British Journal of Pharmacology (1999) 126, 1393–1407
http://www.stockton-press.co.uk/bjp

Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity

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1 Bioactivation of sulphamethoxazole (SMX) to chemically-reactive metabolites and subsequent protein conjugation is thought to be involved in SMX hypersensitivity. We have therefore examined the cellular metabolism, disposition and conjugation of SMX and its metabolites *in vitro*.

2 Flow cytometry revealed binding of *N*-hydroxy (SMX-NHOH) and nitroso (SMX-NO) metabolites of SMX, but not of SMX itself, to the surface of viable white blood cells. Cellular haptenation by SMX-NO was reduced by exogenous glutathione (GSH).

3 SMX-NHOH and SMX-NO were rapidly reduced back to the parent compound by cysteine (CYS), GSH, human peripheral blood cells and plasma, suggesting that this is an important and ubiquitous bioinactivation mechanism.

4 Fluorescence HPLC showed that SMX-NHOH and SMX-NO depleted CYS and GSH in buffer, and to a lesser extent, in cells and plasma.

5 Neutrophil apoptosis and inhibition of neutrophil function were induced at lower concentrations of SMX-NHOH and SMX-NO than those inducing loss of membrane viability, with SMX having no effect. Lymphocytes were significantly ($P<0.05$) more sensitive to the direct cytotoxic effects of SMX-NO than neutrophils.

6 Partitioning of SMX-NHOH into red blood cells was significantly ($P<0.05$) lower than with the hydroxylamine of dapsone.

7 Our results suggest that the balance between oxidation of SMX to its toxic metabolites and their reduction is an important protective cellular mechanism. If an imbalance exists, haptenation of the toxic metabolites to bodily proteins including the surface of viable cells can occur, and may result in drug hypersensitivity.

Keywords: Sulphamethoxazole; hypersensitivity; cellular disposition; haptenation; reduction

Abbreviations: CYS, cysteine; DMSO, dimethylsulphoxide; FITC, fluorescein isothiocyanate; GSH, glutathione; LC-MS, liquid chromatography-mass spectroscopy; MetHb, methaemoglobin; NADPH, β-nicotinamide adenine dinucleotide; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; SMX, sulphamethoxazole; SMX-NHOH, sulphamethoxazole hydroxylamine; SMX-NO, nitroso sulphamethoxazole

Introduction

Sulphonamides cause a variety of unpredictable idiosyncratic drug reactions including fever, lymphadenopathy, skin rashes, hepatitis, nephritis and blood dyscrasias in about 2–3% of patients (Mandell & Sande, 1985). In the U.K., this has resulted in restricted indications for drugs such as co-trimoxazole (Saiag *et al*., 1992; Pirmohamed & Park, 1995), a combination of sulphamethoxazole (SMX) and trimethoprim. The hypersensitivity reactions have been attributed to the sulphonamide component (Cribb *et al*., 1996a).

One of the main uses of co-trimoxazole is in the treatment of *Pneumocystis carinii* pneumonia in HIV-infected patients. In comparative studies with other agents such as dapsone and pentamidine, co-trimoxazole has been found to be the most efficacious, both for acute treatment and for prophylaxis (Smith, 1994). However, the rate of adverse reactions in HIV-infected patients is significantly higher than in seronegative patients severely limiting its usefulness (Carr & Cooper, 1995; Koopmans *et al*., 1995; Pirmohamed & Park, 1995; Tschachler *et al*., 1996). The types of reactions are similar to those observed in HIV-negative individuals and occur within 5–15 days of the start of therapy, although in general, they are more severe (Anonymous, 1995). Virus-induced changes in drug metabolism and drug detoxification, in particular a change in cellular redox potential, immune dysregulation, drug-drug interactions and drug dosage have all been postulated to be responsible for the increased risk of hypersensitivity in HIV-infected patients (Carr & Cooper, 1995; Pirmohamed & Park, 1995). It is important to identify the exact mechanism(s) of SMX hypersensitivity in HIV-infected patients in order to develop strategies to prevent the reactions and thus improve the tolerability of co-trimoxazole.

SMX is metabolized not only to stable metabolites, such as the *N*-acetate and glucuronide but also to a potentially toxic hydroxylamine metabolite (SMX-NHOH), which can undergo further oxidation to a nitroso metabolite (SMX-NO). It has been proposed that SMX-NO is responsible for idiosyncratic toxicity, with the tissue injury occurring *via* an immune-mediated mechanism (Rieder *et al*., 1988; 1989; 1995a,b; Carr *et al*., 1993; Meekins *et al*., 1994; Cribb *et al*., 1996b). In an *in vivo* rat model, both SMX-NHOH and SMX-NO underwent extensive reduction which serves as a detoxication mechanism (Gill *et al*., 1997), indicating that measurement of urinary concentrations of SMX-NHOH – estimated to be about 2% of an ingested dose in man (Gill *et al*., 1996) – may underestimate

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the total level of tissue exposure to the toxic metabolites. Therefore, an imbalance between oxidation of SMX and the reduction of its toxic metabolites back to the parent compound may predispose to toxicity.

In order to investigate further the role of metabolism in SMX toxicity, we have investigated the cellular disposition of SMX and its oxidative metabolites *in vitro*.

Methods

Materials

Bovine and human serum albumin, bromobimane, cysteine (CYS), dicuomarol, d_6-dimethyl sulphoxide (DMSO), dapsone, EDTA, glacial acetic acid, reduced glutathione (GSH), neocuproin, reduced nicotinamide adenine dinucleotide phosphate (NADPH), NADH, *N*-ethyl morpholine, D-L thiolactone homocysteine, o-phenylene diamine hydrochloride, phorbol 12-myristate 13-acetate (PMA), propidium iodide, RNAse [type I-A], SMX, salicyl hydroxamate, sodium azide, sodium citrate, trichloroacetic acid, triethylamine, Tris, Tween 20, trypan blue, FITC-conjugated anti-rabbit IgG antibody and peroxidase-linked anti-rat IgG antibody were obtained from Sigma Chemical Co. (Poole, U.K.). Anti-SMX IgG antibody was kindly donated by Dr A.E. Cribb (Merck Research Laboratories, West Point, PA, U.S.A.). SMX-NHOH and SMX-NO were synthesized according to the method of Naisbitt *et al*. (1996) and found to be >99% pure by liquid chromatography-mass spectroscopy (LC-MS) and NMR (Bruker, 200 MHz spectrometer). Monopoly resolving medium (Ficoll Hypaque, 1.114 g ml^{-1}) and Lymphoprep (1.077 g ml^{-1}) were from ICN Biomedicals (Bucks., U.K.) and Nycomed (Birmingham, U.K.), respectively. All HPLC-grade solvents were purchased from Fischer Scientific (Loughborough, U.K.).

Isolation of peripheral blood cells

Human neutrophils, lymphocytes and red blood cells were isolated from the heparinized venous blood of ten healthy male volunteers (aged 19–45) on a dual density gradient of Monopoly resolving medium and Lymphoprep, as described previously (Naisbitt *et al*., 1997). Neutrophils and lymphocytes were resuspended in Dulbecco's phosphate buffered saline (PBS; pH 7.4) and diluted to the concentration required for each experiment. Both cell types were greater than 98% pure and 97% viable as assessed by Wright's stain and trypan blue dye exclusion, respectively. The isolated red blood cells were washed twice and resuspended in Dulbecco's PBS to produce a haematocrit of 50%. When required, cells were lysed with a sonic probe (Heat systems, Farmingdale, NY, U.S.A.; 0°C, 3×20 s).

Platelet-free plasma was prepared from ice-cold heparinized venous blood by centrifugation at 0°C ($1200 \times g$ for 10 min). All samples were kept on ice and the experiments were initiated within 25 min of venepuncture.

Thiolation of human serum albumin

Human serum albumin (200 mg) was incubated with D,L-thiolactone homocysteine (330 mg) in PBS (4 ml, pH 7.4) and glycine buffer (8 ml, 100 μM glycine in 100 μM sodium chloride, 100 μM sodium hydroxide) for 2 h at room temperature. The thiolated albumin was then washed and concentrated using a Centriprep-10 column ($1500 \times g$ for 2 h; Amicon, Glos., U.K.). The degree of thiolation was determined by the method of Akerboom & Sies (1981); the human serum albumin : thiol ratio increased from 1:0.05–1:42.

Reaction of sulphamethoxazole, sulphamethoxazole hydroxylamine and nitroso sulphamethoxazole with human serum albumin and thiolated human serum albumin

Albumin or thiolated albumin (100 mg ml^{-1}) was incubated with SMX, SMX-NHOH and SMX-NO (1–1000 μM) in PBS (pH 7.2) for 24 h at 37°C. Ninety-six well microtitre plates were coated with 100 μg of each incubation (100 μl) in PBS overnight at 4°C. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBS-Tween) before incubation with anti-SMX IgG antibody (1/100; 100 μl) for 1 h at room temperature. The antibody was raised in rats immunized with SMX-NO alone (Gill *et al*., 1997). The wells were washed three times with PBS-Tween; peroxidase-linked anti-rat IgG antibody in PBS (dilution 1/2500; 100 μl) was then added to each well and incubated for 1 h. The wells were washed again followed by addition of the developing solution (0.1% hydrogen peroxide [30%] and 400 μg ml^{-1} o-phenylenediamine dihydrochloride in citrate phosphate buffer [0.15 M; pH 5.0]) to each well. The reaction was terminated after 20 min by the addition of 25% sulphuric acid (25 μl) and the optical density determined using an automated microplate reader (Dynatech MR 600, Guernsey, U.K.) at an absorbance of 490 nm.

Haptenation of human white blood cells by sulphamethoxazole, sulphamethoxazole hydroxylamine and nitroso sulphamethoxazole

Neutrophils or lymphocytes (1×10^6 ml^{-1}) were incubated with SMX, SMX-NHOH or SMX-NO (1–1000 μM) in PBS (pH 7.4) in the presence or absence of GSH (1 mM) for 1 h at 37°C. The control incubations contained the vehicle (DMSO) only. In other experiments, SMX (2.5 mM) was incubated with neutrophils (1×10^6 ml^{-1}) in the presence of PMA (10 ng ml^{-1}), a cell activating factor (Thompson *et al*., 1989).

Antigen formation on cell surfaces from SMX or its metabolites was determined by flow cytometry as follows. The drug or vehicle-treated control cells (1×10^5) were washed (3×1 ml) in ice-cold PBS (pH 7.4) and centrifuged ($1100 \times g$) prior to incubation with anti-SMX IgG antibody (1:500, 40 μl) for 30 min at 4°C. The cells were washed (0.5 ml) to remove unconjugated anti-SMX antibody, and then incubated with FITC-conjugated anti-IgG antibody (1:50, 40 μl) for a further 30 min at 4°C. After antibody labelling, the cells were re-suspended in 0.5 ml of PBS and kept on ice prior to analysis using an EPICS-XL flow cytometer (Coulter Electronics, Luton, Beds., U.K.). The fluorescence threshold gating was set to give <5% positive cells in the negative control populations. Greater than 3000 cells per sample were analysed in each case. The percentage of cells staining positively for SMX was then calculated as the difference from the negative controls.

Assessment of white and red blood cell toxicity of sulphamethoxazole and its hydroxylamine and nitroso metabolites

Neutrophil and lymphocyte membrane integrity was assessed by trypan blue dye exclusion as described previously (Pirmohamed *et al*., 1991). Cells were incubated with SMX,

SMX-NHOH or SMX-NO (1–1000 μM) in Dulbecco's PBS (1 ml, pH 7.4) in a shaking water bath at 37°C for 1 h. Data represents the percentage of trypan blue stained cells/total number of cells.

