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AN INVESTIGATION INTO THE ROLE OF REACTIVE OXYGEN SPECIES IN THE MECHANISM OF 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE TOXICITY USING NEURONAL CELL LINES

MARGARET LAI, HELEN GRIFFITHS,*† HARDEV PALL,‡ ADRIAN WILLIAMS‡ and JOSEPH LUNEC*

Wolfson Research Laboratories, Queen Elizabeth Medical Centre, Edgbaston, Birmingham B15 2TH; * Molecular Toxicity Group, Clinical Sciences Building, Glenfield Hospital, Groby Road, Leicester LE1 9QP; and ‡Department of Neurology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

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Abstract—The study of oxygen radical generation and effects during 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) metabolism was undertaken in an *in vitro* test system. Three neurochemically discrete neuronal cell lines, B50 (cholinergic) and B65 rat cell lines and SKNSH human neuroblastoma (both catecholaminergic), were exposed to MPTP (0–200 μM). Parallel experiments were performed using reagent H_2O_2, an intermediate which may be generated during MPTP metabolism, to determine whether MPTP and H_2O_2 had any selectivity of toxicity and whether the mechanisms of cell death were similar. MPTP toxicity was shown to be reduced by monoamine oxidase B inhibitors, pargyline ($P < 0.01$) and selegiline ($P < 0.05$), indicating that toxicity was due to metabolism of MPTP rather than the parent compound. Cytotoxicity was also decreased in the presence of antioxidants, most notably in the presence of superoxide dismutase and catalase together ($P < 0.01$), suggesting that reactive oxygen species (ROS) play a role in MPTP-induced cell death. Attempts to determine the intracellular target for oxidative attack did not identify significant levels of lipid peroxidation products, but did demonstrate nucleoid expansion, possibly the result of double stranded DNA breaks induced by ROS.

Oxygen radicals and reactive oxygen species (ROS§) have been implicated as important mediators of ageing and of a variety of human diseases [1, 2]. In recent years much interest has been centred on the role of ROS in neurodegenerative diseases such as Parkinson's disease (PD). PD is associated with a loss of dopamine-containing cells in the zona compacta of the substantia nigra. The exact cause of neuronal degeneration remains unknown, but involvement of environmental or endogenous toxins perhaps via the effects of ROS has been suggested in the aetiology of PD. Accumulating evidence does indeed suggest a role for ROS in the pathophysiology of PD [3, 4].

Until recently, there have been no satisfactory models for the investigation of PD, but the chance discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a by-product in the synthesis of a meperidine type narcotic analgesic, has provided impetus towards further research in PD. It has been shown that MPTP induces Parkinsonism in humans and in other primates by causing degeneration of dopamine containing neurones within the substantia nigra [5, 6], thereby mimicking the clinical as well as pathological features of PD. MPTP itself is not neurotoxic but when biologically activated produces active toxic species. It has been shown that the enzyme monoamine oxidase B (MAO B) oxidizes MPTP to an unstable intermediate, the 1-methyl-4-phenyl-2,3-dihydropyridinium ion ($MPDP^+$), which is then further oxidized to the pyridinium species, the 1-methyl-4-phenyl pyridinium ion (MPP^+) [7]. Pre-treatment with selective inhibitors of MAO B appears to protect against neurotoxicity [8] and suggests that MPTP oxidation plays a crucial role in the neurotoxic process. However, the exact mechanism by which MPTP exerts this effect is still unclear. It is thought that MPP^+ may be responsible for MPTP-induced dopaminergic damage by acting as mitochondrial toxin where it inhibits the oxidation of NAD-linked substrates [9] and thus kills cells by mitochondrial energy deprivation [10]. Alternatively, MPDP metabolism to MPP^+ may produce ROS which cause lethal damage to critical biomolecules. It is postulated that decomposition of MPDP to MPP^+ may involve one-electron oxidation/reduction producing superoxide anion radicals ($O_2^-\cdot$) from molecular oxygen, and electron spin resonance studies have demonstrated the formation of free radical adducts after aerobic MPTP treatment of mouse brain mitochondria [12]. Dismutation of the superoxide anion radical to H_2O_2 and the presence of divalent metal ions subsequently give rise to other more damaging ROS, most notably the hydroxyl radical $OH^\cdot$, which is widely reported as a highly cytotoxic agent. As yet however, the precise

† Corresponding author. Tel. (0533) 871471, ext. 3046; FAX (0533) 879533.

§ Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium ion; MPP^+, 1-methyl-4-phenyl pyridinium ion; PD, Parkinson's disease; ROS, reactive oxygen species; MAO B, monoamine oxidase B; DFX, desferrioxamine; SOD, superoxide dismutase.

contribution of these two mechanisms in MPTP toxicity has not been determined. Many of the studies aimed at elucidating its mechanism of action have been complicated by the use of whole animals. Cellular models have been developed from embryonic rat mesencephalon, but these have a complex array of neuronal cell types and also suffer from the inherent variability of animal studies. Therefore, we have investigated the use of neuronal cells maintained in culture for toxicity testing and examined the role of ROS in MPTP toxicity using three neurochemically discrete cell systems, in an attempt to clarify the mechanism of action of MPTP.

MATERIALS AND METHODS

All tissue culture supplies were from Gibco BRL (Paisley, U.K.). MPTP as the hydrochloride form, MPP^+, H_2O_2 as a 30% solution with added stabilizers, D-mannitol, catalase (EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1) together with clorgyline, pargyline and selegiline for MAO inhibition studies, were obtained from the Sigma Chemical Co. (Poole, U.K.). Desferal (desferrioxamine) was purchased from Ciba Geigy (Horsham, U.K.). CHOD-iodine kit for lipid peroxidation measurements was from Boehringer Mannheim (Lewes, U.K.).

Cell culture. B50 rat neuronal cell lines of cholinergic origin (ECACC No. 85042302), B65 rat neuronal cell lines of dopaminergic origin (ECACC No. 85042305) and SKNSH human neuroblastoma cells were obtained from the PHL Centre for Applied Microbiology & Research (Porton Down, Salisbury, U.K.). Peripheral blood lymphocytes were obtained from healthy volunteers. Ficoll-paque resolving medium (density 1.077 ± 0.001) used for lymphocyte isolation was from Nycommed (As. Oslo, Norway). Cells were cultured in RPMI 1640 medium supplemented with foetal calf serum (10% v/v), L-glutamine (1% v/v) and penicillin (1000 U/mL)/streptomycin (500 U/mL) and grown in a humidified atmosphere of 95% air/5% CO_2 at 37°. Monolayers of neuroblastoma cells were treated with a 0.25% trypsin solution for 5 min at 37° to enable removal from culture flasks. The trypsinized cells were sedimented by centrifugation at 150 *g* for 5 min, resuspended in and washed three times with the culture buffer. The final working concentration of cells was 2×10^6/mL.

Two methods of assessment of cell death were performed; Trypan blue inclusion and [^{3}H]thymidine uptake 24 hr post-toxin presentation. The former is an index of cellular membrane integrity and a late marker of cell death, whilst the latter is an evaluation of the number of cells undergoing cell division and therefore an early indicator of cell death [13]. Both of these methods correlate well in our laboratory ($P < 0.01$) on five data points, and the results are presented in the more conventional form of Trypan blue inclusion.

A minimum of 200 cells were counted per incubate and initial viability prior to experimental use was approximately 95%.

Lymphocyte preparation. Lymphocytes were isolated from fresh whole blood as follows. Fresh venous blood (20 mL) was collected into heparin and diluted 1:2 in RPMI. Two volumes of blood were layered onto one volume of Ficoll-paque separating medium and centrifuged at 300 *g* for 30 min. White cells were harvested from the interface between the separating medium and the serum. The cells were then washed three times with RPMI medium. Cells were resuspended to give a final working concentration of 2×10^6 cells/mL.

H_2O_2, MPP^+ and MPTP toxicity assay. Lymphocytes and neuroblastoma cells were incubated for 24 hr with concentrations of H_2O_2 ranging from 0 to 1 mM and of MPTP ranging from 0 to 200 μM. The extent of cell killing was assessed by Trypan blue inclusion.

