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Induction of Metabolism-Dependent and -Independent Neutrophil Apoptosis by Clozapine

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ABSTRACT

Clozapine, an atypical antipsychotic used in the treatment of refractory schizophrenia, causes neutropenia and agranulocytosis in 3 and 0.8% of patients, respectively. Clozapine undergoes bioactivation to a chemically reactive nitrenium ion, which has been shown to cause neutrophil cytotoxicity. To define further the mechanism of cell death, we have investigated the toxicity of clozapine, its stable metabolites, and its chemically reactive nitrenium ion to neutrophils and lymphocytes. Clozapine was able to induce neutrophil apoptosis at therapeutic concentrations (1–3 μM) only when it was bioactivated to the nitrenium ion. The parent drug caused apoptosis at supratherapeutic concentrations (100–300 μM) only. Neutrophil apoptosis induced by the nitrenium ion, but not by the parent drug itself, was inhibited by antioxidants and genistein and was accompanied by cell surface haptenation (assessed by flow cytometry) and glutathione depletion. Dual-color flow cytometry showed that neutrophils that were haptenated were the same cells that underwent apoptosis. No apoptosis of lymphocytes was evident with the nitrenium ion or the parent drug, despite the fact that the former caused cell surface haptenation, glutathione depletion, and loss of membrane integrity. Demethylclozapine, the major stable metabolite in vivo, showed a profile that was similar to, although less marked than that observed with clozapine. *N*-oxidation of clozapine or replacement of the nitrogen (at position 5) by sulfur produced compounds that were entirely nontoxic to neutrophils. In conclusion, the findings of the study expand on potential mechanisms of clozapine-induced cytotoxicity, which may be of relevance to the major forms of toxicity encountered in patients taking this drug.

Clozapine (Clozaril), an atypical dibenzodiazepine antipsychotic, has several advantages over conventional neuroleptics, including lack of extrapyramidal side effects and increased effectiveness in refractory schizophrenia (Baldessarini and Frankenberg, 1991). Its use, however, is restricted because it causes agranulocytosis in 0.8% of patients (Atkin et al., 1996). Patients on clozapine require monitoring of their neutrophil (polymorphonuclear leukocytes, PMNs) count weekly for 18 weeks, after which the frequency of monitoring can be decreased but not discontinued. Blood count monitoring has reduced the mortality associated with clozapine agranulocytosis (Atkin et al., 1996), because the drug-induced depletion of PMNs is reversible if detected early enough (Gerson, 1994).

The mechanism of the agranulocytosis is unclear (Pirmohamed and Park, 1997). Theoretically, it could be due either to the parent drug or to its stable metabolites; however, these do not seem to be toxic to peripheral or progenitor blood cells at therapeutic concentrations (Pirmohamed and Park, 1997; Williams et al., 1997). Alternatively, the toxicity could be due to toxic metabolites produced from clozapine; such bioactivation has been implicated in agranulocytosis associated with other drugs (Uetrecht, 1992a). Indeed, clozapine does undergo bioactivation to a toxic, chemically reactive nitrenium ion by both P450 (Pirmohamed et al., 1995) and peroxidase (Fischer et al., 1991; Liu and Uetrecht, 1995; Maggs et al., 1995) enzymes. This unstable metabolite covalently binds to cellular protein (Liu and Uetrecht, 1995; Maggs et al., 1995), depletes intracellular GSH (Williams et al., 1997), and leads to PMN and mononuclear leukocyte (MNL) cytotoxicity in vitro at therapeutically relevant concentrations (Williams et al., 1997). The nitrenium ion is also formed in vivo, as demonstrated in an animal model of metabolism (Maggs et al., 1995), and immunochemically, using PMNs from patients on clozapine (Gardner et al., 1998).

It has been postulated that clozapine agranulocytosis is immune-mediated (Uetrecht, 1992b). However, unlike compounds such as aminopyrine, for which anti-drug antibodies

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ABBREVIATIONS: PMN, polymorphonuclear leukocyte; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; PE, phycoerythrin; NAC, *N*-acetyl cysteine; ASC, ascorbic acid; HBSS, Hanks' balanced salt solution; MNL, mononuclear leukocyte; DEM, diethylmaleate; CHX, cycloheximide; MPO, myeloperoxidase H-89, *N*-[2-(*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide; TNF-α, tumor necrosis factor α; HSA, human serum albumin; A+/−, annexin-V positive/negative; PI+/−, propidium iodide positive/negative.

were detected (Moeschlin and Wagner, 1952), evidence that clozapine agranulocytosis is immune-mediated has been scant and inconsistent (Jaunkalns et al., 1992; Guest et al., 1998). Therefore, it is important to consider alternative mechanisms.

We have previously shown that bioactivation of clozapine causes cell death (Williams et al., 1997). More recently, it has been suggested that white cells from patients who had suffered a previous episode of clozapine agranulocytosis are more sensitive to the clozapine nitrenium ion than cells from controls (Gardner et al., 1998b; Tschen et al., 1999). In both studies, cell death was assessed by a vital dye exclusion assay, which cannot distinguish between the different forms of cell death, i.e., apoptosis and necrosis. In this study, we have investigated the effect of clozapine and its metabolites on PMN apoptosis and related this to bioactivation, cell binding, and GSH depletion. The potential implications of our findings are discussed.

Materials and Methods

Chemicals. Clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo[*b*,*e*][1,4]-diazepine), demethylclozapine, and clozapine *N*-oxide were gifts from Novartis Pharmaceuticals; glutathione, *N*-acetyl cysteine (NAC), ascorbic acid (ASC), calphostin-C, EDTA, fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated goat anti-rabbit secondary antibody, horseradish peroxidase (HRP) (type VI), H_2O_2, human serum albumin, monobromobimane, *N*-ethylmorpholine, sodium citrate, Tris-HCl, propidium iodide, RNase A (type I-A), May-Grünwald stain, Giemsa stain, Hanks' balanced salt solution (HBSS), genistein, and cycloheximide were all obtained from Sigma Chemical Co. (Poole, UK). The annexin-V apoptosis detection kit was obtained from Beckman Coulter (Luton, UK). H-89, Mono-poly Resolving Medium (Ficoll Hypaque, 1.114 g/ml), and Lymphoprep (1.077 g/ml) were obtained from ICN Biomedicals (Bucks, UK) and Nycomed (Birmingham, UK). Anti-clozapine antibodies were prepared as described previously (Gardner et al., 1998).

Isolation of Peripheral Blood Cells. MNLs and PMNs were isolated from fresh heparinized venous blood of healthy male volunteers (24–29 years), as described previously (Williams et al., 1997). The purity of the cells using Wright's stain was found to be >95%. The viability of the cells as determined by trypan blue dye exclusion was >98%.

Incubation Conditions. PMN or MNL (1×10^6; purity >95%) were incubated with drugs (1–300 μM) in HBSS (pH 7.4; 1 ml), in the presence and absence of 20 of U HRP and 10 μM H_2O_2. In each case, H_2O_2 was added last to initiate the reaction.

