Stimulation of human T cells with sulfonamides and sulfonamide metabolites

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Background: Exposure to sulfonamides is associated with a high incidence of hypersensitivity reactions. Antigen-specific T cells are involved in the pathogenesis; however, the nature of the antigen interacting with specific T-cell receptors is not fully defined.

Objective: We sought to explore the frequency of sulfamethoxazole (SMX)– and SMX metabolite–specific T cells in hypersensitive patients, delineate the specificity of clones, define mechanisms of presentation, and explore additional reactivity with structurally related sulfonamide metabolites.

Methods: SMX- and SMX metabolite–specific T-cell clones were generated from 3 patients. Antigen specificity, mechanisms of antigen presentation, and cross-reactivity of specific clones were then explored. Low-lying energy conformations of drugs (metabolites) were modeled, and the energies available for protein binding was estimated.

Results: Lymphocytes proliferated with parent drugs (SMX, sulfadiazine, and sulfapyridine) and both hydroxylamine and nitroso metabolites. Three patterns of drug (metabolite) stimulation were seen: 44% were SMX metabolite specific, 43% were stimulated with SMX metabolites and SMX, and 14% were stimulated with SMX alone. Most metabolite-responsive T cells were stimulated with nitroso SMX–modified protein through a hapten mechanism involving processing. In contrast to SMX-responsive clones, which were highly specific, greater than 50% of nitroso SMX–specific clones were stimulated with nitroso metabolites of sulfapyridine and sulfadiazine but not nitrosobenzene. Pharmacophore modeling showed that the summation of available binding energies for protein interactions and the preferred spatial arrangement of atoms in each molecule determine a drug's potential to stimulate specific T cells.

Conclusions: Nitroso sulfonamide metabolites form potent antigenic determinants for T cells from hypersensitive patients. T-cell responses against drugs (metabolites) bound directly to MHC or MHC/peptide complexes can occur through cross-reactivity with the haptenic immunogen. (J Allergy Clin Immunol 2010;125:411-8.)

Key words: *Human, T cells, drug hypersensitivity, drug metabolism*

Hypersensitivity reactions to sulfamethoxazole (SMX) occur in 3% to 8% of patients. In patients with HIV infection, the incidence increased to 50% when the drug was used for prophylaxis,[1,2] which might relate to an altered costimulatory threshold. MHC polymorphisms are not major predisposing factors for SMX hypersensitivity.[3]

Drug-specific T cells have been isolated and characterized in terms of their phenotype and function from hypersensitive patients but not from drug-exposed control subjects,[4-11] indicating that they play an important role in the development of tissue pathology. The nature of the drug interaction with specific T cells has not been fully elucidated. Studies using T-cell clones obtained from patients with hypersensitivity have demonstrated that the parent drug can activate specific T cells through a series of noncovalent binding interactions.[4-6,9,12] This phenomenon, often referred to as the Pharmacological interaction of drugs with immunological receptors (PI) hypothesis,[13] is based on several experimental observations that are incompatible with the widely regarded hapten hypothesis,[14,15] which states that formation of a drug (metabolite)/protein complex is a prerequisite for immune stimulation.

SMX is metabolized to a hydroxylamine (SMX-NHOH) intermediate in human subjects and experimental animals.[16,17] Autooxidation of SMX-NHOH generates the electrophilic metabolite nitroso sulfamethoxazole (SMX-NO), which reacts directly with cysteine and cysteine sulfoxy acid residues on cellular and serum proteins, generating multiple antigenic determinants.[18-24]

SMX-NO is a potent immunogen in experimental models, and intracellular generation in dendritic cells is associated with costimulatory signaling.[10,19,20,25-28] SMX-NO activates T lymphocytes from 90% of drug-naive volunteers after 14 to 35 days of *in vitro* culture. Furthermore, T cells from SMX-NO–immunized animals proliferate in the presence of SMX-NO–modified protein. The T-cell stimulatory capacity of SMX-NO has only been explored in a limited cohort of hypersensitive patients; nevertheless, both skin- and blood-derived lymphocytes can be stimulated with SMX-NO.[5,6,10,29] In contrast to these findings, Schnyder et al[6] found that the vast majority of T cells were stimulated with SMX and, because they were not stimulated with

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Abbreviations used
SD: Sulfadiazine
SMX: Sulfamethoxazole
SMX-NHOH: Sulfamethoxazole hydroxylamine
SMX-NO: Nitroso sulfamethoxazole
SP: Sulfapyridine

SMX-NO, suggested that the primary antigenic determinant might be a noncovalently associated drug MHC or MHC/peptide complex.

The objective of this study was to use synthetic nitroso sulfonamide metabolites to explore further the T-cell stimulatory capacity of SMX and SMX metabolites and show for the first time T-cell receptor cross-reactivity through the formation of stable drug-metabolite protein conjugates that require processing.

