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Comparison of the *In Vitro* Cytotoxicity of Hydroxylamine Metabolites of Sulfamethoxazole and Dapsone*

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ABSTRACT. The differential incidence of adverse drug reactions (ADR) between trimethoprim-sulfamethoxazole and dapsone might be explained, in part, by differences in the inherent toxicity of the hydroxylamine metabolites of sulfamethoxazole and dapsone. To test this hypothesis, the *in vitro* cytotoxicities of sulfamethoxazole hydroxylamine, dapsone hydroxylamine, and monoacetyldapsone hydroxylamine were compared using peripheral blood mononuclear cells (PBMC) from healthy volunteers. After 3 hr of exposure to hydroxylamine metabolites, PBMC were washed thoroughly to remove residual hydroxylamine, and viability was assessed 16 hr later by determination of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion. A concentration-dependent toxicity was observed with each hydroxylamine metabolite. While dapsone hydroxylamine and monoacetyldapsone hydroxylamine were not significantly different, both showed significantly greater cytotoxic potency than sulfamethoxazole hydroxylamine ($P < 0.05$). This differential potency was not a function of differential stability in aqueous medium and was maintained over time. The effects of red blood cells (RBC), impermeable RBC "ghosts," and RBC lysate on hydroxylamine-induced cytotoxicity were determined using a two-compartment dialysis system. Amelioration of hydroxylamine-dependent cytotoxicity occurred when RBC were included in PBMC incubations. This apparent detoxifying effect was markedly greater using RBC lysate in comparison with impermeable "ghosts" ($P < 0.05$). No difference in detoxification was observed between sulfamethoxazole hydroxylamine and monoacetyldapsone hydroxylamine. Differences in the inherent cytotoxicity of their hydroxylamine metabolites do not appear to explain the differential incidence of ADR between trimethoprim-sulfamethoxazole and dapsone. BIOCHEM PHARMACOL **55**;6:803–810, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. hypersensitivity; trimethoprim-sulfamethoxazole; dapsone; *Pneumocystis carinii* pneumonia; hydroxylamine metabolites; *in vitro* cytotoxicity

Trimethoprim-sulfamethoxazole therapy is associated with a high frequency of adverse effects [1], which complicate its use in PCP§ prophylaxis/treatment in AIDS patients. The most serious trimethoprim-sulfamethoxazole-related ADR are the idiosyncratic or hypersensitivity reactions manifested as fever, rash, and sometimes whole organ complications [2]. Interestingly, there is a higher incidence of these ADR in HIV-infected subjects [3, 4], which does not appear to be secondary to PCP or general immunosuppression [5].

While the pathogenesis of these ADR remains unclear, evidence suggests a critical role for bioactivation [6]. Sulfamethoxazole is metabolized primarily by N-acetylation to a nontoxic species, but may also undergo oxidative metabolism to a reactive hydroxylamine. A metabolic imbalance between bioactivation and inactivation pathways has, therefore, been suggested as a predisposing factor in the development of ADR [7]. Indeed, the prevalence of phenotypically slow acetylators has been demonstrated to be higher in sulfonamide-hypersensitive patients [8, 9], in whom a greater fraction of sulfamethoxazole is metabolized by cytochrome P450 to its hydroxylamine metabolite [7, 10, 11]. A role for sulfamethoxazole hydroxylamine in initiating the cascade of events leading to hypersensitivity is supported by the observation that this hydroxylamine causes higher *in vitro* cytotoxicity towards PBMC from sulfonamide-hypersensitive patients than in individuals able to tolerate trimethoprim-sulfamethoxazole [12]. Moreover, Carr *et al.* [13] used a prospective analysis to show higher levels of PBMC cytotoxicity in seven HIV-infected patients who subsequently developed ADR to trimethoprim-sulfamethoxazole, than in five matching sub-

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§ *Abbreviations:* PCP, *Pneumocystis carinii* pneumonia; ADR, adverse drug reactions; PBMC, peripheral blood mononuclear cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RBC, red blood cells; and LC_{50}, concentration causing 50% cytotoxicity.

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jects who developed no hypersensitivity upon the initiation of therapy.

This evidence suggests that the proximate toxin mediating ADR development is the hydroxylamine metabolite, possibly through its spontaneous auto-oxidation to a more reactive nitroso species [14–16]. While a number of other factors certainly play an important role, formation of sulfamethoxazole hydroxylamine may be a major predisposing factor. If the hydroxylamine metabolite is truly the proximate toxin, then arylamines giving rise to the largest amount of hydroxylamine should produce the greatest frequency of adverse effects.