Some PMN incubations also contained the antioxidants GSH, NAC, and ASC (1 mM each), which were added no later than 45 s after the addition of H_2O_2. In other incubations, PMNs were preincubated for 1 h with 40 μM genistein, 0.35 μM cycloheximide, 20 μM H-89, and 0.1 μM calphostin-C. Control incubations containing cells and buffer were also preincubated for 1 h. All drugs were added in dimethyl sulfoxide (1% v/v), which alone did not induce apoptosis.

After 2 h, the tubes were centrifuged (10 min, 650*g*), the supernatant discarded, and the cells resuspended in 1 ml of drug-free HBSS containing HSA (5 mg/ml). Samples were incubated for another 6 h (unless otherwise specified).

For assessment of loss of membrane integrity, after a 2-h incubation in the presence of drug, the cells were pelleted and resuspended in 1 ml of drug-free media containing HSA (5 mg/ml) for another 16 h. Cytotoxicity was assessed by trypan blue dye exclusion (Williams et al., 1997).

Determination of Apoptosis by Morphological Examination. PMNs were assessed for apoptotic changes using Wright's stain (consisting of May-Grünwald and Giemsa) (Watson et al., 1996). Samples (100 μl) were spun (90*g*, 10 min) onto a glass slide using a Shandon-Elliot cyto-centrifuge (London, UK). The slides were air-dried for 30 min before staining and fixing with May-Grünwald solution (0.25% w/v, in methanol; 8 min) and Giemsa solution (0.03% w/v; 18 min). A minimum of 200 cells were examined for morphological changes characteristic of apoptosis (nuclear condensation, vacuolation, and blebbing), with the use of a 40× objective (Axioskop, Zeiss, Germany) (Watson et al., 1996).

Flow Cytometric Determination of Apoptosis by Propidium Iodide Staining. Flow cytometric analysis of apoptotic nuclei was assessed according to the method originally described by Nicoletti et al. (1991). After 8 h, 1×10^6 cells were pelleted by centrifugation (200*g*, 8 min), resuspended in ethanol (1 ml, 70% v/v), and incubated for 30 min at 37°C. The fixed and permeabilized cell suspensions were pelleted, washed in 1 ml of HBSS buffer, and finally resuspended in hypotonic fluorochrome solution (1 ml; 75 μM propidium iodide, 3.4 mM sodium citrate, 1 mM Tris, 100 μM EDTA, and 500 μg/ml type I-A RNase-A). Cells were stored in the dark at 4°C before analysis.

For flow cytometry, the forward and side scatter of cell nuclei were measured simultaneously on a Coulter Epics flow cytometer (Coulter Epics, XL software; Beckman Coulter). The propidium iodide (PI) fluorescence of individual nuclei with an acquisition of FL_3 was plotted against forward scatter, and the data were registered on a log scale. A minimum of 5000 events were collected and analyzed. Apoptotic nuclei were distinguished from normal nuclei by their hypodiploid DNA. The forward threshold was raised to exclude cell debris. Acquisition and analysis were conducted with XL software (Beckman Coulter).

Flow Cytometric Determination of Apoptosis in Neutrophils by Annexin V/Propidium Iodide Double Staining. Cells were analyzed for phosphatidylserine exposure by an annexin-V FITC/propidium iodide double-staining method described by Vermes et al. (1995). Briefly, after incubation of the PMNs for another 6 h in drug-free media, the cells (1×10^6/ml) were washed twice with ice-cold PBS (1 ml; pH 7.4), resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$; 0.5 ml), and FITC-annexin-V (final concentration of 1 μg/ml) and propidium iodide (final concentration 35 μM) were then added. This mixture was incubated in the dark for 10 min at 4°C. A minimum of 5000 cells were then analyzed by bivariate flow cytometry (Coulter Epics, XL software; Beckman Coulter). The combination of these two characteristics permits simultaneous detection of vital cells (A−/PI−), apoptotic cells (A+/PI−), and necrotic cells (A+/PI+).

Detection of Haptenation of Neutrophils with an Anti-Clozapine Antibody. For quantification of haptenation, PMNs (1×10^6; purity >95%) were incubated for 1 h with clozapine (0–10 μM) or metabolites (0–30 μM) in HBSS (1 ml, pH 7.4), in the presence and absence of 20 U of HRP and 10 μM H_2O_2. In each case, H_2O_2 was added last to initiate the reaction. A 200-μl aliquot of the incubation mixture suspended in 1 ml of HBSS) was centrifuged (200*g*, 8 min) to pellet the cells. The cells were washed (2×1 ml) in HBSS. After the supernatant was discarded, anti-clozapine primary antibody (200 μl HBSS, 1:500 v/v) was added to the cells, and samples were incubated for 15 min at 4°C. The cells were resuspended in FITC-conjugated goat anti-rabbit secondary antibody (1:50 v/v) after two washing steps in HBSS and incubated in the dark for 15 min at 4°C. The cells were washed again in 4 ml of HBSS before resuspension in 0.5 ml of HBSS. Samples were stored in the dark at 4°C before analysis.

Simultaneous Determination of Haptenation and Apoptosis in Neutrophils. For the simultaneous measurement of haptenation and apoptosis, initial experiments were designed to identify the optimum time point at which both would be detectable. An apoptosis time course was thus conducted by incubating PMNs (1×10^6; purity >95%) for 1, 2, and 4 h with clozapine (0–10 μM) in 1 ml of HBSS (pH 7.4), in the presence and absence of 20 U of HRP and 10 μM H_2O_2. Cells were then analyzed for phosphatidylserine exposure by an annexin-V FITC/propidium iodide double-staining method as

described by Vermes et al. (1995). A haptenation time course, performed as described above, showed that the degree of haptenation decreased after 2 h, possibly because of internalization of the antigen. Thus haptenation and apoptosis were detected simultaneously at the 2-h time point only.

After the 2-h incubation, a 200-μl aliquot was suspended in 1 ml of HBSS and centrifuged (200*g*, 8 min) to pellet the cells, and anti-clozapine primary antibody (200 μl of HBSS, 1:500 v/v) was added; the samples were then incubated for 15 min at 4°C. The cells were washed twice in HBSS (1 × 2 ml; 1 × 1 ml) and incubated with PE-conjugated goat anti-rabbit secondary antibody (1:50 v/v) for 15 min at 4°C in the dark. After they were washed twice in HBSS, the cells were resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$; 0.5 ml) before the addition of FITC-annexin-V (final concentration of 1 μg/ml). Samples were stored in the dark at 4°C before analysis. As a further control to ensure that fluorescent compensation had been set correctly, samples that had been stained to detect either haptenation or apoptosis only were also prepared.

Flow Cytometric Detection of Haptenation and Apoptosis. For flow cytometric detection of haptenation and apoptosis, the forward and side scatter of the cells were measured simultaneously on a Beckman Coulter flow cytometer (Beckman Coulter). The FITC, PE, and PI fluorescence of cells was acquired on fluorescence channel FL_1, FL_2, and FL_3, respectively. The majority of the cells were gated for analysis, and the forward threshold was raised to exclude cell debris. A minimum of 5000 cells was then analyzed. In each case, the control cells were analyzed first and used to set the parameters for the drug-treated cells.

