medium (Ham’s F12, supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin). Culture slides were incubated at 37°C in an atmosphere of 5% CO$_2$ in air. The following day the medium was discarded and replaced.

Chemicals, according to the following experimental protocol, and cyt-B, at a final concentration of 1 µg/ml (stock solution 0.1 mg/ml in DMSO), were added to the flask 24 h before harvest. Cyt-B is an inhibitor of cytoplasmic division and cells that divide in its presence become binucleated; its concentration was determined in a preliminary experiment, showing that higher concentrations (2 and 3 µg/ml) were toxic to the cultured primary fibroblasts of mice.

**Experimental protocol**

**Antioxidant test.** Three mice were killed and the lungs were individually digested with 0.25% trypsin in culture medium for 80 min at 37°C. The cell suspensions obtained after digestion were plated into sterile Slide Flasks and cultured at 37°C in an atmosphere of 5% CO$_2$ in air. The cell concentration was determined in a preliminary experiment, showing that higher concentrations (2 and 3 µg/ml) were toxic to the cultured primary fibroblasts of mice. The antioxidant enzymes CAT and GPox and their specific inhibitors 3-aminotriazole (3AT), a CAT inhibitor; MS, mercaptosuccinate, a GPox inhibitor. (a) Antioxidant test; (b) inhibitor test, pesticides and inhibitors were given at the same time.

Materials and methods

**Chemicals**

The organophosphate PHO and the organochlorine DED were obtained from Riedel-de Haen and diluted in culture medium and dimethyl sulphoxide (DMSO), respectively; the final concentration of DMSO was 0.1%.

The antioxidant enzymes CAT and GPox and their specific inhibitors 3-aminotriazole (3AT) and mercaptosuccinate (MS), respectively, were obtained from Sigma. The inhibitors were prepared in distilled water and stored at –20°C.

Cyt-B was also obtained from Sigma.

**Cell cultures**

Lungs of 10–12-week-old CBA male mice, killed by cervical dislocation, were rinsed in phosphate-buffered saline (PBS), cut into small pieces and digested with 0.25% trypsin in culture medium for 80 min at 37°C. The cell suspensions obtained after digestion were plated into sterile Slide Flasks (Nunc) containing 3 ml of complete medium (Ham’s F12, supplemented with 15% fetal calf serum, 1% l-glutamine and 1% penicillin/streptomycin). Culture slides were incubated at 37°C in an atmosphere of 5% CO$_2$ in air. The following day the medium was discarded and replaced.

Chemicals, according to the following experimental protocol, and cyt-B, at a final concentration of 1 µg/ml (stock solution 0.1 mg/ml in DMSO), were added to the flask 24 h before harvest. Cyt-B is an inhibitor of cytoplasmic division and cells that divide in its presence become binucleated; its concentration was determined in a preliminary experiment, showing that higher concentrations (2 and 3 µg/ml) were toxic to the cultured primary fibroblasts of mice.

**Experimental protocol**

**Antioxidant test.** Three mice were killed and the lungs were individually trypsinized as described above. The cell suspensions were mixed and a final volume of 27 ml obtained by adding fresh medium, was split into nine flasks. When cell growth was satisfactory, i.e. when cells were near to confluence, the pesticides (PHO, 80 µg/ml; DED, 25 µM) and the antioxidant enzymes (0.1 µg/ml), alone or in combination, were added to the cultures, at the same time as cyt-B. The doses of pesticides were selected through preliminary experiments performed with PHO and DED at final concentrations of 60, 80, 100 and 120 µg/ml and 15, 25, 35 and 45 µM, respectively. On this basis, we chose doses that induced a statistical increase in nuclear damage with a minor effect on cellular growth, which was heavily compromised at higher drug concentrations.

**Table I.** Mean frequencies of BN and MN in untreated and treated mouse fibroblasts obtained from four independent cultures for each experimental point

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Chemical</th>
<th>Total no. of cells</th>
<th>BN (mean ± SD)</th>
<th>MN/1000 BN (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DED</td>
<td>PHO</td>
</tr>
<tr>
<td>(a)</td>
<td>CAT</td>
<td>6335 8347 8621</td>
<td>0.66 ± 0.01</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>GPox</td>
<td>6469 8220 7502</td>
<td>0.63 ± 0.03</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>3AT</td>
<td>6115 8238 9274</td>
<td>0.66 ± 0.02</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>7781 18 403 19 005</td>
<td>0.59 ± 0.01</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>(b)</td>
<td>3AT+MS</td>
<td>14 599 19 348 20 161</td>
<td>0.44 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
</tbody>
</table>

The bars indicate SD. Here we report only the statistical analysis performed to verify the effect of CAT and GPox on MN induced by DED and PHO.