Inhibition by MAO inhibitors. Neuroblastoma were incubated for 24 hr in the presence of MPTP (200 μM) and the specific MAO inhibitors, 1 μM pargyline, clorgyline or selegiline. Cell viability was then assessed immediately post-incubation.

ROS inhibitor assay. SKNSH cells were incubated with either 1 mM H_2O_2 or 200 μM MPTP in the presence of the antioxidants catalase (27,000 U/mL), mannitol (50 mM), SOD (3000 U/mL) or desferrioxamine (DFX, 0.5 mM). Cell viability was assessed 24 hr later.

Lipid peroxide assay. To investigate whether ROS produced as a result of MPTP metabolism by neurones exert their toxicity by initiating cell membrane lipid peroxidation, lipid peroxide measurement in the cell suspension was undertaken using the method of El Saadani *et al.* [14]. Briefly, 100 μL of cell suspension was mixed with 1 mL colour reagent from the CHOD-iodide kit and was allowed to stand in the dark at ambient temperature for 30 min prior to absorbance measurement at 365 nm.

Flow cytometry. The effects of MPTP and H_2O_2 on neuronal cells were analysed using a Becton Dickinson fluorescence activated cell sorter (FACS) in conjunction with a PDP II based microcomputer. Cells which have been treated with MPTP (200 μM) or H_2O_2 (1 mM) were examined for damage to their DNA using an adaptation of the method of flow cytometry [15]. To extract the nucleoids, 10^6 cells were suspended in ice-cold buffer (1 mL) comprising of 2 M NaCl, 10 mM Tris (hydroxymethyl) methylamine, 10 mM EDTA plus Triton X-100 (0.5%). Each sample was stained with ethidium bromide and analysed.

All experiments were carried out in triplicate and repeated three times. Statistical analyses were carried out using the Student's *t*-test or $r \times c$ Chi square test where appropriate.

RESULTS

Concentration–response curve of cell killing by H_2O_2

The effects of the reactive oxygen species H_2O_2 on lymphocytes have been well documented for lymphocyte function and viability [16]. Hence all experiments on MPTP were carried out in parallel with H_2O_2. This allowed a comparison to be made of their effects to determine whether they induced similar end points of damage and thus to clarify the role of ROS in MPTP neurotoxicity.

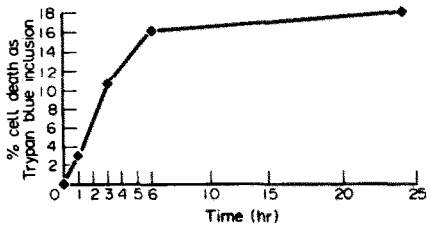

Fig. 1. The kinetics of oxidative cell death in B65 cells. Cells were exposed to 200 μM H_2O_2, and cell death determined by Trypan blue inclusion after defined times.

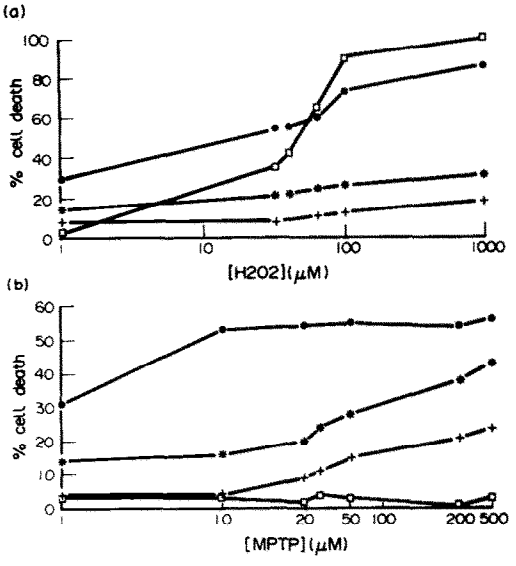

Fig. 2. H_2O_2 and MPTP induced cell death. Lymphocytes and neuronal cells were incubated with varying concentrations of toxin for 24 hr prior to determination of cell death by Trypan blue inclusion. Results are expressed as means and the SD was always less than 4%. (•) SKNSH, (+) B50 (*) B65, (□) lymphocytes.