METHODS

Donors' characteristics

Lymphocytes were isolated from the blood of 3 HIV-negative patients with a history of different clinical phenotypes of hypersensitivity to SMX (see Table E1 in this article's Online Repository at www.jacionline.org for clinical information) and 3 HIV-negative drug-exposed volunteers. Hypersensitive patients with different forms of hypersensitivity were selected to explore whether metabolite-specific T cells exist in the circulation of patients with a spectrum of cutaneous hypersensitivity reactions. Volunteers receiving SMX (12-14 days) for the treatment of urinary tract infections did not experience the clinical features of hypersensitivity. Approval for the study was obtained from the Liverpool local research ethics committee; informed written consent was obtained from each donor.

Chemicals and generation of antigen-presenting cells, T-cell lines, and clones

Autologous EBV-transformed B-cell lines were used as antigen-presenting cells. Antigen-specific T cells were enriched by culturing lymphocytes with SMX, SMX-NHOH, and SMX-NO. After 14 days, T cells were cloned by means of serial dilution. Methods describing the generation of antigen-presenting cells, T-cell clones, and sulfonamide metabolites can be found in the Methods section of this article's Online Repository at www.jacionline.org.

Lymphocyte proliferation

Proliferation of patients' lymphocytes (0.15×10^6 per well in 96-well, U-bottomed, cell culture plates; total volume, 200 μL) against SMX (197-3,150 μmol/L), sulfapyridine (SP; 201-3,212 μmol/L), and sulfadiazine (SD; 200-3,200 μmol/L; all 50–800 μg/mL); SMX-NHOH, SP hydroxylamine, and SD hydroxylamine (all 20–80 μmol/L); and SMX-NO, nitroso SP, and nitroso SD (all 20–80 μmol/L) was measured by using the lymphocyte transformation test.[30,31] After a 6-day incubation (37°C at 5% CO_2), proliferative responses were calculated as the stimulation index (counts per minute in drug-treated cultures/counts per minute in dimethyl sulfoxide–treated cultures; a stimulation index >2 is considered positive) by the addition of tritiated thymidine for 16 hours. Experiments were performed in triplicate with lymphocytes isolated from 2 separate blood donations.

Specificity of T-cell clones

SMX-, SMX-NHOH–, and SMX-NO–responsive T-cell clones were tested for additional reactivity against the parent drug and metabolites. Each clone (0.5×10^5/well) was incubated with antigen-presenting cells (0.1×10^5/well) and SMX (197–3,150 μmol/L; 50–800 μg/mL), SMX-NHOH (20–80 μmol/L), or SMX-NO (20–80 μmol/L). Proliferation was measured by using tritiated thymidine incorporation, as described above. The following terms are used consistently throughout the manuscript to describe clone specificity: *responsive*, which is used to describe whether a clone is stimulated with a particular antigen (this term does not infer specific activity); *specific*, which is used to describe whether a clone responds to 1 particular antigen or a series of antigens; *additional reactivity*, which is used to describe when a clone is responsive toward both the parent drug and metabolites; and *cross-reactivity*, which is used to describe when reactivity is detected between different sulfonamides or sulfonamide metabolites.

Determination of the involvement of processing and covalent adduct formation in the stimulation of antigen-specific T-cell clones

The involvement of processing in SMX and SMX metabolite presentation to T-cell clones was determined by chemically fixing antigen-presenting cells with glutaraldehyde (0.05% for 30 seconds). The role of protein adduct formation in SMX (metabolite) responses was evaluated first by the addition of glutathione (1 mmol/L) to the proliferation assay and second by pulsing antigen-presenting cells with SMX (787 μmol/L; 200 μg/mL), SMX-NHOH (80 μmol/L), or SMX-NO (80 μmol/L) for 1 hour before washing and addition of the pulsed antigen-presenting cells to the proliferation assay.

Cross-reactivity of T-cell clones

SMX-responsive T-cell clones were incubated with antigen-presenting cells and titrated concentrations of SD (400-1,600 μmol/L) or SP (402–1,606 μmol/L; both 100–400 μg/mL). SMX metabolite–responsive T-cell clones were incubated with either SP hydroxylamine and SD hydroxylamine or nitroso SP, nitroso SD, and nitrosobenzene (20–80 μmol/L). Proliferation was measured based on incorporation of tritiated thymidine, as described above.

Estimation of the summation of individual binding energies associated with nitrosobenzene, nitroso sulfamethoxazole, and the drugs SMX, SD, and SP

Methods describing the generation of low-lying energy conformations of nitrosobenzene, nitroso sulfamethoxazole (SMX-NO), SMX, SD, and SP and estimated binding energies can be found in the Methods section of this article's Online Repository.

Statistical analysis

The Mann-Whitney test was used for comparison of control and test values.

RESULTS

Lymphocytes from SMX-hypersensitive patients are stimulated with structurally related sulfonamides and sulfonamide metabolites

Lymphocytes from all 3 hypersensitive patients but not the control subjects were found to proliferate in the presence of SMX, SMX-NHOH, and SMX-NO. Lymphocytes from each patient were also stimulated with SP, whereas SD stimulated lymphocytes from Patient 1 and Patient 2 but not Patient 3 (stimulation index <2). Lymphocytes from patients 1 and 3 were also stimulated with hydroxylamine and nitroso metabolites of SP and SD (see Table E1).