**Fig. 1.** The antioxidant test. MN were scored on 1000 BN cells from mouse lung fibroblasts and data are the means of four replicate plates. Bars indicate SD. Here we report only the statistical analysis performed to verify the effect of CAT and GPox on MN induced by DED and PHO.

**Fig. 2.** The antioxidant inhibitor test. MN were scored on 1000 BN cells from mouse lung fibroblasts and data are the means of four replicate plates. The bars indicate SD. Here we report only the statistical analysis performed to verify the effect of CAT and GPox inhibitors on MN induced by DED and PHO. 3AT, 3-aminotriazole, a CAT inhibitor; MS, mercaptosuccinate, a GPox inhibitor.
The role of oxidative stress by pesticides

Increase in BN cells in the combined treatments was not against the cytotoxicity induced by pesticides, although the with CAT and GPox. These antioxidant enzymes protected in BN cells was shown in cultures simultaneously treated with untreated and treated cultures (Fig. 2). Net increase values were compared. Bars indicate SD. 3AT, 3-aminotriazole, a catalase inhibitor; MS, mercaptosuccinate, a glutathione peroxidase inhibitor.

Inhibitor test. Four mice were killed as described above. A final volume of 36 ml was split into 12 flasks. As described above, when cell growth became satisfactory, the pesticides (at the same concentrations used in the antioxidant test) and the antioxidant enzyme inhibitors (MS, 0.5 mM; 3AT, 40 mM), alone or in combination, were added to the cultures, at the same time as cyt-B. In a similar experiment, the inhibitors were added 12 h before the pesticides.

Slide processing and staining
The cells were rinsed twice with PBS and fixed in 3:1 methanol/acetic acid for 3 min. The slides were then removed, air dried and stained with 7% Giemsa in phosphate buffer. The evaluation of micronucleus frequencies was limited to only BN cells and was assessed by light microscopy on 1000 BN cells for each experimental point in four independent cultures. Slides were scored at 100× magnification.

Statistical analysis was performed on mean frequencies obtained from pooling data from four replicate cultures and parametric criteria (Student’s t-test), after assessing the normality of the distribution and homogeneity of the variance, were used.

Apoptosis assay
Apoptotic cells undergo changes in nuclear morphology that can be visualized using the DNA fluorochrome Hoechst 33258. After 24 h exposure to chemicals, without cyt-B, the cells were fixed in 3:1 methanol/acetic acid and incubated for 30 min with Hoechst 33258 (1 μg/ml in PBS) at 37°C. Thereafter, the cells were washed with 0.1 M PBS and nuclear morphology was observed under a fluorescence microscope (Nikon, Japan). Fibroblasts with condensed chromatin at the nuclear periphery or nuclear fragmentation with a reduction in nuclear size were considered typical features of apoptosis. The apoptosis percentage was determined by counting apoptotic and non-apoptotic nuclei in at least 200 cells for each experimental point in triplicate cultures.

Results
As former studies (Hai et al., 1995; Klauning et al., 1995, 1998; Bachowski et al., 1997) suggested that the toxic effects of organophosphates and DED may be mediated by ROS, we looked into the effect of the antioxidant enzymes CAT and GPox, and their specific inhibitors, on the frequency of MN induced by DED and PHO. For each experimental point four independent cultures were established and the mean values are shown in Table I, which also reports the frequencies of BN cells, as a marker of in vitro proliferation. A significant difference in the frequency of BN cells was observed between untreated and treated cultures (P < 0.01), except for treatments with CAT and GPox. These antioxidant enzymes protected against the cytotoxicity induced by pesticides, although the increase in BN cells in the combined treatments was not significant. In contrast, a highly statistically significant decrease in BN cells was shown in cultures simultaneously treated with pesticides and MS, alone or with 3AT, with regard to those exposed to pesticide alone (P < 0.0001). The addition of 3AT caused a significant decrease in BN cells only in the case of PHO (P < 0.0001).

Figure 1 shows the effects of the antioxidant enzymes on pesticide-induced DNA damage. Both pesticides induced a significant increase in MN (P < 0.001) at the doses tested. Neither CAT nor GPox added alone to the cultures significantly changed the frequencies of spontaneous MN. When the antioxidant enzymes were added at the same time as the pesticides, the damage induced by DED decreased significantly in the presence of GPox (P = 0.001), while CAT, although counteracting DED-induced damage, did not produce a statistically significant decrease (P = 0.058). In contrast, the PHO-induced micronucleus frequency was significantly decreased by both enzymes, mostly by GPox (P<sub>PHO+CAT</sub> = 0.012; P<sub>PHO+GPox</sub> < 0.001).

