Is Hydroxylamine-Induced Cytotoxicity a Valid Marker for Hypersensitivity Reactions to Sulfamethoxazole in Human Immunodeficiency Virus-Infected Individuals?

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ABSTRACT

Hypersensitivity (HS) reactions to sulfonamides and sulfones continue to limit their use in human immunodeficiency virus (HIV)-infected individuals. In vitro cytotoxicity of hydroxylamine metabolites toward peripheral blood mononuclear cells (PBMCs) has been proposed as a marker for these HS reactions. To test the validity of this in vitro system, we determined the selective susceptibility of PBMCs from HIV-infected patients to the cytotoxic effects of hydroxylamine metabolites of sulfamethoxazole (SMX) and dapsone (DDS). Concentration-cytotoxic response data were collected using PBMCs from 12 sulfa-HS (10 SMX-HS and 2 SMX/DDS-HS) and 10 sulfa-tolerant HIV-infected individuals. Although sulfamethoxazole hydroxylamine (SMX-NOH) and dapsone hydroxylamine (DDS-NOH) both caused concentration-dependent increases in cell death, DDS-NOH was significantly more potent in each subject (P < .0001). A comparison of a variety of mean data for sulfa-HS and -tolerant patient populations failed to demonstrate the increased susceptibility of PBMCs from HS patients, noted by others, to either SMX-NOH or DDS-NOH. Moreover, any trend toward an increased susceptibility of PBMCs from HS patients was eliminated when adjusted for control cell death. PBMCs from sulfa-HS patients showed significantly greater susceptibility to the stress of short term in vitro incubation (P < .02). Mean (S.D.) vehicle control cell death values were 24.1% (7.6%) for HS patients and 17.1% (4.4%) for tolerant patients. No significant correlation was observed between hydroxylamine-induced or control cell death and any of the recorded clinical parameters. Although several potential reasons are proposed to explain the disparity with past investigations, the data suggest that in vitro cytotoxicity is not a valid marker for HS reactions in HIV-infected individuals using currently accepted experimental procedures.

Adverse drug reactions (ADRs) associated with trimethoprim-sulfamethoxazole (TMP-SMX) complicate its use against Pneumocystis carinii pneumonia in human immunodeficiency virus (HIV)-infected individuals. The most unmanageable of these reactions are hypersensitivity (HS) reactions, which generally manifest as fever and morbilliform, cutaneous reactions within 7 to 14 days of therapy but may develop into severe, even life-threatening, cutaneous and systemic reactions (Cribb et al., 1996a). Furthermore, the reported incidence of HS reactions to TMP-SMX is dramatically higher in the HIV-infected population than in any other population studied (Kovaks et al., 1984; Medina et al., 1990; Beumont et al., 1996). Unfortunately, the pathogenesis of SMX-induced HS reactions is not fully understood. Current dogma asserts that oxidative metabolism of SMX to a reactive hydroxylamine metabolite (SMX-NOH) renders it capable of binding critical macromolecules, thereby leading to a direct toxic effect and/or an indirect immune response directed against modified (or haptenated) self-proteins (Rieder et al., 1988, 1989; Uetrecht et al., 1993; Cribb et al., 1996b). An imbalance between drug/metabolite bioactivation and detoxification, caused by genetically or environmentally induced alterations, has consequently been proposed as a critical predisposing factor. However, given the magnitude of health-related and financial risks associated with drug-induced adverse events (Bates et al., 1997; Lazarou et al., 1998), there is a significant need to be able to accurately predict which individuals may be inherently more susceptible to these ADRs.

Nearly 20 years ago, Spielberg and coworkers developed a novel in vitro lymphocyte assay aimed at investigating pos-