Generation of T-cell clones after stimulation of lymphocytes with SMX and SMX metabolites

A total of 480 antigen-specific T-cell clones were generated from the hypersensitive patients. Of these, 128 were identified from

TABLE I. Origin, phenotype, and specificity of T-cell clones from SMX-hypersensitive patients

	Initial drug exposure	n	Proliferation (cpm)		Clones analyzed (n)	Phenotype (%)		Cross-reactivity (%)	
			Control	Antigen		CD4	CD8	SMX	SMX-NO
Patient 1	SMX	18	7703 ± 4301	30,386 ± 20,189	5	100	0	–	20
	SMX-NO	26	7557 ± 4584	29,648 ± 21,096	12	100	0	75	–
	SMX-NHOH	19	10102 ± 5946	41,185 ± 26,021	12	100	0	58	–
Patient 2	SMX	96	371 ± 319	1,044 ± 9,160	10	90	1	–	40
	SMX-NO	75	324 ± 314	6,915 ± 9,151	8	100	0	38	–
	SMX-NHOH	54	511 ± 394	12,168 ± 15,314	15	94	6	60	–
Patient 3	SMX	14	3123 ± 2628	21,101 ± 33,472	7	72	28	–	43
	SMX-NO	92	2111 ± 2033	14,459 ± 11,699	16	87	13	25	–
	SMX-NHOH	86	1,957 ± 1,526	14,621 ± 10,588	16	87	13	25	–

SMX-stimulated lymphocytes and 352 from SMX metabolite–stimulated lymphocytes. The number of SMX- and SMX metabolite–responsive T-cell clones isolated from each patient and their origin, phenotype, and specificity are summarized in Table I. Zero, 6%, and 15% of clones expressed high levels of $CD8^+$ from patients 1 (exanthema), 2 (drug rash with eosinophilia and systemic symptoms), and 3 (Stevens-Johnson syndrome), respectively. One hundred one well-growing $CD4^+$ and $CD8^+$ clones (22 from SMX, 43 from SMX-NHOH, and 36 from SMX-NO cultures) were subsequently characterized. Antigen-specific T-cell clones were not identified from volunteer blood.

Antigen specificity of SMX- and SMX metabolite–responsive T-cell clones

Three patterns of reactivity were seen with clones from each hypersensitive patient (Fig 1). First, 44 metabolite-specific T-cell clones were detected. These clones were stimulated with SMX-NHOH and SMX-NO but not the parent compound. Proliferative responses were detected with metabolite concentrations greater than a threshold of 10 μmol/L. Dose-response curves showed a sharp increase in the slope, with maximal responses detected at 40 to 80 μmol/L; higher metabolite concentrations inhibited the proliferative response. Second, 14 clones were stimulated with the parent compound alone. Finally, 43 clones displayed activity against both SMX and SMX metabolites. Each pattern of reactivity was detected with $CD4^+$ and $CD8^+$ clones.

Response of drug- and drug metabolite–specific T-cell clones toward pulsed antigen-presenting cells

SMX metabolite–specific $CD4^+$ and $CD8^+$ T-cell clones were stimulated to proliferate with both SMX-NHOH– and SMX-NO–pulsed antigen-presenting cells. The strength of the induced proliferative response did not differ when soluble SMX metabolites and SMX metabolite–pulsed antigen-presenting cells were used as a source of antigen (Fig 2, *A*). In contrast, SMX-specific $CD4^+$ and $CD8^+$ clones that do not display additional reactivity against SMX metabolites were not stimulated with SMX- or SMX metabolite–pulsed antigen-presenting cells.

Glutathione significantly reduced the proliferative response of SMX metabolite–specific $CD4^+$ and $CD8^+$ T-cell clones (Fig 2, *B*). However, stimulation of SMX-specific T-cell clones was not affected by the addition of glutathione.

Involvement of processing in presentation of SMX and SMX metabolites to T cells

Glutaraldehyde prevents antigen processing but not the presentation of preprocessed antigen or antigens that bind directly to MHC molecules or MHC/peptide complexes. The majority (16/23) of SMX metabolite–specific clones were stimulated to proliferate with SMX-NO through a classical pathway involving antigen processing. Although only low numbers of $CD8^+$ clones were tested, several clones were shown to be responsive to processed peptides derived from SMX-NO–modified protein. In contrast, the response of SMX-specific $CD4^+$ and $CD8^+$ T-cell clones was not altered by fixation of antigen-presenting cells (Fig 2, *C*).

Response of T-cell clones showing reactivity against SMX and SMX metabolites toward antigen-pulsed and glutaraldehyde-fixed antigen-presenting cells

In agreement with previous studies,[5] the response of $CD4^+$ and $CD8^+$ T-cell clones showing reactivity against SMX and SMX metabolites was not blocked with glutathione (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). T-cell clones were stimulated to proliferate with SMX-NHOH– and SMX-NO–pulsed but not SMX-pulsed antigen-presenting cells (see Fig E1, *B*). Fixation experiments show that SMX stimulates clones through a direct interaction with immunologic receptors, whereas SMX-NO stimulates a proliferative response through both processing-dependent and independent pathways (see Fig E1, *C*).

