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CELLULAR TOXICITY OF SULFAMETHOXAZOLE REACTIVE METABOLITES—I

INHIBITION OF INTRACELLULAR ESTERASE ACTIVITY PRIOR TO CELL DEATH

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Abstract—Reactive metabolites produced by oxidative metabolism of the parent compound are considered responsible for the toxicity of a number of drugs, including idiosyncratic reactions to sulfonamide antibiotics. Using sulfamethoxazole hydroxylamine (SMX-HA) as a model compound, we report the use of a pH-sensitive fluorescent probe, 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF), to identify early subcellular targets of chemically synthesized, toxic drug metabolites in peripheral blood mononuclear cells. When toxicity was assessed with this probe immediately after a 2-hr drug challenge, SMX-HA produced a concentration-dependent decrease in cellular fluorescence which was not accompanied by the development of compromised cell membrane integrity until 18 hr later. Dissipation of pH gradients across the cell membrane with nigericin and monensin demonstrated that decreased intracellular pH was only a small component of SMX-HA-induced toxicity. Loading cells with BCECF 30 min prior to SMX-HA challenge produced only a 3% decrease in cellular fluorescence at an SMX-HA concentration of 1 mM, whereas addition of BCECF after drug challenge resulted in a 71% decrease in fluorescence, consistent with a direct drug effect on cellular esterase activity. This was confirmed by monitoring BCECF cleavage in cell lysates in the presence and absence of SMX-HA. These studies demonstrate that inhibition of cellular esterase activity accounted for the observed loss of cellular fluorescence after drug exposure. Since changes in cellular fluorescence at 2 hr correlated well with cell death at 18 hr, we conclude that SMX-HA inhibition of intracellular esterase activity is an early event in the process that terminates in metabolite-induced cell death.

Idiosyncratic drug reactions are potentially serious side-effects of antimicrobial therapy with sulfonamides. This hypersensitivity-like syndrome is characterized by the onset of fever, rash and lymphadenopathy 10–14 days after initiation of treatment, and may be accompanied by hepatic, renal, bone marrow, cardiovascular, pulmonary or central nervous system toxicity [1, 2].

Pathogenic mechanisms of these reactions are not clear, although hapten formation and subsequent autoimmune responses have been suggested: covalent binding of reactive intermediates to nucleophilic groups on cellular proteins would generate drug–protein conjugates which could lead directly to cell death or be recognized as an immunogen, stimulating an immune response [3].

Sulfonamides are normally acetylated to non-toxic metabolites by *N*-acetyltransferase, a polymorphic enzyme whose activity is inherited as an autosomal recessive trait [4]. These drugs can also be oxidatively metabolized to toxic intermediates capable of covalent binding to macromolecules. Studies with murine hepatic microsomes previously suggested cytochrome P450-mediated production of a reactive species which could be detoxified by glutathione and *N*-acetylcysteine [5]. While such observations are consistent with the hapten model of idiosyncratic reactions, definitive proof is lacking. For example, diagnosis of sulfonamide-specific tissue damage by the detection of antibodies directed against cellular neoantigens has not been reported to date.

Sulfonamide (and perhaps many other) idiosyncratic drug reactions may reflect altered generation of toxic metabolites, altered cellular detoxification capacity, or both. An altered ability to generate or detoxify reactive metabolites may predispose a susceptible individual to idiosyncratic reactions. To approach these questions experimentally, we have developed an *in vitro* model for the measurement of drug-induced cytotoxicity in peripheral blood mononuclear cells (PBMC†) from relevant patients. When challenged with appropriate sulfonamide metabolites, PBMC from hypersensitive patients show increased toxicity relative to controls with obligate carriers demonstrating intermediate sensitivity. Interestingly, all patients studied were found to be slow acetylators [1].

The reactive metabolite involved in sulfonamide hypersensitivity reactions may be an arylhydroxylamine [6] or possibly its nitroso derivative [7].

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† Abbreviations: PBMC, peripheral blood mononuclear cells; BCECF, 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein; SMX, sulfamethoxazole; SMX-HA, sulfamethoxazole hydroxylamine; PI, propidium iodide; and pH_i, intracellular pH.

To better understand the cell biology of drug metabolite toxicity, we have previously developed an automated, high throughput fluorescent-based viability assay with a wide and linear assay range. This method can utilize a variety of recently developed fluorescent probes for cellular and subcellular cytosolic functions and their modulation under stress [8]. In this report, we use one such probe, 2',7'-biscarboxyethyl 5(6)-carboxyfluorescein (BCECF), to explore early subcellular targets of synthetic drug metabolites. BCECF is commonly used as an intracellular pH indicator [9, 10]. The membrane permeant acetoxymethyl ester (BECEF-AM) is hydrolyzed by intracellular esterases to the highly charged fluorescent form [9]. Flow cytometry demonstrates that cells retaining BCECF uniformly exclude the nucleic acid-binding dye propidium iodide (PI) which requires loss of membrane integrity to enter the cell [8].