Apoptosis and necrosis are morphologically distinct modes of cell death (Searle *et al*., 1982). While apoptosis occurs prior to and at lower toxicant dose than necrotic cell death (Raffray & Cohen, 1997), cell selectivity of toxicants can stem from the apoptotic and necrotic thresholds at which different cells die, site of bioactivation/detoxification or site of toxicant accumulation. For these reasons, lymphocyte and neutrophil apoptosis was quantified morphologically and by flow cytometry at lower concentrations than that required to cause necrotic cell death. Cells (1×10^6) were incubated with SMX, SMX-NHOH or SMX-NO (1–300 μM) in Dulbecco's PBS (pH 7.4). Drugs were added in DMSO, which as a 1% solution (v v^{-1}) did not induce apoptosis. Some incubations also contained GSH (1 mM). After 2 h, the tubes were centrifuged ($650 \times g$; 10 min) to pellet the cells. The supernatants were discarded and the cells were resuspended in 1 ml of drug-free Dulbecco's PBS containing human serum albumin (5 mg ml^{-1}). Samples were incubated for a further 6 h prior to analysis.

Cells were assessed for characteristic morphologic changes using Wright's stain. Stained cells were viewed under a light microscope (Shandon Elliot, London, U.K.) for apoptotic structure (Watson *et al*., 1996b). At least 200 cells were examined.

Flow cytometric evaluation of apoptotic nuclei was assessed according to the method originally described by Nicoletti *et al*. (1991). Cells were pelleted by centrifugation ($200 \times g$; 8 min), resuspended in 1 ml of 70% ethanol (v v^{-1}) and stored for 30 min at 37°C. The fixed cell suspensions were then centrifuged, washed with Dulbecco's PBS (1 ml) and finally resuspended in 1 ml of hypotonic fluorochrome solution (propidium iodide, 50 μg ml^{-1}; sodium citrate, 3.4 mM; Tris, 1 mM; EDTA, 100 μM; RNAse [type I-A], 500 μg ml^{-1}). The cells were kept overnight in the dark (4°C) prior to FACScan analysis on a Becton Dickinson flow cytometer (Becton Dickinson, CA, U.S.A.). Propidium iodide fluorescence (FL-2) of individual nuclei was registered on a logarithmic scale. At least 5000 nuclei were counted and analysed on a Hewlett Packard (HP 9000) computer using Lysis II software.

The ability of SMX, SMX-NHOH and SMX-NO (1–300 μM) to inhibit the neutrophil oxidative burst was determined using the nitroblue tetrazolium slide test and an indirect procedure that involves inhibition of eugenol-induced GSH depletion by the test compounds. The experimental protocols have been described previously (Naisbitt *et al*., 1997).

Red blood cell toxicity was assessed by measuring the amount of methaemoglobin (MetHb) formed when cells (50% haematocrit) were incubated with SMX-NHOH and SMX-NO, according to the method of Harrison & Jollow (1986).

Determination of NF-κB and AP-1 levels in lymphocytes

Freshly isolated lymphocytes were incubated in serum-free RPMI 1640 medium containing glutamine (2 mM) and penicillin (50 units ml^{-1})/streptomycin (50 μg ml^{-1}) at a density of $1-3 \times 10^6$ cells ml^{-1}. After 24 h cells were incubated in the presence of SMX-NO (1–1000 μM) for a further 1 h in cell culture tubes (37°C, 5% CO_2). NF-κB and AP-1 DNA binding activity was determined using the Promega Gel Shift Assay System (Promega, Southampton, U.K.). The double-stranded DNA oligonucleotide probes used contained the following sequences:

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NF-κB 5′-AGT TGA GGG GAC TTT CCC AGG C-3′
AP-1 5′-CGC TTG ATG AGT CAG CCG GTA A-3′
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Oligonucleotide probes were [γ-^{32}P]-ATP (Amersham International, Amersham, U.K.) end-labelled with T4 polynucleotide kinase. Binding reactions consisted of nuclear protein (2 μg), 5× binding buffer and labelled NF-κB or AP-1 oligonucleotide probe (50,000 c.p.m.). Competitive mobility shift reactions included unlabelled NF-κB or AP-1 oligonucleotide probe (1.75 pmol) to characterize the DNA binding specificity. Following incubation for 30 min at room temperature, samples were loaded onto a 6% polyacrylamide gel and electrophoresis was performed at 150 V for 2.5 h. The gels were then dried and exposed to photographic film. The results are expressed as the integrated optical density of each band. All experiments were carried out with cells from three individuals.

Reaction of sulphamethoxazole, sulphamethoxazole hydroxylamine and nitroso sulphamethoxazole with biological thiols

To determine the relative importance of intracellular and extracellular thiols in the detoxification of SMX-NHOH and SMX-NO, we investigated their reaction with CYS (the most abundant extracellular thiol in plasma; Eck *et al*., 1989), and compared it to their reaction with GSH (a predominantly intracellular thiol), which has been described previously (Cribb *et al*., 1991). SMX (50 μM) or its metabolites (SMX-NHOH and SMX-NO; 50 μM) were incubated with CYS (100 μM) in Dulbecco's PBS (1 ml) at 37°C for 0.2, 1, 8 or 20 h. Aliquots (25 μl) of the reaction mixture were analysed at each time-point by LC-MS, using a previously described protocol (Gill *et al*., 1996). SMX and SMX-NHOH were identified from authentic standards, whereas any reaction intermediates were identified from their parent molecular ion peak and retention times quoted previously (Naisbitt *et al*., 1996).

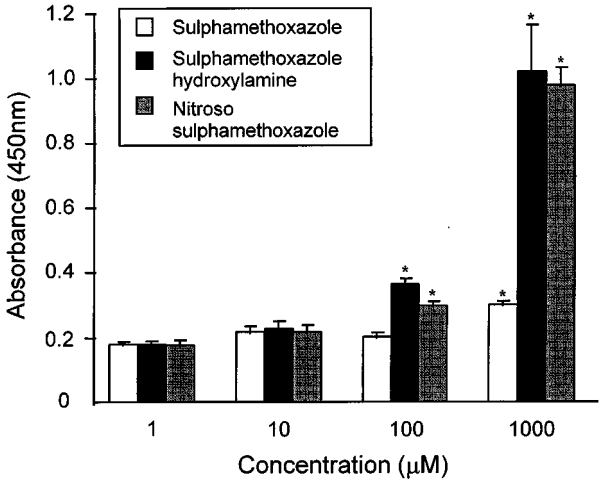

Figure 1 Haptenation of thiolated human serum albumin by SMX, SMX-NHOH or SMX-NO (1–1000 μM) after a 24 h incubation. Results are expressed as the mean absorbance above the background values for three experiments. Statistical analysis performed by comparing the relative absorbance at different concentrations of the compounds with that of the control value ($^*P<0.05$).

Two major difficulties were encountered with the use of LC-MS. Firstly, the concentration of SMX-NO throughout the reaction could not be measured because it only produces a very weak protonated molecule; and second, absolute quantification of the amounts of products formed is not possible. To overcome these problems, the reactions of SMX, SMX-NHOH and SMX-NO (10 mM) with CYS in d_6-DMSO were analysed by proton NMR. DMSO as a solvent has the added advantage of stabilizing SMX-NHOH, thus allowing the analysis of reaction intermediates over a longer time period (i.e., 96 h; Naisbitt *et al*., 1996). The amount of each intermediate formed was quantified by measurement of the integral peak height from each spectrum throughout the reaction period. All reaction intermediates were identified from NMR spectra of standard compounds.

The reactions of SMX, SMX-NHOH and SMX-NO with CYS and GSH were also determined by fluorescence HPLC. The compounds (1–300 μM) were incubated with either CYS or GSH (3 μM) in Dulbecco's PBS (1 ml) at 37°C for 20 min. Free sulphydryl groups were measured as described previously, using the fluorescent probe bromobimane (Cotgreave & Moldeus, 1986; Pirmohamed *et al*., 1996). The reaction between SMX-NO (30 μM) and a mixture of CYS (10 μM) and GSH (10 μM) in Dulbecco's PBS was analysed by a similar method. Incubations were carried out at 37°C and thiol levels were measured at intervals between 0–60 min.

Depletion of glutathione and cysteine from neutrophils, lymphocytes and plasma

Intact or lysed peripheral white blood cells (0.5×10^6 cells/incubation) were incubated (final volume 1 ml) in Dulbecco's PBS at 37°C for 1 h with SMX, SMX-NHOH or SMX-NO (1–400 μM). The experiments with neutrophils were performed both in the presence and absence of PMA (10 ng ml^{-1}). PMA

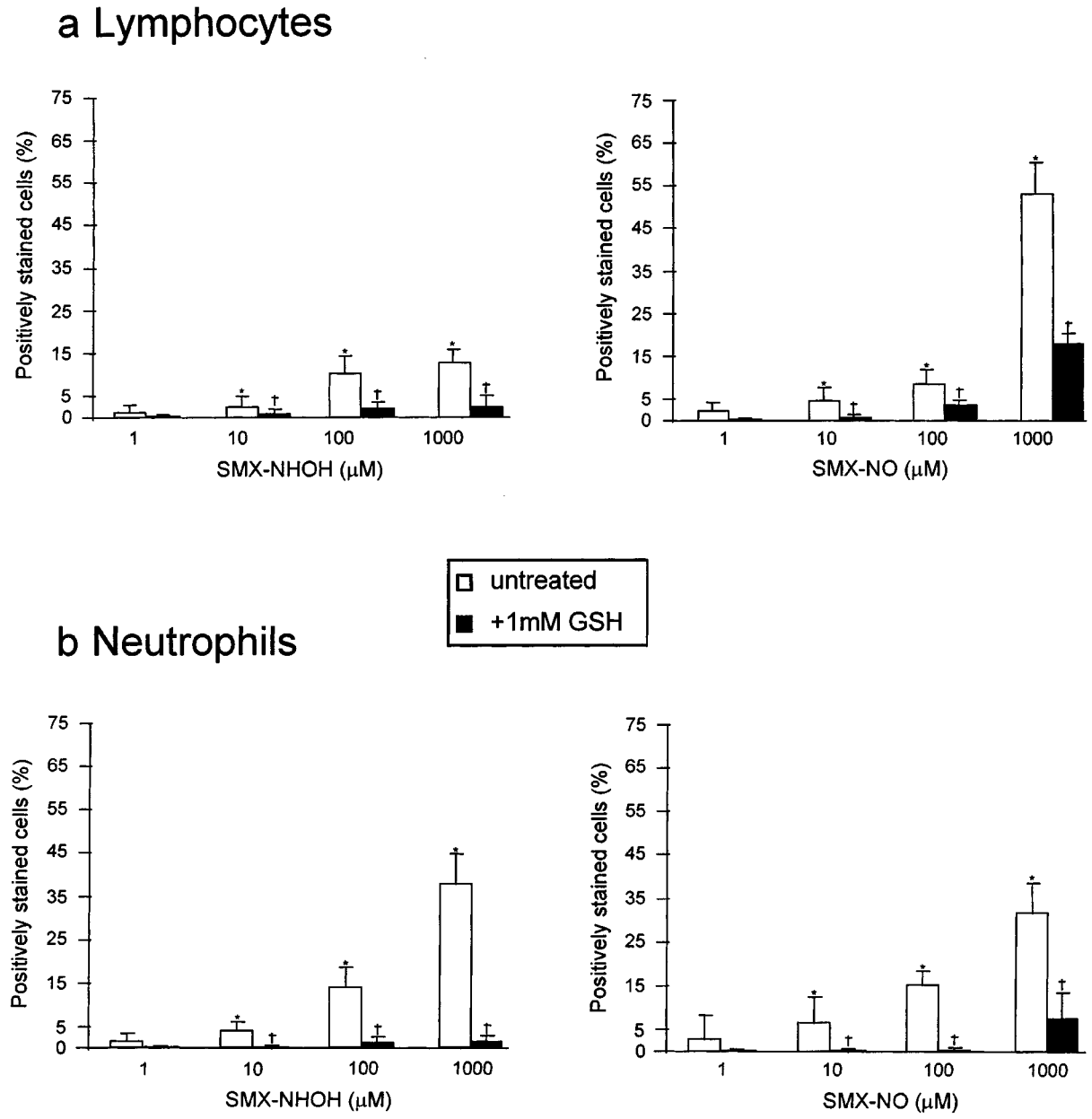

Figure 2 Haptenation of lymphocytes (a) and neutrophils (b) after incubation with either SMX-NHOH or SMX-NO for 1 h in the presence or absence of GSH (1 mM). The results are expressed as the percentage of positively stained cells from four experiments. Statistical analysis compares the ability of the drugs to haptenate cells with that of the control value (*$P<0.05$) and the effect of GSH on cellular staining at each drug concentration (†$P<0.05$).

stimulates the neutrophil respiratory burst, a phenomena known to oxidize almost any compound with an easily oxidizable functional group (Uetrecht, 1992). Protein was precipitated by the addition of 100% trichloroacetic acid (10 μl) and thiol levels were measured using bromobimane (Cotgreave & Moldeus, 1986; Pirmohamed *et al*., 1996). Plasma thiol depletion was analysed by a similar method except that 1 ml of ice-cold plasma was added to each incubation instead of the cells.