Dapsone is an alternative agent used for PCP therapy in AIDS patients [4, 17] that is also associated with a high incidence of ADR [18, 19]. Approximately 20–35% is excreted as dapsone hydroxylamine [20], and an unknown fraction as monoacetyldapsone hydroxylamine. In contrast, only 3% of a sulfamethoxazole dose is excreted as its hydroxylamine metabolite [11, 12, 21]. However, despite this 10-fold excess of hydroxylamine in comparison with sulfamethoxazole, dapsone therapy is associated with a significantly *lower* incidence of ADR [4, 22]. For example, Medina *et al.* [4] reported a 57% incidence of ADR with trimethoprim-sulfamethoxazole, but only a 30% incidence with trimethoprim-dapsone. Though sulfamethoxazole forms a smaller amount of hydroxylamine, it actually produces a higher frequency of ADR.

The differential incidence of ADR between trimethoprim-sulfamethoxazole and dapsone presents a unique paradigm in which to investigate factors contributing to the occurrence of these hypersensitivity reactions. The observed disparity might result from differences in the inherent toxicity of reactive metabolites or, alternatively, from differences in detoxification. The studies described herein provide a direct comparison of the *in vitro* toxicity of sulfamethoxazole hydroxylamine, dapsone hydroxylamine, and monoacetyldapsone hydroxylamine, which may provide insight into the pathogenesis of these hypersensitivity reactions.

MATERIALS AND METHODS

Materials

Accuspin™ System Histopaque®-1077 was obtained from SIGMA Diagnostics, and Spectra/Por® 1.1 Biotech Sterile Membrane was obtained from SPECTRUM. Dapsone hydroxylamine and monoacetyldapsone hydroxylamine were synthesized as described previously [23]. Sulfamethoxazole hydroxylamine was synthesized as described by Rieder *et al.* [24]. Product identity was confirmed for each hydroxylamine by NMR and i.r. spectroscopy. Purity, determined by HPLC, was found to be >97%. Sulfamethoxazole, dapsone, sulfamethazine, HEPES, BSA, MTT, and DMSO were all obtained from the Sigma Chemical Co. Remaining chemicals were purchased from Sigma, Fisher Chemical, or the J. T. Baker Chemical Co. Solvents obtained from Fisher Chemical and Curtain Matheson Scientific were of HPLC grade.

Study Subjects

Normal volunteers were recruited from the staff and students of the Department of Pharmaceutical Sciences at Wayne State University. Written informed consent was obtained from all volunteers prior to collecting 60 mL of whole blood into heparin-containing Vacutainer® tubes. The study was approved by the Human Investigation Committee of Wayne State University.

Stability of Hydroxylamines in HEPES Medium

The stability of sulfamethoxazole hydroxylamine and the stability of dapsone hydroxylamine (750 μM) in HEPES medium (pH 7.4) were compared using the HPLC method of Coleman *et al.* [25]. Briefly, 25-μL samples of sulfamethoxazole hydroxylamine or dapsone hydroxylamine in HEPES medium were injected onto the HPLC at regular time intervals over 7 hr. Elution was conducted on a Waters μBondapak C_{18} column using a mobile phase of water: acetonitrile:acetic acid:triethylamine (80:20:1:0.05) at a flow rate of 1.2 mL/min. Sulfamethazine was used as an internal standard, and UV absorbance at 254 nm was quantified using an electronic integrator (Hewlett Packard 3396A). Metabolite half-life ($T_{1/2}$) was calculated by linear regression of log peak area ratio versus time data where $T_{1/2} = 0.693/k_d$, and k_d is the first order rate constant for degradation.

Isolation of PBMC

PBMC were isolated from heparinized whole blood by centrifugation over Histopaque®-1077 according to the manufacturer's instructions (SIGMA Diagnostics). Following three washes in PBS (pH 7.4), PBMC were suspended in HEPES medium and then centrifuged over 20% sucrose (300 *g*; 10 min) to remove platelets. After an additional two washes in PBS, PBMC were suspended in HEPES medium, and cell counts and viability were assessed by trypan blue dye exclusion.