Based on the characteristics of the fluorescent probe, it was conceivable that decreased intracellular pH, esterase activity, compromised cell membrane permeability or some combination of the three parameters could represent early sequelae of metabolite exposure triggering the process eventually leading to cell death. Our data indicate that one main target of SMX-HA is intracellular esterase activity. The ability to use fluorescent probes of cytosolic functions and events preceding cell death is of considerable promise for studies of drug toxicity.

METHODS

Cell preparation. Human PBMC were isolated from fresh, heparinized blood of healthy volunteers using Histopaque 1077 (Sigma Diagnostics, St. Louis, MO) gradients [11] and resuspended in HEPES-buffered salt medium (sodium-HEPES: 15 mM HEPES [*N*-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid], pH 7.4; 125 mM NaCl; 6 mM KCl; 1.2 mM $MgSO_4 \cdot 7H_2O$; 1 mM NaH_2PO_4; 1 mM $CaCl_2$; and 10 mM glucose). After platelets were removed with a 20% sucrose gradient, PBMC were washed and adjusted to 10^6/mL in sodium-HEPES. For some experiments, cells were prepared in HEPES with K^+ (130 mM) as the sole monovalent cation (potassium-HEPES). All procedures were carried out at room temperature.

Toxicity assay. SMX-HA was synthesized according to the method of Rieder *et al.* [6] and was 90% pure by HPLC. The major contaminants were identified as the parent compound, sulfamethoxazole (SMX), and the nitro derivative of SMX. SMX and SMX-HA stock solutions were prepared in anhydrous dimethyl sulfoxide (DMSO) (Aldrich Chemical Co., Milwaukee, WI); storage at room temperature, unprotected from light, did not result in degradation to the nitroso- or azoxy forms. PBMC (2×10^4/well) in sodium-HEPES were incubated in a 96-well unidirectional, vacuum filtration plate (Baxter Healthcare Corp., Pandex Division, Mundelein, IL) for 2 hr at 37° in the presence of various concentrations of SMX-HA. Assay incubations contained 0.001% fluorescent reference particles in a total assay volume of 50 μL [8]. The final concentration of DMSO (2%) was not associated with detectable toxicity.

BCECF-AM (Molecular Probes, Inc., Eugene, OR) was dissolved in DMSO to a final concentration of 1 mg/mL. For most experiments, the BCECF-AM stock was further diluted with sodium-HEPES and added to the wells of filtration plates (final concentration = 1 μg/mL) during the last 30 min of a 2-hr drug challenge. In some cases, PBMC were incubated with 1 μg/mL BCECF-AM for 30 min at 37°, centrifuged and resuspended in sodium-HEPES prior to drug challenge. For both dye-loading methods, the amount of additional DMSO added to the cells was 0.1%. After the 2-hr drug challenge, inert polystyrene microspheres (8.0 μm; Baxter) were added to each well. Using an automated protocol, the filtration plates were washed and vacuumed, and the fluorescence of viable cells was read by front surface fluorimetry in a Screen Machine™ (Baxter). BCECF fluorescence (excitation/emission: 485 nm/535 nm) is expressed relative to the fluorescence of reference particles (Epicon™, Baxter-Pandex) with excitation and emission wavelengths of 590 nm/620 nm respectively. The ratio of BCECF fluorescence to internal standard under these assay conditions is linear over a range of approximately 100 to 20,000 PBMC [8]. SMX-HA-induced changes in cellular BCECF fluorescence are expressed relative to vehicle (DMSO) controls.

Assessment of cell membrane integrity. PI was prepared in phosphate-buffered saline (PBS, 0.5 mg/mL; Sigma), and a 10-μL aliquot was added to cells (final concentration 5 μg/mL) following drug challenge. Toxicity, the number of non-viable or PI-stained cells expressed as a percentage of total cells present, was determined with a Zeiss epifluorescence microscope. Estimates of SMX-HA toxicity have been corrected for baseline (DMSO) toxicity.