To establish the kinetics of cell death and the neurotoxicity of H_2O_2, oxidative cytotoxicity was assessed in B65 neuroblastomas with increasing time. Figure 1 illustrates the rate of increase in cell death with time. For subsequent experiments, lymphocytes were used as a cell type control and neuronal cell lines were incubated with reagent H_2O_2 over the range 0–1 mM. These concentrations of H_2O_2 induced a pattern of cell killing as indicated by Trypan blue inclusion which increased in a dose-dependent manner (Fig. 2a). However, there was no significant difference observed in the total cell concentration. Of the cell types, lymphocytes were shown to be the most susceptible to H_2O_2 cytotoxicity with 90% cell death at 200 μM H_2O_2 (Fig. 2a). The B50 rat cholinergic neuronal cells were the most resistant (15% cell death at 1 mM H_2O_2 vs 3% in controls, $P < 0.05$). The B65 rat dopaminergic neurones were less resistant than B50 cells (20% cell death at 1 mM H_2O_2 compared to 4% in controls, $P < 0.02$) whilst the human SKNSH cells showed a much higher degree of susceptibility to 1 mM H_2O_2 (80% cell death, $P < 0.001$). A concentration of 1 mM H_2O_2 was significantly toxic to all cell types

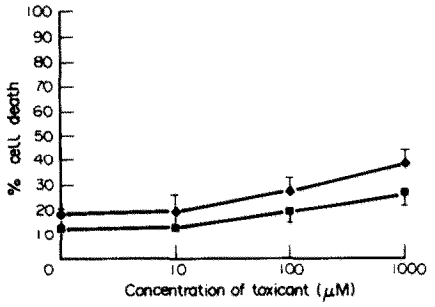

Fig. 3. A comparison of the relative toxicities of MPTP and MPP^+ on B65 cells. Cells were incubated with each toxicant (400 μM) in the absence of foetal calf serum for 24 hr, and cell death was assessed by Trypan blue inclusion. Each data point represents the mean of triplicate analyses, and the SD are shown as error bars. (◆) MPTP, (■) MPP^+.

and was used for subsequent experiments, as a positive control model for ROS effects on neuronal cells.

Concentration–response curve for MPTP toxicity

Similarly, to examine the cytotoxic effects of MPTP and to establish a working concentration, lymphocytes (cell type control) and neuronal cell lines were incubated with MPTP (0–200 μM) for 24 hr. No effect of MPTP was seen in the lymphocyte preparations over this concentration range (Fig. 2b). Of the three neuronal cell lines, B50 cells were the most resistant (28% cell death at 200 μM, $P < 0.02$), 35% of B65 cells were non-viable after 24 hr ($P < 0.01$) whilst SKNSH cells were the most vulnerable to MPTP toxicity, incurring 50% cell death at 200 μM ($P < 0.01$). A concentration of 200 μM MPTP was used for subsequent inhibitor experiments.

Figure 3 illustrates that there was no significant difference in toxicity between MPTP and MPP^+ ($P > 0.05$ for all data points).

Monoamine oxidase inhibition

Experiments were carried out to examine indirectly, whether MAO B was present in the neuronal cell lines and to investigate the importance of MAO B in MPTP toxicity in this isolated cellular system.

Two specific MAO B inhibitors, pargyline and selegiline (both at 1 nM) were used. A pronounced effect against MPTP toxicity was observed as compared to the controls (Fig. 4). All three cell lines showed a significant reduction in cell death with the addition of pargyline ($P < 0.01$) or selegiline ($P < 0.01$). Clorgyline, which does not exhibit MAO B specificity, also exerted a significant protective effect against MPTP toxicity in all three cell lines (Fig. 5) where $P < 0.01$, and also reduced spontaneous cell death in control cultures of B50 and B65 cells.

