Neutrophil Cytotoxicity of the Chemically Reactive Metabolite(s) of Clozapine: Possible Role in Agranulocytosis

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ABSTRACT

Clozapine is associated with a 0.8% incidence of agranulocytosis. Bioactivation of an unstable protein-reactive metabolite, identified as a nitrenium intermediate, has been implicated in the toxicity. In this study, we investigated whether the reactive metabolite is cytotoxic toward polymorphonuclear leukocytes and mononuclear leukocytes using horseradish peroxidase and H2O2 to generate the metabolite in situ. In the absence of a full metabolizing system (i.e., lack of horseradish peroxidase and/or H2O2), clozapine (0–100 μM) and its stable metabolites were not cytotoxic. With a full metabolizing system, both clozapine (30 μM) and demethylclozapine exhibited cytotoxicity toward polymorphonuclear leukocytes (50.7 ± 7.7% and 17.6 ± 1.2% cell death, respectively) and mononuclear leukocytes (36.6 ± 2.1% and 24.6 ± 4.1%, respectively), whereas clozapine N-oxide was not cytotoxic. Exogenous glutathione (GSH), N-acetylcysteine and ascorbic acid all protected the cells. Bioactivation of clozapine and demethylclozapine, but not the N-oxide, was accompanied by depletion of intracellular GSH. [14C]Clozapine was metabolized to the previously identified C6 and C9 glutathionyl conjugates; GSH conjugates were also detected when demethylclozapine and clozapine N-oxide were bioactivated by horseradish peroxidase and H2O2. In conclusion, using a novel in vitro assay, we have shown that clozapine and its stable metabolites are not cytotoxic per se but are bioactivated to cytotoxic metabolites. The cytotoxic metabolite of clozapine is identical to the protein-reactive metabolite that has been characterized previously. These cytotoxic metabolites may play an important role in the pathogenesis of clozapine agranulocytosis; the mechanism by which this occurs is currently being investigated.

Clozapine is a dibenzodiazepine antipsychotic whose main advantages include the lack of extrapyramidal adverse effects such as tardive dyskinesia (Baldessarini and Frankenburg, 1991) and its effectiveness in otherwise “treatment-resistant” patients (Kane et al., 1988). Despite these advantages, the use of clozapine is restricted because of a relatively high incidence (0.8%) of agranulocytosis (Alvir and Lieberman, 1994; Lieberman and Safferman, 1992). The nitrenium ion has been implicated in the pathogenesis of the agranulocytosis, although the exact mechanism by which toxicity occurs is unclear. As has been postulated for other types of reactive metabolites (Park et al., 1992, 1995), agranulocytosis may arise from an interaction of the stable metabolites, demethylclozapine and clozapine N-oxide (Gauch and Michaelis, 1971). Additionally, several glucuronide and sulfate conjugates have been detected in rodent bile (Zhang et al., 1996). It has been suggested that N-demethylclozapine is more toxic than clozapine toward myeloid cells, but this was only observed at concentrations higher than those achieved therapeutically (Gerson et al., 1994).

Recently, we have shown in rodents that clozapine undergoes extensive bioactivation in vivo by the detection of several GSH conjugates (Maggs et al., 1995). In man, clozapine can also be metabolized to a reactive intermediate by hepatic P450 enzymes (Pirmohamed et al., 1995), myeloid cells (Maggs et al., 1995) and peripheral blood PMN (Liu and Uetrecht, 1995; Maggs et al., 1995). MPO, the major enzyme present in PMN, can bioactivate clozapine to a radical cation and then to a nitrenium ion (Fischer et al., 1991; Uetrecht, 1992). The nitrenium ion has been implicated in the pathogenesis of the agranulocytosis, although the exact mechanism by which toxicity occurs is unclear.