Cross-reactivity of T-cell clones with SD, SP, and their hydroxylamine and nitroso metabolites

Greater than 50% of SMX metabolite-responsive $CD4^+$ T-cell clones were stimulated with SP metabolites, SD metabolites, or both (see Fig E2 in this article's Online Repository at www.jacionline.org). The majority of cross-reactive clones proliferated in the presence of SMX, SP, and SD metabolites; however, certain clones were stimulated with SMX and SP metabolites but not SD metabolites or SMX and SD metabolites but not SP metabolites. Nitrosobenzene did not stimulate SMX metabolite–responsive T-cell clones. One SMX metabolite–responsive $CD8^+$ T-cell clone was incubated with and shown to be stimulated by SMX, SP, and SD metabolites.

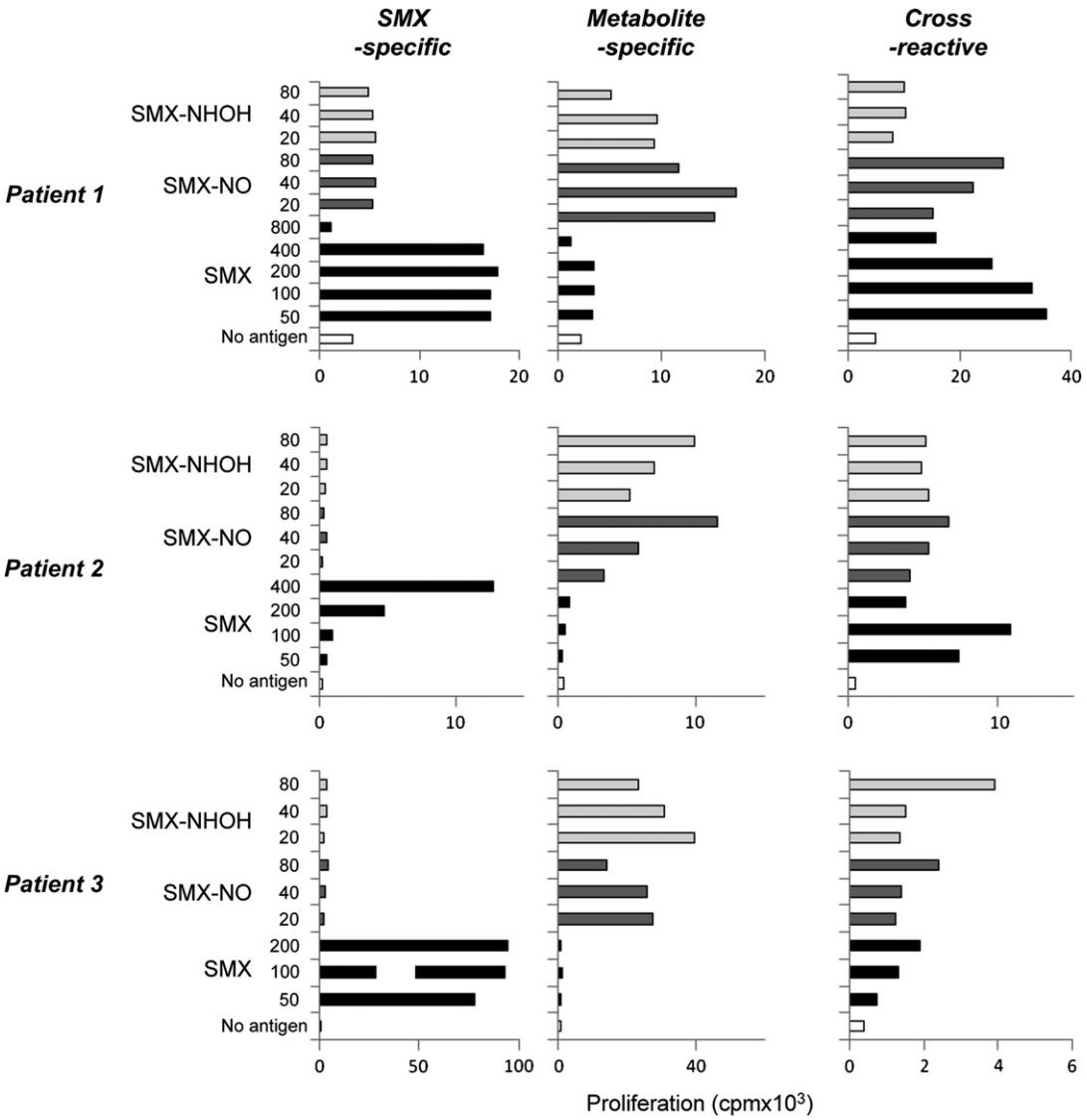

FIG 1. Stimulation of individual SMX-specific, SMX metabolite–specific, and cross-reactive T-cell clones from 3 patients (1, 2, and 3) with SMX (197–1,575 μmol/L; 50-400 μg/mL) and SMX metabolites (20–80 μmol/L). T-cell clones were incubated with SMX, SMX-NHOH, or SMX-NO and irradiated autologous B-lymphoblastoid cell lines for 48 hours to measure proliferation. Results are presented as the mean tritiated thymidine incorporation. Differences in replicate cultures at each antigen concentration were less than 15%.