Effects of ROS inhibitors

To examine the hypothesis that ROS produced as a result of MPTP metabolism were mediating the observed cytotoxicity in SKNSH cells, the use of

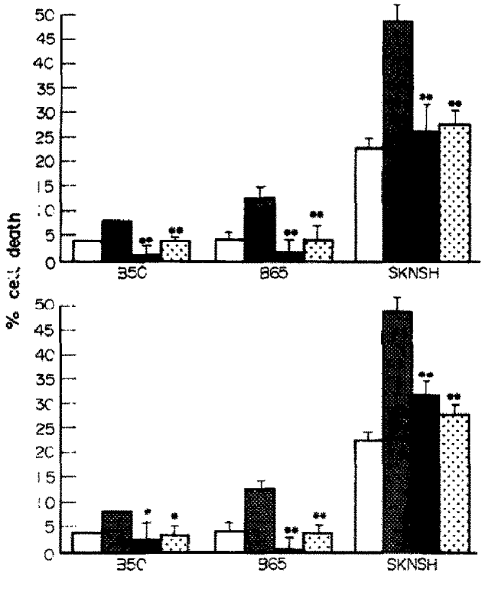

Fig. 4. (a) Pargyline and (b) selegiline on MPTP toxicity. Neuronal cells were exposed to 200 μM MPTP in the presence of 1 μM MAO B inhibitor for 24 hr, after which cell death was determined by Trypan blue inclusion. Data are expressed as the means + SD of triplicate analyses. * Represents $P < 0.05$ and ** represents $P < 0.01$ against MPTP + cells. (□) cells; (■) cells + MPTP; (■) cells + (a) pargyline/(b) selegeline; (▨) cells + MPTP + (a) pargyline/(b) selegeline.

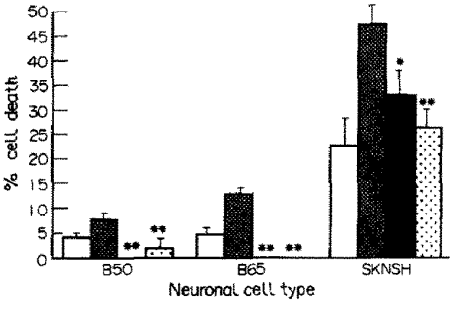

Fig. 5. The effect of clorgyline on MPTP toxicity. Neuronal cells were exposed to MPTP in the presence or absence of clorgyline for 24 hr prior to estimation of cell death by Trypan blue inclusion. Results are expressed as the mean + SD of triplicate analyses. * Represents $P < 0.05$ and ** represents $P < 0.01$ against MPTP + cells. (□) Cells alone, (■) cells + MPTP, (■) cells + MPTP + clorgyline, (▨) cells + clorgyline.

antioxidants and free radical scavengers in the experimental cultures were investigated. Cells were incubated with MPTP (200 μM) or H_2O_2 (1 mM) in the presence or absence of one of four inhibitors of ROS, mannitol (50 mM), DFX (0.5 mM), SOD (3000 U/mL) or catalase (27,000 U/mL) and the results are shown in Fig. 6. Normally, mannitol scavenges $OH^{\cdot}$ radicals with a rate constant of $1.0 \times 10^9\ M^{-1}\ sec^{-1}$. In these experiments, data were statistically analysed by the $r \times c$ Chi squared test, comparing the protective effects of ROS scavengers with the antioxidant blank, and the scavenger + toxicant-treated cells. Mannitol afforded no significant protection ($P > 0.1$) against either MPTP or

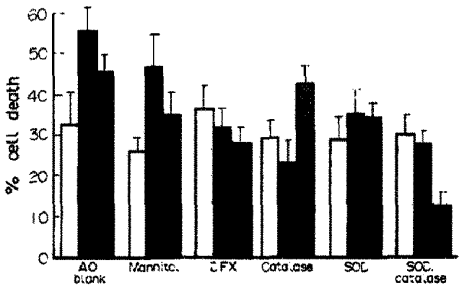

Fig. 6. The effects of ROS inhibitors on SKNSH cell death induced by MPTP and H_2O_2. SKNSH cells were co-incubated with ROS inhibitors and toxicant for 24 hr prior to analysis of cell death by Trypan blue inclusion. Results are expressed as the means of triplicate analyses + SD, and are analysed with respect to the antioxidant blank (AO blank). (□) Control, (■) + H_2O_2, (■) + MPTP.