ABBREVIATIONS: HRP, horseradish peroxidase; MPO, myeloperoxidase; PMN, polymorphonuclear leukocytes; MNL, mononuclear leukocytes; clozapine, 8-chloro-11-[[4-methyl-1-piperazinyl]-5H-dibenzo[b,e][1,4]diazepine; GSH, glutathione; NAC, N-acetylcysteine; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; HEPES, (N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid]); TNF-α, tumor necrosis factor-α; HSA, human serum albumin; PMA, phorbol myristate acetate.
nitrenium ion with a cellular macromolecule, which leads to either direct toxicity or indirect immune-mediated toxicity (Liu and Uetrecht, 1995; Maggs et al., 1995; Safferman et al., 1992).

To date, all studies have used irreversible protein binding and adduct formation as indicators for bioactivation of clozapine. There have been no studies that have addressed the issue of the functional toxicity of the putative nitrenium ion to PMN. Using a liver microsomal metabolizing system, it has been shown (Pirmohamed et al., 1995) that clozapine undergoes bioactivation to a metabolite that is cytotoxic to MNL. However, studies in our laboratory have shown that this assay cannot reliably be used to assess toxicity to the target cell (i.e., the PMN or its precursors) because microsomes bind to the surface. Although the chemically reactive metabolite can be synthesised, it has a short half-life, estimated to be <1 min (Liu and Uetrecht, 1995), making it difficult to use in direct cytotoxicity assays.

To address the issue of whether the reactive metabolite of clozapine is cytotoxic, we have developed a novel in vitro assay in which the in situ generation of the reactive metabolite is coupled to an assessment of PMN and MNL viability and chemical characterization of the metabolism of clozapine. Peripheral blood neutrophils have been used as surrogates for their precursors in the bone marrow, which seem to be the major target for the toxicity of clozapine (Pirmohamed and Park, 1997).

### Materials and Methods

\[^{14}C\]Clozapine (164 μCi/mg, radiochemical purity 98% by HPLC), unlabeled clozapine, demethylclozapine and clozapine-N-oxide were gifts from Sandoz Pharmaceuticals (Basle, Switzerland). HRP (type VI), MPO, reduced GSH, NAC, ascorbic acid, gentamycin, bromobimane, N-ethyl morpholine and trichloroacetic acid were purchased from Sigma Chemical Co. (Poole, UK). All solvents were of HPLC grade and were products of Fisher Scientific plc (Loughborough, UK). Mono-poly Resolving Medium (Ficoll Hypaque, 1.114 g/ml) and Lymphoprep (1.077 g/ml) were from ICN Biomedicals (Bucks, UK) and Nycomed (Birmingham, UK), respectively.

#### Isolation of human peripheral blood cells.

To prevent contamination by micro-organisms, MNL and PMN were isolated from fresh heparinized venous blood of healthy male volunteers (24–29 years) in a Class II Biohazard Cabinet with a vertical laminar air flow. Fresh heparinized venous blood of healthy male volunteers (24–29 years) in a Class II Biohazard Cabinet with a vertical laminar air flow (Gelaire BSB 4A, Flow Laboratories, Milan, Italy). The buffers were stored for no more than 15 min in a Class II Biohazard Cabinet with a vertical laminar air flow (Gelaire BSB 4A, Flow Laboratories, Milan, Italy). The buffers were stored for no more than 15 min in a Class II Biohazard Cabinet with a vertical laminar air flow. Fresh heparinized venous blood of healthy male volunteers (24–29 years) in a Class II Biohazard Cabinet with a vertical laminar air flow (Gelaire BSB 4A, Flow Laboratories, Milan, Italy). The buffers were stored for no more than 15 min in a Class II Biohazard Cabinet with a vertical laminar air flow.

#### Determination of depletion of intracellular GSH.

To determine whether clozapine or its metabolites depleted intracellular GSH in PMN and MNL, the fluorescent probe monobromobimane, which binds to intracellular GSH, was used (Cotgreave and Moldeus, 1986). The cells (5 × 10⁶ cells/incubation) were incubated with either clozapine, demethylclozapine or clozapine N-oxide (0–30 μM) in HEPES buffer at 37°C for 1 hr in the presence or absence of HRP (20 units) and H₂O₂ (10 μM). The total incubation volume was 1 ml.