Five of 14 SMX-responsive T-cell clones were stimulated to proliferate with SD. Two of these clones were also stimulated with SP.

Estimation of binding energy associated with SMX-NO, nitrosobenzene, and the drugs SMX, SD, and SP

Pharmacophore models demonstrate that 2 parameters, the summation of available binding energies for drug-protein interactions and the preferred spatial arrangement of atoms in each molecule, determine a compound's potential to interact with specific T-cell receptors (Fig 3).

Comparison of the preferred low-energy 3-dimensional spatial arrangement of SMX-NO with SMX, SD, and SP revealed that the arrangement of atoms within each compound differed, and therefore cross-reacting drugs must adopt more strained confirmations to interact with specific T cells. Adding together the energy values associated with each moiety provided an estimated total binding energy for each compound. The summation of available energies for SMX, SD, and SP MHC/MHC/peptide–T-cell receptor binding was found to be 121, 145, and 115 kj/mole, respectively.

Assuming that the nitroso group of SMX-NO associates covalently with MHC or a peptide embedded in the MHC-binding cleft, the binding energy associated with the remainder of the structure (121 kj/mole) is available for T-cell receptor binding. The binding energy associated with nitrosobenzene, which does not stimulate SMX-NO–specific clones, was 35 kj/mole.

These *in silico* studies indicate that it is possible for a drug MHC (peptide) complex could arise that has comparable binding energies for 3 sulfonamides and similar affinities with respect to binding.

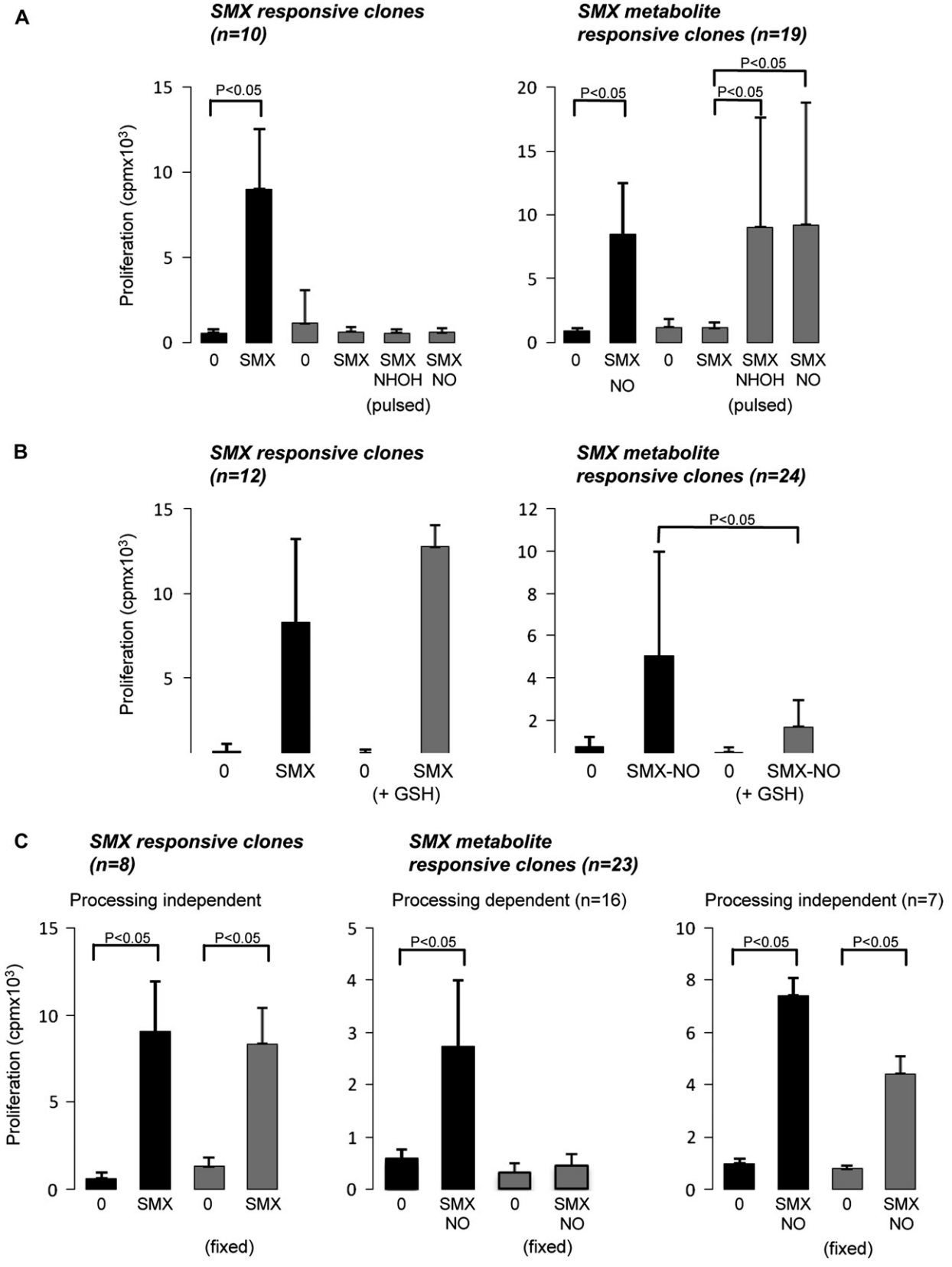

FIG 2. The response of SMX- and SMX metabolite–specific T-cell clones toward drug (metabolite)–pulsed antigen-presenting cells **(A)**, antigen-presenting cells incubated with drug (metabolites) in the presence and absence of glutathione **(B)**, and glutaraldehyde-fixed antigen-presenting cells **(C)**. Results are presented as the mean ± SD tritiated thymidine incorporation.