The basal levels of the thiols were measured prior to the incubation and in the absence of any of the compounds. Additionally, thiol stability after isolation of the plasma and cells was assessed between 0–5 h. The results are presented as the mean of three separate incubations conducted in triplicate. GSH and CYS standard curves were constructed between 0.5 and 50 μM.

Reduction of sulphamethoxazole hydroxylamine and nitroso sulphamethoxazole by the individual components of peripheral blood

Isolated neutrophils, lymphocytes (5×10^6 cells) and plasma (1 ml) were incubated with SMX-NHOH and SMX-NO (30 μM) in a shaking water bath at 37°C in Dulbecco's PBS (pH 7.4). The neutrophil incubations were carried out in the presence or absence of PMA (10 ng ml^{-1}). After 1 h, the reaction was stopped by placing the tubes on ice. The internal standard, dapsone (5 μM), was added. In some experiments, neutrophils were lysed by repeated freeze thawing. Extraction was performed with ethyl acetate (2×3 ml). The combined extracts were evaporated to dryness under nitrogen, reconstituted in mobile phase (100 μl) and analysed by HPLC using the system described above.

Further experiments were carried out to investigate the role of nitroso reductase enzymes in the reduction of SMX-NO. Neutrophils ($5 \times 10^3 - 5 \times 10^6$) were incubated with SMX-NO (30 μM) in a shaking water bath at 37°C in Dulbecco's PBS (pH 7.4) for 1 h in the presence or absence of the reductase inhibitors salicyl hydroxamate (1 mM), dicuomarol (10 μM), neocuproin (1 mM), or sodium azide (1 mM) (Somerville *et al*., 1995). SMX-NHOH and SMX formation was measured by HPLC.

Incubations containing red blood cells (0.5 ml, 50% haematocrit) and SMX-NHOH (1–100 μM) or SMX-NO (30 μM) were carried out as above and terminated after 1 h by placing the tubes on ice. Internal standard was added to the samples, and cells were lysed by the addition of 0.5 ml distilled water. Following extraction with ethyl acetate (2×3 ml), the samples were reconstituted in mobile phase (100 μl) and analysed by HPLC.

SMX-NHOH and SMX-NO were also incubated for 1 h in either Dulbecco's PBS, ethyl acetate or mobile phase (37°C), and analysed by HPLC to measure any spontaneous reduction. All incubations were performed in triplicate using cells from four individuals.

Red blood cell accumulation of sulphamethoxazole hydroxylamine and dapsone hydroxylamine

Freshly drawn blood from healthy volunteers was collected into potassium-EDTA tubes containing GSH (1 mM). GSH was added to stabilize the hydroxylamine and prevent auto-oxidation to the nitroso metabolite (Mitra *et al*., 1996). SMX-NHOH (10 μM) or dapsone hydroxylamine (10 μM) were incubated with blood (0.5 ml) at 37°C for various times (10, 20, 40, 60 and 100 min). Samples were then microcentrifuged for 1 min ($13{,}000 \times g$) and an aliquot of the plasma was removed into a silanized glass tube into which the internal standard [SMX (5 μM) for dapsone hydroxylamine incubations and dapsone (5 μM) for SMX-NHOH incubations] was added. Extraction was performed using ethyl acetate (2×3 ml). The solvent was evaporated to dryness and samples were reconstituted in methanol : distilled water (1:1 v v^{-1}) containing GSH (1 mM), and analysed using HPLC with UV (Gill *et al*., 1996) and electrochemical detection (Antdec Decade Electrochemical Detector, Protech Scientific Ltd, Manchester, U.K.). The limit of detection was 0.025 nmole (dapsone hydroxylamine) and 0.08 nmole (SMX-NHOH) for UV detection and 1 pmole (dapsone hydroxylamine and SMX-NHOH) for electrochemical detection.

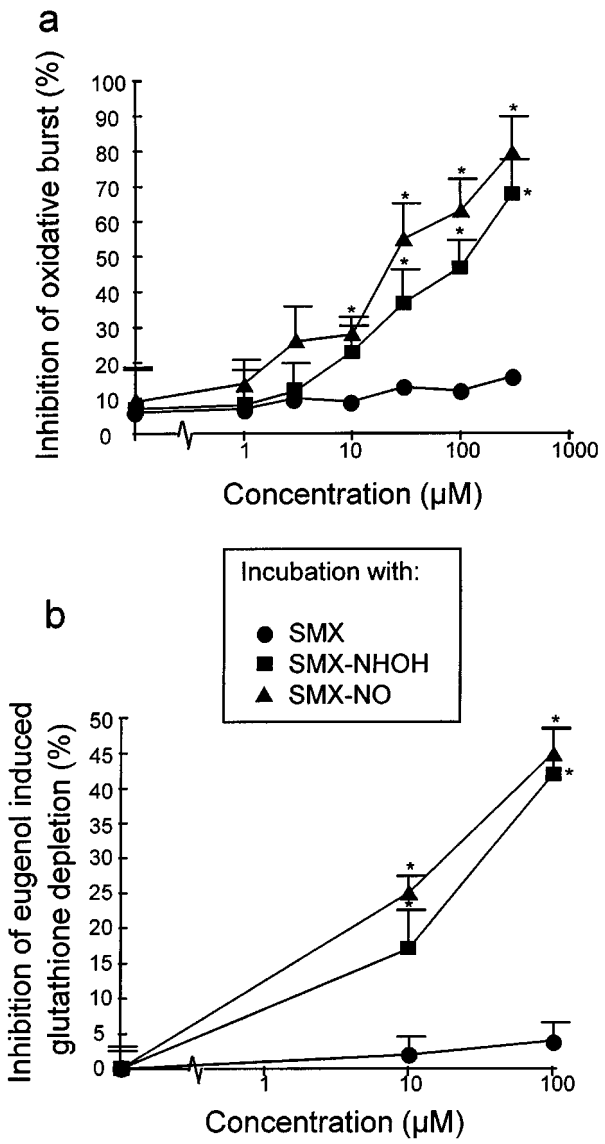

Figure 3 (a) Inhibition of neutrophil oxidative metabolism and (b) inhibition of eugenol-induced GSH depletion in PMA-stimulated neutrophils by SMX and its oxidative metabolites. The respiratory burst was measured using the nitroblue tetrazolium slide test. GSH levels were measured using bromobimane, by fluorescence HPLC. The results represent the mean ± s.d. of three experiments carried out in triplicate. Statistical analysis was performed by comparing the different concentrations of compounds required to inhibit the respiratory burst with that of solvent alone (*$P < 0.05$).

Statistical analysis

All values are expressed as the mean ± s.d. All values to be compared were analysed for non-normality using the Shapiro-Wilk test. Values were often found to be non-normally distributed, and therefore, the Mann-Whitney test was used for comparison of the two groups, accepting $P<0.05$ as significant.

Results

Haptenation of human serum albumin by sulphamethoxazole and its metabolites

Incubation of human serum albumin with SMX, SMX-NHOH and SMX-NO did not result in significant binding at any of the concentrations tested. There was a concentration-dependent increase in binding to thiolated albumin with both SMX-NHOH and SMX-NO (Figure 1), while SMX itself only showed binding at the highest concentration (1000 μM) tested.

Flow cytometric analysis of cellular haptenation by sulphamethoxazole and its metabolites

Haptenation of SMX, SMX-NHOH and SMX-NO to cell surfaces was determined by flow cytometry. Incubation of cells (both lymphocytes and neutrophils) with SMX-NHOH and SMX-NO resulted in a concentration-dependent increase in surface antigen formation (Figure 2). In contrast, there was no detectable antigen formation when SMX (1–1000 μM) was incubated with cells. Co-incubation of the cells with GSH (1 mM) significantly ($P<0.05$) reduced cellular antigen formation at each concentration (Figure 2). Haptenation to the surface of lymphocytes on incubation with SMX-NO (1000 μM; 52.9 ± 6.6% positively stained cells) was reduced by 66% (18.0 ± 5.3) in the presence of CYS (1 mM) and 67% (17.7 ± 6.9%) in the presence of GSH (1 mM). Incubation of neutrophils with SMX (2.5 mM) in

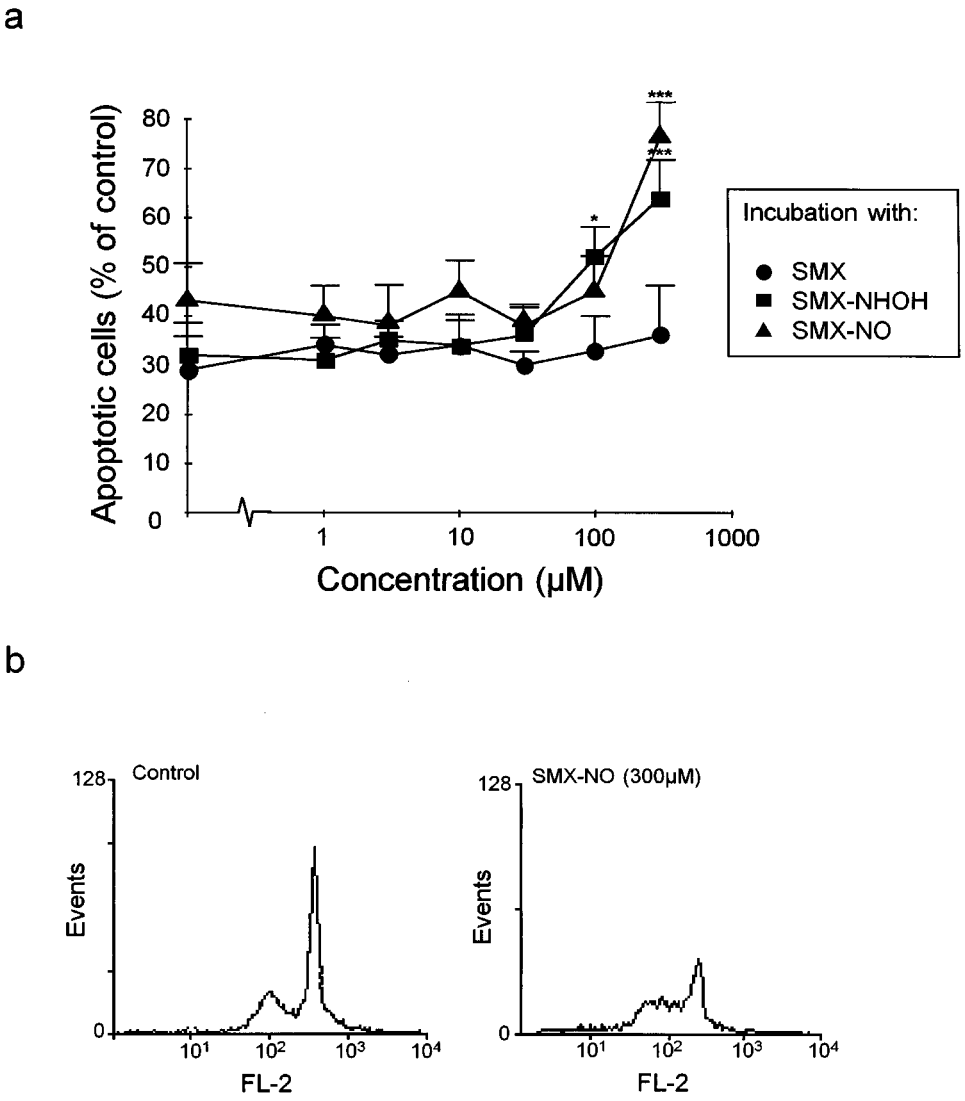

Figure 4 (a) Neutrophil apoptosis after incubation with SMX, SMX-NHOH and SMX-NO (1–300 μM). Apoptosis was measured by flow cytometric evaluation of propidium iodide stained nuclei. Results represent the mean of three experiments carried out in triplicate. Statistical analysis was performed by comparing the ability of different concentrations of compound to induce apoptosis with that of solvent alone (*$P<0.05$). (b) A typical trace obtained from the flow cytometer in the presence and absence of SMX-NO (300 μM).

the presence of PMA (10 ng ml^{-1}) produced significant binding in two of the six individuals tested (5 and 10% positively stained cells).