H_2O_2 stressed cells compared to the antioxidant blank (Fig. 6). It did not show any effect on cell viability in the absence of MPTP or H_2O_2. DFX significantly reduced the extent of killing in cells treated with MPTP ($P < 0.01$) and H_2O_2 ($P < 0.02$) as compared to the antioxidant blank. In addition, cells were pre-incubated with DFX for 24 hr prior to toxicant exposure. Results (data not shown) indicated that there was no difference in the protective effect of DFX with pre-incubation. Cells treated with catalase were significantly protected from H_2O_2 toxicity ($P < 0.01$) since the reagent H_2O_2 provides an immediate substrate for the catalase. Catalase alone had no effect on cell viability, and did not afford any significant protection against MPTP-induced toxicity. Finally, MPTP-stressed human neuroblastoma cells were treated with SOD to determine whether toxicity was exacerbated by generation of H_2O_2, or inhibited. It was found that SOD did not significantly protect against MPTP toxicity ($P > 0.1$), nor did it potentiate toxicity. However, when both SOD and catalase were added to the incubation, protective effects were shown to be highly significant compared to SOD alone ($P < 0.01$).

Flow cytometry of treated neuronal cells

Studies were carried out to identify targets for ROS attack. One such macromolecular target is DNA, the integrity of which can be monitored by flow cytometry. For this experiment, B65 cells were incubated with MPTP (200 μM) or H_2O_2 (1 mM) for 24 hr before being lysed for nucleoid analysis. Nucleoids are histone-free DNA molecules without intact scaffolding proteins but in which the overall looped structure of the chromosomes is retained. Conformational damage induced in nucleoids was detected by staining with ethidium bromide and passing through a laser cytometer. Two parameters, forward scatter and red fluorescence, were analysed. Varying concentrations of ethidium bromide were added to the nucleoids from those cells treated with MPTP or H_2O_2. The median channel number was calculated for each concentration of ethidium bromide and it was found that a concentration between 2 and 5 μg/mL ethidium bromide gave the best scatter profiles (Table 1). At these

Table 1. The effect of H_2O_2 and MPTP on nucleoid structure

Concn of EB (μg/mL)	Control		+ Hydrogen peroxide		+ MPTP	
	MV	RF	MV	RF	MV	RF
50	91.84	133.65	85.14	144.24	88.78	143.23
20	114.02	95.65	141.98	99.7	104.81	112.97
5	107.78	27.59	160.66	41.25	173.32	52.18
2	90.10	15.21	133.27	24.02	155.17	34.33

B65 cells were incubated in the presence of H_2O_2 (1 mM) or MPTP (200 μM) for 24 hr. Cells were lysed in disruption buffer to extract the nucleoids and stained with ethidium bromide (EB) prior to analysis by FACS using the method of flow cytometry [15]. The results represent the median forward scatter (MV) indicative of conformational damage, and red fluorescence (RF) indicating DNA strand breakage for the extracted nucleoids of stressed cells.

Table 2. Lipid peroxide formation in neuronal cells exposed to H_2O_2 and MPTP

Concentration (μM)	A_{365} for B50	A_{365} for B65
MPTP		
0	0.085 + 0.007	0.076 + 0.007
10	0.073 + 0.003	0.071 + 0.009
100	0.1 + 0.003	0.069 + 0.002
H_2O_2		
0	0.085 + 0.001	0.076 + 0.002
100	0.11 + 0.004	0.074 + 0.007
1000	0.156 + 0.003	0.078 + 0.004

Stressed cell suspensions were analysed for the presence of peroxides [14] by monitoring the spectrophotometric reduction of iodine at 365 nm.

Results are expressed as the mean of triplicate analyses + SD.

concentrations, there was a marked shift from a low scatter profile in control cells to a high scatter profile in the treated cells as shown by an increase in median channel number indicating the occurrence of DNA damage.

Lipid peroxide analysis

Lipid peroxidation measurements determined using a CHOD-iodine kit gave very low levels of lipid peroxides with the cell cultures both pre- and post-incubation (Table 2). An increasing trend of lipid peroxide levels was seen for both MPTP and H_2O_2, but low levels were detected and no significance was seen.