Modulation of intracellular pH. The contribution of alterations in intracellular pH to the observed SMX-HA-induced changes in cellular fluorescence were determined using potassium or sodium ionophores [12]. pH gradients across the cell membrane were dissipated with either nigericin (4 μM) or monensin (4 μM). PBMC were prepared as described in potassium-HEPES or in sodium-HEPES for the nigericin and monensin, respectively, and challenged with SMX or SMX-HA. After incubation with BCECF-AM, ionophore was added followed by polystyrene microspheres, and the cellular fluorescence was read immediately.

Determination of cellular esterase activity. Intact PBMC or homogenates prepared by sonication and low-speed centrifugation were incubated for 90 min at 37° with SMX, SMX-HA or DMSO. BCECF-AM (1 μg/mL) was then added to each incubation mixture and the esterase activity was estimated with continuous fluorescence measurement for 30 min at 37° using excitation/emission wavelengths of 485 nm/535 nm, respectively, in a Hitachi model 400 fluorimeter. The initial rate of fluorescence generation and the ultimate fluorescence achieved were corrected for nonspecific hydrolysis of the probe in sodium-HEPES.

RESULTS

Effect of SMX and SMX-HA on membrane

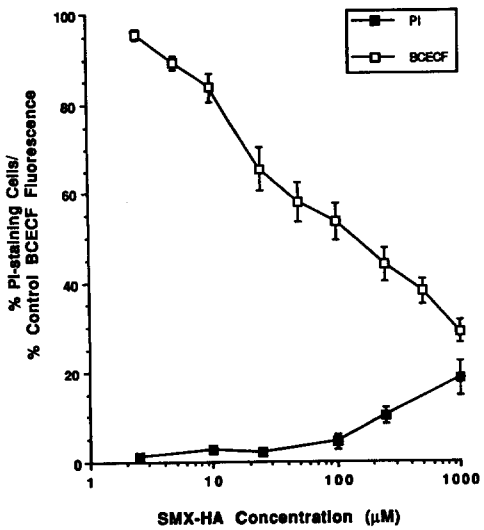

Fig. 1. Contribution of compromised membrane integrity to SMX-HA-induced decreases in cellular fluorescence. PBMC were challenged with SMX-HA for 2 hr, and viability was assessed with BCECF and the Screen Machine, or by propidium iodide exclusion and fluorescence microscopy immediately post challenge. Propidium iodide data are expressed as the percentage of stained (non-viable) cells. BCECF data represented the fluorescence of SMX-HA-treated cells relative to DMSO-treated control cells. Control fluorescence was routinely 1 fluorescence unit per cell. Data are the means (± 1 SEM) of 10 experiments.

integrity. Previous flow cytometry studies demonstrated that PBMC exposed to SMX-HA could be divided into exclusive populations of non-viable (PI^+) and viable ($BCECF^+$) cells [8]. Compromised membrane integrity which allows the entry of extracellular PI and binding to nucleic acid substrates reflects irreversible damage in prelytic cells. To determine whether membrane integrity was a target of SMX-HA early in the toxic process, PBMC from ten volunteers were exposed (2 hr; 37°) to various concentrations of SMX-HA. BCECF-AM was added during the last 30 min of the incubation and the fluorescence of viable cells was measured in the Screen Machine. PI was added to aliquots of cells immediately upon completion of the incubation period.

Cellular BCECF fluorescence was decreased by SMX-HA in a concentration-dependent manner with 1000 μM SMX-HA producing approximately a 75% decrease relative to the DMSO control (Fig. 1). In contrast, when corrected for toxicity of DMSO alone (~5%), the percentage of PI^+ cells was negligible at SMX-HA concentrations ≤ 100 μM, increased to 10.1 ± 1.8% (mean ± SEM) at 250 μM and 18.4 ± 3.9% at 1000 μM. When aliquots of exposed cells were incubated overnight and then examined, as many as 90% of the cells stained positively with PI indicating irreversible damage. At the highest SMX-HA concentration, over 50% of cells had undergone lysis and were not available for measurement (data not shown).

These observations suggest that within the 2-hr period monitored, SMX-HA induces a dramatic change in cell function(s) measured by BCECF. Damage to these cellular functions precedes the terminal prelytic events measured by PI along the pathway ultimately leading to cell death.