Cellular toxicity of sulphamethoxazole hydroxylamine and nitroso sulphamethoxazole

The toxicity of the oxidative metabolites of SMX was assessed in neutrophils, lymphocytes and red blood cells. In neutrophils, we assessed both functional and structural toxicity. Both SMX-NHOH and SMX-NO caused a concentration-dependent inhibition of neutrophil function as assessed by the nitroblue tetrazolium slide test and inhibition of eugenol-induced GSH depletion (Figure 3a and b), while the parent compound had no effect.

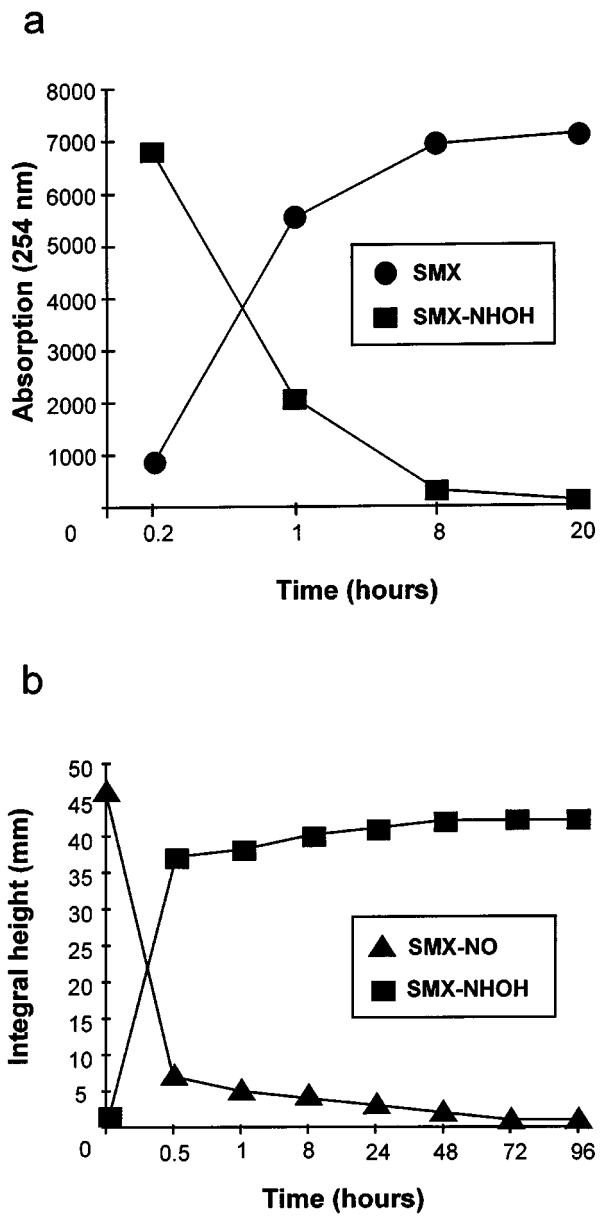

Figure 5 Ratio of product formation/disappearance in the reaction of SMX-NO with CYS (a) under aqueous conditions, analysed by LC-MS and (b) in DMSO, analysed by NMR. The concentration of SMX-NO could not be measured by LC-MS, while DMSO enhances the stability of SMX-NHOH (see text).

Cell death was initially assessed by trypan blue dye exclusion as a measure of membrane integrity. Death of neutrophils after a 1 h incubation above background values ($<5\%$) was only seen with 1000 μM SMX-NO ($7.3\pm1.0\%$), but not with SMX or SMX-NHOH. Lymphocytes in contrast to neutrophils were significantly ($P<0.05$) more sensitive to the direct cytotoxic effects of SMX-NO (1000 μM) with $46.8\pm2.8\%$ cells unable to exclude trypan blue after a 1 h incubation ($P<0.05$ when compared to a background value of $<5\%$).

In order to determine the mode of cell death, we went on to investigate whether these metabolites induced apoptosis. Freshly isolated neutrophils have a limited life span, after which time they die by apoptosis. The background rate of apoptosis was equivalent to previous studies (Cox, 1995; Watson *et al.*, 1996a). Both SMX-NHOH and SMX-NO (100–300 μM) increased the rate of spontaneous neutrophil apoptosis as assessed by flow cytometry (Figure 4a) and morphological evaluation (data not shown). This was reduced to background values on incubation with GSH (1 mM; data not shown). No apoptosis was observed with SMX (1–300 μM) or lower concentrations of SMX-NHOH (1–30 μM) and SMX-NO (1–30 μM). Figure 4b illustrates a typical trace obtained from the flow cytometer in the presence and absence of SMX-NO (300 μM). Lymphocytes in contrast, did not undergo apoptosis.

Red blood cell toxicity was assessed by measurement of MetHb. MetHb formation with SMX-NHOH was concentration-dependent, with $26.1\pm6.4\%$ (control, $1.2\pm1.0\%$) MetHb being observed at the highest concentration studied (100 μM SMX-NHOH). MetHb formation after incubation of red blood cells with SMX-NO (30 μM; $4.9\pm0.8\%$) was lower ($P<0.05$) than that observed with an equimolar concentration of SMX-NHOH ($10.2\pm3.1\%$).

Effect of nitroso sulphamethoxazole on redox sensitive transcription factors NF-κB and AP-1 in mononuclear leukocytes

SMX-NO (1000 μM) caused a decrease in basal levels of NF-κB in lymphocytes after 1 h (control, 1779 ± 213 arbitrary units; drug treated, 968 ± 359 arbitrary units; $P<0.05$). In contrast, incubation of SMX-NO (1000 μM) with these cells produced a strong induction of AP-1 (control, 242 ± 193 arbitrary units; drug treated, 1157 ± 196 arbitrary units; $P<0.05$). Neither NF-κB nor AP-1 levels were altered at lower SMX-NO concentrations (1–100 μM).

Reaction of sulphamethoxazole, sulphamethoxazole hydroxylamine and nitroso sulphamethoxazole with thiols

Figure 5a and b show the reaction of SMX-NO with CYS analysed by LC-MS and NMR, respectively. The initial product was SMX-NHOH; this largely (95%) underwent further reduction to SMX during the first 8 h of the incubation. Semi-mercaptal and sulphonamide conjugates which are formed when SMX-NO reacts with GSH and other biological thiols (Cribb *et al.*, 1991; Naisbitt *et al.*, 1996) were not detected. SMX was the only product when SMX-NHOH and CYS were incubated in buffer (results not shown).

SMX-NHOH and SMX-NO (1–300 μM) both caused concentration-dependent depletion of CYS (3 μM) and GSH

(3 μM) (Figure 6a and b), while SMX had no effect. The reaction between SMX-NO (30 μM) and CYS (10 μM) was rapid resulting in 92.4$\pm$3.2% depletion within 1 min. In contrast, the reaction with GSH was significantly slower than with CYS ($P<0.05$), with 63.6$\pm$5.4% depletion being observed after 1 min. CYS was completely depleted after 10 min, while a significant amount of GSH remained for a further 20 min.

Thiol depletion from human neutrophils, lymphocytes and plasma by sulphamethoxazole and its metabolites

The basal intracellular GSH concentration measured in 20 individuals (3.2–5.6 nmoles/10^6 cells) was similar to that reported previously (Cotgreave & Moldeus, 1986; Pirmohamed *et al*., 1996). CYS was present almost exclusively in plasma, with control values (7.1–15.0 μM, measured in ten individuals) again being similar to those reported previously (Mills & Lang, 1996). In control incubations, there was no significant decrease in either the CYS or GSH levels for the duration of the experiment (1 h), although 2.5 h after cell isolation, CYS levels were reduced by 24–37%.

Neither SMX-NHOH nor SMX-NO (up to 400 μM) depleted GSH in PMA-stimulated and unstimulated neutrophils. In contrast, GSH depletion ($P<0.05$) was observed in lymphocytes with both SMX-NHOH and SMX-NO at concentrations of 10 μM or greater. GSH depletion was significantly greater when neutrophils were lysed than when they were intact ($P<0.05$); the concentration of SMX-NHOH and SMX-NO which caused 50% depletion in lysed cells was 11.1$\pm$1.4 μM and 2.9$\pm$0.6 μM, respectively, and greater than 90% depletion was observed at 30 μM (Figure 7a and b).