### Comparison of metabolism of clozapine by MPO and HRP.

Clozapine (30 μM) was incubated at 37°C for 2 hr with either MPO (1 unit) or HRP (20 units), as described previously (Fischer et al., 1991), and H₂O₂ (10 μM) in HEPES buffer (pH 7.4; final incubation volume 1 ml). The reaction was initiated by the addition of H₂O₂, and GSH (1 mM) was added within 30 sec. After 2 hr, methanol (1 ml) was added to each tube. The solutions were then evaporated to dryness under a stream of nitrogen at 37°C, reconstituted in methanol/water (1:1,300 μl) and analyzed by HPLC as described previously (Maggs et al., 1995).

#### Bioactivation of clozapine and its metabolites by HRP and hydrogen peroxide and determination of cytotoxicity.

PMN and MNL were incubated with clozapine, demethylclozapine and clozapine N-oxide in 15-ml plastic conical tubes in an agitating water bath for 2 hr at 37°C. They (1 × 10⁶ cells/ml) were incubated with drug (0–100 μM) in the presence or absence of HRP (20 units), H₂O₂ (10 μM), GSH (1 mM), NAC (1 mM) and ascorbic acid (1 mM). Reactions were initiated with H₂O₂ and after 2 hr, the tubes were centrifuged (10 min, 650 × g) to pellet the cells. The supernatants were decanted and the cells were resuspended in 1 ml of drug-free HEPES buffer containing HSA (5 mg/ml) and gentamycin (50 μg/ml). Samples were then placed in an incubator at 37°C. Cytotoxicity was assessed after 16 hr by trypan blue dye exclusion, as described previously (Riley et al., 1988). Cytotoxicity was also assessed by the LDH release assay, and this correlated with trypan blue dye exclusion (data not shown).

Chemical characterization of the metabolites of clozapine. PMN and MNL were incubated with [¹⁴C]clozapine in 15-ml glass test tubes in an agitating water bath for 2 hr at 37°C. A typical incubation contained either PMN or MNL (1 × 10⁶ cells/ml), [¹⁴C]clozapine (0–30 μM, 0.1 μCi) in 1 ml of HEPES buffer in the presence or absence of HRP (20 Units), H₂O₂ (10 μM) and GSH (1 mM). After 2 hr, the test tubes were centrifuged (10 min, 750 × g) to pellet the cells. The cells were then resuspended as above and cytotoxicity was assessed after 16 hr by trypan blue dye exclusion. The supernatants were decanted, and 1 ml of methanol was added to each. They were then evaporated to dryness under a stream of nitrogen at 37°C, reconstituted in methanol/water (1:1, 300 μl) and analyzed by HPLC as described previously (Maggs et al., 1995).

In other experiments, unlabeled clozapine and its stable metabolites clozapine-N-oxide and demethylclozapine (each 30 μM) were incubated with HRP (20 units), H₂O₂ (10 μM) and GSH (1 mM) in the presence or absence of PMN (1 × 10⁶ cells/ml) in 1 ml of HEPES buffer. Clozapine (30 μM) was also incubated with HRP (20 units) and H₂O₂ (10 μM) in the absence of both cells and GSH. Hydrogen peroxide was added to start the reaction and GSH was added no later than 40 sec after the H₂O₂. After 2 hr, the tubes containing cells were centrifuged (10 min, 650 × g) and the supernatant removed. Methanol (1 ml) was added to the supernatant, which was evaporated to dryness under a stream of nitrogen at 37°C and reconstituted in 150 μl of ethanol/water (1:1, v/v) for analysis by HPLC and LC-MS.

The conditions for HPLC and LC-MS have been described previously (Maggs et al., 1995). Briefly, samples were eluted from a 5-μm Nucleosil C₁₈ column (25 × 0.32 mm; Phenomenex, Macclesfield, Cheshire, UK) with gradients of acetonitrile in 6 mM ammonium formate, pH 3.5: 20% to 40% over 15 min (gradient I), 10% to 25% over 15 min and 25% to 55% over 20 min (gradient II). The flow rate was 0.75 ml/min.