Figure 2 shows data obtained from one of the two experiments performed to verify the effect of the antioxidant inhibitors 3AT and MS, which are specific for CAT and GPox, respectively. The inhibitors were tested alone or in combination with the pesticides and were added at the same time. The addition of the single inhibitors to the cultures induced a significant increase in the micronucleus frequencies (P < 0.01) in respect of the spontaneous values. When both

<table>
<thead>
<tr>
<th>Chemical</th>
<th>DED (mean ± SD)</th>
<th>PHO (mean ± SD)</th>
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<tr>
<td>CAT</td>
<td>21.0 ± 2.0</td>
<td>16.0 ± 2.6</td>
</tr>
<tr>
<td>GPox</td>
<td>18.0 ± 1.0</td>
<td>14.0 ± 2.0</td>
</tr>
<tr>
<td>3AT</td>
<td>29.0 ± 2.6</td>
<td>34.7 ± 1.5</td>
</tr>
<tr>
<td>MS3</td>
<td>65.0 ± 3.0</td>
<td>62.0 ± 2.6</td>
</tr>
<tr>
<td>AT+MS</td>
<td>73.0 ± 1.0</td>
<td>72.0 ± 2.0</td>
</tr>
</tbody>
</table>

Basal level of apoptosis was 2.4 ± 0.1. DED, dieldrin; PHO, phosphamidon; CAT, catalase; GPox, glutathione peroxidase. 3AT, 3-aminotriazole, a catalase inhibitor; MS, mercaptosuccinate, a glutathione peroxidase inhibitor.

Table II. Mean percentage of apoptotic cells induced by treatments with pesticides alone or in combination with antioxidant enzymes or their inhibitors.

* P ≤ 0.05; ** P ≤ 0.01 by Student’s t-test

Fig. 3. Comparison between observed and expected values obtained summing the MN induced by single chemicals in the inhibitor test (see Figure 2). Net increase values were compared. Bars indicate SD. 3AT, 3-aminotriazole, a catalase inhibitor; MS, mercaptosuccinate, a glutathione peroxidase inhibitor.

Fig. 4. Pre-treatment with antioxidant inhibitors. The inhibitors were added to cultures 12 h before pesticides. MN were scored on 1000 BN cells from mouse lung fibroblasts and data are the means of four replicate plates. The bars indicate SD. Also in this case, we report only the statistical analysis performed to verify the effect of CAT and GPox inhibitors on MN induced by DED and PHO. 3AT, 3-aminotriazole, a catalase inhibitor; MS, mercaptosuccinate, a glutathione peroxidase inhibitor.

* P ≤ 0.05 by Student’s t-test
inhibitors were tested together, the increase was not significant, probably owing to the wide standard deviation. The frequency of pesticide-induced MN did not rise significantly when the single inhibitors were added to the system, excluding the experimental case DED+3AT ($P = 0.0003$). In the other cases, the induced damage surprisingly decreased, although the decrease was statistically significant only for the PHO+MS experiment ($P < 0.01$). When both inhibitors were assayed together with the pesticides, a significant decrease in MN was observed only when they were tested with PHO ($P = 0.02$). Nevertheless, if the effects of simultaneous exposure to pesticides and inhibitors were compared to expected values (obtained from the sum of MN induced by the single chemicals), these latter were always higher, with significant differences when MS, alone or with 3AT, was added with the pesticides. The comparison was performed on the net increase values, obtained by subtracting spontaneous values from treated ones (Figure 3).

A further experiment was performed with pretreatment of the cultures with the inhibitors 12 h before the pesticides (Figure 4). In this case significant differences were found with respect to frequencies from cultures treated with pesticide alone, when MS or MS+3AT were added to cultures together with PHO ($P_{MS} = 0.05; P_{MS+3AT} = 0.04$).

Finally, apoptosis was assessed by Hoechst 33258 staining of cells from cultures treated for 24 h with pesticide alone or in the presence of the antioxidant enzymes or their inhibitors and without cyt-B. Figure 5 shows the treated cells that displayed nuclear condensation and fragmentation, reflecting morphological changes commonly observed in apoptotic cells. Data on mean percentages of apoptotic cells detected in three independent cultures are reported in Table II. Both DED and PHO were able to induce apoptosis with regard to the control ($P = 0.0001$); apoptosis decreased significantly when DED and PHO were tested in presence of CAT ($P_{DED+CAT} = 0.013; P_{PHO+CAT} = 0.002$) and GPox ($P_{DED+GPox} = 0.001; P_{PHO+GPox} = 0.001$). A highly significant increase in apoptotic cells was found for experiments in which the GPox inhibitor MS was tested with a single pesticide, alone or with 3AT ($P = 0.0001$), while 3AT did not cause a significant increase in apoptotic cells.