In Vitro *Cytotoxicity*

The toxicities of sulfamethoxazole hydroxylamine, dapsone hydroxylamine, and monoacetyldapsone hydroxylamine toward PBMC were compared using the *in vitro* assay described previously [7, 26]. Following the distribution of PBMC in HEPES medium to microplate wells (4.0×10^5/well), hydroxylamines in DMSO were added to give final concentrations ranging from 0 to 4000 μM (200 μL volume/well). Each concentration was tested in quadruplicate, and DMSO was maintained at <4% (v/v), including controls. After incubation for 3 hr at 37°/5% CO_2, PBMC were washed three times using HEPES medium with BSA

(5 mg/mL). Supernatant was removed entirely each time to eliminate residual metabolite. PBMC in HEPES medium with BSA were then incubated for an additional 16 hr before assessment of viability via the MTT assay (described below).

Time Course of Hydroxylamine-Dependent Cytotoxicity

Exposure to hydroxylamine metabolites (1500 μM) and cell washes were performed as described above. Subsequently, cell viability was determined at 2, 5, 10, and 20 hr by comparing MTT conversion in test wells to controls. Separate microplates, each with independent controls, were used for every time point. All incubations were performed in quadruplicate.

Preparation of Impermeable RBC "Ghosts"

To assess the detoxifying contribution of RBC lysate versus membrane uptake, impermeable RBC "ghosts" were prepared by a modification of the procedure of Steck and Kant [27]. Briefly, RBC pelleted from whole blood were washed three times in PBS. Aliquots of RBC were taken for either dilution in HEPES medium (50% hematocrit) or hemolysis in distilled water (1:1, v/v), the lysate being collected after centrifugation (2300 *g*; 10 min). The remainder of packed RBC were mixed thoroughly in 5 mM sodium phosphate (pH 8.0) containing 1 mM magnesium sulfate at 4°. Under these conditions, RBC hemolyze but then spontaneously "reseal," retaining only a portion of their cytoplasmic contents [27]. After centrifugation at 22,000 *g* for 10 min, the "ghost" pellet was washed twice in PBS and then diluted to 50% hematocrit in HEPES medium. Visual inspection revealed that hemoglobin was still present, although in significantly diminished quantities.

Effects of RBC, Impermeable "Ghosts," and RBC Lysate on PBMC Cytotoxicity

The detoxifying effects of RBC, "ghosts," and lysate were determined using a two-compartment *in vitro* system described previously [28, 29]. Briefly, semi-permeable cellulose dialysis membrane (molecular weight cut-off, 8000) was used to separate two dialysis apparatus chambers. Side A contained PBMC plus the appropriate hydroxylamine, while side B contained only PBMC, RBC, "ghosts," or lysate in HEPES medium. A total dialysis apparatus volume of 700 μL, containing <4% DMSO (v/v), was used to determine the hydroxylamine concentration. The dialysis apparatus was incubated in a 37° water bath rotating at 8 rpm for 3 hr. PBMC were subsequently removed from dialysis chambers and washed 2–3 times in PBS, and then aliquots were placed into microplate wells to give eight replicates per condition. After overnight incubation, MTT conversion was assessed as described below.

MTT Assay for Cell Viability

Cell viability was determined by modification of the mitochondrial dehydrogenase MTT assay described previously [30], wherein only metabolically active cells are able to catalyze the reduction of MTT to yield an insoluble, colored formazan product. Optimization of the assay protocol provided the following conditions: After overnight incubation of PBMC, wells were replenished with fresh medium, and 20-μL aliquots of MTT (5 mg/mL) were added to each well. Previous reports of the susceptibility of the assay to pH changes and interfering agents [31] necessitated this modification. Following a 3-hr incubation at 37°/5% CO_2, unreacted MTT was removed to prevent hindrance of formazan solubilization in DMSO (200 μL/well) [32]. After plate mixing to ensure complete solubility, optical density was read at 560 nm using a V_{max} kinetic microplate reader (Molecular Devices). Then cytotoxicity was calculated as the percent reduction in absorbance (minus background) relative to controls.

Statistical Analysis

Discordant data in repeat determinations of MTT conversion were identified and rejected using established parameters of the Q-test [33]. Results are presented as means ± SD where appropriate. Statistical significance was determined using either paired *t*-tests or ANOVA, pairwise comparisons being performed according to the Student–Newman–Keuls method. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Preliminary investigation of hydroxylamine-induced cytotoxicity revealed considerable interference with the MTT assay caused by residual hydroxylamine. When MTT was incubated with sulfamethoxazole hydroxylamine or dapsone hydroxylamine in the absence of PBMC, a concentration-dependent increase in optical density was observed (Fig. 1). Reaction of hydroxylamines with MTT caused a nonenzymatic reduction to the colored product, thereby yielding high optical density values that interfere with assay results. Thus, for all subsequent experiments, repeat washing was necessary to ensure removal of all residual hydroxylamine.