### Determination of depletion of intracellular GSH.

To determine whether clozapine or its metabolites depleted intracellular GSH in PMN and MNL, the fluorescent probe monobromobimane, which binds to intracellular GSH, was used (Cotgreave and Moldeus, 1986). The cells (5 × 10⁶ cells/incubation) were incubated with either clozapine, demethylclozapine or clozapine N-oxide (0–30 μM) in HEPES buffer at 37°C for 1 hr in the presence or absence of HRP (20 units) and H₂O₂ (10 μM). The total incubation volume was 1 ml.

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² M. Pirmohamed, unpublished data.
Monobromobimane (3 mM) in N-ethyl morpholine (50 mM, pH 8.0, 100 μl) was added to the incubations, which were then left in the dark at 37°C for 5 min. Protein was precipitated with 100% trichloroacetic acid (w/v) (10 μl) and sedimented by centrifugation (750 × g, 3 min). Aliquots (50 μl) of the supernatant were analyzed by fluorescence chromatography, as described previously (Pirmohamed et al., 1996).

**Statistical analysis.** The results are presented as mean ± S.E.M. Statistical analysis was performed by the Mann-Whitney U test, accepting P < .05 as significant.

**Results**

Direct cytotoxicity of clozapine and its metabolites. Clozapine alone did not exhibit any cytotoxicity toward either MNL or PMN (fig. 1A) up to a concentration of 100 μM. Similar results were obtained for demethylclozapine (fig. 1B) and clozapine N-oxide (fig. 1C).

**Metabolism of clozapine by MPO and HRP.** Clozapine was metabolized by MPO (1 unit) to C6 glutathionyl clozapine (6.2 ± 0.5% conversion) only, whereas HRP (20 units) produced both C6 glutathionyl clozapine (21.0 ± 1.1%) and C9 glutathionyl clozapine (4.0 ± 0.7%). These metabolites were identified by coelution with glutathionyl clozapine isomers, which have been characterized previously (Maggs et al., 1995). Given the higher turnover achieved with the concentration of HRP used, all of the further studies were performed using an activating system comprising HRP and H2O2.

**HRP/hydrogen peroxide-mediated metabolism-dependent cytotoxicity.** In the presence of both HRP and H2O2, clozapine was bioactivated to a metabolite that showed concentration-dependent cytotoxicity (fig. 1A) toward both MNL and PMN (P < .05 at all concentrations compared with solvent control). There was no difference in the sensitivity of the MNL and PMN up to a concentration of 10 μM clozapine, whereas at 30 μM clozapine, PMN were significantly (P < .05) more sensitive. Significant cytotoxicity was also observed at concentrations (3 μM) of clozapine achieved therapeutically (Sayers and Amsler, 1977). When the drug was incubated with an incomplete activating system (i.e., in the absence of either HRP or H2O2), no cell death (above background values) was observed. Additionally, in the absence of the drug (i.e., solvent control) but in the presence of a full activating system, again there was no increase in cell death above background values.

In the presence of the full activating system, demethylclozapine, like clozapine, also showed a concentration-dependent cytotoxicity. However, it was less cytotoxic than clozapine at all concentrations (fig. 1B). In contrast, clozapine N-oxide did not exhibit any cytotoxicity up to a concentration of 30 μM in the presence of a full activating system (fig. 1C).

**Effect of biological modifiers on HRP/hydrogen peroxide-mediated clozapine cytotoxicity.** The cytotoxicity of clozapine and demethylclozapine toward both cell types was reduced to background values when either GSH, NAC or ascorbic acid was included in the incubation (fig. 2, A and B).

**Chemical identification of the metabolites of clozapine.** After incubation of [14C]clozapine (10 μM; 0.1 μCi) with HRP, H2O2 and either cell type, analysis of the supernatant by radiometric HPLC revealed a small peak which coeluted with C6-glutathionylclozapine (table 1). We have previously characterized this metabolite by LC-MS (Maggs et al., 1995). When GSH (1 mM) was added to the incubation, the size of this peak increased significantly (P < .05) with both MNL and PMN (table 1). A second peak was visible only when GSH was present, and coeluted with the previously identified C9-glutathionylclozapine adduct. There was no metabolism of clozapine when either the HRP or H2O2 was omitted from the incubations. A typical HPLC chromatogram obtained when [14C]clozapine was incubated in the presence of HRP and hydrogen peroxide is shown in figure 3A and, with GSH present, figure 3B.