Determination of GSH Depletion. Cells (1×10^6/ml) were incubated with clozapine (0–30 μM) in the presence and absence of activation (HRP and H_2O_2) for 1 h, after which the fluorescent probe, monobromobimane (100 μl, 3 mM), was added in *N*-ethylmorpholine (50 mM, pH 8). After a further 5-min incubation, trichloroacetic acid (100% w/v) was added to precipitate the protein. Samples were frozen until analysis by fluorescent HPLC as described previously (Cotgreave and Moldeus, 1986).

Comparison of the Effect of Diethylmaleate on PMN Apoptosis and GSH Depletion. To determine the role of GSH depletion in inducing PMN apoptosis, we also compared the effects of diethylmaleate (DEM) on both GSH depletion and PMN apoptosis with those observed with clozapine. DEM (1–1000 μM) was incubated with PMN by itself in a manner similar to that described for clozapine, and apoptosis and GSH depletion were determined as described earlier.

Statistical Analysis. Data are presented as the mean ± S.E. from at least three different experiments (each performed in triplicate). Statistical analysis (Kruskal-Wallis test) was performed by comparing incubations containing different concentrations of the drug with the solvent control, accepting $P < .05$ as significant.

Results

Effect of Clozapine on PMN Apoptosis. Apoptosis was assessed morphologically, and by flow cytometry using propidium iodide staining (Nicoletti et al., 1991) and annexin-V (Vermes et al., 1995). The background (i.e., in the absence of drug or solvent) rates of PMN apoptosis ranged from 5 to 35%; this is consistent with previous studies (Payne et al., 1994; Cox, 1995; Watson et al., 1996).

When clozapine alone was incubated with PMNs, an increase in apoptosis was observed only at suprapharmacological concentrations (100–300 μM) (Fig. 1A). The results obtained with PI staining correlated with morphological examination and phosphatidylserine exposure.

To test for the ability of the reactive nitrenium ion to induce apoptosis, we used an in situ metabolite generating system comprising HRP and H_2O_2; this has previously been shown by liquid chromatography-mass spectroscopy to convert clozapine to the toxic nitrenium metabolite in a concentration-dependent manner (Maggs et al., 1995). Incubation of either HRP or H_2O_2 (or both together) in the absence of clozapine did not induce PMN apoptosis. However, the nitrenium ion induced PMN apoptosis at clozapine concentrations ranging from 0.1 to 3 μM (Fig. 1A), which correspond to therapeutic concentrations in vivo (Jann et al., 1993). Concentrations of clozapine above 3 μM in the presence of an activating system produced necrotic cell death. This was confirmed by the annexin-V assay, which showed a concentration-dependent increase in apoptosis in the presence of an activating system, up to a clozapine concentration of 3 μM (Fig. 2). At higher clozapine concentrations, the proportion of necrotic cells increased. Morphological assessment was also consistent with the above, showing a concentration-dependent increase in PMN apoptosis in the presence of an activating system (33.1 ± 2.3, 61.2 ± 4.6, and 61.0 ± 5.8%, at clozapine concentrations of 0, 1, and 3 μM, respectively; $P < .05$), with the mode of cell death changing to necrosis at 10 μM.

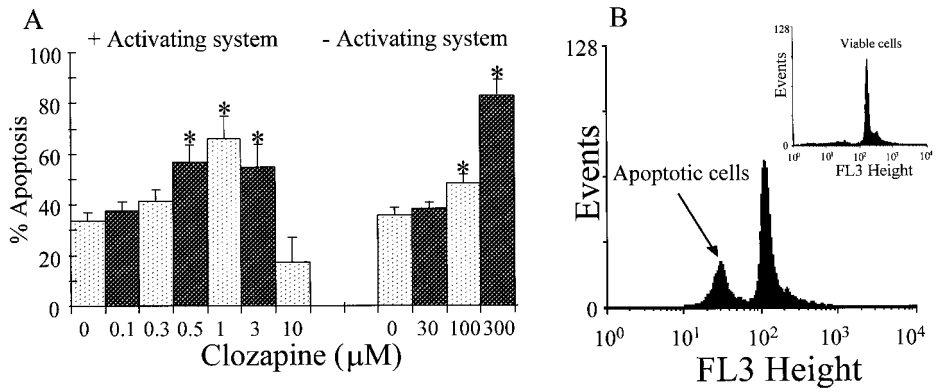

Fig. 1. Flow cytometric evaluation of clozapine-induced PMN apoptosis by propidium iodide staining. A, dose response of clozapine-induced apoptosis in the presence and absence of the activating system. PMNs (1×10^6) were incubated for 2 h with clozapine in the presence or absence of 20 U of HRP and 10 μM H_2O_2; they were then incubated in drug-free medium for another 6 h. Apoptosis was determined by flow cytometry (see *Materials and Methods*). The data are presented as the mean ± S.E. from three different experiments (carried out in triplicate). Statistical analysis (Kruskal-Wallis test) was performed by comparing incubations containing different concentrations of the drug with the solvent control: *$P < .05$. B, typical trace obtained from the flow cytometer: 0.5 μM clozapine was incubated with PMNs in the presence of HRP/H_2O_2 (inset shows the control incubation).

Effect of Antioxidants on Clozapine-Induced PMN Apoptosis. GSH, NAC, and ASC all inhibited apoptosis caused by the toxic metabolite of clozapine, but did not affect apoptosis mediated by high concentrations of clozapine itself (Fig. 3).

Effect of Apoptosis Modulators on Clozapine-Induced PMN Apoptosis. It is known that thiol-oxidizing agents can alter apoptotic rates (Watson et al., 1996) by increasing tyrosine phosphorylation (Yousefi et al., 1994). Preincubation with 40 μM genistein, a tyrosine kinase inhibitor (Watson et al., 1996), inhibited apoptosis induced by the toxic nitrenium metabolite of clozapine (Table 1) to a greater extent than that caused by high concentrations (>100 μM) of clozapine (Table 1). Cycloheximide, the protein synthesis inhibitor, significantly increased PMN apoptosis in the absence of clozapine (from 28.2 ± 0.5 to 39.2 ± 1.5% in the absence and presence of cycloheximide, respectively) but did not increase apoptosis induced by the nitrenium ion (37.5 ± 4.3 and 39.8 ± 6.4% at 3 μM clozapine in the absence and presence of cycloheximide, respectively) and clozapine itself (94.1 ± 2.5 and 94.8 ± 2.1% in the absence and presence of cycloheximide, respectively).

It has previously been shown that apoptosis of smooth muscle cells induced by nitric oxide donors can be regulated by the activity of the signal transduction systems involving the protein kinases A and C (Nishio and Watanabe, 1997). In our experiments, the protein kinase A inhibitor, H-89 (20 μM), and the protein kinase C inhibitor, calphostin-C (0.1 μM), neither enhanced nor inhibited PMN apoptosis induced by CLZ (both in the presence and absence of activation; data not shown).