Effect of SMX-HA on pH_i. One manifestation of the SMX-HA-induced alterations in cell function(s) measured by BCECF could be cytosolic acidification. Initially, the relationship between BCECF fluorescence and intracellular pH was determined in PBMC pH-clamped with nigericin [13]. PBMC were loaded with 1 μg/mL BCECF for 30 min at 37° and aliquots resuspended in fresh potassium-HEPES pH adjusted with 5 N KOH to values ranging from 7.2 to 7.8. Each aliquot was divided in half with one-half receiving nigericin (4 μM final concentration using a 10 mM stock in ethanol) and the other receiving an equivalent amount of ethanol. Suspensions of 8.0 μm polystyrene microsomes were prepared at each pH value and added to the respective wells prior to fluoresence measurement as described. In the presence of nigericin, there was a linear increase in cellular fluorescence with pH ($r^2 = 0.923$; P = 0.002) compared to vehicle alone ($r^2 = 0.083$; P = 0.580). Consistent, reproducible pH_i changes could also be measured following addition of the Na^+ ionophore monensin (4 μM) to BCECF-loaded cells (data not shown).

The contribution of alterations in pH_i on the SMX-HA-induced changes in cellular BCECF fluorescence was assessed by adding nigericin (final concentration 4 μM) or monensin (4 μM) at the end of the 2-hr drug challenge, 5 min prior to fluorescence measurement. If the SMX-HA-induced decrease in cellular fluorescence was due solely to decreased pH_i, dissipation of the pH gradient should return cellular fluorescence to control (DMSO only) levels.

Figure 2 demonstrates that dissipation of pH gradients across the cell membrane with nigericin (panel A) or monensin (panel B) resulted in only slight changes in the relationship between cellular BCECF fluorescence and SMX-HA concentration. SMX was not associated with any significant change in cellular fluorescence (panel A). The addition of monensin was accompanied by an approximately 10–12% *increase* in BCECF fluorescence, indicating dissipation of the pH gradient across the cell membrane and intracellular alkalinization, but had essentially no effect on the relationship between SMX-HA concentration and BCECF fluorescence (panel B). Therefore, it appears that cytosolic acidification represents at best a relatively minor component of early SMX-HA-induced events.

Effects on esterase activity. SMX-HA-induced effects on intracellular esterase activity were determined in two ways. First, PBMC were loaded with BCECF prior to or 90 min after addition of various concentrations of SMX-HA. In each case, the duration of dye-loading was 30 min, and fluorescence of the vehicle-treated cells was comparable. Dye-loading prior to drug challenge (Fig. 3) was not associated with any appreciable change in cellular fluorescence (3% reduction at 1 mM SMX-HA), whereas dye-loading after drug challenge resulted in the characteristic log-linear decrease in fluorescence noted in Figs. 1 and 2. Since metabolite effects are

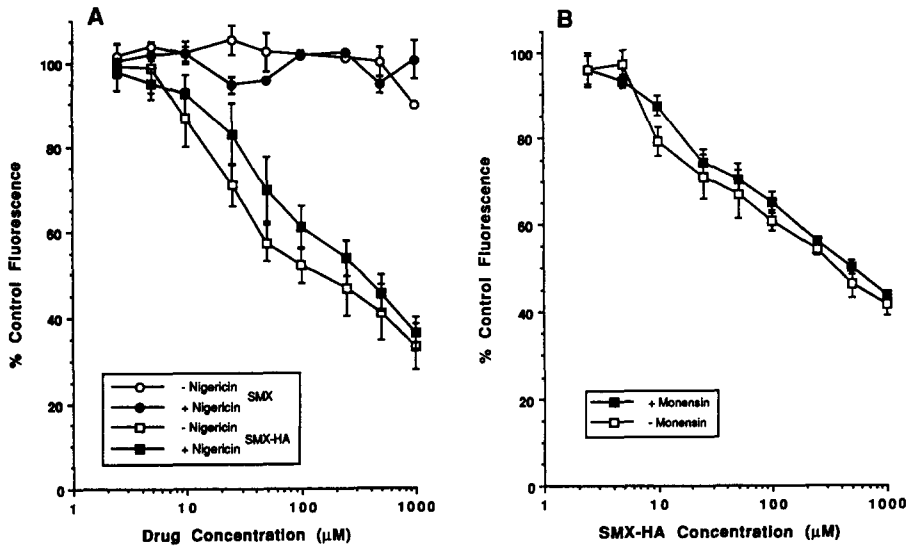

Fig. 2. Contribution of changes in intracellular pH to the observed SMX-HA-related decrease in cellular fluorescence. PBMC were challenged with SMX (●, ○) or SMX-HA (■, □) for 2 hr at 37°, and BCECF (1 μg/mL) was added for the final 30 min of the incubation period. Immediately prior to fluorescence measurement, pH gradients across the cell membrane were dissipated by ionophore addition (solid symbols): 4 μM nigericin (panel A) or 4 μM monensin (panel B). Control wells (open symbols) received vehicle only; fluorescence values were typically 2.0–2.5 × 10^4 fluorescence units per well. Data are the means (± 1 SEM) of triplicate experiments.