ABBREVIATIONS: ADR, adverse drug reaction; DDS, dapsone; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; HBSS, Hanks’ balanced salt solution; HIV, human immunodeficiency virus; HS, hypersensitivity; LC50, concentration causing 50% cell death; PBMC, peripheral blood mononuclear cell; TMP, trimethoprim; SMX, sulfamethoxazole; NOH, hydroxylamine.
sible defects in cellular detoxification processes (Spielberg, 1980; Spielberg and Gordon, 1981; Spielberg et al., 1981). Because data suggested that lymphocytes lacked the ability to significantly generate reactive metabolites but possessed a full range of detoxifying capabilities, increased in vitro cytotoxicity, in the presence of a complete bioactivating system, was believed to indicate a reduced ability to detoxify these reactive species. They hypothesized then that increased cell death signified an increased susceptibility to ADRs as a direct result of defects in detoxification capabilities. Recurrent findings wherein in vivo HS reactions correlate with the susceptibility of patients' peripheral blood mononuclear cells (PBMCs) to metabolism-dependent in vitro cytotoxicity support this hypothesis (Shear et al., 1986; Wolkenstein et al., 1995; Gardner et al., 1998; Kearns et al., 1998; Tabatabaei et al., 1998). Moreover, sulfonamide-induced HS reactions reportedly correlate with in vitro toxicity caused by synthetic SMX-NOH in both retrospective and prospective studies (Rieder et al., 1989; Carr et al., 1993). These data support the hypothesized need for a balance between bioactivation and detoxification processes and suggest that the ability of in vitro cytotoxicity to assess detoxification capabilities may allow its use as a biomarker for HS reactions.

Despite the aforementioned evidence, questions remain regarding the validity of this in vitro test system. First, attempts at correlating in vitro toxicity with genotypic or phenotypic differences in epoxide hydrolase, glutathione transferase, and other detoxifying enzymes have yet to firmly associate a defect in cellular detoxification with in vivo HS reactions (Riley et al., 1991; Gaedigk et al., 1994). Second, although some studies have presented evidence suggesting a selective susceptibility of PBMCs to reactive metabolites of the causative drug (anticonvulsants or sulfonamides) rather than nonspecific toxins (Spielberg et al., 1981; Wolkenstein et al., 1995), other investigations have shown varying degrees of cross-reactivity among agents used for similar therapeutic purposes (Shear and Spielberg, 1988; Gennis et al., 1991; Kearns et al., 1998). These studies question the specificity of this in vitro test system.

Our recent work exploring the differential incidence of HS reactions between SMX and dapsone (DDS), an alternative but structurally similar agent used in HIV-infected patients, also raises some interesting questions as to how and why in vitro toxicity and in vivo HS reactions relate. Although SMX is associated with a significantly higher frequency of HS reactions (Lee et al., 1989; Medina et al., 1990; Pertel and Hirschtick, 1994), hydroxylamine metabolites of DDS have been shown to be inherently more toxic than SMX-NOH in vitro (Reilly et al., 1998). One study, in particular, reported a 57% incidence of ADRs with TMP-SMX but only a 30% incidence with TMP-DDS (Medina et al., 1990). Thus, the drug (i.e., DDS) that is metabolized to species that are significantly more toxic and bioactivated to a quantitatively comparable or greater degree (Coleman et al., 1990; Cribb and Spielberg, 1992) actually causes a significantly lower frequency of ADRs. Importantly, many individuals hypersensitive to SMX do not develop HS reactions to DDS (Blum et al., 1992; Jorde et al., 1993), suggesting a high degree of selectivity in the manifestation of an HS response.

In vitro cytotoxicity is truly a valid marker for sulfonamide-induced HS reactions, these observations suggest that it should be capable of identifying SMX-susceptible, but DDS-tolerant patients, while at the same time distinguishing individuals susceptible to HS reactions caused by both agents. In order to test this hypothesis, we compared the cytotoxic effects of SMX-NOH and DDS-NOH toward PBMCs from HIV-infected individuals with and without a previous HS reaction to SMX. Although the results described herein do not support previous reports suggesting that this in vitro test is a specific and/or selective marker for HS reactions, we did observe an increased susceptibility of PBMCs from HS patients to generalized external stress, at least during relatively short-term in vitro incubations.

Materials and Methods

Chemicals. Hydroxylamine metabolites of DDS and SMX were synthesized as described previously (Rieder et al., 1988; Vage et al., 1994) and determined by HPLC to be >97% pure. Accupin System-Histopaque 1077, HEPEES, penicillin/streptomycin, dimethyl sulfoxide (DMSO), and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Hanks' balanced salt solution (HBSS; without phenol red) was obtained from Life Technologies (Grand Island, NY), and 96-well tissue culture treated polystyrene microplates were obtained from Rainin Instruments (Woburn, MA). The fluorescent nucleic acid dye YO-PRO-1 iodide was obtained from Molecular Probes (Eugene, OR). All remaining chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical Co.