Comparison of the cytotoxicity induced by sulfamethoxazole hydroxylamine, dapsone hydroxylamine, and monoacetyldapsone hydroxylamine was performed using PBMC isolated from five normal volunteers (three Caucasian men, one Asian man, one Caucasian woman; age range, 23–31 years). A concentration-dependent increase in toxicity was observed with each metabolite, sulfamethoxazole hydroxylamine being significantly less potent in every instance. Solubility limitations prevented determination of maximal cytotoxicity; therefore, data from 20–80% cytotoxicity were analyzed by linear regression to determine LC_{50} values

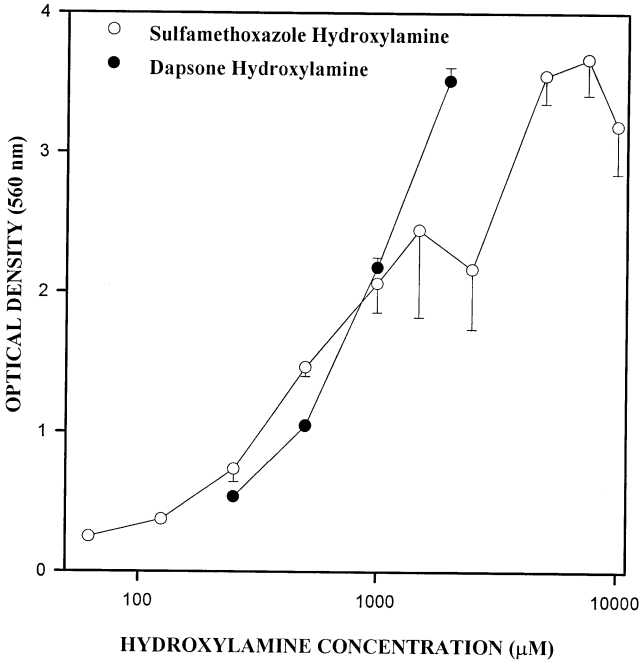

FIG. 1. Effect of increasing concentrations of sulfamethoxazole hydroxylamine and dapsone hydroxylamine on MTT conversion in the absence of PBMC. Optical density values at 560 nm are indicative of MTT conversion to a colored product. Data points represent the means ± SD of triplicate determinations.

for each subject (Table 1). Despite the wide inter-individual variability, a within subjects pairwise comparison (Student–Newman–Keuls method) showed a significant difference between the hydroxylamine metabolites of dapsone and sulfamethoxazole hydroxylamine, but not between dapsone hydroxylamine and monoacetyldapsone hydroxylamine ($P < 0.05$). To determine if this difference was a consequence of differential degradation of hydroxylamine metabolites, the stability of sulfamethoxazole hydroxylamine and dapsone hydroxylamine in HEPES medium was analyzed by HPLC. Half-lives of 96 and 108 min were determined for dapsone hydroxylamine and sulfamethoxazole hydroxylamine, respectively. Thus, differential stability in buffer does not appear to contribute significantly to the observed differences in cytotoxic potency.

We also investigated the time course of cytotoxicity to determine the validity of assessing PBMC viability 16 hr post-removal of residual hydroxylamine. After exposure to 1500 μM sulfamethoxazole hydroxylamine, dapsone hydroxylamine, or monoacetyldapsone hydroxylamine, PBMC cytotoxicity was determined over time. At each interval, the hydroxylamine metabolites of dapsone caused greater cytotoxicity than sulfamethoxazole hydroxylamine (Fig. 2A). Similar differences were also observed at time points extended to 24 hr (data not shown). Interestingly, we observed an increase in optical density values from both experimental and control cells over time. This led us to hypothesize that PBMC may require a period of recovery, after isolation, but before hydroxylamine exposure, as a means to stabilize enzyme activity assessed in the MTT assay. Thus, in an adaptation of the previous time course study, PBMC were isolated, allowed to recover overnight (16 hr), and then exposed to 1500 μM concentrations of hydroxylamine before assessment of viability over time (Fig. 2, B and C). While percent cytotoxicity levels were fairly stable over time (Fig. 2B), analysis of mean optical density values showed that MTT conversion by controls increased to a greater degree than did PBMC exposed to hydroxylamines (Fig. 2C). Again, sulfamethoxazole hydroxylamine showed lower cytotoxicity than dapsone hydroxylamine or monoacetyldapsone hydroxylamine.