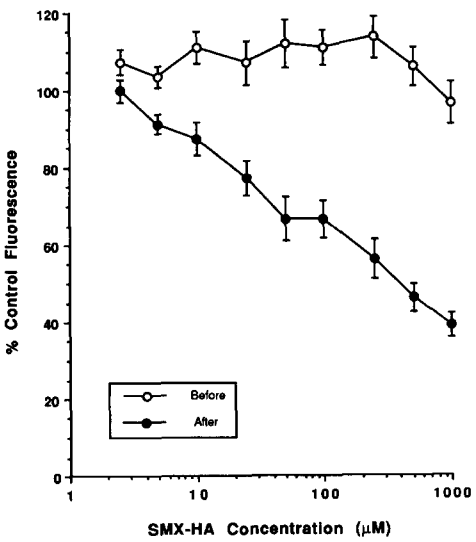

Fig. 3. Comparison of SMX-HA effects in PBMC loaded with BCECF-AM before and after drug challenge. PBMC were loaded with 1 μg/mL BCECF-AM for 30 min at 37° prior to (○) or during the last 30 min (●) of a 2-hr SMX-HA challenge, and cellular fluorescence was measured in the Screen Machine. Control fluorescence was 2.0–2.5 × 10^4 fluorescence units per well. The discrepancy in the data obtained with the two dye-loading protocols is suggestive of an effect on esterase activity. Data are the means (± 1 SEM) of three experiments.

not apparent when cells are dye-loaded prior to drug challenge, these data are strongly indicative of an effect on cellular esterase activity as an early target of SMX-HA.

To better characterize the kinetics of SMX-HA effects on esterase activity, the generation of cellular BCECF fluorescence was monitored in a spectrofluorimeter. Experimental conditions were similar to those used for endpoint measures in vacuum filtration plates. PBMC or cell sonicates (total volume 1.5 mL) were challenged with DMSO, SMX (1 mM) or SMX-HA (10 μM, 100 μM, 1 mM) for 90 min at 37° (Figs. 4 and 5). A 1-mL aliquot was transferred to a polystyrene cuvette, BCECF-AM (1 μM final concentration) was added, and the progression of fluorescence was monitored for 30 min. For intact cells, the fluorescence curves showed an initial lag period which tended to decrease with increasing reaction rate (Fig. 4). The reaction rate was linear for the first 5–6 min after which the rate of BCECF-AM hydrolysis began to decrease. Substrate depletion could not account for the observed decrease in reaction rate as a 5-fold increase in BCECF-AM concentration did not produce any appreciable increase in the rate (data not shown). Sonication of cells was associated with a marked reduction of esterase activity (14.3 ± 1.4% that of intact cells) despite sonicating and maintaining the sonicate on ice (Fig. 5). In general, however, the effect of SMX-HA pretreatment on the progression of cellular fluorescence with time was similar to that observed with intact cells.

With both intact cells and sonicates, SMX-HA was associated with a concentration-dependent decrease

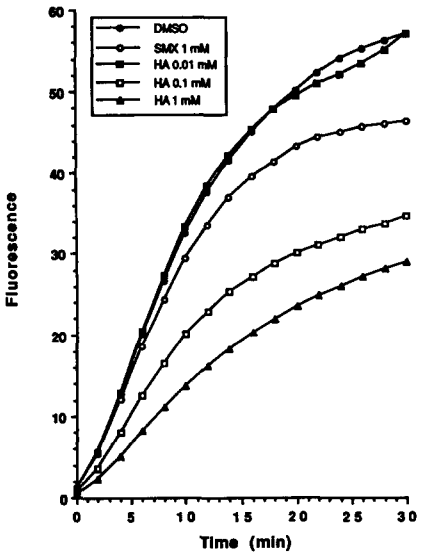

Fig. 4. Effect of SMX-HA on cellular esterase activity: Fluorimeter studies with intact cells. Intact PBMC were challenged with DMSO (●), 1 mM SMX (○), or 10 μM (■), 100 μM (□) or 1 mM (▲) SMX-HA as described in the legends to Figs. 1–3 except that after 90 min, a 1-mL aliquot was transferred to a polystyrene cuvette, BCECF (1 μg/mL) was added and the rate of fluorescence was monitored in a Hitachi 2000 fluorimeter for the final 30 min of the incubation period. Data presented are representative of three replicate experiments.