**Discussion**

ROS may be involved in the toxicity of various pesticides (Banerjee et al., 2001), including DED and the organophosphates (Hai et al., 1995; Bachowski et al., 1997; Kitazawa et al. 2001). A cell defends itself against ROS by elaborate

Fig. 5. Hoechst 33258 staining of pesticide-induced apoptosis in mouse lung fibroblasts. The figure represents the typical patterns observed in untreated cells (a) and cells treated with: (b) pesticide alone (PHO or DED); (c) pesticide + antioxidant enzyme (CAT or GPox) or (d) pesticide + MS. No cyt-B was added to the system. Apoptosis was visualized with a fluorescent microscope (100× magnification).
systems of biological defence. These systems include the inducible antioxidant enzymes, such as superoxide dismutase, CAT and GPox, which suppress the formation of free radicals.

The purpose of this study was to clarify the nature of the genotoxicity of DED and PHO, taking into account the possible role of oxidative stress that results from an imbalance between ROS and antioxidant systems in the target cell. To this end, we investigated the possible modulation of DED- and PHO-induced DNA damage, utilising two preventive antioxidant enzymes, CAT and GPox, and their specific inhibitors 3AT and MS.

In a previous in vivo study in mice (Cicchetti et al., 1999) we demonstrated that both pesticides are able to induce a dose-dependent increase in MN via a clastogenic mechanism. In the current work we confirmed in vitro the genotoxicity of the two pesticides and also their contribution to the induction of apoptotic cell death, as reported in the literature (Akbarsha and Sivasamy, 1997; Kitazawa et al., 2001).

The addition to cultures of CAT and GPox at the same time as PHO reduced the numbers of induced MN, and GPox also reduced the damage induced by DED; both antioxidant enzymes protected against apoptosis induced by these pesticides. Regarding DED-induced apoptosis, Kitazawa et al. (2001) recently demonstrated that it was significantly attenuated by superoxide dismutase, another antioxidant enzyme that scavenges superoxide anions.

The inhibition of CAT and GPox by their specific inhibitors 3AT and MS led to the expected increase in MN only in the experiment with DED+3AT; in the other cases we detected a surprising decrease in MN. The comparison between observed values and expected ones (obtained summing MN induced by the single chemicals) showed significant differences when MS, alone or with 3AT, was added with the pesticides. Addition of the inhibitors 12 h before the pesticides did not give results different from co-treatment.

Thus, the data from our experiments surprisingly seem to indicate that the greater the protection offered by the antioxidant enzyme (GPox) against pesticide damage, the lower is the frequency of MN scored in the presence of its specific inhibitor. We think that the inhibition of GPox in particular determines such an increment and accumulation of intracellular damage, in that the cell dies or cannot complete its reproduction. In any case, the cells cannot reach mitotic telophase, when MN are scored in BN cells. In reality we observed a drastic decrease in BN cells in the cultures simultaneously treated with the inhibitor of GPox and pesticides.

There are various cell cycle checkpoints which monitor the genome and may also arrest the cell cycle in response to DNA damage (Hartwell and Weinert, 1989; Elledge, 1996). Sometimes, cell growth arrest is transient and it is utilised by cells to protect themselves against acute stress, allowing time for DNA repair (Wiese et al., 1995; Davies, 1999).

Another mechanism that protects against the transmission of damaged DNA is apoptosis, a mode of programmed cell death. Increasing evidence provides support that ROS, and the resulting oxidative stress, play a pivotal role in apoptosis (Kannan and Jain, 2000; Simon et al., 2000). Recent studies have demonstrated that ROS serve as a signal for induction of apoptosis by activating an array of cell signalling molecules, such as caspases and kinases (Junn and Mouradian, 2001). Our data from the apoptotic assay confirmed that the pesticide-induced apoptosis was mediated by ROS and allowed us to conclude that the decrease in micronucleus frequencies with respect to expected values was due to a reduced survival of cells that showed the typical changes of apoptotic death. In fact, the highest increase in apoptotic cells (60–70%) was observed when an unexpected decrease in micronucleus frequency was detected, i.e. when MS was tested, alone or with 3AT, in combination with the pesticides.

In conclusion, our data show an involvement of the preventive antioxidant enzymes CAT and GPox in defence against the genotoxic events induced by PHO and DED, suggesting that the two pesticides can damage DNA through the generation of ROS and therefore of oxidative stress. This is especially important since oxidative stress is thought to play a role in carcinogenesis by causing damage to DNA, besides inducing a very wide spectrum of genetic, metabolic and cellular responses.

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References


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