DISCUSSION

Chronic exposure to neurotoxins has been proposed to be an underlying cause in the aetiology of the neurodegenerative diseases, where a common mechanism, such as the generation of ROS, is ultimately responsible for cell death. In idiopathic Parkinson's disease, excessive formation of destructive ROS, through oxidative metabolism of dopamine or a lack of detoxification, in critical regions of the brain may play a role in the pathogenesis of the disease. Herein, we have investigated the effects of two cytotoxins, MPTP and H_2O_2, on neuronal cells maintained in culture. Maximal cell death was achieved within 24 hr of toxin addition, after which background cell death rose significantly. Control cells also exhibited differences in stability over the 24 hr culture; the dopaminergic cells were much more labile than cholinergic cells or lymphocytes, and this may reflect their higher concentration of auto-oxidizable substrates such as dopamine. In examining the effects of MPTP and H_2O_2 on cell viability, a similar pattern of susceptibility was seen; the B50 rat cholinergic cells were the most resistant, after which the B65 dopaminergic cells and the SKNSH human neuroblastoma cells were the most susceptible. Lymphocytes on the other hand were only affected by H_2O_2. The inability of MPTP to exert a toxic effect in lymphocytes is likely to be due to the lack of MAO B in lymphocytes required to metabolize the protoxicant [17] or because of a lack of an analogous dopamine uptake mechanism to transport $MPDP^+$ intracellularly. In general, the rat cell lines appear to be much more refractory to the effects of H_2O_2 than human cells, and this may reflect differences in endogenous antioxidant status. In support of this, Kawamura *et al.* [18] have recently reported high levels of Mn-SOD in neuroblastoma cell lines.

In examining the effects of MPTP on neuronal cell lines, the most striking observation is the comparative lack of susceptibility of rat derived lines when compared to the human line. This supports the findings reported in animal systems, where primates are much more sensitive to MPTP toxicity than rodent species [19]. The observation that the cell lines B65 and SKNSH are more susceptible to damage than the B50 cholinergic cell lines may reflect the dopaminergic properties of these differentiated neurones, which include a specific sodium-dependent high affinity dopamine uptake mechanism and may also be affected by the activity of MAO B in these cultures. MPP^+ can enter nigrostriatal dopaminergic neurones via the same dopamine uptake system, thereby interfering with the normal neurotransmitter uptake [20] and enhancing toxicity. It has been postulated that if it is the MPTP metabolites which are the ultimate toxicants, then the use of MAO B inhibitors would reduce toxicity. MAO B inhibitors have been used successfully in the clinical management of PD, and are highly lipophilic so they can be easily taken up by neurones. Indeed, we found that the neuronal cell lines were protected from MPTP in the presence

of pargyline ($P < 0.01$) and selegiline ($P < 0.05$). This confirmed the hypothesis that in cell culture as in experimental animals, MPTP metabolism by MAO B is responsible (at least in part) for exerting the final toxic effect. The protective effect afforded by clorgyline may be explained by some cell-stabilizing properties which are not related to MAO inhibitory activity, since control cultures also showed significant protection against spontaneous cell death.

Decomposition of $MPDP^+$ to MPP^+ in part may involve one-electron oxidation/reduction [21] providing a pathway by which $O_2^-\cdot$ may be generated and subsequently produce other ROS. Alternatively, the disruption of mitochondrial integrity can lead to the leakage of reducing equivalents onto molecular oxygen, thereby generating $O_2^-\cdot$. Since MPTP and MPP^+ appear to exert similar neurotoxic activity in these systems, it is likely that both mechanisms may be important in their cytotoxicity.