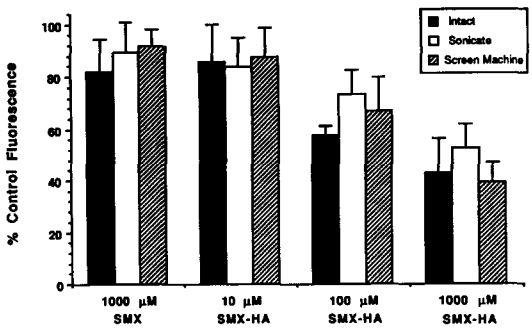

Fig. 6. SMX-HA-induced decrease in cellular esterase activity: Comparison of fluorimeter data with cell concentration fluorescence. SMX-HA toxicity was estimated from the fluorimeter data presented in Fig. 4 by comparison of initial reaction rate and total fluorescence after correcting for spontaneous hydrolysis of the dye. Data from intact PBMC (solid columns) and cell sonicates (open columns) were compared to cell concentration fluorescence results obtained with the Screen Machine (Fig. 3; stippled columns). For CCF experiments, control fluorescence was 2.0–2.5 × 10^4 fluorescence units per well. For the fluorimetry data, total fluorescence was 65–80 and 15–20 arbitrary fluorescence units for intact cells and cell sonicates respectively. Results are the means (± 1 SEM) of three experiments.

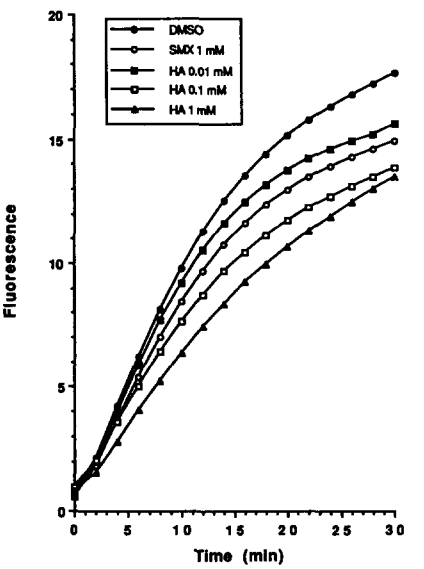

Fig. 5. Effect of SMX-HA on cellular esterase activity: Fluorimeter studies in cell sonicates. A volume of cell sonicate equivalent to the aliquot of cells used for the experiments presented in Fig. 4 was challenged with DMSO (●), 1 mM SMX (○), or 10 μM (■), 100 μM (□) or 1 mM (▲) SMX-HA. After 90 min of drug challenge, a 1-mL aliquot was transferred to a polystyrene cuvette, BCECF (1 μg/mL) was added, and the rate of fluorescence was monitored in a Hitachi 2000 fluorimeter for the final 30 min of the incubation period. Data presented are representative of three replicate experiments.

in both the initial rate of hydrolysis as well as a decrease in the total fluorescence produced in the 30-min period. Estimates of toxicity based on initial reaction rate correlated well with those based on the total fluorescence generated ($r^2 = 0.889$); P = 0.0001). When corrected for the minor spontaneous hydrolysis of BCECF-AM in buffer only, both fluorimeter-based estimates of SMX-HA toxicity were comparable to the vacuum filtration data (Fig. 6). The overall metabolite effect determined from the cell sonicate data was slightly less than that determined from intact cells confirming that the latter includes the effects of changes in membrane integrity and small changes in pH_i. In contrast, SMX-HA-induced changes in BCECF fluorescence generated by cell sonicates is solely a reflection of esterase activity since the cell membrane is no longer a factor. In this respect, it should be noted that the SMX-HA effect is unlikely to be simply a competitive inhibition since the rate of BCECF-AM hydrolysis by cell sonicates required preincubation with the reactive metabolite. The simultaneous addition of SMX-HA and BCECF-AM to intact cells or cell sonicates without preincubation with the reactive metabolite resulted in no alteration of rate or extent of fluorescence generation compared to BCECF-AM alone (data not shown).

Toxicity at 2 hr vs 24 hr. Cumulatively, the data presented above suggested that within the first 2 hr of SMX-HA exposure, BCECF served more as an indicator of cell damage and perhaps impending cell death than as a measure of ultimate cell death. To further explore the relationship between cell damage and cell death, SMX-HA-induced changes in