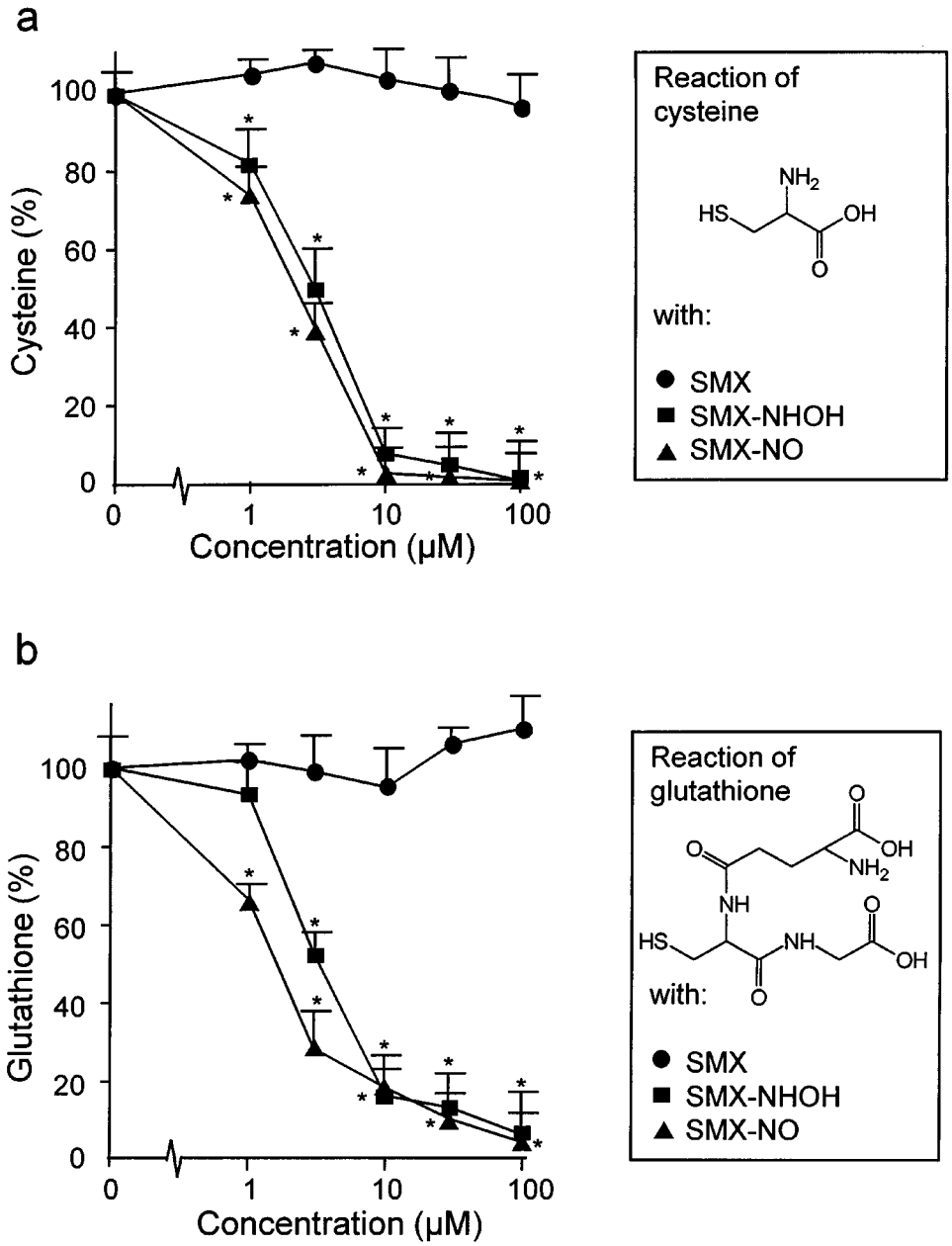

Figure 6 Reaction of CYS (a) or GSH (b) with SMX and its metabolites. Unreacted sulphydryl concentrations, expressed as percentage of thiol depletion in the absence of drug, were measured by fluorimetric HPLC following conjugation with bromobimane, after incubation of SMX, SMX-NHOH or SMX-NO (1–100 μM) for 1 h with either CYS (3 μM) or GSH (3 μM). The results represent the mean of three experiments conducted in triplicate. Statistical analysis was performed by comparing the depletion of thiols in the absence and presence of SMX or its metabolites (*$P<0.05$).

Both SMX-NHOH and SMX-NO caused a concentration-dependent depletion of CYS in plasma. At the highest concentration (400 μM) tested, SMX-NHOH and SMX-NO caused 82.3 ± 4.7 and 90.3 ± 3.1% depletion, respectively, when compared with the control values (Figure 7c). SMX itself did not deplete either GSH or CYS in plasma, intact or lysed cells.

Reduction of sulphamethoxazole hydroxylamine and nitroso sulphamethoxazole by the individual components of peripheral blood

There was no spontaneous reduction of SMX-NHOH or SMX-NO after a 1 h incubation in either buffer, mobile phase

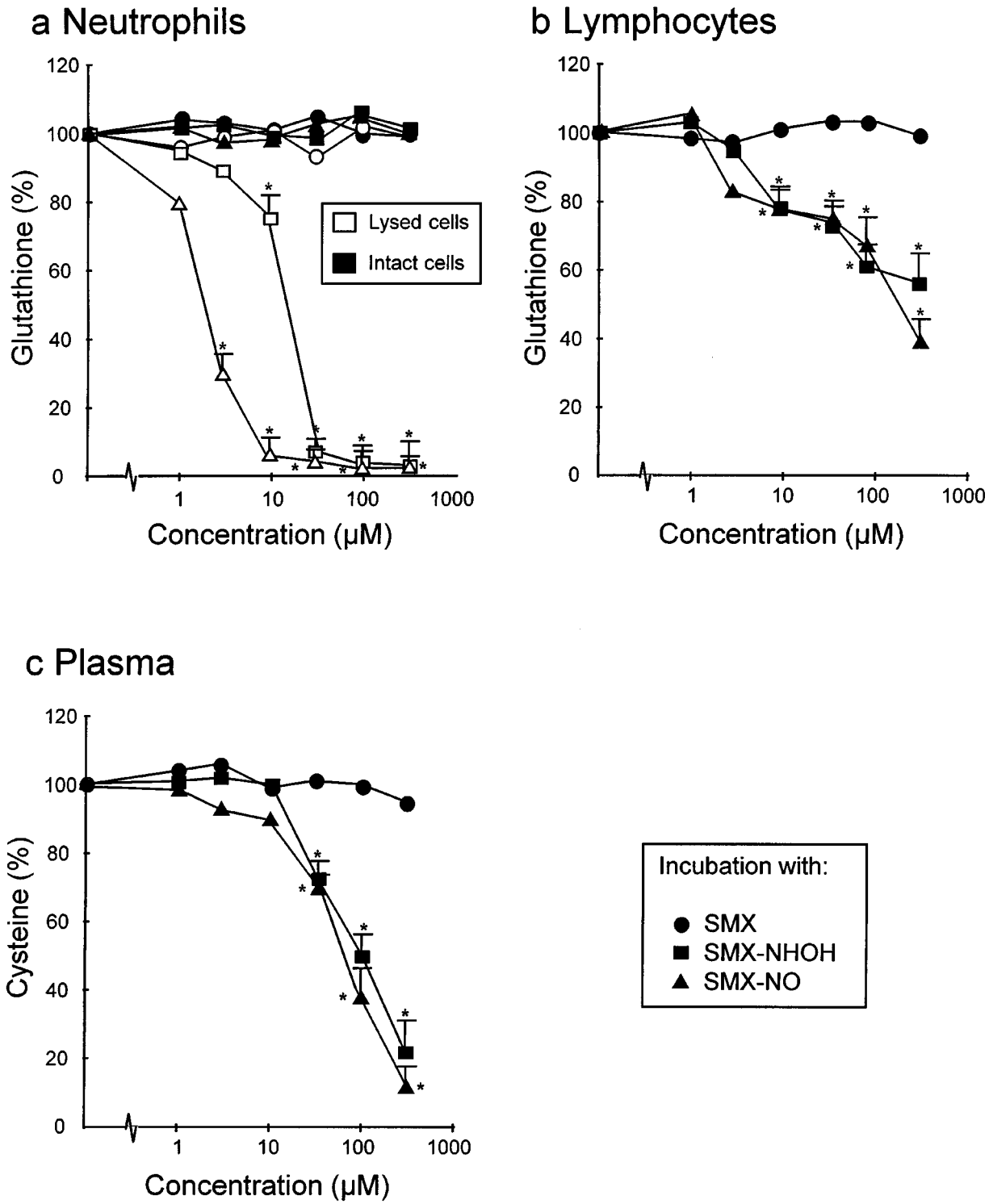

Figure 7 Depletion of CYS and GSH from cells and plasma after incubation with SMX and its metabolites. Thiol levels in neutrophils, lysed neutrophils (0.5×10^6; a), lymphocytes (0.5×10^6; b), and plasma (1 ml; c) were measured with fluorescent HPLC detection using bromobimane after incubation with either SMX, SMX-NHOH or SMX-NO (1–400 μM). Results represent the mean of three experiments carried out in triplicate. Statistical analysis was performed by comparing depletion at different concentrations of drug with that in solvent alone ($^*P < 0.05$). Error bars have been omitted for clarity when there was no significant depletion.

Table 1 Reduction of 30 μM SMX-NHOH or SMX-NO by human neutrophils, lymphocytes, red blood cells and plasma after 1 h

	%Reduction		
	$NHOH \rightarrow NH_2$	$N=O \rightarrow NH_2$	$N=O \rightarrow NHOH$
Neutrophils + PMA	3.0 ± 0.8	1.8 ± 0.6	35.3 ± 3.6
Neutrophils − PMA	2.6 ± 0.2	1.7 ± 0.4	41.0 ± 7.2
Lysed neutrophils	2.5 ± 0.4	7.8 ± 0.6	3.2 ± 2.6
Lymphocytes	2.9 ± 1.1	2.8 ± 0.5	49.3 ± 5.4
Lysed lymphocytes	2.4 ± 0.3	7.9 ± 0.3	3.1 ± 0.3
Red blood cells	12.1 ± 2.4	6.2 ± 1.3	17.2 ± 3.6
Plasma	4.8 ± 1.1	16.3 ± 3.6	45.0 ± 10.3

[a]Control values (<1%) are subtracted from the tabulated values.

or solvent. All components of peripheral blood were able to reduce SMX-NHOH to SMX, and SMX-NO to the SMX-NHOH and SMX (Table 1). Intact neutrophils, lymphocytes and plasma were equipotent at reducing the nitroso metabolite back to the hydroxylamine, while red blood cells were most potent at reducing SMX-NHOH back to SMX. Lysis of neutrophils decreased the capacity for reduction of SMX-NO to SMX-NHOH by 12–16 fold.

SMX is converted to the electrophilic hydroxylamine metabolite in neutrophils (Cribb *et al*., 1990). The reaction is thought to be catalyzed by myeloperoxidase. *In vitro* activation of myeloperoxidase requires an artificial stimuli such as phorbol esters or fMLP. In this study PMA did not affect the cells capacity for reduction.

The reduction of SMX-NO to SMX-NHOH was dependent on cell concentration, with less than 20% reduction being observed at 0.5×10^6 neutrophils/ml (Figure 8a). Salicyl hydroxamate, dicoumarol and sodium azide caused a significant decrease in the reduction of SMX-NO to SMX-NHOH ($P<0.05$), while neocuprin had no effect (Figure 8b). The inhibitors had no effect on SMX formation.

Accumulation of sulphamethoxazole hydroxylamine and dapsone hydroxylamine into red blood cells

Dapsone hydroxylamine was taken up rapidly into the red blood cells, with 92.9 ± 0.8% partitioning into the cells over 90 min. In comparison, the amount of SMX-NHOH taken up into red cells significantly ($P<0.05$) lower (33.1 ± 13.1%; Figure 9).