One conventional method by which ROS toxicity can be determined involves the use of antioxidants. Of the antioxidants used to examine whether ROS were involved in mediating MPTP toxicity, DFX and the simultaneous presentation of catalase and SOD significantly protected against damage ($P < 0.01$) as compared to the antioxidant control. SOD alone and mannitol failed to protect against cell death. This latter observation suggests that the hydroxyl free radical is either not involved in the mechanism of toxicity of H_2O_2 or MPTP, or that these radicals are generated in sites where the mannitol has no access to them (for instance in site-specific locations within nuclear DNA or within mitochondria). Walker *et al.* [22], have recently reported the lack of effect of SOD on mitochondrial function after redox cycling between MPP^+ and $MPDP^+$. However, examination of the effects of SOD alone cannot preclude the generation of ROS, since our studies have also shown that DFX, a powerful chelator of Fe(II) [23], protected cells against MPTP and H_2O_2 toxicity probably by removing any available iron that would otherwise catalyse the production of $OH\cdot$ radicals via the Haber–Weiss reaction. Catalase and SOD alone failed to provide antioxidant protection with stressed human-derived cells; however, the simultaneous presence of both catalase and SOD gave rise to highly significant levels of protection ($P < 0.01$) in the human cell line. SOD catalyses the dismutation of two molecules of $O_2^-\cdot$ to form one molecule of H_2O_2. We predicted therefore that SOD alone would be more likely to enhance toxicity, however, this effect was not seen, and may be due to a non-specific scavenging effect of the SOD protein. The additional presence of catalase catalytically eliminates H_2O_2 thus lowering the overall steady state level of ROS generated, and protecting against toxicity. Since the antioxidant status, particularly related to glutathione, is compromised in PD, this work suggests that antioxidant supplementation may protect against toxicity. This conclusion is supported by a recent report by Spina *et al.* [24], who have shown that brain-derived neurotrophic factor can protect against MPP^+ toxicity by increasing the activity of glutathione reductase.

Whilst intra-neuronal uptake of DFX remains equivocal, SOD and catalase are too large to be taken up and are believed to exert largely their effects extracellularly. The protection afforded by antioxidants in our system supports the hypothesis that MPTP auto-oxidation or MAO-catalysed oxidation by contaminating glial cells with subsequent generation of ROS occurs extra-neuronally, as postulated by Singer and Ramsay [25].

In order to identify the lethal event(s) involved in MPTP and H_2O_2 toxicity, two critical cellular targets were considered; the lipid membrane and nuclear DNA. Lipid peroxidation is a classic index of ROS damage. The novel method employed to measure lipid peroxidation in this study is thought to be one of the most sensitive and reliable means of measuring lipid peroxides, being able to detect levels of less than 1 μM. However, very low levels at the limit of detection were obtained in the cell incubate, and thus no significant difference was observed between stressed cells versus controls. This supports the previous observations that MPTP fails to induce lipid peroxidation *in vivo* [26]. Moreover, it has been postulated that lipid peroxidation is an end stage event which proceeds rapidly after lethal events have occurred, irrespective of the mechanism of cell death [27].

One important mechanism involved in cell death is irreparable DNA damage. Strand breakage and modification of DNA by ROS [28] has been demonstrated after exposure to H_2O_2 in the presence of iron cations [29]. Therefore, we examined the nuclear changes after exposure to toxin using the method of flow cytometry developed by Milner *et al.* [15]. Two parameters were measured; forward light scatter, indicating structural changes, and red fluorescence, measuring the extent of strand breakage. The results obtained suggest that cells incubated with H_2O_2 or MPTP incurred some form of DNA damage since nucleoids from the cells exhibited a shift from a low scatter profile to a population of higher scatter properties. This was shown by an increase in median channel number from control cells to treated cells. These observations are typical of ROS-mediated DNA strand breakage, and may constitute a lethal event if the template is destroyed. However, it is not possible to conclude unequivocally from these data whether DNA damage is a cause or merely a contributory factor in the cytotoxic mechanism of MPTP.

In summary, we have shown that dopaminergic neuronal cells are more susceptible than cholinergic cells to the toxic effect of both MPTP and H_2O_2. Furthermore, using cell lines, we have confirmed the observations seen in whole organisms, that human-derived cells are more susceptible to oxidative toxicity than are rat-derived cells. MAO B inhibition experiments indicate that this toxicity arises following MPTP metabolism and antioxidant studies indicate that ROS appear to be involved in mediating cytotoxicity. The finding that the generation of ROS may be involved in the mechanism of cell death is of importance in the context of PD, where the search for an endogenous or environmental protoxin which generates ROS may provide some clues as to the aetiology of the disease. Such culture systems may be of value in general screening of neurotoxins and

in studying the therapeutic potential of novel agents in PD.

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