Effect of Demethylclozapine, Clozapine *N*-Oxide, and SDZ 105-402 on PMN Apoptosis. To investigate the role of drug bioactivation, we went on to investigate the ability of the stable metabolites and an analog of clozapine to induce apoptosis. The major stable metabolite, demethyl-

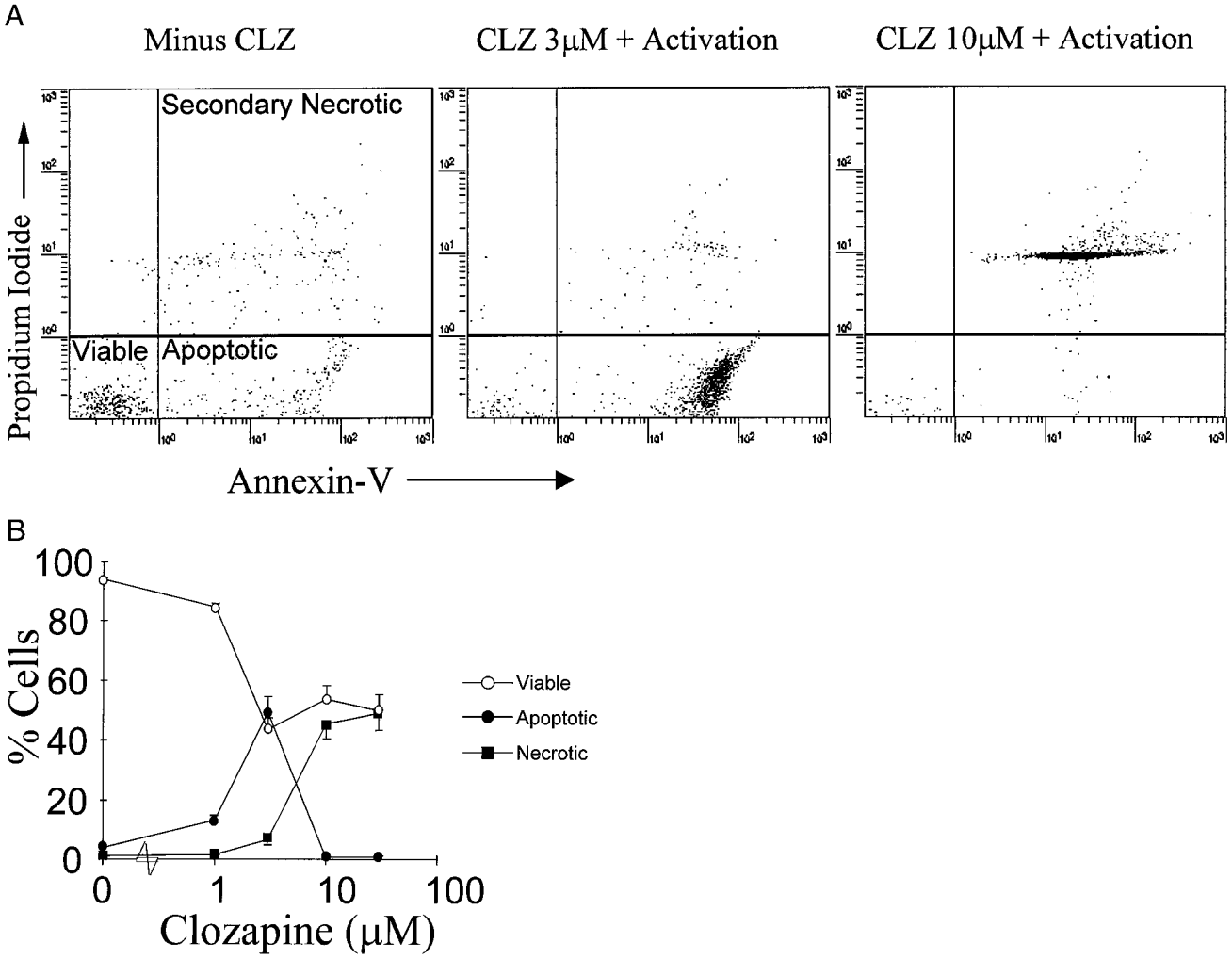

Fig. 2. Annexin-V detection of external phosphatidylserine residues on PMN cell membranes. PMNs were incubated with clozapine (1–100 μM) in the presence of HRP/H_2O_2. After 2 h they were resuspended in drug-free HBSS containing HSA (5 mg/ml) for another 6 h and then washed in ice-cold HBSS before being resuspended in binding buffer (Beckman Coulter; see *Materials and Methods*). Prepared annexin-V FITC conjugate (see *Materials and Methods*) was added along with propidium iodide. PMNs were left on ice and incubated for 10 min in the dark. Cells were then analyzed by flow cytometry. A, flow cytometric traces showing differential staining of PMN populations at different concentrations of clozapine. The graphs show changes in the mode of cell death of PMNs in response to increasing concentrations of clozapine in (B) the presence of an activating system. Statistical analysis (Kruskal-Wallis test) was performed by comparing incubations in the presence of drug to those in the absence of drug. The data were significant for apoptosis at all drug concentrations, whereas for necrosis, significance was achieved at 10 and 30 μM (*$P < .05$; * have been omitted for the sake of clarity).

clozapine (Jann et al., 1993), produced a concentration-dependent increase in apoptosis up to 300 μM when bioactivated with HRP and H_2O_2, although this was less than that observed with clozapine bioactivation (Fig. 4A). Demethylclozapine alone, like clozapine, produced apoptosis only at high concentrations (100–300 μM) (Fig. 4A). The polar *N*-oxide metabolite of clozapine did not induce apoptosis in a concentration-dependent manner in the presence or absence of HRP and H_2O_2 up to 300 μM, although the apoptosis was greater in the absence of activation (Fig. 4B).

To further investigate the role of clozapine bioactivation in apoptosis, we went on to study the clozapine analog SDZ 105-402 (Fig. 4C), which cannot be oxidized by activated neutrophils to the nitrenium ion (Uetrecht et al., 1997). In our system, SDZ 105-402 did not accelerate PMN apoptosis above background levels up to a concentration of 30 μM in the presence of an external bioactivating system (i.e., HRP and H_2O_2), although as with clozapine, it did induce apoptosis at high concentrations (100–300 μM) when incubated alone with PMN (Fig. 4C). In contrast with clozapine, SDZ 105-402 (1–300 μM) neither depleted intracellular glutathione nor exhibited cytotoxicity toward neutrophils in both the presence and absence of HRP and H_2O_2 (data not shown).

Haptenation of Neutrophils by Clozapine and Its Stable Metabolites. Flow cytometry with an anti-clozapine antibody showed that PMN became haptenated at clozapine concentrations above 1 μM in the presence of the activating system, i.e., HRP and H_2O_2 (Table 2). In contrast, in the absence of activation, no haptenation was observed (10 μM; Table 2).