Study Subjects. Initially, two healthy, HIV-negative volunteers from the staff of the Department of Pharmaceutical Sciences were recruited to evaluate the reproducibility of the in vitro cytotoxicity assay. HIV-infected individuals were then recruited from the Detroit Medical Center AIDS Clinic by reviewing patient charts for a documented history of HS reactions to SMX and DDS. Criteria for a delayed-type HS reaction to SMX or DDS included the development of a rash, with or without fever, >4 days after initiation of therapy that subsequently abated on the discontinuation of drug use. Subjects with an immediate-type allergic reaction were excluded from the study. Supporting evidence of a true delayed-type HS reaction was obtained by a research nurse (M.J.F.) based on chart review, patient interview, and, when necessary, consultation with the patient's primary care physician. After obtaining written informed consent from each patient, approximately 60 ml of whole blood was drawn into Vacutainer tubes (Becton Dickinson, Franklin Lake, NJ) containing sodium heparin. Samples were then transported to the research laboratory on ice within 0.5 h for subsequent analysis. In addition to recording the sex, race, age, symptoms and approximate time of the documented HS reaction, and concurrent medications for each subject, recent CD4⁺, CD8⁺, lymphocyte, and neutrophil counts were obtained if available. All aspects of the study were approved by the Human Investigation Committees at Wayne State University and Harper Hospital.

PBMC Isolation and Hydroxylamine Exposure. PBMCs were separated from whole blood using Histopaque-1077 according to the manufacturer's instructions. After two washes in HBSS containing 15 mM HEPES, contaminating erythrocytes were lysed by a 5-min incubation in 0.85% NH₄Cl, and the subsequent PBMC pellet was washed two additional times. PBMCs were then suspended in HBSS containing 15 mM HEPES, and cell counts and viability were determined by trypan blue dye exclusion.

PBMCs were diluted further in HBSS containing 15 mM HEPES and distributed to microplate wells (1.75 × 10⁵ viable PBMCs/well). Hydroxylamine solutions were prepared in DMSO and added to wells to give final concentrations ranging from 0 to 1500 μM for DDS-NOH and 0 to 4000 μM for SMX-NOH. The highest concentrations were at the limit of each metabolite's solubility. PBMC incubations were maintained at 37°C and continued for 3 h. Concen-
trations were tested in quadruplicate (200 μl volume/well), and DMSO was maintained at 1% (v/v).

**Determination of Cell Death.** After a 3-h incubation with or without hydroxylamines, time-dependent increases in cell death were determined using the impermeable, nucleic acid-binding fluorescent dye YO-PRO-1 as described previously (Tabatabaei et al., 1997). Because each microplate well acts as its own control, it has the unique advantage of incorporating any autolysis that may occur, while eliminating slight variations in cell number as well as dye binding and fluorescence (Tabatabaei et al., 1997). Briefly, PBMCs were pelleted by centrifugation at 400 g for 10 min, and incubation medium was replaced with complete assay medium containing HBSS with 15 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, and 4 μM YO-PRO-1. Initial fluorescence (A) was then determined using a CytoFluor Series 4000 Fluorescence Multi-Well Reader (PerSeptive Biosystems, Framingham, MA) with excitation and emission set at 485 nm (20-nm bandpass) and 530 nm (25-nm bandpass), respectively. Incubations were continued at 37°C within the microplate reader, and fluorescence was monitored automatically at 1-h intervals (X) over 16 h. Final or maximal fluorescence (B) was determined within 3 h of permeabilizing cells with the addition of Triton X-100 (final concentration, 0.1%), and cytotoxicity was then calculated as percent cell death = 100 [(X – AB) / A].

**Statistical Analysis.** Discordant data in repeat determinations of cell death within each subject were identified using established parameters of the Q-test (Shoemaker et al., 1989). Results are presented as mean (S.D.) where appropriate. Pharmacodynamic parameters, including LC₅₀ values, were derived from concentration-cell death response data for each patient with the use of regression analysis. Comparisons between groups were made with the Student's t test, and significant correlations were detected using the Pearson product moment correlation test. In each case, a value of P < .05 was considered statistically significant.