**Determination of the extent of bioactivation.** When clozapine, demethylclozapine and clozapine N-oxide (30 μM) were incubated individually with HRP, H2O2 and GSH in the
Metabolism-dependent depletion of intracellular GSH by clozapine and its metabolites. There was no GSH depletion observed when either clozapine, demethylclozapine or clozapine N-oxide were incubated alone with either PMN or MNL (fig. 5). Similarly, there was no GSH depletion when clozapine was incubated with either HRP or H₂O₂ in isolation. In the presence of the full activating system (i.e., HRP and H₂O₂), in contrast, clozapine resulted in a concentration-dependent depletion of intracellular GSH in both MNL and PMN (fig. 5; P < .05 at all concentrations compared with incubations without drug). Demethylclozapine also caused GSH depletion, but this was significantly (P < .05) less than that observed with clozapine. Clozapine N-oxide, however, did not cause any depletion of GSH in either PMN or MNL in the presence of both HRP and H₂O₂ (fig. 5).

Discussion

The purpose of this study was to investigate the relative abilities of clozapine and its stable and reactive metabolite(s) to cause damage to PMN at therapeutically relevant concentrations to gain an insight into the mechanism(s) of clozapine-induced agranulocytosis.

Assessment of the cytotoxic potential of the parent drug and its stable metabolites was performed by incubating the respective compounds with MNL and PMN. In accordance with a previous study (Gerson et al., 1994), we found that clozapine and its major stable metabolites were not directly toxic even at relatively high concentrations. Assessment of whether drug-derived chemically reactive metabolites are cytotoxic is not as straightforward largely because these metabolites are, by definition, unstable and therefore cannot be isolated. Thus, to date, it has not been possible to determine whether the nitrenium ion which has been postulated to be the chemically reactive metabolite derived from clozapine is cytotoxic.

To ascertain cytotoxicity of chemically reactive metabolites, an in vitro cytotoxicity assay in which such metabolites can be generated in situ by a liver microsomal metabolizing system has been devised (Riley et al., 1988; Spielberg, 1980). Using such an assay, we have previously shown that clozapine was bioactivated by human and phenobarbitone-induced mouse microsomes to a metabolite that was cytotoxic toward MNL (Pirmohamed et al., 1992). A similar investigation has also been conducted by Tschen et al. (1996) using a rat liver microsomal system. However, studies in our laboratory showed that a microsomal system could not be used to assess PMN cytotoxicity because the microsomes bound to the PMN surface and caused nonspecific cell death. Therefore, the

Table 1

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<th>Parameter assessed</th>
<th>Mononuclear leukocytes</th>
<th>Polymorphonuclear leukocytes</th>
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<tr>
<td></td>
<td>−Glutathione (+μM)</td>
<td>+Glutathione (+μM)</td>
</tr>
<tr>
<td>C6-Glutathionyl clozapine formation</td>
<td>9.7 ± 0.3</td>
<td>45.6 ± 2.0</td>
</tr>
<tr>
<td>C9-Glutathionyl clozapine formation</td>
<td>—</td>
<td>83.3 ± 0.3</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>40.2 ± 4.2</td>
<td>5.5 ± 1.0</td>
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The major purpose of this study was to devise an assay that could be used to assess cytotoxicity of the nitrenium ion of clozapine toward PMN.