The stable metabolites of clozapine were also assessed for their ability to haptenate PMN cell membranes. Demethylclozapine showed significant ($P < .05$) haptenation at 10 μM only in the presence of the activating system (Table 2), but this was significantly less ($P < .05$) than that observed with clozapine. Clozapine *N*-oxide did not show any haptenation in the presence or absence of the activating system (Table 2).

Detection of Haptenation and Apoptosis in Neutrophils. Experiments were initially performed to identify the optimum times for simultaneous detection of both apoptosis and haptenation. Apoptosis was detected with the annexin-V assay; this showed that there was no apoptosis after 1 h, but there was a significant increase in apoptosis, but not necrosis, after 2 h (Fig. 5). Because haptenation was also detectable at 2 h, this time point was chosen for further experiments.

PMNs, in the presence of clozapine plus an external activating system, showed a significant ($P < .05$) increase in both haptenation and apoptosis at 10 μM. The cells that became haptenated were the same cells that were observed to undergo apoptosis (Fig. 6). In the absence of an external activating system, there was no haptenation or apoptosis seen above background values.

Effect of Diethylmaleate on PMN Apoptosis, GSH Depletion, and Cytotoxicity. To further investigate the role of oxidative stress within the PMN, the effect of the model GSH-depleting agent, DEM, was investigated. DEM forms a thioether conjugate with GSH in a reaction that is catalyzed by GSH-*S*-transferase (Watson et al., 1996). Apoptosis and cytotoxicity were measured simultaneously after 8 h by the annexin-V/propidium iodide assay. There was a concentration-dependent increase in apoptosis and depletion of GSH over 8 h (Fig. 7); however, GSH depletion occurred at concentrations that were lower than those needed to induce apoptosis. There was no necrosis observed with DEM.

Effect of Clozapine on MNL Haptenation, Apoptosis, GSH Depletion, and Cytotoxicity. MNL did not undergo apoptosis in the presence (3.8 ± 1.0% at 30 μM CLZ) or absence (4.8 ± 1.8% at 300 μM CLZ) of a CLZ activating system. In contrast, as with PMNs, the nitrenium ion (but not clozapine itself) leads to haptenation, GSH depletion, and loss of membrane integrity, as assessed by trypan blue dye exclusion (Fig. 8B and Table 3).

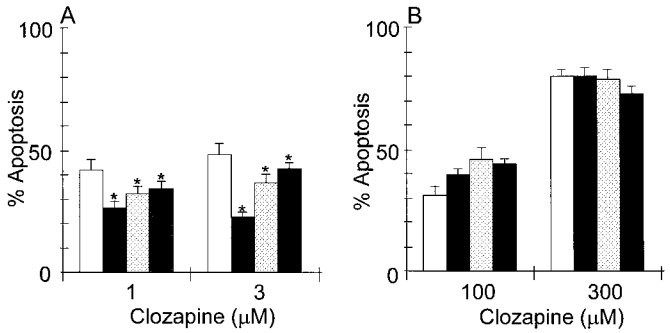

Fig. 3. Effect of antioxidants on clozapine-induced apoptosis. Antioxidants were incubated with clozapine in the presence (A) and absence (B) of an activating system and analyzed by flow cytometry, using propidium iodide staining. Clozapine was incubated in the absence of antioxidants (□) or in the presence of 1 mM GSH (■), 1 mM NAC (hatched squares), or 1 mM ASC (gray squares). Data are presented as the mean ± S.E. of triplicates of three different experiments. Statistical analysis (Kruskal-Wallis test) was performed by comparing incubations in the presence and absence of antioxidants: *$P < .05$ Incubation of antioxidants with PMNs in the absence of clozapine did not alter the rate of apoptosis above background values.

Discussion

The PMN is a cell that is destined to undergo apoptosis, with the life span of a circulating cell being between 8 and 20 h (Payne et al., 1994). Essentially our results show that clozapine accelerates this process in vitro. Two different concentration-dependent mechanisms seem to be operating: at therapeutic drug concentrations, apoptosis was only observed when clozapine underwent bioactivation to its nitrenium ion (the bioactivation-dependent pathway), whereas suprapharmacological concentrations were needed for the parent drug itself to induce apoptosis (metabolism-indepen-

TABLE 1

Effect of preincubation of PMN with genistein in the presence and absence of the activating system

Effect of preincubation with genistein (40 μM) for 1 h on clozapine-induced apoptosis in the presence or absence of the activating system. Genistein was preincubated for 1 h with PMNs before the addition of clozapine and was compared to incubations in the absence of genistein. Data are presented as the mean ± S.E. of three different triplicate experiments. Statistical analysis (Kruskal-Wallis test) was performed by comparing incubations in the presence and absence of genistein.

% Apoptosis					
+ Activating System			− Activating System		
Clozapine conc (μM)	−Genistein	+Genistein	Clozapine conc (μM)	−Genistein	+Genistein
0	35 ± 2	34 ± 9	0	34 ± 11	34 ± 6
0.1	49 ± 11	46 ± 10	30	45 ± 13	26 ± 5
1	55 ± 7	26 ± 8*	300	74 ± 6	65 ± 16

* $P < .05$.

dent pathway). Antioxidants and genistein inhibited the bioactivation-dependent pathway but had no effect on the metabolism-independent pathway, suggesting a role for oxidative stress (discussed below) and tyrosine phosphorylation (Yousefi et al., 1994), respectively, in the pathogenesis of the former pathway.

The role of metabolism in inducing apoptosis was studied further by the investigation of the stable metabolites of clozapine and an analog of clozapine that is refractory to metabolic bioactivation. In accordance with our previous study of clozapine-induced cytotoxicity (Williams et al., 1997), we found that demethylclozapine was less potent than clozapine in inducing apoptosis via the bioactivation-dependent pathway, probably reflecting a lower cellular uptake, because turnover to its nitrenium metabolite was similar to that of clozapine. The *N*-oxide, which forms a nitrenium ion incapable of traversing the cell membrane (Williams et al., 1997), did not induce a concentration-dependent increase in apoptosis with or without an activating system. Interestingly, SDZ 105-402, which cannot form a nitrenium ion (Uetrecht et al., 1997), did not induce apoptosis in the presence of an activating system, but was able to induce apoptosis via the metabolism-independent pathway at higher (clozapine equivalent) concentrations. Taken together, the results show that bioactivation of clozapine to the nitrenium ion is an important pathway for inducing apoptosis in vitro.

To generate the nitrenium ion, we used a potent oxidizing system comprising H_2O_2 and HRP, rather than MPO, the enzyme found in neutrophils. This was done to ensure efficient metabolic turnover to the nitrenium ion, which is significantly greater than that observed with MPO (Williams et al., 1997). However, it is important to note that the metabolites produced from clozapine (Fischer et al., 1991) and other compounds (Eastmond et al., 1986) by the two enzymes are qualitatively the same, with both enzymes capable of catalyzing one- and two-electron oxidations via one-electron transfer (Babior, 1984). The advantage of the HRP system was that it permitted investigation of the effect of clozapine and its nitrenium ion on four parameters in the PMN (apoptosis, necrosis, GSH depletion, and haptenation; Fig. 8A) and allowed a comparison with the effects on MNLs (Fig. 8B). Thus, whereas it was possible to induce PMN apoptosis at therapeutic clozapine concentrations, MNLs remained resistant to apoptosis. In contrast, necrosis was seen at higher clozapine concentrations with both PMNs and MNLs; with PMNs, the necrosis was secondary to the apoptosis and may be a consequence of the overwhelming insult suffered by the cell on exposure to high clozapine concentrations resulting in changes in the intracellular ATP concentration (Leist et al., 1997). The differential effects on PMNs and MNLs are interesting to note because there was depletion of intracellular GSH and cell surface haptenation with both cells, in accordance with the fact that the mechanism of cell toxicity varies according to cell type.