Discussion

The demonstration of cell-mediated immunity (Mauri-Hellweg *et al*., 1995) and anti-SMX antibodies (Daftarian *et al*., 1995) in hypersensitive patients and the presence of SMX-substituted proteins in patient sera (Meekins *et al*., 1994) are indicative of an immune pathogenesis for the toxicity associated with the drug. Bioactivation of SMX to the hydroxylamine and thence to the nitroso metabolite is thought to be the initial step in the pathogenesis of hypersensitivity reactions (Rieder *et al*., 1995a,b; 1988; Carr *et al*., 1993), with subsequent formation of drug-associated antigen by drug-protein conjugation. Alternatively it has been postulated that the drug, or drug metabolite, exerts a direct immunotoxic effect on cells to produce a hypersensitivity reaction (Schnyder *et al*., 1997). We have therefore explored the relationship between drug-antigen formation, drug (metabolite) disposition, and cell-function, by use of

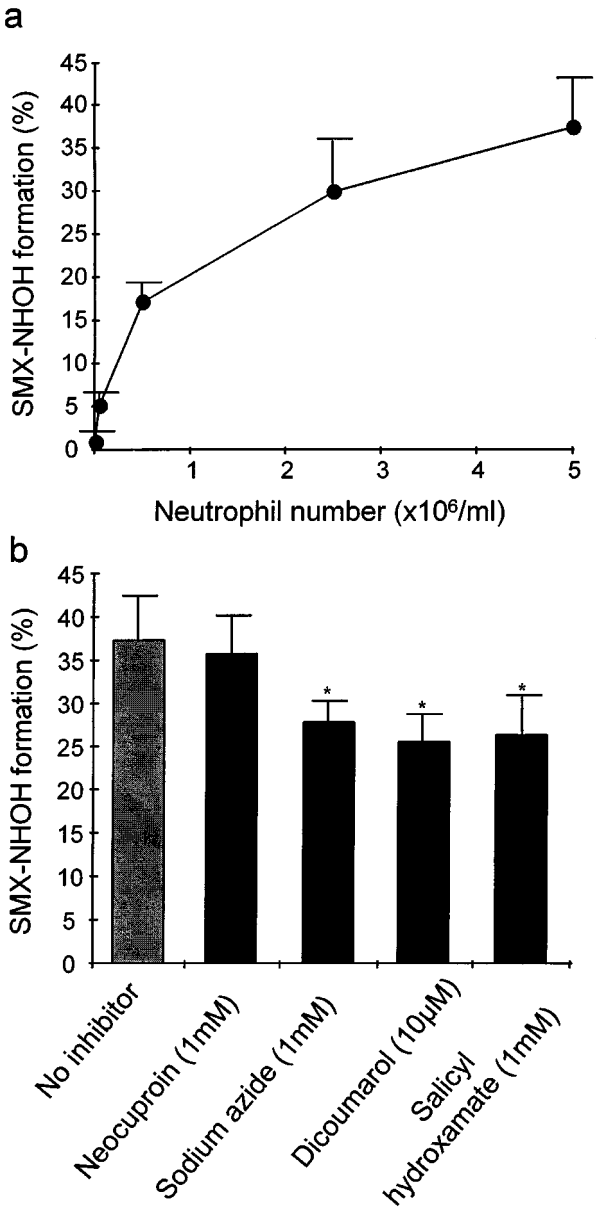

Figure 8 (a) Reduction of SMX-NO to SMX-NHOH by neutrophils ($0.005-5 \times 10^6$ ml^{-1}) in Dulbecco's PBS. (b) Inhibition of neutrophil (5×10^6 ml^{-1}) mediated reduction of SMX-NO (30 μM) after co-incubation of the cells with neocuproin (1 mM), sodium azide (1 mM), dicoumarol (10 μM) or salicyl hydroxamate (1 mM). SMX-NHOH formation was measured by HPLC with UV detection. Results represent the mean of three experiments carried out in duplicate. Statistical analysis was performed by comparing SMX-NHOH formation in the presence and absence of the inhibitors (*$P<0.05$).

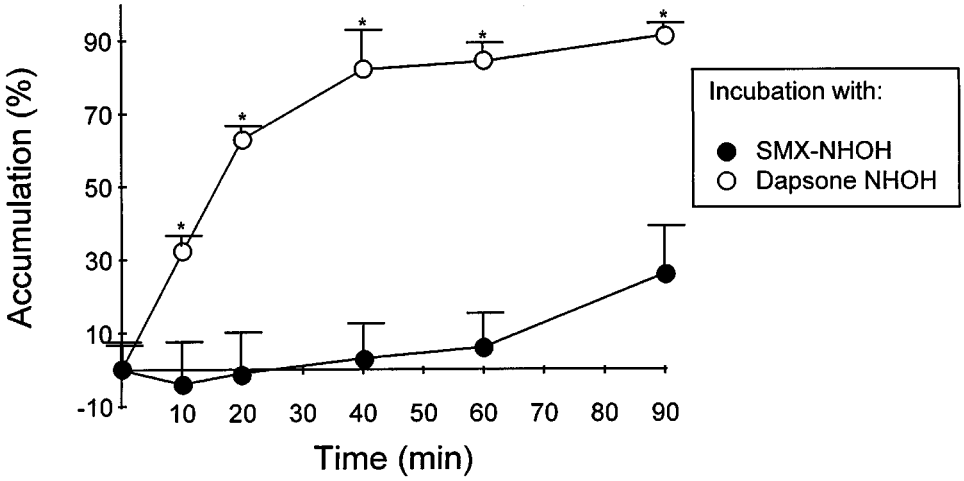

Figure 9 Time-dependent accumulation of dapsone hydroxylamine (10 μM) and SMX-NHOH (10 μM) by red blood cells. The hydroxylamines were measured by HPLC with UV and electrochemical detection. Results represent the mean of four experiments carried out in triplicate. Statistical analysis was performed by comparing the difference in the cellular uptake of dapsone hydroxylamine and SMX-NHOH (*$P<0.05$).

two accessible human cell types, neutrophils and lymphocytes.

Use of flow cytometry, in conjunction with a specific anti-SMX antibody, clearly demonstrated the ability of both SMX-NHOH and SMX-NO, but not SMX itself, to bind to the outer surface of neutrophils and lymphocytes in a concentration-dependent fashion. Haptenation was greater with SMX-NO. Binding of SMX-NHOH is presumed to involve autoxidation to SMX-NO (Cribb *et al*., 1991) which can react with CYS but not with other amino acids (Naisbitt *et al*., 1996). In contrast, there was detectable binding to albumin only when the protein was chemically modified to increase its free thiol content. Furthermore, an increase in extracellular GSH (or CYS) blocked cell-haptenation by SMX-NO.

Cell-surface haptenation was detectable after exposure to drug metabolite concentrations of 10 μM which may occur after a therapeutic dose of SMX (Mitra *et al*., 1996). Our results are in accordance with the finding that there are high levels of protein thiols expressed extracellularly in cells such as lymphocytes (Lawrence *et al*., 1997). It is important to note that haptenation can occur without loss of cell membrane integrity, depletion of intracellular GSH or detectable perturbation of redox sensitive transcription factors. The evidence for this is 3 fold. First, the system used to detect cell-surface haptenation will distinguish between viable and non-viable cells, and haptenation was clearly occurring on viable cells. Second, although SMX-NO and SMX-NHOH are toxic to lymphocytes, loss of membrane integrity was only observed at concentrations of 100 μM and greater. In addition, concentrations of SMX-NO required to induce apoptosis (100–300 μM) and necrosis (1000 μM) in neutrophils, are likely to be outside the range achieved with therapeutic doses of SMX. Lymphocytes were more sensitive to the direct cytotoxic effects of SMX-NO, but did not undergo apoptosis. The differential extent of toxicity in neutrophils and lymphocytes may be related to the significant GSH depletion observed with high concentrations of SMX-NO in lymphocytes. SMX itself was non-toxic to either cell type. Third, we investigated the effect on the redox sensitive transcription factors, AP-1 and NF-κB, known to be modified under situations of cellular stress and initiation of an immune response (Angel & Karin, 1991; Baeuerle & Henkel, 1994). Although SMX-NO did cause changes in the electrophoretic mobility of the AP-1 and NF-κB probes, consistent with altered protein binding, these changes only occurred at concentrations above those seen therapeutically (i.e., 1000 μM) and appeared to coincide with perturbation of membrane integrity. Similar results have been reported in the liver, after administration of hepatotoxic doses of paracetamol to mice (Blazka *et al*., 1996; 1997).

These findings show that SMX-NO can haptenate cells without loss of viability. Such viable cell-drug conjugates can function as a potent antigenic stimulus *in vivo*. For example, dinitrophenyl conjugated to lymphocytes at extremely low hapten densities was shown to elicit hypersensitivity reactions to a significantly greater extent than similar amounts of dinitrophenyl conjugated to serum proteins (Sjoeberg *et al*., 1978). Additionally, lymphocytes pre-treated with the reactive metabolite of halothane, were found to be highly immunogenic when administered to rabbits (Hastings *et al*., 1995). Conjugation of external cellular proteins may lead to internalization, antigen processing and, ultimately, to presentation of drug-peptide conjugates which are then recognized by specific $CD8^+$ T-cells (Park *et al*., 1998), thus providing a mechanism for drug hypersensitivity reactions. Haptenation of viable cells is essential for each of these processes to function unimpaired.

The ability of SMX, and its oxidative metabolites to interact selectively with target cells, will be partly dependent on the balance between various pathways of drug metabolism. In the liver, acetylation and glucuronidation represent direct detoxification processes whereas *N*-hydroxylation represents bioactivation. Accordingly, relative dysfunction of either of these phase II biotransformations may predispose to drug toxicity (Shear *et al*., 1986). Although the liver is the major site of hydroxylamine formation, hepatotoxicity is only rarely associated with SMX (Cribb *et al*., 1996a). GSH and the GSH transferase enzymes are known to be important in the prevention of severe hepatotoxicity. The fact that GSH levels in the liver are higher than in other cells may explain why SMX-induced hepatotoxicity is rarely observed in the clinical situation. Chemically reactive products of oxidative drug metabolism generally have a short biological half-life and are not expected to reach significant concentrations at a site distant from where they are formed. The hydroxylamine can be detected in plasma, while SMX-NO cannot, reflecting the instability and shorter half-life of the latter.

There is extensive reduction of both SMX-NO and SMX-NHOH in *in vivo* animal models (Gill *et al*., 1997). We have shown that SMX-NO undergoes reduction in plasma, with concomitant depletion of thiols *in vitro*. Thus, plasma concentrations of CYS, the major circulating thiol [total levels reach 300 μM (Mills & Land, 1996)] in blood may normally play a role in preventing the autoxidation of circulating SMX-NHOH to a protein-reactive species *in vivo*. Conversely, a disease-induced depletion of plasma thiols, as observed in HIV-positive patients (Eck *et al*., 1989; Staal *et al*., 1992; Helbling *et al*., 1996; Walmsley *et al*., 1997), will increase the possibility of cell-surface haptenation and thus provide one reason for the greatly increased incidence of SMX hypersensitivity in this group of patients.

The results of our previous study demonstrated that intracellular thiols, principally GSH, can effect reduction of SMX-NO (to both SMX and SMX-NHOH) by a non-enzymatic process (Naisbitt *et al*., 1996). The spontaneous reaction of thiols with hydroxylamines and nitroso-derivatives is dependent on thiol concentration, pH and the electronic properties of the aromatic ring substituents of the drug (Ellis *et al*., 1992). The reaction is initiated by nucleophilic attack by GSH on the nitroso moiety resulting in the formation of an unstable semi-mercaptal intermediate which, depending on the conditions, can yield either a stable sulphonamide conjugate, a hydroxylamine or the parent amine (Cribb *et al*., 1991; Naisbitt *et al*., 1996). The experiments undertaken in this study show that CYS is also capable of rapid and complete reduction of SMX-NO. Indeed, the rate of reaction was more rapid than that observed with equimolar concentrations of GSH. In contrast to the reaction with GSH which produced a complex series of reaction intermediates, no thiol conjugates were identified. However, the mechanism of reduction is likely to be similar. The thiol/thiolate ion ratio of GSH and CYS at physiological pH [approximately 63 : 1 (GSH/GS^-); 8 : 1 (CYS/CYS^-)] may explain the lack of conjugate formation and enhanced reactivity of CYS towards SMX-NO (Kosower & Kosower, 1976). These findings have been observed by other groups working with reactive electrophiles such as quinones, diethyl maleate and diethyl fumarate (Murty & Penning, 1992; Kubal *et al*., 1995). Kubal *et al*. (1995) suggested that donation of a proton from the amine group of CYS may enhance product formation, while in turn the amide linkage between CYS and glutamate in GSH may result in stabilization of the intermediate products of conjugation.