**Results**

The membrane-impermeant, nucleic acid-binding fluorescent dye YO-PRO-1 was selected as a high-capacity, objective, and easy-to-use indicator of PBMC cell death. In validation of the assay use with PBMCs from HIV-negative, healthy volunteers, sensitivity and reproducibility were found to be extremely high (data not shown). Moreover, hydroxylamine-induced cell death data collected over a 4-week period suggested that intraindividual variability in cytotoxicity values using YO-PRO-1 was negligible (Fig. 1). These data strongly supported its use in subsequent patient studies.

Over the course of the study, blood samples were obtained from 24 HIV-infected individuals. Two sulfonamide-tolerant patients were eliminated from further analysis due to the inability to obtain adequate PBMC isolation. Relevant patient characteristics of the 22 remaining patients are given in Table 1. All 10 sulfonamide-tolerant patients were currently taking TMP-SMX as prophylaxis against P. carinii pneumonia. Of the 12 patients who had experienced a sulfonamide-induced HS reaction, 10 patients were tolerant of subsequent DDS therapy and 2 experienced an HS reaction as well. Documentation of HS reactions included some combination of rash and/or fever developing 4 days or later after the initiation of therapy. Interestingly, however, 1 patient did not develop symptoms of an HS reaction until approximately 1 year after beginning TMP-SMX, although no clear reasons for this delay could be established. No significant difference existed between the age, race, sex, CD4⁺, CD8⁺, lymphocyte, or neutrophil counts of each patient group.

Comparison of the cytotoxic potency of DDS-NOH and SMX-NOH was evaluated using freshly isolated PBMCs from each HIV-infected individual. Data within each patient revealed a concentration-dependent increase in toxicity caused by both hydroxylamines, with SMX-NOH being significantly less potent in each case (P < .0001; data not shown). However, a comparison of the inherent toxicity of each hydroxylamine between patient groups did not reveal significant differences. An initial comparison of PBMC toxicity at 1 mM
TABLE 1
Relevant patient characteristics of HIV+ subjects with and without HS reactions to sulfonamides and sulfones

<table>
<thead>
<tr>
<th></th>
<th>Sulfas-HS</th>
<th>Sulfas/Sulfone-HS</th>
<th>Sulfas-Tolerant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n)</td>
<td>10</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>6/4</td>
<td>1/1</td>
<td>8/2</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>37.1 (6.0)</td>
<td>39.5</td>
<td>40.6 (7.9)</td>
</tr>
<tr>
<td>Range (yr)</td>
<td>28–48</td>
<td>38–41</td>
<td>33–58</td>
</tr>
<tr>
<td>Race</td>
<td>7 African-Americans, 3 Caucasians</td>
<td>1 Caucasian, 1 Hispanic</td>
<td>9 African-Americans, 1 Caucasian</td>
</tr>
<tr>
<td>Time since HS reaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (yr)</td>
<td>1–7</td>
<td>1–3</td>
<td>None</td>
</tr>
<tr>
<td>CD4⁺ count</td>
<td>234.6 (338.5)</td>
<td>60</td>
<td>178.2 (112)</td>
</tr>
<tr>
<td>Range</td>
<td>0–1040</td>
<td>1–119</td>
<td>20–340</td>
</tr>
<tr>
<td>CD4⁺/CD8⁺ ratio</td>
<td>10.1 (9.6)</td>
<td>4</td>
<td>11.8 (7.0)</td>
</tr>
<tr>
<td>Range</td>
<td>0–26</td>
<td>1–7</td>
<td>2–22</td>
</tr>
<tr>
<td>CD8⁺ count</td>
<td>991 (847.1)</td>
<td>1037</td>
<td>874 (411.9)</td>
</tr>
<tr>
<td>Range</td>
<td>114–2538</td>
<td>NA⁺</td>
<td>374–1820</td>
</tr>
<tr>
<td>CD8⁺ count/CD8⁺ ratio</td>
<td>49.9 (7.7)</td>
<td>61</td>
<td>57 (14.9)</td>
</tr>
<tr>
<td>Range</td>
<td>36–58</td>
<td>NA⁺</td>
<td>22–71</td>
</tr>
<tr>
<td>Neutrophil count</td>
<td>0.218 (0.237)</td>
<td>0.115</td>
<td>0.251 (0.214)</td>
</tr>
<tr>
<td>Range</td>
<td>0.2–4.7</td>
<td>0–1.7</td>
<td>0.2–2.6</td>
</tr>
<tr>
<td>Neutrophil count⁺</td>
<td>3.2 (2.6)</td>
<td>2.3</td>
<td>2.2 (1.5)</td>
</tr>
<tr>
<td>Range</td>
<td>91.4–102</td>
<td>1.5–3.1</td>
<td>1.1–6.1</td>
</tr>
</tbody>
</table>