It might be argued that this oxidizing system is too potent and does not reflect the in vivo situation, particularly because the GSH depletion that is seen in our cellular systems has not been accounted for in vivo. This, however, presupposes that the mechanism of clozapine-induced apoptosis is due solely to GSH depletion and an increase in cellular oxidative stress. It is known that the reactive oxygen species plays a significant role in the induction of the apoptotic cascade (Buttke and Sandstrom, 1994) by activating the caspase enzyme system and causing accumulation of P53 (Buttke and Sandstrom, 1994). Although oxidative stress, caused by the depletion of GSH, is likely to play a role in the mechanism of clozapine-induced apoptosis, two lines of evidence from our study suggest that other mechanisms are also important. First, comparison of the GSH depletion and apoptosis induced by DEM and clozapine showed that at concentrations where there was equivalent GSH depletion (30% at 1 μM clozapine and 10 μM DEM), clozapine caused apoptosis, whereas DEM did not. Thus simple GSH depletion is unlikely by itself to be entirely responsible for the apoptosis. Furthermore, higher concentrations of clozapine (and therefore its reactive metabolite) caused necrosis, whereas DEM, despite causing almost complete GSH depletion, was not associated with necrosis. It is also interesting to note that in a recent study investigating the toxicity of benzene to NAD(P)H/quinone oxidoreductase-transfected HL-60 cells, a

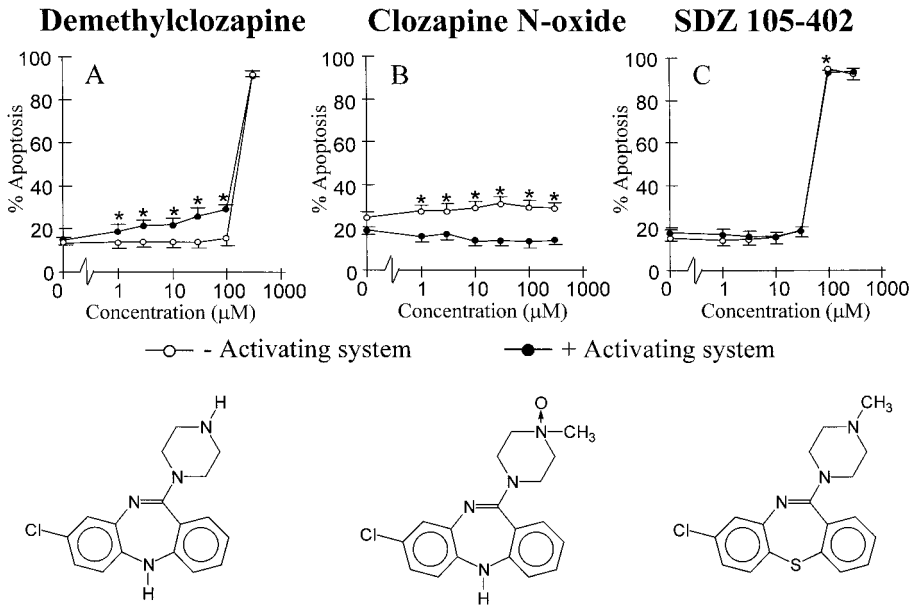

Fig. 4. Effect of clozapine analog and stable metabolites on PMN apoptosis. Flow cytometric analysis of apoptosis caused by the stable metabolites of clozapine (A and B) and an analog (SDZ 105-402) of clozapine (C) was performed in the presence and absence of the activating system (HRP and H_2O_2). Details are presented in *Materials and Methods*. Data are presented as the mean ± S.E. of quadruplicate incubations. Statistical analysis (Kruskal-Wallis test) was performed by comparing incubations containing the activating system to those without activation: $^*P < .05$.

reduction in apoptosis was accompanied by an increase in GSH depletion (Wiemels et al., 1999). Second, simultaneous assessment of apoptosis and cellular binding showed that haptenation was greater in those cells undergoing apoptosis, providing a link between cell binding and functional toxicity. Although it is possible that the haptenated cells may have become depleted of GSH, it is also important to note that haptenation of PMN does occur in vivo in patients on chronic clozapine therapy (Gardner et al., 1998). It is possible such binding may hasten the death of the PMNs in vivo; clearly, this is an area that needs additional study.

Whether PMN apoptosis induced by clozapine is an acceleration of the natural process of aging or is due to a separate pathway is unclear. The fact that PMNs are highly resistant to any attempts to accelerate the normal process of apoptosis (Payne et al., 1994) and the fact that classical pro-apoptotic agents such as steroids actually inhibit apoptosis in PMNs (Cox, 1995) suggest that a distinct pathway of apoptosis is being activated in these particular cells. Furthermore, in accordance with this, cycloheximide accelerated PMN apoptosis in the absence of the drug, as has been observed previously (Whyte et al., 1997), but did not have the same effect when the drug was present in the incubations.

Treatment with clozapine for a year results in neutropenia in 1.5 to 2.9% of patients and agranulocytosis in 0.8% of patients (Gerson, 1993; Atkin et al., 1996). The mechanisms of both forms of neutrophil toxicity are unclear (Pirmohamed and Park, 1997; Guest et al., 1998). It is has been suggested that the neutropenia and agranulocytosis are due to different mechanisms (Gerson, 1994). It is possible that apoptosis may play a role in the pathogenesis of both forms of toxicity. With regard to clozapine agranulocytosis, it can be postulated that the cellular target will be a more committed neutrophil precursor, as well as mature peripheral PMNs, and for the neutropenia, the main target would be the peripheral blood PMNs. Both of these scenarios would be consistent with the bone marrow appearances in patients with neutrophil toxicity (Chengappa et al., 1996). Indeed, it has recently been suggested that aplastic anemia induced by benzene and remoxipride is due to apoptosis of CD34+ cells (Ross et al., 1996; McGuiness et al., 1999). Clearly, the role of apoptosis and whether it occurs in vivo need to be investigated.