Intracellular reduction of SMX-NO could provide an important cellular defence mechanism against the direct toxic effects of the metabolite. We therefore examined the metabolism of SMX-NO by both lymphocytes and neutrophils. Cell-concentration dependent reduction of SMX-NO to SMX-NHOH was observed without depletion of intracellular GSH. SMX was not formed in the reaction. The reduction was blocked to some extent by specific inhibitors of alcohol dehydrogenase (salicyl hydroxamate,

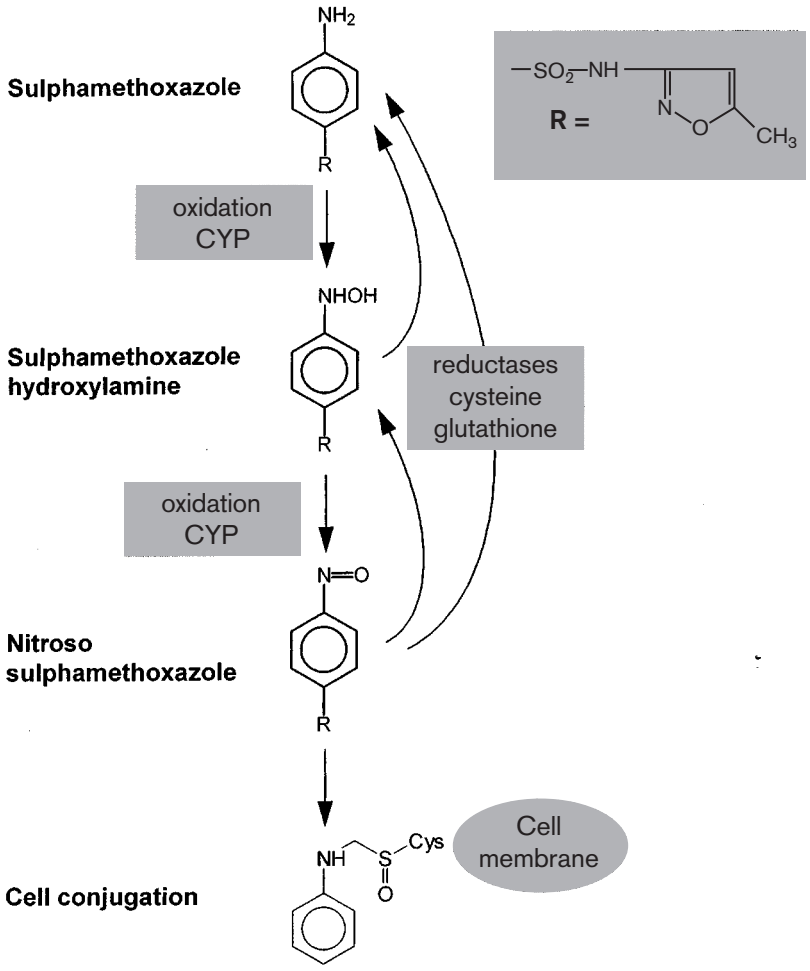

Figure 10 Scheme illustrating the proposed mechanism of SMX hypersensitivity reactions involving sequential cytochrome P450-mediated oxidations to the reactive nitroso metabolite which can bind irreversibly to either circulating or, as seen in this study, to cell surface proteins, to elicit an immune-mediated reaction. Defence mechanisms involving reduction of the reactive metabolites back to the parent compound, either enzymatically or by thiol compounds, are also indicated.

dicoumarol and sodium azide) (Somerville *et al*., 1995), an enzyme previously characterized in human neutrophils (Gotoh *et al*., 1989). Based on reductase activity and inhibitor studies, Kuwada *et al*. (1980) and others (Horie & Ogura, 1980) have also demonstrated alcohol dehydrogenase-dependent *C*-nitroso reduction. In contrast, when cells were lysed there was extensive consumption of GSH and reduction of SMX-NO to the parent amine was observed. Thus a combination of both enzymatic and non-enzymatic processes may serve to protect cells from intracellular chemical damage from SMX-NO. These findings identify an important extrahepatic detoxification mechanism, which if deficient, may determine susceptibility to SMX hypersensitivity. Previous studies have demonstrated that hepatic enzymes can also effect the reduction of SMX-NHOH to SMX (Cribb *et al*., 1995); an NADH-dependent hydroxylamine reductase is responsible.

Finally, there is the question of cellular accumulation. SMX accumulates in white cells, with intracellular levels increasing by up to 130 fold in infected neutrophils compared with resting cells (Climax *et al*., 1986). Intracellular accumulation, together with inhibition of neutrophil function as assessed by inhibition of eugenol-induced GSH depletion and inhibition of oxidative metabolism, may be of importance for the antimicrobial activity of SMX. Previous studies by our group have shown that red blood cells selectively accumulate dapsone hydroxylamine (Tingle & Park, 1993), thus providing an explanation for the cell-selective toxicity observed with this drug; i.e., MetHb and oxidative haemolysis. Indeed, circulating plasma levels of dapsone hydroxylamine cannot be detected (Rhodes *et al*., 1995) and there is little exposure of peripheral white blood cells to this toxic metabolite. By contrast, SMX-NHOH is not taken up avidly by red blood cells. Therefore, the differential uptake by red blood cells of dapsone hydroxylamine may serve to protect against hypersensitivity, and thus provide one reason for the markedly different clinical toxicological profiles of the two structurally related anti-infectives, SMX and dapsone.

In conclusion, we have demonstrated in an *in vitro* model, the propensity of a metabolite of SMX, SMX-NO, to haptenate cell-surface proteins on viable cells. In addition, we have found that under normal physiological conditions a number of processes would serve to prevent such chemical modification of autologous cells, with endogenous thiols playing a key role in drug metabolite detoxication (Figure 10). However, if the normal balance between drug bioactivation and metabolite bioinactivation, both hepatic and peripheral, is disturbed, haptenation of cell surfaces could lead to an immune response. Such observations are of particular relevance to AIDS patients who require treatment with this drug despite its adverse reaction profile, and in whom the detoxication processes of glucuronidation (Esteban *et al*., 1997), acetylation (Lee *et al*., 1993) and reduction by thiols (Naisbitt *et al*., unpublished observation) are known to be impaired. Studies are underway to measure cell haptenation in such patients with, and without, hypersensitivity.

The authors would like to express their thanks to Miss Helen Powell, Dr James Maggs and Dr Stephen Madden for assistance with molecular biology, mass spectrometry and analytical techniques respectively. We also acknowledge the Depts. of Immunology and Veterinary Pathology (The University of Liverpool) for use of their flow cytometers. Anti-SMX IgG antibody was kindly donated by Dr A.E. Cribb (Merck Research Laboratories, West Point, PA, U.S.A.). Thanks are also extended to the Wellcome Trust, the Medical Research Council and the NHS Executive North West (grant RDO/25/19) for funding PhD studentships. B.K.P. is a Wellcome Principal Fellow.

References

AKERBOOM, T.P.M. & SIES, H. (1981). Enzymatic and non-enzymatic assays of glutathione. *Methods in Enzymology,* **77,** 379.

ANGEL, P. & KARIN, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta.,* **1072,** 129–157.

ANONYMOUS (1995). Revised indications for co-trimoxazole (Septrin, Bactrim, various genetic preparations). *Current Problems in Pharmacovigilance,* **21,** 6.

BAEUERLE, P.A. & HENKEL, T. (1994). Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.,* **12,** 141–179.

BLAZKA, M.E., BRUCCOLERI, A., SIMEONOVA, P., GERMOLEC, D.R., PENNYPACKER, K.R. & LUSTER, M.I. (1996). Acetaminophen-induced hepatotoxicity is associated with early changes in AP-1 DNA binding activity. *Res. Comm. Mol. Pathol. Pharmacol.,* **92,** 259–273.

BLAZKA, M.E., GERMOLEC, D.R., SIMEONOVA, P., BRUCCOLERI, A., PENNYPACKER, K.R. & LUSTER, M.I. (1997). Acetaminophen-induced hepatotoxicity is associated with early changes in NF-κB and NF-IL6 DNA binding activity. *J. Inflamm.,* **47,** 138–150.

CARR, A. & COOPER, D.A. (1995). Pathogenesis and management of HIV-associated drug hypersensitivity. *AIDS Clin. Rev.,* **96,** 65–97.

CARR, A., TINDALL, B., PENNY, R. & COOPER, D.A. (1993). *In vitro* cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. *Clin. Exp. Immunol.,* **94,** 21–25.

CLIMAX, J., LENEHAN, T.J., LAMBE, R., KENNY, M., CAFFREY, E. & DAARRAGH, A. (1986). Interaction of antibacterial agents with human peripheral blood leucocytes: Uptake and intracellular localisation of certain sulphonamides and trimethoprims. *J. Antimicrob. Agents Chemother.,* **17,** 489–498.

COTGREAVE, I.A. & MOLDEUS, P. (1986). Methodologies for the application of monobromobimane to the simultaneous analysis of soluble and protein bound thiol components of biological systems. *J. Biochem. Biophys. Methods,* **13,** 231–249.

COX, G. (1995). Glucocorticoid treatment inhibits apoptosis in human neutrophils - separation of survival and activation outcomes. *J. Immunol.,* **154,** 4719–4725.

CRIBB, A.E., LEE, B.L., TREPANIER, L.A. & SPEILBERG, S.P. (1996a). Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials. Clinical syndromes and pathogenesis. *Adv. Drug React. Toxicol. Rev.,* **15,** 9–50.

CRIBB, A.E., MILLER, M., LEEDER, J.S., HILL, J. & SPIELBERG, S.P. (1991). Reactions of nitroso and hydroxylamine metabolites of sulphamethoxazole with reduced glutathione: implications for idiosyncratic toxicity. *Drug. Metab. Dispos.,* **19,** 900–906.

CRIBB, A.E., MILLER, M., TESORO, A. & SPIELBERG, S.P. (1990). Peroxidase-dependent oxidation of sulfonamides by monocytes and neutrophils from humans and dogs. *Mol. Pharmacol.,* **38,** 744–751.

CRIBB, A.E., NUSS, C.E., ALBERTS, D.W., LAMPHERE, D.B., GRANT, D.M., GROSSMAN, S.J. & SPIELBERG, S.P. (1996b). Covalent binding of sulphamethoxazole reactive metabolites to human and rat liver subcellular fractions assessed by immunochemical detection. *Chem. Res. Toxicol.,* **9,** 500–507.

CRIBB, A.E., SPIELBERG, S.P. & GRIFFIN, G.P. (1995). N_4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. *Drug Metab. Dispos.,* **23,** 406–414.

DAFTARIAN, M.P., FILION, L.G., CAMERON, W., CONWAY, B., ROY, R., TROOPER, F. & DIAZ-MITOMA, F. (1995). Immune response to sulphamethoxazole in patients with AIDS. *Clin. Diagnostic Lab. Immunol.,* **2,** 199–204.

ECK, H.P., GMUNDER, H., PETZOLDT, D., DANIEL, V. & DROGE, W. (1989). Low concentrations of acid soluble thiol in the plasma of HIV-1 infected patients. *Biol. Chem. Hoppe-Seyler,* **370,** 101–108.

ELLIS, M.K., HILL, S. & FOSTER, P.M.D. (1992). Reactions of nitrosobenzenes with biological thiols: Identification and reactivity of glutathion-S-yl conjugates. *Chem. Biol. Interact.,* **82,** 151–163.

ESTEBAN, A., PEREZMATEO, M., BOIX, V., GONZALEZ, M., PORTILLA, M. & MORA, A. (1997). Abnormalities in the metabolism of acetaminophen in patients infected with the human immunodeficiency virus (HIV). *Methods Findings Exp. Clin. Pharmacol.,* **19,** 129–132.

GILL, H.J., HOUGH, S.J., NAISBITT, D.J., PIRMOHAMED, M., KITTERINGHAM, N.R. & PARK, B.K. (1997). The relationship between the disposition and the immunogenicity of sulphamethoxazole in the rat. *J. Pharmacol. Exp. Ther.,* **282,** 1375–1382.

GILL, H.J., MAGGS, J.L., MADDEN, S., PIRMOHAMED, M. & PARK, B.K. (1996). The effect of fluconazole and ketoconazole on the metabolism of sulphamethoxazole. *Br. J. Clin. Pharmacol.,* **42,** 347–353.