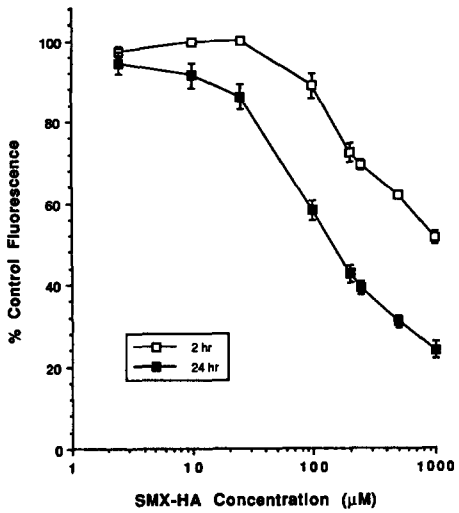

Fig. 7. Comparison of SMX-HA-induced effects at 2 hr and 24 hr. PBMC were challenged with SMX-HA for 2 hr and incubated with BCECF-AM (1 μg/mL) during the final 30 min of the incubation period (□) or 22 hr later (■). Changes in cellular fluorescence at 2 hr (predominantly esterase activity) correlated with changes at 24 hr (predominantly decreased membrane integrity; Ref. 8) as evidenced by the parallel relationship between the two curves and regression of 24 hr toxicity (dependent variable) against cell damage at 2 hr ($r^2 = 0.813$; P = 0.0001). Control fluorescence was 2.0–2.5 × 10^4 fluorescence units per well. Data are the means (± 1 SEM) of 10 experiments.

BCECF fluorescence were compared following a 2-hr incubation and after overnight incubation. Assessment of cell viability with BCECF 24 hr after SMX-HA treatment has been shown previously to be related to viable cell number as measured by tetrazolium salts and dye-exclusion methods [8]. PBMC from ten volunteers were challenged with SMX-HA and incubated with 1 μg/mL BCECF-AM for 30 min after 2 hr or overnight (24-hr) incubations. There was a clear relationship between the 2-hr data and the 24-hr data as evidenced by the parallel shift of the 24-hr concentration–response curve (Fig. 7). Regression of the 24-hr data (dependent variable) against the 2-hr data ($r^2 = 0.813$; P = 0.0001) confirmed this relationship, suggesting that cellular functions altered during the 2-hr drug exposure ultimately were associated with cell death.

DISCUSSION

The series of biochemical events between exposure of cells to toxic compounds and ultimate cell death is an area of intense interest. In the case of acetaminophen toxicity, early studies demonstrated an association between covalent binding of an oxidative metabolite to tissue macromolecules and subsequent tissue necrosis. Manipulations which increased or decreased toxicity such as P450 inducers and inhibitors and nucleophiles produced corresponding changes in the extent of covalent binding [14]. The mechanism(s) of the cytotoxic effects is less clear, and a causal relationship between covalent binding/adduct formation and cell death is currently under debate [15]. In another example, halothane is metabolized to a reactive acyl halide which forms a covalent adduct with an intracellular carboxylesterase. Antibodies in the sera of patients with halothane hepatitis recognize neoantigens expressed as a consequence of covalent modification of the enzyme [16]. The cellular consequences of electrophilic attack on the carboxylesterase are not currently known, nor is the pathogenicity of such antibodies established. Nevertheless, the observations do delineate cellular esterase functions as one target of toxic metabolites within the framework of hypersensitivity reactions.

Identification of the putative reactive metabolite and knowledge of its cellular sites of action can provide insight into the mechanism(s) of cytotoxicity. PBMC from individuals with a history of hypersensitivity reactions to sulfamethoxazole are more susceptible to the cytotoxic effects of SMX-HA than PBMC isolated from controls [6]. This suggested that this metabolite may be the proximal toxin mediating sulfonamide hypersensitivity. Understanding the mechanism of SMX-HA cytotoxicity is essential for determining a cellular defect(s) which predisposes susceptible individuals to idiosyncratic toxicity.

During development of our fluorescence-based cytotoxicity assay, it became apparent that alterations in cellular BCECF fluorescence under the conditions of the SMX-HA toxicity assay could be a composite function of effects on membrane integrity, pH_i and esterase activity. BCECF is generally accepted as an attractive fluorescent probe for measuring pH_i [13, 17]. Our flow cytometric data [8] attest to its utility as an indicator of cell membrane integrity and have been confirmed by continuous visual monitoring with digitized video microscopy [18]. BCECF has also proven superior to other fluorescein derivatives for flow cytoenzymological studies of cellular esterase activity [19].