Furthermore, it is important that any mechanism explain two key features of clozapine agranulocytosis. First, what is the nature of individual susceptibility, inasmuch as only 0.8% of patients are affected? It is known that PMNs exhibit a heterogeneous response to agents such as TNF-α and bMLP (Balazovich et al., 1996), which may partly reflect differences in cell maturity (Payne et al., 1994). Susceptibility to clozapine agranulocytosis has been linked to variations in the genes for TNF-α (Turbay et al., 1997) and heat shock proteins (Corzo et al., 1995); these bioactive proteins have been shown to modulate apoptosis (Takano et al., 1998), and thus a complex interaction between either of these pathways, and the pathway responsible for clozapine apoptosis may influence individual susceptibility. An area that must be considered in relation to individual susceptibility is the role of the bcl-2 family. Bcl-2 itself inhibits apoptosis (Korsmeyer et al., 1995) and is differentially expressed, according to the maturity and differentiation of the PMN precursors, being absent or expressed in low amounts in mature PMNs (Hockenbery et al., 1991) and in primitive hemopoietic stem cells (Park et al., 1995). This may be one reason why MNLs, which express high amounts of bcl-2, were not susceptible to apoptosis induced by clozapine (Hockenbery et al., 1991; Park et al., 1995). A recent study has suggested that a determinant of PMN apoptosis may be the balance between the expression of Bax (a pro-apoptotic protein) and Mcl-1 (an anti-apoptotic protein) (Moulding et al., 1998). It is important to note that Mcl-1 levels are inducible by cytokines (Chao et al., 1998), the concentrations of which are known to be modulated by clozapine (Sperner-Unterweger et al., 1993). The effect of clozapine on Mcl-1 is currently being studied as a susceptibility factor for neutrophil toxicity.

Second, why does toxicity occur mostly within the first 3 months of drug administration? The reason for this is unknown and is being addressed in longitudinal studies in patients starting clozapine therapy. An area that needs investigating is whether there is a disturbance in the oxidant-antioxidant balance, which, combined with the susceptibility factor, leads to agranulocytosis. Such a mechanism has been proposed for apoptosis induced by benzene in the bone marrow, where the susceptibility of $CD34^+$ progenitor cells is

TABLE 2

Comparison of direct and activation-induced binding of clozapine, demethylclozapine, and clozapine *N*-oxide to PMN cell surface

Comparison of the binding of clozapine, demethylclozapine, and clozapine *N*-oxide to the PMN cell surface in the presence and absence of extracellular activating system. Data represent the mean ± S.E. of triplicate incubations from a single individual (demethylclozapine and clozapine *N*-oxide) and triplicate incubations from three different individuals (clozapine). Statistical analysis was performed by comparing incubations of the compounds in the presence and absence of the activating system at the same concentrations.

Concentration (μM)	Clozapine		Demethylclozapine		Clozapine *N*-oxide	
	−AS[a]	+AS	−AS	+AS	−AS	+AS
0	32 ± 2	28 ± 1	10 ± 3	14 ± 3	10 ± 3	14 ± 5
1	25 ± 3	26 ± 2	16 ± 3	9 ± 3	9 ± 5	7 ± 6
3	24 ± 3	64 ± 4*	9 ± 2	18 ± 9	12 ± 4	7 ± 3
10	21 ± 4	79 ± 2*	11 ± 5	37 ± 10*	11 ± 8	8 ± 7

[a] AS, activating system.
* $P < .05$, Kruskal-Wallis test.

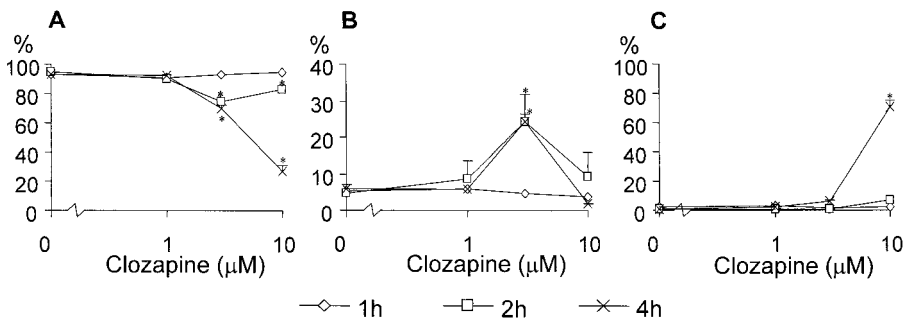

Fig. 5. Time course of clozapine-induced apoptosis and necrosis. Time course of PMN viability (A), apoptosis (B), and necrosis (C) in the presence of clozapine plus extracellular activating system assessed by the combination of annexin-V and PI. Data represent the mean ± S.E. of triplicate incubations. Statistical analysis was performed by comparing incubations in the presence and absence of clozapine: *$P < .05$, Kruskal-Wallis test.