The nitrenium ion of clozapine can be generated by coin-cubation with hypochlorous acid (Liu and Uetrecht, 1995). However, use of hypochlorous acid produced unacceptably high background cytotoxicity (data not shown). Both HRP and MPO (Fischer et al., 1991) can also metabolize clozapine to a chemically reactive species. These peroxidases are capable of catalyzing both one- and two-electron oxidations via one-electron transfer and through the production of hypochlorous acid (Babior, 1984; Eastmond et al., 1986). The metabolites produced by HRP are qualitatively similar to those produced by MPO (Eastmond et al., 1986). With clozapine, we have recently shown that the metabolites produced by HRP are identical to those produced by both PMN and PMN precursors that have been activated by PMA (Maggs et al., 1995). Additionally, the metabolic turnover to the GSH conjugates with HRP (20 units; 9.8 μmol/l) was higher than with MPO (1 unit; 3 μmol/l). Therefore, we used HRP in conjunction with hydrogen peroxide to determine whether the reactive metabolite was cytotoxic. Our results are consistent with the reactive nitrenium metabolite being cytotoxic toward both MNL and PMN, and indeed, cytotoxicity was seen at concentrations much lower than those needed with microsomes (where turnover to the GSH conjugates is comparatively lower, 0.3 μmol/l; Pirrmohamed et al., 1995). The turnover in the present system was also higher than that seen when PMN are activated by PMA (0.2 μmol/l; Maggs et al., 1995). Moreover, in this study, cytotoxicity was seen at therapeutic clozapine concentrations. Interestingly, cytotoxicity was also accompanied by depletion of GSH. In fact, GSH depletion occurred at concentrations which were lower than that needed for cytotoxicity. GSH depletion was not seen when PMN were stimulated by PMA (Maggs et al., 1995) which may have been a consequence of the lower turnover. Taken together, the results of the present study indicate that conjugation with GSH serves as a detoxication mechanism, and when GSH is depleted below a threshold level, binding to PMN protein may initiate cellular death. In support of this, exogenous GSH afforded protection to the cells. The role of

**Table 2**

Conjugation of clozapine and its stable metabolites with glutathione (1 mM) in the presence and absence of neutrophils

<table>
<thead>
<tr>
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<th>Conversion to C6-glutathionyl clozapine</th>
<th>Conversion to C9-glutathionyl clozapine</th>
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<tr>
<td></td>
<td>+ Neutrophils</td>
<td>− Neutrophils</td>
</tr>
<tr>
<td>Clozapine</td>
<td>25.5 ± 2.4</td>
<td>26.9 ± 1.0</td>
</tr>
<tr>
<td>Demethylclozapine</td>
<td>28.7 ± 0.8</td>
<td>18.2 ± 3.3</td>
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<tr>
<td>Clozapine N-oxide</td>
<td>23.1 ± 0.1</td>
<td>19.1 ± 3.6</td>
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**Fig. 3.** HPLC chromatograms (gradient I; refer to Materials and Methods) of clozapine in the presence of an activating system (in the presence of cells) either with (B) or without (A) GSH (1 mM).

**Fig. 4.** Ion-current chromatograms of metabolites of clozapine (30 μM) incubated with HRP (20 units) and hydrogen peroxide (10 μM) (in the absence of cells) either in (A) the presence of GSH (1 mM; m/z 632) or (B) in its absence (m/z 343). The gradient system used was gradient II (see Materials and Methods). Ions were protonated molecules.
GSH in preventing cell death has been examined previously in relation to anticancer agents (O’Brien and Tew, 1996). Indeed, an increase in cellular GSH content is one of the major mechanisms by which cancer cells develop resistance to chemotherapeutic agents (O’Brien and Tew, 1996). In accordance with previous studies (Maggs et al., 1996), chemical analysis of the incubations showed that bioactivation of clozapine was accompanied by the formation of C6-glutathionyl clozapine and both C6- and C9-glutathionyl clozapine without and with the addition of exogenous GSH, respectively. The amount of conjugate formed was equivalent to the degree of GSH depletion observed.

In addition to GSH, both NAC and ascorbic acid also protected cells. The cytoprotection afforded by ascorbic acid is particularly interesting since it is known that schizophrenics have decreased ascorbic acid concentrations (Suboticanec et al., 1990), and it has previously been suggested that ascorbic acid supplementation may prevent clozapine agranulocytosis (Fischer et al., 1991).