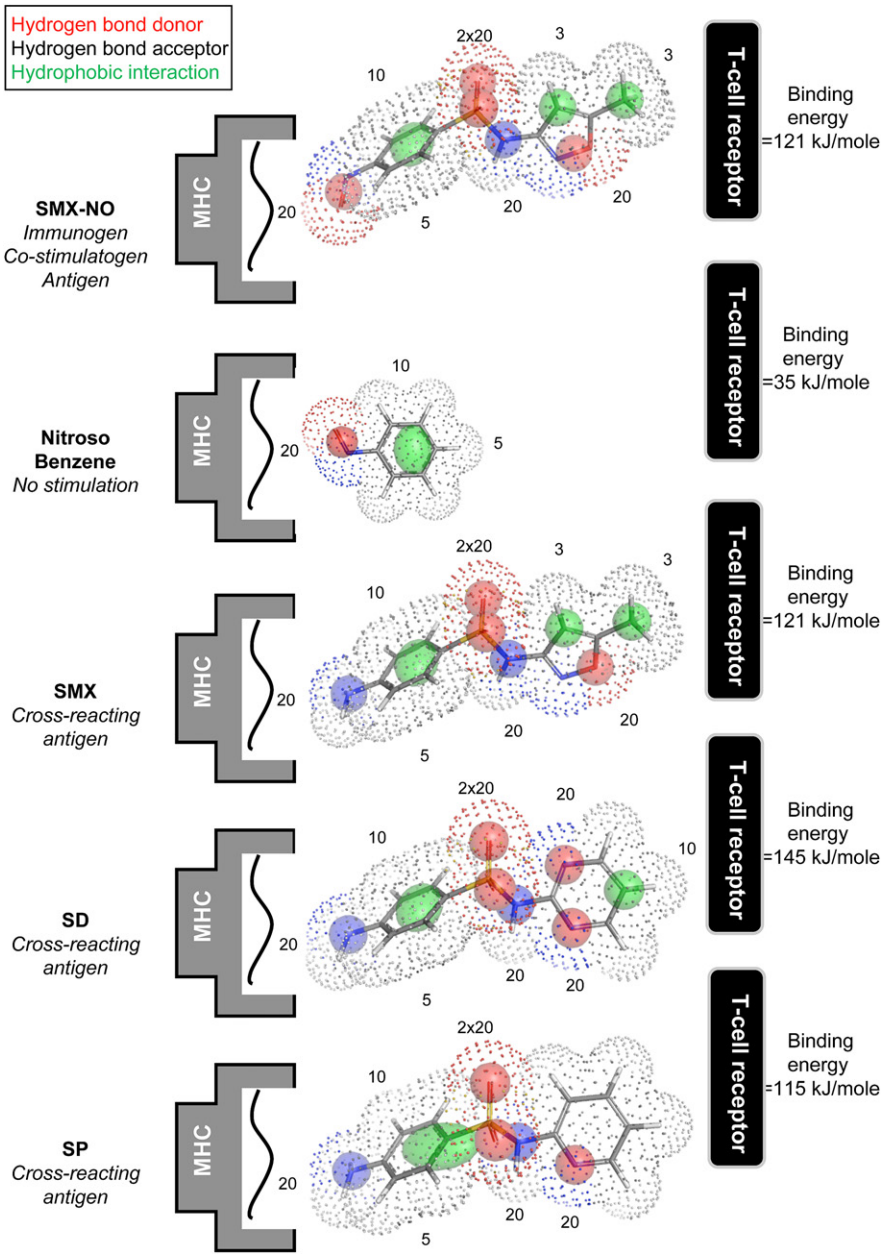

FIG 3. Low-energy conformations and intermolecular forces associated with SMX-NO, nitrosobenzene, SMX, SD, and SP and the contribution of the different structural moieties. Calculated binding energies for SMX-NO and nitrosobenzene exclude the energy associated with the covalent interaction between the nitroso moiety and protein.