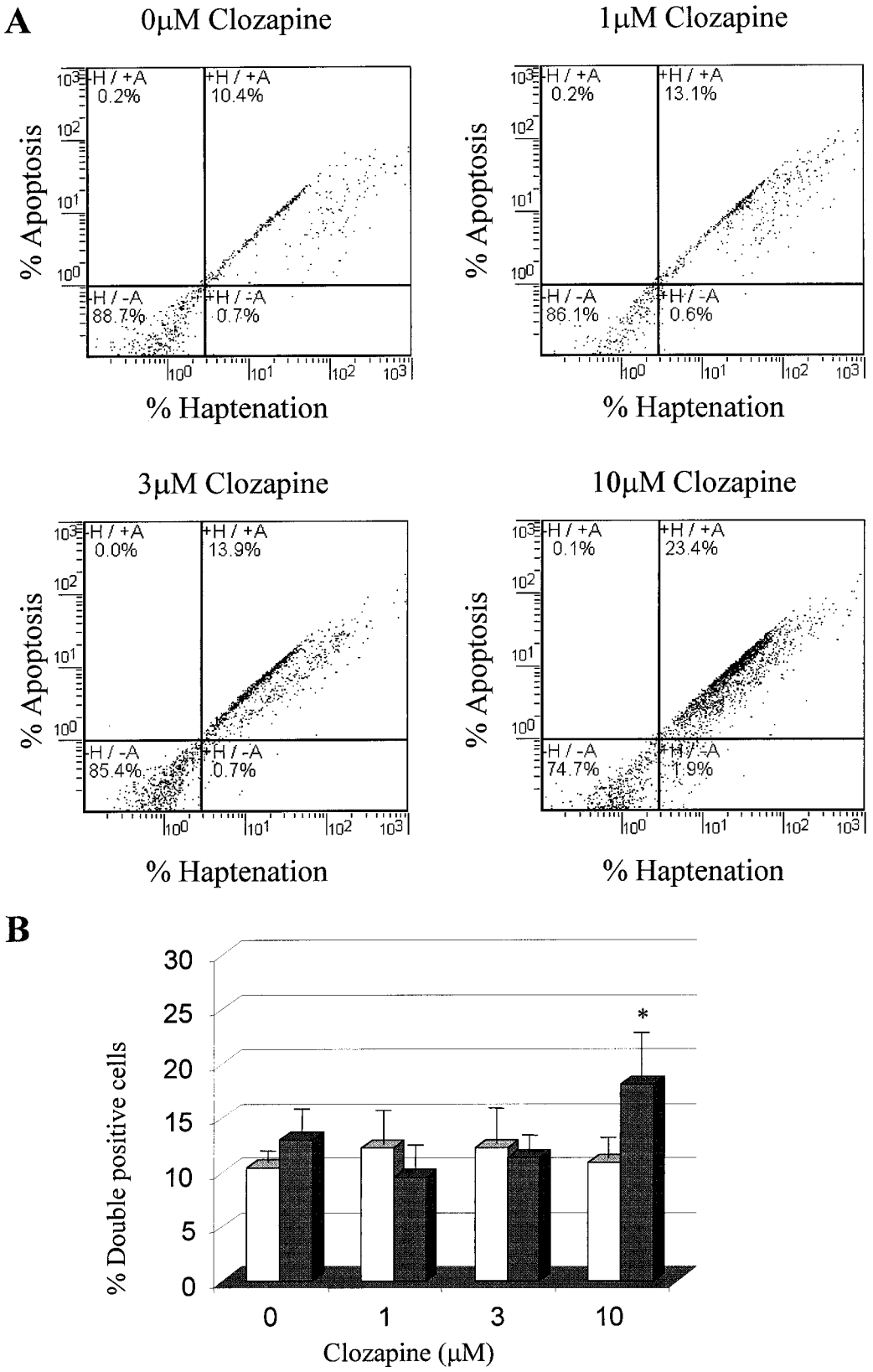

Fig. 6. Two-color flow cytometric assessment of apoptosis and haptenation. A, typical traces obtained from the flow cytometer after incubation of PMN with clozapine in the presence of extracellular activating system. PMNs were then dual stained with annexin-V to detect apoptosis and with an anti-clozapine antibody to detect bound drug. B, graph illustrating the percentage of cells that have become stained for both apoptosis and bound drug. Open columns represent PMN incubated with drug alone, shaded columns represent PMN incubated with drug in the presence of the activating system. Data represent the mean ± S.E. of triplicate incubations. Statistical analysis (Kruskal-Wallis test) was performed by comparing incubations in the presence and absence of the activating system: $^*P < .05$.

thought to be a function of the balance between peroxidase activity (bioactivation) and quinone reductase activity (bioinactivation) (Ross et al., 1996). An oxidative stress-related mechanism has also been implicated in the apoptosis of bone marrow precursor cells by remoxipride (McGuiness et al., 1999), a drug known to cause aplastic anemia (Philpott et al., 1993).

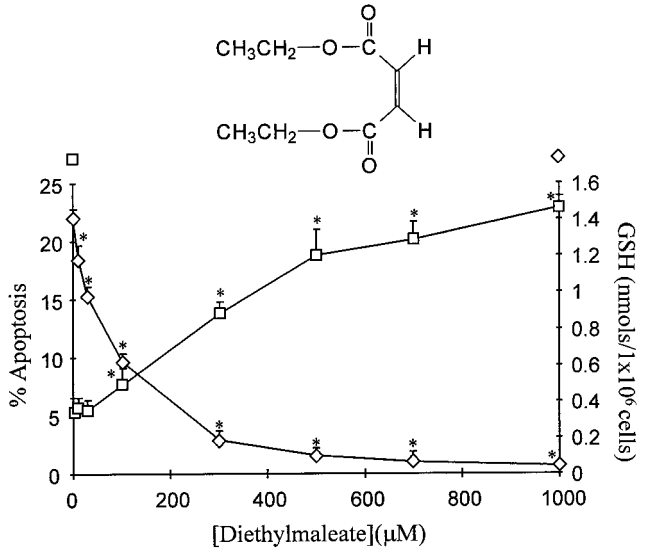

Fig. 7. Effect of incubation of PMNs with DEM on GSH depletion and apoptosis. GSH depletion was assessed by a fluorescent HPLC method, and apoptosis was assessed by an annexin-V/PI method. The inset shows the structure of DEM. Data represent the mean ± S.E. of quadruplicate incubations from a single individual. Statistical analysis (Kruskal-Wallis test) was performed by comparing incubations in the presence and absence of DEM: *$P < .05$.

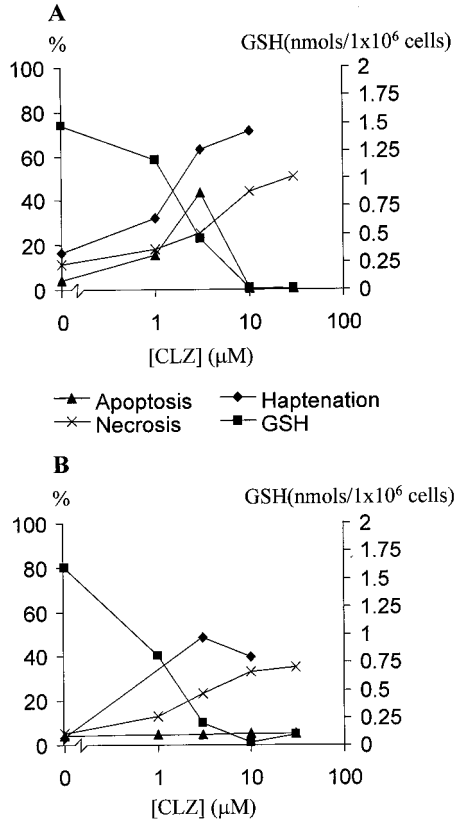

Fig. 8. Comparison of PMN (A) and MNL (B) haptenation, GSH depletion, apoptosis, and necrosis induced by clozapine in the presence of an extracellular activating system. Data have been described earlier or represent the mean of triplicate incubations carried out on cells from three different individuals. Error bars are excluded for the sake of clarity.

TABLE 3

Comparison of clozapine-induced apoptosis, GSH depletion, and cytotoxicity in PMNs and MNLs

Comparison of apoptosis, cytotoxicity, and GSH depletion in PMNs and MNLs at clozapine concentrations of 0, 1, and 3 μM in the presence of an extracellular activating system. Data represent the mean ± S.E. of triplicate incubations. Statistical analysis was performed by comparing incubations of the compounds in the presence and absence of the activating system at the same concentrations.

	CLZ (μM)	Apoptosis (%)	GSH depletion (% of control)	Cytotoxicity (%)
PMN	0	33 ± 1	100	11 ± 1
	1	66 ± 8*	69 ± 9*	17 ± 7*
	3	54 ± 8*	19 ± 1*	25 ± 12*
MNL	0	3 ± 1	100	4 ± 1
	1	4 ± 1	72 ± 12*	13 ± 2*
	3	4 ± 2	23 ± 12*	22 ± 5*

* $P < .05$, Kruskal-Wallis test.

In conclusion, the findings of the study expand on potential mechanisms of clozapine-induced cytotoxicity, which may be of relevance to the major forms of toxicity encountered in patients taking this drug. Induction or acceleration of neutrophil (and their precursors) apoptosis by the nitrenium metabolite must be considered alongside the potential for an immunological mechanism (Uetrecht, 1992b). Further studies are under way to define the exact pathways for apoptosis in vitro and to identify how these interact with the identified susceptibility factors in patients, and whether the in vitro findings can be linked directly to the clinical characteristics of the different forms and severities of the toxicity.

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