An alternative mechanism that may contribute to the differential sensitivity between sulfamethoxazole and dapsone is a difference in detoxification of reactive metabolites. For instance, previous studies have shown that RBC may detoxify dapsone hydroxylamine [34]. Whether a similar protective effect occurs with sulfamethoxazole hydroxylamine is unknown. Thus, we also assessed the effect of RBC on hydroxylamine-induced *in vitro* cytotoxicity using a bicompartmental dialysis system. Inclusion of RBC in PBMC incubations with hydroxylamines resulted in a marked decrease in cytotoxicity (Fig. 3). Pairwise comparison (paired *t*-test) of cytotoxicity values showed a significant difference between sulfamethoxazole hydroxylamine and sulfamethoxazole hydroxylamine with RBC ($P < 0.02$) and a borderline difference between monoacetyldap-

TABLE 1. LC_{50} values calculated from linear regression analysis of log concentration versus percent cytotoxicity curves (20–80% toxicity) generated for each subject

	LC_{50} (μM)		
Subject No.	Dapsone hydroxylamine	Monoacetyldapsone hydroxylamine	Sulfamethoxazole hydroxylamine
1	242	115	686
2	231	327	1722
3	332	441	1639
4	162	280	766
5	659	1795	3946
Geometric mean	288	384	1424
Arithmetic mean ± SD	325 ± 196	592 ± 683	1752 ± 1317

Sulfamethoxazole hydroxylamine LC_{50} was significantly different from both dapsone hydroxylamine and monoacetyldapsone hydroxylamine ($P < 0.05$). The hydroxylamine metabolites of dapsone were not significantly different from one another.

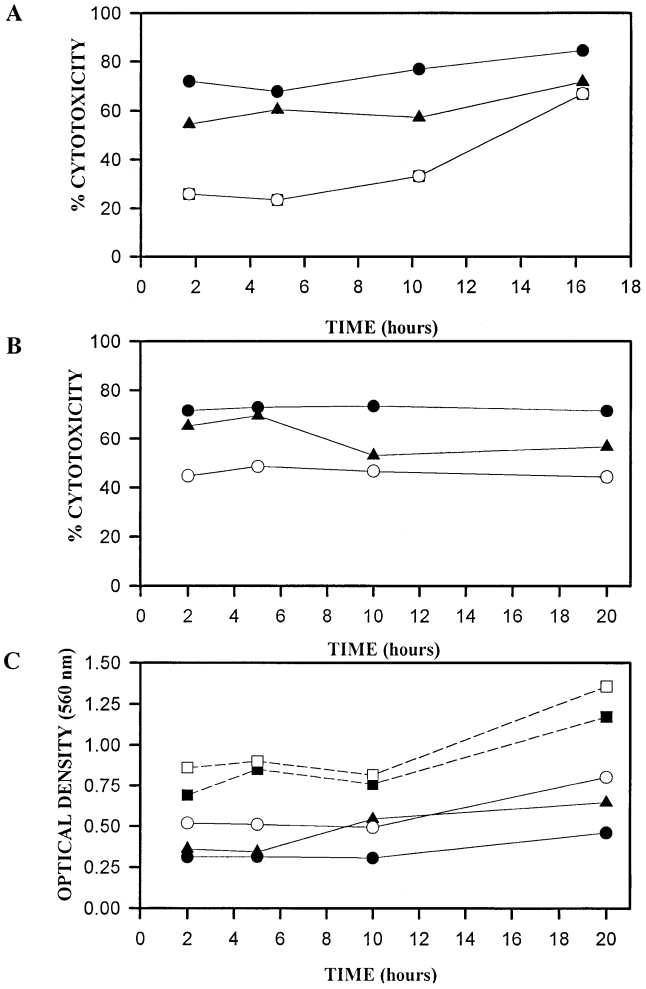

FIG. 2. Time course of *in vitro* cytotoxicity caused by sulfamethoxazole hydroxylamine (○), dapsone hydroxylamine (●), and monoacetyldapsone hydroxylamine (▲). After isolation, PBMC were either (A) immediately exposed to hydroxylamines (1500 μM, 3 hr) and then viability was assessed over time, or (B and C) incubated for 16 hr before hydroxylamine exposure and viability assessment at 2, 5, 10, and 20 hr. Incubations at all time points were performed in quadruplicate using separate microplates, each containing their own control, and viability was assessed by MTT conversion. Results presented as percent cytotoxicity (A and B) were calculated as the percent reduction in absorbance relative to controls. Raw optical density values (C) simply indicate the amount of colored formazan product. Open and closed squares (□, ■) represent mean optical density values for two independent controls.