GOTOH, Y., SUMIMOTO, H. & MINAKAMI, S. (1989). Formation of 20-oxoleukotrine B4 by an alcohol dehydrogenase isolated from human neutrophils. *Biochim. Biophys. Acta.,* **1043,** 52–56.

HARRISON, J.H. & JOLLOW, D.J. (1986). Role of aniline metabolites in aniline-induced haemolytic anaemia. *J. Pharmacol. Exp. Ther.,* **238,** 1045–1054.

HASTINGS, K.L., THOMAS, C., BROWN, A.P. & GANDOLFI, A.J. (1995). Trifluoroacetylation potentiates the humoral response to halothane in the guinea pig. *Immunopharm. Immunotox.,* **17,** 201–213.

HELBLING, B., VON OVERBECK, J. & LAUTERBURG, B.H. (1996). Decreased release of glutathione into the systemic circulation of patients with HIV infection. *Eur. J. Clin. Invest.,* **26,** 38–44.

HORIE, S. & OGURA, Y. (1980). Studies on the enzymatic reduction of C-nitroso compounds. IV. Partial purification and kinetic properties of porcine heart C-nitrosoreductase. *J. Biochem.,* **88,** 1141–1150.

KOOPMANS, P.P., VAN DER VEN, A.J.A.M., VREE, T.B. & VAN DER MEER, J.W.M. (1995). Pathogenesis of hypersensitivity reactions to drugs in patients with HIV infection: allergic or toxic? *AIDS,* **9,** 217–222.

KOSOWER, N.S. & KOSOWER, E.M. (1976). Functional aspects of glutathione disulphides and hidden forms of glutathione, In *Glutathione: Metabolism and Function*. ed. Arias, I.M. & Jakoby, W.B. pp. 159–174, Raven Press, New York.

KUBAL, G., MEYER, D.J., NORMAN, R.E. & SADLER, P.J. (1995). Investigations of glutathione conjugation *in vitro* by 1H NMR spectroscopy. Uncatalysed and glutathione transferase-catalysed reactions. *Chem. Res. Toxicol.,* **8,** 780–791.

KUWADA, M., HORIE, S. & OGURA, Y. (1980). Studies on the enzymatic reduction of C-nitroso compounds. II. Multiple forms of liver C-nitrosoreductase and the identity with alcohol dehydrogenase. *J. Biochem.,* **88,** 859–869.

LAWRENCE, D.A., SONG, R. & WEBER, P. (1997). Surface thiols of human lymphocytes and their changes after *in vitro* and *in vivo* activation. *J. Leukoc. Biol.,* **60,** 611–618.

LEE, B.L., WONG, D., BENOWITZ, N.L. & SULLAM, P.M. (1993). Altered patterns of drug metabolism in patients with acquired immunodeficiency syndrome. *Clin. Pharmacol. Ther.,* **53,** 529–535.

MANDELL, G.L. & SANDE, M.A. (1985). Anti-microbial agents: Sulfonamides, trimethoprim-sulfamethoxazole and agents for urinary tract infections, In *The Pharmacological Basis of Therapeutics*. ed Gilman, A.G., Goodman, L.S., Rall, T.W. & Marud, R. pp. 1095–1114, Macmillan Publishing Co., New York.

MAURI-HELLWEG, D., BETTENS, F., MAURI, D., BRANDER, C., HUNZIKER, T. & PICHLER, W.J. (1995). Activation of drug specific CD4+ and CD8+ T cells in individuals allergic to sulphonamides, phenytoin, and carbamazepine. *J. Immunol.,* **155,** 462–472.

MEEKINS, C.V., SULIVAN, T.J. & GRUCHALLA, R.S. (1994). Immunological analysis of sulphonamide drug allergy: identification of sulphamethoxazole substituted human serum proteins. *J. Allergy Clin. Immunol.,* **94,** 1017–1024.

MILLS, B.J. & LANG, C.A. (1996). Differential distribution of free and bound glutathione and cyst(e)ine in human blood. *Biochem. Pharmacol.,* **52,** 401–406.

MITRA, A.K., THUMMEL, K.E., KALHORN, T.F., KHARASCH, E.D., UNADKAT, J.D. & SLATTERY, J.T. (1996). Inhibition of sulfamethoxazole hydroxylamine formation by fluconazole in human liver microsomes and healthy volunteers. *Clin. Pharmacol. Therap.,* **59,** 332–340.

MURTY, V.S. & PENNING, T.M. (1992). Polycyclic aromatic hydrocarbon (PAH) ortho-quinone conjugate chemistry: kinetics of thiol addition of PAH ortho-quinones and structures of thioether adducts of naphthalene-1,2-dione. *Chem. Biol. Interact.,* **84,** 169–188.

NAISBITT, D.J., O'NEILL, P.M., PIRMOHAMED, M. & PARK, B.K. (1996). Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: Implications for immune-mediated toxicity. *Bioorg. Med. Chem. Let.,* **6,** 1511–1516.

NAISBITT, D.J., RUSCOE, J.E., WILLIAMS, D.P., O'NEILL, P.M., PIRMOHAMED, M. & PARK, B.K. (1997). Disposition of amodiaquine and related antimalarials in human neutrophils: Implications for drug design. *J. Pharmacol. Exp. Ther.,* **280,** 884–893.

NICOLETTI, I., MIGLIORATI, G., PAGLIACCI, M.C., GRIGNANI, F. & RICARDI, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods,* **139,** 271–276.

PARK, B.K., PIRMOHAMED, M. & KITTERINGHAM, N.R. (1998). The role of drug disposition in drug hypersensitivity: A chemical, molecular and clinical perspective. *Chem. Res. Toxicol.,* **11,** 969–988.

PIRMOHAMED, M., COLEMAN, M.D., HUSSAIN, F., BRECKENRIDGE, A.M. & PARK, B.K. (1991). Direct and metabolism dependent toxicity of sulphasalazine and its principal metabolite towards human erythrocytes and leucocytes. *Br. J. Clin. Pharmacol.,* **32,** 303–310.

PIRMOHAMED, M. & PARK, B.K. (1995). Drug reactions in HIV-infected patients. *Postgrad. Doctor,* **18,** 438–444.

PIRMOHAMED, M., WILLIAMS, D.P., TINGLE, M.D., BARRY, M., KHOO, S., O'MAHONY, C., WILKINS, E., BRECKENRIDGE, A.M. & PARK, B.K. (1996). Intracellular glutathione in the peripheral blood cells of HIV-infected patients: failure to show a deficiency. *AIDS,* **10,** 501–507.

RAFFRAY, M. & COHEN, G.M. (1997). Apoptosis and necrosis in toxicology: A continuum or distinct modes of cell death? *Pharmacol. Ther.,* **75,** 153–177.

RHODES, L.E., TINGLE, M.D., PARK, B.K., CHU, P., VERBOV, J.L. & FRIEDMANN, P.S. (1995). Cimetidine improves the therapeutic toxic ratio of dapsone in patients on chronic dapsone therapy. *Br. J. Dermotol.,* **132,** 257–262.

RIEDER, M.J., KRAUSE, R. & BIRD, I.A. (1995a). Time-course of toxicity of reactive sulphonamide metabolites. *Toxicology,* **95,** 141–146.

RIEDER, M.J., KRAUSE, R., BIRD, I.A. & DEKABAN, G.A. (1995b). Toxicity of sulphonamide-reactive metabolites in HIV-infected, HTLV-infected and non-infected cells. *J. Acq. Imm. Defic. Synd. Hum. Retrovirol.,* **8,** 134–140.

RIEDER, M.J., UETRECHT, J.P., SHEAR, N.H., CANNON, M., MILLER, M. & SPIELBERG, S.P. (1989). Diagnosis of sulphonamide hypersensitivity reactions by *in vitro* rechallenge with hydroxylamine metabolites. *Ann. Intern. Med.,* **110,** 286–289.

RIEDER, M.J., UETRECHT, J.P., SHEAR, N.H. & SPIELBERG, S.P. (1988). Synthesis and *in vitro* toxicity of hydroxylamine metabolites of sulphonamides. *J. Pharmacol. Exp. Ther.,* **244,** 724–728.

SAIAG, P., CAUMES, E., CHOSIDOW, O., REVUZ, J. & ROUJEAU, J.C. (1992). Drug-induced toxic epidermal necrolysis (Lyell syndrome) in patients infected with the human-immunodeficiency-virus. *J. Am. Acad. Dermatol.,* **26,** 567–574.

SCHNYDER, D., MAURI-HELLWEG, D., ZANNI, M.P., BETTENS, F. & PICHLER, W.J. (1997). Direct MHC-dependent presentation of the drug sulphamethoxazole to human $\alpha\beta$ T cell clones. *J. Clin. Invest.,* **100,** 136–141.

SEARLE, J., KERR, J.F.R. & BISHIP, C.J. (1982). Necrosis and apoptosis: Distinct modes of cell death with fundamentally different significance. *Pathol. Annu.,* **17,** 229–259.

SHEAR, N.H., SPIELBERG, S.P., GRANT, D.M., TANG, B.K. & WILLOW, W. (1986). Differences in the metabolism of sulphonamides predisposing to idiosyncratic toxicity. *Ann. Intern. Med.,* **105,** 179–184.

SJOEBERG, B., SUMERSKA, T., BINNS, R.M. & BALFOUR, B.M. (1978). Contact sensitivity in the pig. II. Induction by intralymphatic infusion of DNP-conjugated cells. *Int. Arch. Allergy Appl. Immunol.,* **57,** 114–125.

SMITH, G.H. (1994). Treatment of infections in the patient with acquired immunodeficiency syndrome. *Arch. Intern. Med.,* **154,** 949–973.

SOMERVILLE, C.C., NISHINO, S.F. & SPAIN, J.C. (1995). Purification and characterisation of nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.,* **177,** 3837–3842.

STAAL, F.J.T., ELA, S.W., ROEDERER, M., ANDERSON, M.T., HERZENBERG, L.A. & HERZENBERG, L.A. (1992). Glutathione deficiency and human immunodeficiency virus infection. *Lancet,* **339,** 909–912.

THOMPSON, D., CONSTANTIN-TEODOSIU, D., NORBECK, K., SVENSSON, B. & MOLDEUS, P. (1989). Metabolic activation of eugenol by myeloperoxidase and polymorphonuclear leukocytes. *Chem. Res. Toxicol.,* **2,** 186–192.

TINGLE, M.D. & PARK, B.K. (1993). The use of a three compartment in vitro model to investigate the role of hepatic drug metabolism in drug-induced blood dyscrasias. *Br. J. Clin. Pharmacol.,* **36,** 31–39.

TSCHACHLER, E., BERGSTRESSER, P.R. & STINGL, G. (1996). HIV related skin diseases. *Lancet,* **348,** 659–663.

UETRECHT, J.P. (1992). The role of leukocyte generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions. *Drug Metab. Rev.,* **24,** 299–366.

WALMSLEY, S.L., WINN, L.M., HATTISON, M.L., UETRECHT, J.P. & WELLS, P.G. (1997). Oxidative stress and thiol depletion in plasma and peripheral blood lymphocytes from HIV-infected patients: toxicological and pathological implications. *AIDS,* **11,** 1689–1697.

WATSON, R.W.G., REDMOND, H.P., WANG, J.H. & BOUCHIER-HAYES, D. (1996a). Mechanisms involved in sodium arsenite-induced apoptosis of human neutrophils. *J. Leukoc. Biol.,* **60,** 625–632.

WATSON, R.W.G., REDMOND, H.P., WANG, J.H., CONDRON, C. & BOUCHIER-HAYES, D. (1996b). Neutrophils undergo apoptosis following ingestion of *Escherichia coli*. *J. Immunol.,* **156,** 3986–3992.

(Received July 2, 1998
Revised October 9, 1998
Accepted January 7, 1999)