aData presented as mean (S.D.) except for sulfa/sulfone-HS subjects, where average values are reported.

bNot available (NA) because CD8⁺ counts were only obtained from one of two patients.

cRepresents data obtained from one patient.

DDS-NOH or SMX-NOH, meant to mimic an earlier study by Carr et al. (1993), demonstrated the substantial variability in PBMC susceptibility among HIV-infected individuals. Mean (S.D.) cell death values for sulfa-HS and sulfa-tolerant patients, respectively, 16 h after hydroxylamine exposure were 94.9% (13.1%) and 85.1% (14.6%) for DDS-NOH and 61.8% (22.8%) and 55.6% (13.1%) for SMX-NOH. In light of the high variability, these differences were not significantly different (P > .16, P > .48, respectively).

A more complete analysis using mean data for the entire concentration-cytotoxic response curve also demonstrated higher levels of cell death toward PBMCs from sulfa-HS patients, although these differences were statistically insignificant except at high concentrations of DDS-NOH and the lowest concentration of SMX-NOH (P < .05; Fig. 2). Linear regression of concentration-response data from each patient, which permitted comparison of pharmacodynamic parameters including LC₅₀ values (Fig. 3), also failed to demonstrate a significant correlation between in vitro cytotoxicity and in vivo HS. The only significant correlation observed between LC₅₀ (concentration causing 50% cell death) values and any of the recorded clinical parameters (CD4⁺, CD8⁺, lymphocyte, or neutrophil counts) was between LC₅₀ values for sulfa-tolerant patients and their respective lymphocyte count (P < .05; data not shown).

Moreover, although our patient recruitment was able to capture only two double-HS patients (intolerant of TMP-SMX and DDS), the apparent failure to discriminate between sulfa-HS and sulfa-tolerant patients suggests that this in vitro test would not permit the more specific discrimination between sulfa-HS and sulfa/sulfone-HS patient populations.

An inability to reproduce the correlation between in vitro and in vivo results noted in previous studies prompted us to investigate possible reasons for this disparity. The standard experimental protocol used in these earlier studies involved the determination of terminal or maximal toxicity 16 h after exposure to hydroxylamine metabolites. Because the possibility existed that toxicity at an earlier time point could be predictive of HS reactions, cell death was evaluated at 1-h intervals over the normal 16-h incubation period. Figure 4 shows the time-dependent increase in cell death caused by DDS-NOH and SMX-NOH in sulfa-HS and sulfa-tolerant patient groups. Although the data suggest that PBMCs from HS patients are more susceptible to hydroxylamine-induced toxicity from DDS-NOH and SMX-NOH, significant interindividual variability precludes these differences from reaching statistical significance (error bars not shown). Moreover, no time appears to be any more predictive than the 16-h postexposure time point used previously.

A careful analysis of data obtained from each subject also suggested that when cell death values were corrected for background cell death, differences between patient groups were completely eliminated at the 16-h postexposure time point (Fig. 5). There was no statistically significant difference at earlier time points (e.g., 7-h postexposure) due to the substantial variability among patients (Fig. 5). However, in a direct comparison of control cell death in the presence of vehicle alone (1% DMSO), PBMCs from HIV-infected, sulfa-HS patients demonstrated a significantly greater susceptibility to the stress of short-term in vitro culture than did cells from sulfa-tolerant patients (P < .02; Fig. 6). Mean (S.D.) control cell death values for sulfa-HS and sulfa-tolerant patients were 24.1% (7.6%) and 17.1% (4.4%), respectively. A similar significant difference existed between patient groups using controls without vehicle, because DMSO caused no appreciable toxicity alone (data not shown). There was no significant correlation between control cell death and any of the recorded clinical parameters for either patient group (data not shown).