DISCUSSION

T cell–mediated drug hypersensitivity reactions are an important health issue and a cause of drug discontinuation and morbidity and mortality in patients.[32] Antibacterial sulfonamides are a class of drugs associated with hypersensitivity reactions and represent the model most widely studied to explore the chemical basis of immune activation.[33]

In initial experiments lymphocytes from 3 hypersensitive patients were found to proliferate *ex vivo* with SMX, SMX-NHOH, and SMX-NO (see Table E1). By generating T-cell clones, it was possible to explore the relative frequency of drug- and drug metabolite–specific T cells residing in the peripheral circulation. It is important to note that SMX metabolite concentrations (20-80 μmol/L) were optimized to detect a proliferative response. Calculations based on ingested dose and levels of oxidative metabolites secreted in urine estimate that patients might be exposed to metabolite concentrations of 5 to 15 μmol/L. However, it must be noted that this form of analysis likely greatly misjudges cellular exposure because it does not account for the possibility of drug accumulation, covalent binding to tissues, or organ-specific metabolic activation.[34] Our previous collaborative studies with Pichler and colleagues,[5,6] which detected only low numbers of metabolite-responsive T cells from hypersensitive patients, were performed with logarithmic

increases in metabolite concentration. It is now recognized that SMX-NO concentrations of 10 μmol/L and less are suboptimal, whereas concentrations of 100 μmol/L have an inhibitory effect on T-cell proliferation. Thus previous reports might have underestimated the importance of metabolite-responsive T cells in SMX hypersensitivity, and this is the reason for revisiting this issue.

A panel of 480 primarily $CD4^+$ T-cell clones was generated. The response of 78% (373 clones) of the clones was directed against SMX metabolites (Table I). From experiments on 101 well-growing clones, 3 patterns of antigen-specific T-cell stimulation were discernable.

First, the 44 clones were metabolite specific. Dose-response curves show a steep incline at concentrations of greater than 10 μmol/L, indicating that T cells respond strongly to SMX metabolites once a threshold has been surmounted. Second, 14 clones were stimulated with the parent compound alone. Finally, 43 clones were stimulated with SMX and both SMX metabolites. Our previous reports assessing T cells derived from SMX- and SMX-NO–driven cultures[5,6] demonstrated that less than 2% of clones were metabolite specific, whereas 4.5% were stimulated with SMX and SMX metabolites.

SMX-NHOH– and SMX-NO–pulsed antigen-presenting cells were found to stimulate SMX metabolite–specific T-cell clones. A 1-hour incubation of antigen-presenting cells with SMX metabolites, but not SMX, is associated with the formation of membrane adducts that become internalized by a caveolae-dependent process.[20,21,27] Glutathione pretreatment, which prevents protein adduct formation,[5,22] significantly reduced the strength of the proliferative response. Furthermore, stimulation of 16 of 23 clones was blocked when T cells were incubated with SMX metabolites and fixed antigen-presenting cells. Thus processed peptides derived from SMX-NO–conjugated protein stimulate the majority of metabolite-specific clones. The nitroso moiety of SMX-NO binds covalently to cysteine residues on protein to generate a protein adduct. After processing, linear peptides derived from the protein adduct associate with MHC molecules and, in union with the SMX metabolite, provide a binding interaction with specific T-cell receptors. SMX-NO–specific T cells were not stimulated to proliferate in the presence of nitrosobenzene, the simplest aromatic nitroso compound, which generates neoepitopes by means of formation of cysteinyl sulfoxy acids and haptenic epitopes by binding covalently to cysteine residues on protein.[35] Thus the intermolecular forces associated with the sulfonamide group and the 5-methylisoxazole side chain of SMX (Fig 3; totaling 90 kj/mole) are involved in the hapten-modified peptide-binding interaction with MHC molecules and specific T-cell receptors.

In agreement with the PI hypothesis, low numbers of T-cell clones with specificity for noncovalently associated SMX were identified from all 3 patients. SMX competes with the folic acid precursor *p*-aminobenzoic acid for binding sites on the enzyme dihydropteroate synthetase to exert a pharmacologic effect. Because a binding interaction is critical for pharmacologic and presumably immunologic activity, *in silico* modeling was also used to estimate the summation of available binding energies for SMX/protein complex formation. The binding energy associated with the SMX structure (121 kj/mole) equates to the strength of a weak covalent bond or several coordinate linkages, which are involved in the stimulation of nickel-specific T cells from patients with contact allergic dermatitis.[36-38] Thus assuming MHC/MHC/peptide complexes express complementary binding sites in the correct spatial arrangement, it is feasible chemically for SMX to interact directly with MHC/MHC/peptide complexes and activate specific T cells.

Several T-cell clones were stimulated with SMX and SMX metabolites (Fig 1) through both processing-dependent and independent pathways (see Fig E1). Discriminating between different pathways of drug stimulation with these clones is somewhat difficult. Glutathione does not inhibit SMX metabolite–specific stimulation because it generates SMX-NHOH and SMX, which themselves are stimulatory through direct MHC/MHC/peptide binding.

In contrast to maculopapular exanthemas that are predominantly mediated by $CD4^+$ T cells, severe cutaneous reactions show a strong drug-specific $CD8^+$ T-cell migration to the skin.[29] In the present study approximately 5% and 15% of clones generated from patients WJ and BB, patients presenting with severe forms of hypersensitivity, expressed high levels of $CD8^+$. Although the number of $CD8^+$ clones analyzed was relatively small, both SMX- and SMX metabolite–responsive $CD8^+$ T-cell clones with similar reactivity patterns to $CD4^+$ T cells were characterized. SMX-NO has been shown to bind to serum and cell-membrane proteins in *in vitro* cell assays.[18,21] Thus peptides derived from processed serum protein adducts presumably represent antigenic determinants for $CD4^+$ clones, whereas cellular adducts are likely antigenic determinants for $CD8^+$ T cells. To address this issue, in ongoing experiments we are investigating the relationship between selective adduct formation and the development of divergent immune responses.