An analysis of the contribution of membrane integrity, cytosolic acidification and esterase activity to the observed SMX-HA-induced decrease in cellular BCECF fluorescence provided insight into both the toxic process and the detection method. The cell concentration fluorescence (CCF) data presented provide evidence for a predominant effect on esterase activity which correlated well with toxicity estimates based on viable cell number following a 24-hr incubation. The results of the spectrofluorimetric assays with whole cells could also be a composite of effects on membrane integrity, pH_i and esterase activity. Furthermore, dye leakage into the relatively more alkaline extracellular medium could lead to an underestimation of the inhibitory effects of SMX-HA on the esterase activity, a problem which does not exist in the CCF assays since extracellular fluid is removed prior to fluorescence detection. By conducting experiments with cell sonicates, substrate influx and efflux, pH gradients and permeability barriers were no longer factors. The results of these experiments were comparable to the whole cell CCF data strengthening the evidence that observed decreases in BCECF fluorescence were due primarily to inhibition of esterase activity.

The kinetics of BCECF-AM hydrolysis by cellular esterases in our spectrofluorimetric experiments were similar to those reported by Dive *et al.* [19] in terms of the characteristic lag phase and linearity of fluorescence generation. This group also found that the rate of hydrolysis by cell sonicates was about 20% that of whole cells comparing favorably to the value of 15% in our experiments. The reason for decreased esterase activity in sonicates is not known, but may reflect enzyme inactivation by sonication or release of an endogenous inhibitor. This phenomenon is apparently unique to BCECF-AM [19] leading these authors to speculate that BCECF-AM may be metabolized by different esterase isozymes than fluorescein diacetate or carboxyfluorescein diacetate.

The biological function of the "non-specific" carboxylesterases is largely unknown although they are reportedly involved in steroid and fatty acid metabolism, and may be important in the maintenance of cell membrane integrity [20, 21]. If correct and applicable, then inhibition of esterase activity by oxidative SMX metabolites may represent an early indicator of eventual cell lysis as suggested by the correlation between cell damage at 2 hr and cell death at 24 hr.

Carboxylesterases are also involved in xenobiotic detoxification and in the activation of ester and amide prodrugs [22]. They also have been demonstrated to metabolize acetylated derivatives of arylamine carcinogens to DNA binding intermediates [23]. Antibodies in the sera of halothane hepatitis patients are directed against new epitopes expressed as a result of conformational changes in a 59 kD carboxylesterase upon covalent attachment of the reactive trifluoroacetyl group [16].

Esterases have been implicated in the lysis of target cells by cytotoxic T-lymphocytes and natural killer cells. Although the mechanism is largely unknown, it is thought to involve the secretion of granules which contain lytic molecules [24]. In addition to a Ca^{2+}-dependent pore-forming protein or perforin [25], these granules also contain serine esterases identified by their ability to cleave *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester [26, 27]. This enzyme activity is inhibited by the serine esterase inhibitor phenylmethylsulfonyl fluoride (PMSF) [28] as is the esterase activity responsible for BCECF-AM cleavage [19]. Based on this information, one would expect that incubation of cytotoxic effector cells with SMX-HA might reduce the esterase-mediated component of cytolytic activity. Data in the accompanying paper confirm this hypothesis and suggest a high sensitivity of natural killer cell fractions to SMX-HA [29]. The extent to which other granular lytic components are affected by SMX-HA is currently unknown. The role of this inhibitory function in sulfonamide hypersensitivity reactions will require further investigation.

At present, the mechanism of SMX-HA-induced inhibition of esterase activity is unknown. Current evidence indicates that the nitroso metabolite rather than SMX-HA is the toxic species [7]. Regardless, candidate nucleophilic sites for electrophilic attack could presumably include cysteine, serine, lysine or histidine residues. While the active site serine and histidine residues would be attractive nucleophilic sites for mechanistic purposes, such an interaction between SMX-HA and the esterase molecule is purely speculative at this time. It is interesting to note that isocyanate degradation products of the carcinostatic nitrosoureas 1,3-bis[2-chloroethyl]-1-nitrosourea (BCNU) and 1-[2-chloroethyl]-3-cyclohexyl-1-nitrosourea (CCNU) are potent inhibitors of serine proteases [30, 31] and serine esterases [19, 32]. In each case the active site serine has been identified as the carbamoylated residue. The similarity between the inhibitory effects of these compounds and SMX-HA (SMX-NO) remains to be determined.

In summary, inhibition of cellular esterase activity appears to be an early target function and thus a sensitive indicator of SMX-HA-induced cell damage. This esterase inhibition precedes eventual loss of membrane integrity but the data presented in this paper are insufficient to imply a causal relationship. Neither the mechanism of this form of toxicity nor its relationship to sulfonamide hypersensitivity reactions is currently understood. Identification of the ultimate reactive species and elucidation of the specific cellular targets are essential to determining the metabolic event(s) predisposing susceptible individuals to hypersensitivity reactions to sulfonamides and related compounds.

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