Although no difference was noted among CD4⁺, CD8⁺, lymphocyte, or neutrophil counts between each patient group, the generally accepted flux in overall health of HIV-infected patients also prompted us to speculate that intraindividual variability may significantly affect subsequent analysis of the validity of this in vitro test system. Therefore, three patients were recruited for a second determination of hydroxylamine-induced PBMC toxicity (Fig. 7). With LC₅₀ values...
and control cell death values used as indicators of the potential variability, the data do suggest that in vitro cytotoxicity is subject to significant intraindividual variability. In particular, the ability of control cells to overcome the stress of isolation and short-term in vitro culture appears to have a substantial impact on any subsequent analysis suggestive of an increased susceptibility.

**Discussion**

Despite significant efforts to elucidate biological and/or environmental factors that increase susceptibility to the adverse effects of TMP-SMX, the potential severity of these effects continue to limit its use. Viral-induced alterations in immune responsiveness, glutathione status, drug metabolism, and detoxification mechanisms have all been proposed as critical predisposing factors (Park et al., 1998). However, our current understanding of the increased susceptibility of HIV-infected individuals to sulfonamide-induced ADRs is severely lacking.

One observation that had heretofore been unshaken was that PBMCs from sulfonamide-HS patients appeared to be inherently more susceptible to the toxic effects of reactive hydroxylamine metabolites in vitro (Shear and Spielberg, 1985; Shear et al., 1986; Rieder et al., 1989; Carr et al., 1993; Wolkenstein et al., 1995). Previous studies even suggested the predictive and specific nature of this test (Carr et al.,

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**Fig. 2.** Mean hydroxylamine concentration-cell death data from HIV-infected patients. PBMCs from HIV-infected individuals with or without a previous HS reaction were exposed to hydroxylamines for 3 h (quadruplicate samples per concentration), and fluorescence was determined hourly over the next 16 h. Results shown are the mean (S.D.) cell death values for each concentration of DDS-NOH (A) and SMX-NOH (B) at 19 h using PBMCs from 12 sulfa-HS and 10 sulfa-tolerant patients. *, significantly different from sulfa-tolerant patients, \( P < .05 \).

**Fig. 3.** Comparison of the cytotoxic potency of hydroxylamines toward PBMCs from HIV-infected individuals. Isolated PBMCs from patients hypersensitive to SMX but tolerant of DDS (sulfa-HS), hypersensitive to both SMX and DDS (sulfa/sulfone-HS), and tolerant of SMX (sulfa-tolerant) were incubated with increasing concentrations of DDS-NOH (A) or SMX-NOH (B) for 3 h, and percent cell death was determined over the next 16 h. LC\(_{50}\) values were derived from 18-h concentration-cytotoxic response data for each subject using linear regression analysis. NS, not significantly different.
Although mechanistic reasons for this association between in vitro and in vivo events remain to be determined, the data suggested that hydroxylamine-induced cytotoxicity was a marker for sulfonamide-induced HS reactions in HIV-infected and noninfected individuals.

Interestingly, however, it was unknown whether PBMCs from sulfonamide-HS individuals might also be more susceptible to hydroxylamine metabolites of other arylamines. In particular, we questioned how PBMCs from sulfa-HS patients would respond to the cytotoxic effects of hydroxylamine metabolites of DDS, an alternative agent to TMP-SMX used by AIDS patients. This was a critical question to ask in assessing the validity of this in vitro system because many individuals unable to tolerate TMP-SMX do not experience HS reactions to DDS.

Evaluation of the validity of in vitro cytotoxicity as a marker for HS reactions first necessitated that the assay system be capable of handling large numbers of samples with both objectivity and high sensitivity. Our previous work with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) indicated that replicate samples may be highly variable and that redox-active compounds, such as reactive hydroxylamine metabolites, can nonspecifically react with MTT, further confounding assay results (Reilly et al., 1998). Moreover, because calculated toxicity levels are dependent on the relative enzymatic activity of controls, direct comparisons between patients becomes problematic. Therefore, we selected the impermeable, nucleic acid-binding fluorescent dye YO-PRO-1 initially used by Tabatabaei et al. (1997) as a rapid and more sensitive indicator of cell death. In addition to the limited variability noted within experiments, little intraindividual variability was observed using PBMCs from two HIV-negative volunteers over a 4-week period (Fig. 1).