In this study, we also examined whether demethylclozapine and clozapine N-oxide, the two major stable metabolites of clozapine, underwent bioactivation to cytotoxic metabolites. Our results suggest that bioactivation of demethylclozapine may also contribute to the pathogenesis of the agranulocytosis (Fig. 5). The putative N4-demethylated nitrenium ion also exhibited cytotoxicity but this was less than that observed with the toxic metabolite of clozapine, and was prevented by thiol and ascorbic acid. GSH depletion was also observed, but again this was less than with clozapine. In contrast, with clozapine N-oxide, no cytotoxicity or GSH depletion was observed on incubation with HRP and H2O2. It was initially thought that the N-oxide did not undergo bioactivation to the respective nitrenium ion. However, HPLC and LC-MS analysis showed the formation of GSH adducts with the N-oxide indicating that it was being bioactivated. Toxicity and GSH depletion may not have been seen with clozapine N-oxide because of the increased polarity of the N4-nitrogen which may be preventing the molecule from crossing the cell membrane. If that is the case, then it would indicate that the reactive metabolites of clozapine and demethylclozapine are crossing the membrane and causing toxicity by interacting with intracellular protein, although clearly this requires further investigation.

The cytotoxicity induced by the reactive metabolite of clozapine may not necessarily be due to a direct interaction with essential cellular proteins but may be due to an indirect effect on cellular function. For example, binding to the PMN cell membrane may cause the release of cytokines such as TNF-α (Pollmacher et al., 1996), which then induce cell death in PMN or their precursors. The role of TNF-α in particular merits further investigation for three reasons: first, it has recently been shown that clozapine increases the plasma levels of TNF-α along with the TNF receptors p55 and p75 (Pollmacher et al., 1996). Second, in hepatocytes, GSH depletion can sensitize the cells to TNF-α mediated cytotoxicity (Xu and Czaja, 1996). Third, clozapine agranulocytosis has recently been shown to be associated with various polymorphisms in the TNF-α gene (Turbay et al., 1996); these polymorphisms are known to modulate TNF-α secretion (Wilson and Duff, 1995). Clozapine also causes a dose-dependent decrease in GM-CSF release in bone marrow cultures (Sperner-Unterweger et al., 1993), and thus the use of GM-CSF in treating clozapine agranulocytosis (Pirmohamed and Park, 1997) is a logical therapeutic manoeuvre. It is important to note that in our study we have used cell death as an endpoint, and have not distinguished between the two major forms of cell death, apoptosis and necrosis (Corcoran et al., 1994). TNF-α is known to induce apoptosis (Takeda et al., 1993) while GM-CSF inhibits apoptosis (Brach et al., 1992). Thus, the possible combination of GSH depletion (as a result of drug bioactivation), reduced GM-CSF production and increased TNF-α production, could predispose to clozapine-induced agranulocytosis by enhancing apoptosis. This is currently being investigated in our laboratory.

Although our data suggest that the pathogenesis of clozapine-induced agranulocytosis is due to direct effects of its reactive metabolites on PMN and their precursors, the involvement of immune mechanisms cannot be completely discounted. It is possible that in vivo there is binding of the
reactive metabolites of clozapine to PMN cell surfaces which is not enough to cause cell death but may act as a potent immunogenic stimulus and initiate an immune response. However, to date, there has been no convincing evidence to implicate an immune mechanism in clozapine agranulocytosis (Pirmohamed and Park, 1997).

In summary, we have shown that clozapine and demethylclozapine are bioactivated in vitro to chemically reactive nitrenium metabolites that cause PMN cytotoxicity at drug concentrations that can be achieved in vivo. Whether bioactivation occurs in vivo in humans is unknown but is suggested by studies performed in rodents (Maggs et al., 1995). The mechanism by which bioactivation of clozapine causes PMN toxicity in vivo still requires further investigation. In particular, it is important to define the individual susceptibility factors and what determines the time lag to toxicity in the clinical situation and to relate these to the bioactivation of the drug and cytotoxicity of the chemically reactive metabolite observed in this study. These areas are currently being investigated.

Acknowledgments

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PARK, B. K., PIRMOHAMED, M., KITTERINGHAM, N. R.: Idiosyncratic drug reactions:


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