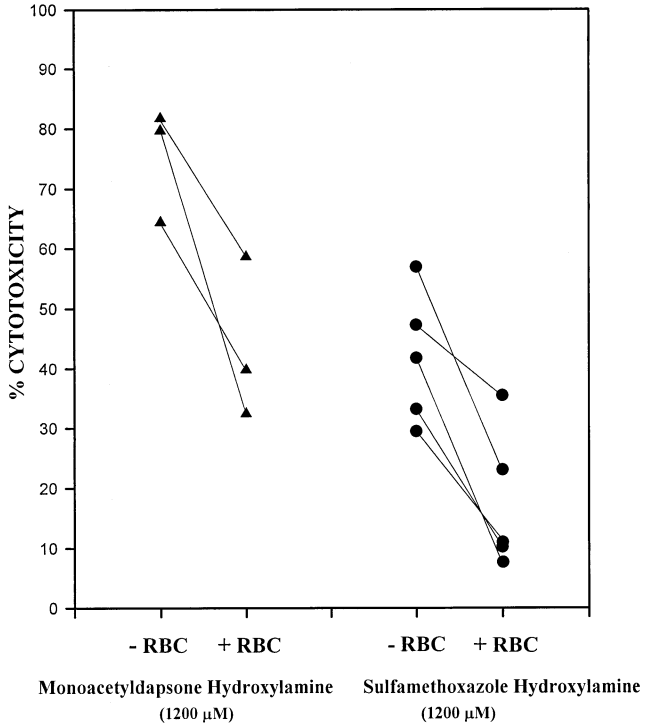

FIG. 3. Effect of red blood cells (RBC) on hydroxylamine-dependent *in vitro* cytotoxicity. Viability was assessed by MTT conversion, and cytotoxicity values were calculated from the percent reduction in optical density values relative to control. Data are presented as the means of eight replicates using PBMC from normal volunteers.

sone hydroxylamine and monoacetyldapsone hydroxylamine with RBC ($P = 0.056$). Using mean optical density values, a significant difference was determined in both instances ($P < 0.05$, data not shown). As observed previously, the difference in cytotoxicity between sulfamethoxazole hydroxylamine and monoacetyldapsone hydroxylamine was also statistically significant ($P < 0.05$). Importantly, no significant difference in cytotoxicity existed between PBMC separated by the dialysis membrane, indicating that the metabolites originally added to only one chamber were able to rapidly traverse through the membrane, equilibrate throughout both chambers, and maintain their cytotoxic potency.

To assess whether the detoxifying function of RBC was a consequence of simple hydroxylamine uptake into RBC, or redox cycling with intracellular components, we also compared the ability of intact RBC to counteract sulfamethoxazole hydroxylamine-induced toxicity to that of impermeable RBC "ghosts" and RBC lysate (Fig. 4). Co-incubation with lysate had the most pronounced detoxifying effect, causing a 53.5 ± 26.6% increase in optical density in comparison with PBMC exposed to sulfamethoxazole hydroxylamine alone. This increase in MTT conversion was significantly greater than PBMC co-incubated with "ghosts" (15.3 ± 16.4% increase, $P < 0.05$), but not PBMC co-incubated with RBC (38.6 ± 17.3% increase).

DISCUSSION

The unpredictable nature of idiosyncratic hypersensitivity reactions to trimethoprim-sulfamethoxazole poses a serious therapeutic dilemma in the treatment of AIDS patients with PCP. While evidence suggests that bioactivation to reactive species occurs prior to any adverse effect, the pathogenesis of these reactions remains unclear [6]. Understanding the mechanism of these ADR should allow determination of specific factors that might predispose individuals to an ADR.