Our subsequent comparison of the susceptibility of PBMCs from HIV-infected individuals with and without a prior HS reaction failed to support previous investigations. Regardless of the method of data analysis or the time of cell death determinations, PBMCs from sulfa-HS patients did not show.

Fig. 4. Time course of PBMC cell death in HIV-infected individuals. After a 3-h incubation with DDS-NOH (A) or SMX-NOH (B), fluorescence was monitored at 1-h intervals over the next 16 h using YO-PRO-1 (see Materials and Methods). Results are presented as the mean cell death values of representative hydroxylamine concentrations from 12 sulfa-HS (closed symbols) and 10 sulfa-tolerant patients (open symbols). Error bars are excluded for clarity of presentation.

Fig. 5. Comparison of the time course of PBMC cell death with and without correction for control cell death. After a 3-h incubation with DDS-NOH or SMX-NOH, fluorescence was monitored at 1-h intervals over the next 16 h using YO-PRO-1 (see Materials and Methods). Results are presented as the mean cell death values, with and without appropriate subtraction of vehicle control cell death, caused by 1 mM DDS-NOH (A) or SMX-NOH (B) using PBMCs from 12 sulfa-HS (closed symbols) and 10 sulfa-tolerant (open symbols) patients. Statistical significance was not observed at any time point due to significant variability. Error bars are excluded for clarity of presentation.
significantly greater susceptibility to hydroxylamine-induced cell death. That is not to say that a trend toward increasing susceptibility was not observed. As shown most clearly in Figs. 2 and 4, mean cell death values at each concentration of DDS-NOH and SMX-NOH were greater in sulfa-HS patients than in sulfa-tolerant patients. However, the extensive variability among patients (see Fig. 3) prevented these values from reaching statistical significance, thereby precluding the use of these results to discriminate between patient groups. Even in previous studies in which significance was noted, extensive variability was also observed (Carr et al., 1993; Wolkenstein et al., 1995). Most notably, Wolkenstein et al. (1995) observed a positive lymphoid cell death test (10% above baseline) in only 8 of 13 patients with a sulfonamide-induced cutaneous drug reaction and in only 5 of 13 with an anticonvulsant-induced reaction. Similarly, our results suggest that the degree of susceptibility is highly patient dependent in that select patients were clearly more susceptible to hydroxylamine-induced toxicity (see Fig. 3).

The original purpose of this study was to test the validity of the use of in vitro cytotoxicity as a marker for HS reactions by determining whether sulfa-HS patients, sulfa-tolerant patients, and individuals hypersensitive to both SMX and DDS differed in their susceptibility to SMX-NOH and DDS-NOH. Unfortunately, we were unable to capture a significant number of patients with HS reactions to both agents. However, in light of our apparent inability to differentiate sulfa-HS from sulfa-tolerant patients, it seems highly unlikely that a larger patient population would allow us to differentiate between the two HS groups.

Interestingly, any trend toward a difference between patient groups was completely eliminated when cell death values were corrected for differences in control cell death (Fig. 5). In fact, control cell death values were the only measures that achieved statistical significance between patient groups (Fig. 6). This raises a serious issue in regard to aforementioned studies in which in vitro toxicity correlated with in vivo HS (Shear and Spielberg, 1985; Shear et al., 1986; Rieder et al., 1989; Carr et al., 1993; Wolkenstein et al., 1995). These studies used trypan blue dye exclusion and/or MTT reduction as indicators of cytotoxicity, both of which fail to account for cells that autolyse. This may lead to an underestimation of control cell death. Based on these data, we propose that a portion of the significant differences noted between HS and control patients in previous studies might actually be due to differences in control cell death that could not detected with these less-sensitive assay procedures.
These data also suggest that there may be more of a generic defect in the ability to resist external stress than had been described previously (Spielberg et al., 1981; Wolkenstein et al., 1995), making select individuals more susceptible to adverse reactions from a variety of drugs.

Additional lines of evidence may help to explain our inability to correlate in vitro cytotoxicity with in vivo HS. Unlike previous studies, the majority of our patient population were black i.v. drug abusers. A recent report suggests that black race may provide a protective effect against sulfonamide-induced HS reactions (Lehmann et al., 1998). If indeed in vitro toxicity and in vivo HS are related, it is theoretically possible that race may also play a role in PBMC susceptibility in this in vitro system. Data to support such a hypothesis are currently lacking.

In further contrast to many of the previous studies noted, the manifestations of ADRs among our sulfa-HS patients were relatively minor, including only mild cutaneous reactions with or without a concomitant febrile response. In those earlier studies, reactions were much more severe, resulting in toxic epidermal necrolysis, Stevens-Johnson syndrome, and whole organ complications (Spielberg et al., 1981; Shear et al., 1986; Gennis et al., 1991; Wolkenstein et al., 1995). Wolkenstein et al. (1995) found no correlation between the severity of cutaneous reactions and lymphoid cell death, although reactions in all of their patients were sufficiently severe to require hospitalization. The question must therefore be raised of whether the severity of HS reactions affects the magnitude of in vitro cytotoxic responses. Although other studies have included sulfa-HS patients with relatively mild reactions, comparable to the present analysis, yet have still shown increased toxicity, the possibility remains that our inability to demonstrate increased susceptibility of sulfa-HS patients may be related in part to the decreased severity of their reaction.

Another recent report suggests that in vitro susceptibility may wane with increasing time from the documented HS event (Guest et al., 1998). In a study of the susceptibility of neutrophils from patients with clozapine-induced agranulocytosis to reactive clozapine metabolites, these investigators observed a time-dependent disappearance in the differential toxicity between HS patients and controls. This becomes a critical issue in the present study because all of our patients were recruited >1 year after their documented HS reaction (see Table 1). Carr et al. (1993) had previously reported a mean time lapse since HS reactions of >1 year, although they provided no indication of the range within their patient population. In another study in which consistent results were observed years after recovery from ADRs, the severity of HS reactions was substantially greater than in any of our patients (Shear et al., 1986). Taken together, these data suggest that the severity of adverse reaction and the time elapsed since HS events may be critical factors in determining the magnitude of susceptibility to in vitro toxicity. Contrary to previous speculation, increased toxicity of cells from HS patients may depend less on their inherent susceptibility and more on their physiological status at the time of testing. In this regard, data from Fig. 7 indicate that the overall ability of PBMCs to resist external stress under control conditions may change with time. This flux will have a tremendous impact on subsequent analysis of metabolite susceptibility.

Much of our most recent work has focused on the differential incidence of ADRs to SMX and DDS as a paradigm in which to investigate the role of hydroxylamine metabolites in the manifestation of drug reactions. It is worth noting, therefore, the apparent difference in the time course of toxicity caused by SMX-NOH and DDS-NOH (Fig. 4). DDS-NOH appears to cause a much more rapid death response in comparison with SMX-NOH. Interestingly, we have also observed dramatic differences in the toxicity of these hydroxylamines toward human epidermal keratinocytes (Reilly et al., 1999a). Based on these data, one might speculate that the mechanism of toxicity and/or mechanism of detoxification differs between the reactive species of SMX and DDS. We and others have recently reported differences in the handling of SMX-NOH and DDS-NOH by human erythrocytes (Naisbit et al., 1999; Reilly et al., 1999b). Although further work is needed, these data do support the suggestion that mechanistic differences in the effects of hydroxylamine metabolites may contribute to the differential incidence of ADRs between SMX and DDS.

Despite previous reports demonstrating a correlation between in vitro cytotoxicity and in vivo HS, we have been unable to detect a metabolite-specific increased susceptibility of PBMCs from HIV-infected HS individuals. Instead, we report a nonspecific increased susceptibility of PBMCs from HS patients to the stress of short-term in vitro incubation. This finding may contribute to the increased frequency of ADRs in HIV-infected individuals to a wide range of chemically unrelated drugs. Although there may be multiple reasons for our inability to reproduce previous findings, the data do suggest that in vitro cytotoxicity does not appear to be a specific or sensitive marker for sulfonamide-induced HS reactions in HIV-infected patients.

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