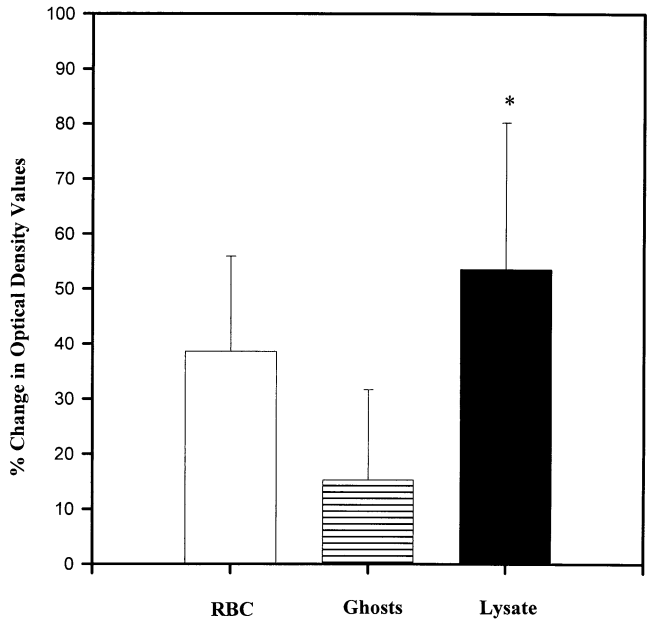

FIG. 4. Comparison of the effects of RBC, impermeable RBC "ghosts," and RBC lysate on the *in vitro* cytotoxicity of PBMC from healthy volunteers (N = 5). PBMC separated from RBC, "ghosts," and lysate by semi-permeable dialysis membrane were exposed to 1200 μM sulfamethoxazole hydroxylamine for 3 hr. After a 16-hr recovery period and 3-hr incubation with MTT, the percent change in optical density values was calculated relative to PBMC exposed to sulfamethoxazole hydroxylamine alone. The results represent the means ± SD of five individuals, where eight replicates were performed for incubations with each individual. Note that negative percent change values attributed to assay variability were included as 0% to prevent their disproportionate contribution to mean value calculations. Key: (*) significantly different from "ghosts", $P < 0.05$.

Despite the similar metabolic fates of sulfamethoxazole and dapsone, the fact that they are associated with a differential incidence of ADR [4] provides an interesting model in which to probe the role of hydroxylamine metabolites in adverse reactions to these drugs. Using *in vitro* exposure to PBMC as a model system [7, 26], a number of investigators have documented that the *in vitro* cytotoxicity of sulfamethoxazole hydroxylamine is associated with the occurrence of hypersensitivity *in vivo* [12, 13]. This suggests that sulfamethoxazole hydroxylamine plays a role in ADR development. Moreover, the hydroxylamine metabolites of dapsone are also known to cause *in vitro* cytotoxicity [25, 34]. We, therefore, hypothesized that differences in the inherent toxicity of sulfamethoxazole and dapsone hydroxylamine metabolites might help explain the differential incidence of ADR.

Determination of cell viability by analysis of MTT conversion has shown good correlation with other methods [24]. However, initial investigations demonstrated the necessity for optimization of the assay. In particular, we found that residual hydroxylamine was capable of converting MTT to a colored product (Fig. 1), thereby giving a false indication of cell viability. It was essential, therefore, to wash all hydroxylamine from incubations, replenishing cells with fresh medium to stabilize pH as described previously [31].

Using PBMC from normal individuals, dapsone hydroxylamine and monoacetyldapsone hydroxylamine were found to have significantly greater cytotoxic potency than sulfamethoxazole hydroxylamine (Table 1). Preliminary studies using the membrane-impermeant nucleic acid dye YO-PRO-1 [35] as a measure of hydroxylamine-induced cytotoxicity demonstrated a similar difference in cytotoxicity between these metabolites (unpublished data). This indicates that the difference in cytotoxic potency is not simply a function of a differential effect on mitochondrial dehydrogenase activity, but rather a true difference in the incidence of cell death. This observed difference is actually the opposite of the *in vivo* situation where sulfamethoxazole is associated with a higher incidence of ADR [4]. The lack of significant difference between dapsone hydroxylamine and monoacetyldapsone hydroxylamine supports previous findings where they were shown to be equitoxic in their formation of methemoglobin [23]. Levels of cytotoxicity caused by sulfamethoxazole hydroxylamine also agree with previous reports [13, 36], although Rieder *et al.* [24] has reported as high as 62% toxicity at 400 μM concentrations, in contrast to approximately 20–25% toxicity observed in our studies. Reasons for this discrepancy are unclear but may reflect differences in experimental conditions or, alternatively, the differential susceptibility of cells isolated from different populations.

To determine if the instability of hydroxylamine metabolites contributed to this differential potency *in vitro*, we compared the degradation of sulfamethoxazole hydroxylamine and dapsone hydroxylamine in HEPES medium over time. Previous studies in our laboratory have shown no difference in stability between dapsone hydroxylamine and monoacetyldapsone hydroxylamine [23]. Similarly, no significant difference in degradation half-life between sulfamethoxazole hydroxylamine and dapsone hydroxylamine was found. Therefore, while a similar *in vitro* comparison must be performed using PBMC from sulfonamide hypersensitive subjects, these data suggest that the differential incidence of ADR between dapsone and trimethoprim-sulfamethoxazole cannot be explained by an inherently greater toxicity of sulfamethoxazole hydroxylamine.

It was also important to determine if the observed potency difference was due to the arbitrary selection of a single time point for determination of viability. Using a protocol identical to our cytotoxicity experiments, we determined MTT conversion at 2, 5, 10, and 20 hr (Fig. 2). The relative constancy of the differences in cytotoxicity over time indicates an inherent difference in cytotoxic potency between these agents. This evidence seems to contradict the notion of a non-specific reaction of hydroxylamines with critical cellular macromolecules as the initiating event in ADR development. According to this hypothesis, the more potent metabolite (dapsone hydroxylamine or monoacetyldapsone hydroxylamine) would cause an immediate effect based upon its rapid binding to

cell components. However, as the less potent sulfamethoxazole hydroxylamine binds to more and more critical molecules, differences in cytotoxicity should decrease over time. Maintenance of differential potency over time, therefore, suggests the possibility of more specific interactions within cells.

These data also suggest that MTT conversion may be an early, sensitive indicator of sulfamethoxazole hydroxylamine-inducing toxicity. MTT conversion by control PBMC increased more dramatically than PBMC exposed to hydroxylamines (Fig. 2C), suggesting that this exposure somehow interferes with normal enzymatic recovery within cells. In correlating membrane and mitochondrial dysfunction, Rieder *et al.* [24] demonstrated that sulfamethoxazole hydroxylamine toxicity was not a mere artifact. Therefore, this inhibition of enzyme recovery or function upon hydroxylamine exposure may actually precede loss of membrane integrity and cell death. While this question warrants further study, support is provided by Leeder *et al.* [37], who demonstrated inhibition of intracellular esterase prior to cell death.

An alternative explanation for this differential incidence of ADR may also be differences in detoxification. *In vivo*, reactive metabolites can interact with any number of cell types, such as PBMC and RBC. This becomes significant when the interaction between reactive species and various cell types in close proximity to each other causes radically different effects. In particular, RBC have been shown to have a protective effect towards PBMC in reducing dapsone hydroxylamine-induced cytotoxicity [34]. Importantly, this does not appear to be a generalized phenomenon, since RBC were shown to have *no* protective effect on the toxicity of procainamide or primaquine hydroxylamine metabolites [34]. The effect of RBC on sulfamethoxazole hydroxylamine-induced cytotoxicity had not been investigated. We, therefore, hypothesized that the incidence of ADR may be higher with sulfamethoxazole due to lower or absent detoxification by RBC.

Using a bicompartmental dialysis system [28, 29], we found that inclusion of RBC caused similar decreases in toxicity caused by sulfamethoxazole hydroxylamine or monoacetyldapsone hydroxylamine (Fig. 3). Further characterization of this phenomenon, comparing intact RBC to lysate and impermeable "ghosts," suggested that the observed detoxifying effect was due to the co-oxidation of hemoglobin, since inclusion of RBC lysate produced the greatest protection against cytotoxicity (Fig. 4). The detoxification observed with "ghosts" was likely caused by residual hemoglobin present after hemolysis and "resealing" of RBC and, therefore, was not surprising. Coleman and Jacobus [38] have shown that dapsone hydroxylamine is reduced to the parent compound with a proportional increase in methemoglobin formation. While methemoglobin formation has not been demonstrated directly with sulfamethoxazole hydroxylamine, we observed the characteristic darkening of RBC in all incubations, which suggests that methemoglobin is formed.

Coleman [39] has suggested that RBC are able to sequester dapsone metabolites, allowing their release from cells over an extended period. Washed RBC previously exposed to dapsone hydroxylamine release enough hydroxylamine to cause RBC and PBMC toxicity [40]. In this way, RBC have been proposed to act as a conduit, delivering reactive dapsone hydroxylamine to tissues where hapten formation may occur [41]. It is possible that differences in this sequestration with sulfamethoxazole hydroxylamine may partially explain differences in ADR incidence, although data to support such a phenomenon are lacking.

In conclusion, we have shown the inherent toxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone to be the opposite of what is expected, based upon the frequency of adverse effects *in vivo*. No difference in the detoxification of these hydroxylamines by RBC was observed. Dispositional differences, differences in the lipophilicity (which would influence the ability to cross biological membranes), or further bioactivation of sulfamethoxazole hydroxylamine, dapsone hydroxylamine, and monoacetyldapsone hydroxylamine may provide alternative explanations for the differential incidence of ADR. Confirmation of these results by comparison of the toxicity of these hydroxylamine metabolites using PBMC from trimethoprim-sulfamethoxazole hypersensitive and tolerant patients is currently in progress.

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