Collectively, these data raise the intriguing possibility that peptides derived from SMX-NO protein conjugates represent high-affinity antigens for T cells. In *in vitro* experiments only a small percentage of SMX-NO binds covalently to protein. The majority is consumed by competing reduction, oxidation, and self-conjugation reactions.[10,20,27] Thus T cells are likely to be responsive toward incredibly low metabolite-derived antigen concentrations. In contrast, SMX is present at millimole per liter concentrations for the duration of the experiment and will ligate and saturate all available binding sites. These conditions produce large numbers of densely coated but weakly associated SMX/MHC peptide complexes with somewhat lower affinity for SMX-NO–specific T-cell receptors. In this scenario SMX can stimulate hapten-specific T cells through molecular or structural mimicry of hapten/peptide complexes, epitope spreading, or both.

Hydroxylamine and nitroso metabolites of SD and SP were synthesized and tested for their ability to stimulate T cells to investigate whether cross-reactivity through protein complex formation can explain the development of multiple allergic reactions to structurally related sulfonamides. In contrast to SMX-specific T-cell clones, which display limited responses toward SD and SP, 53% (27/51 clones) of SMX metabolite–responsive clones displayed reactivity toward nitroso metabolites of SD and SP. Pharmacophore modeling of preferred low-energy conformations of SMX, SD, and SP show that although the summation of intermolecular forces for each compound is similar, cross-reacting drug antigens must adopt twisted higher energy structures to mimic the binding interaction of SMX at specific T-cell receptors. These data demonstrating high levels of T-cell receptor cross-reactivity with nitroso sulfonamides displaying different sulfonamide side chains have important clinical ramifications. They show the clear potential for hypersensitivity reactions to develop to different drug structures within the same

chemical class through metabolite formation and targeting of identical binding sites on protein.

In conclusion, SMX-NO was found to form potent antigenic determinants for T cells from hypersensitive patients presenting with different clinical phenotypes of SMX hypersensitivity. T-cell responses against SMX were detected less frequently and might be dependent on the formation of densely coated but weakly associated SMX MHC/MHC/peptide T-cell receptor complexes that cross-react with the haptenic immunogen. In view of the fact that our study was limited to 3 patients with disparate forms of cutaneous hypersensitivity, generalization of the findings to all sulfonamide hypersensitivity reactions should be done with caution. Nevertheless, it appears that previous reports have underestimated the importance of metabolite-specific T-cell responses in SMX hypersensitivity.

We thank the blood donors for their generosity.

Clinical implications: T-cell receptor cross-reactivity with different nitroso sulfonamides reveals a potential mechanism for the development of hypersensitivity reactions to different drug structures through metabolite formation and protein binding.

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METHODS

Chemicals and generation of autologous antigen-presenting cells, T-cell lines, and clones

EBV-transformed B-cell lines, used as antigen-presenting cells, were generated by incubating blood lymphocytes with supernatant from the EBV-producing cell line B9-58. The medium used for culture of EBV-transformed B cells was RPMI-1640 supplemented with 10% FCS (Gibco, Paisley, Scotland) and 25 mmol/L HEPES buffer but no L-glutamine, transferrin, or antibiotics. Cyclosporin A was added for the first 2 weeks of culture to prevent EBV-induced T-cell growth.

Antigen-specific T cells were enriched by culturing lymphocytes (1×10^6 in 330 μL) from 3 patients with SMX (197, 394, and 797 μmol/L; 50-200 μg/mL), SMX-NHOH (20, 40, and 80 μmol/L), and SMX-NO (20, 40, and 80 μmol/L). IL-2 was added on days 6 and 9 to maintain antigen-specific proliferation. After 14 days, T cells were cloned by means of serial dilution with established methodology.[E1] T cells (0.5×10^5) were incubated with antigen-presenting cells (0.1×10^5) and the drug or metabolite to which the lymphocytes were originally exposed to test the specificity of the clones. After 48 hours, tritiated thymidine (0.5 μCi) was added, and 16 hours later, proliferation was measured by means of scintillation counting. The proliferative response of clones is expressed as counts per minute in antigen-stimulated and unstimulated cultures. The CD phenotype of specific clones was measured by means of flow cytometry.

Nitroso and hydroxylamine metabolites of SMX, SD, and SP were synthesized according to recently established methods (Castrejon et el, unpublished date).[E2] The compounds were found to be greater than 95% pure when assessed by means of nuclear magnetic resonance and liquid chromatography–mass spectrometry.

Medium for lymphocyte culture and T-cell cloning

Culture medium consisted of RPMI-1640 supplemented with pooled heat-inactivated human AB serum (10% vol/vol), HEPES (25 mmol/L), L-glutamine (2 mmol/L), transferrin (25 μg/mL), streptomycin (100 μg/mL), and penicillin (100 U/mL).

Estimation of the summation of individual binding energies associated with nitrosobenzene, SMX-NO, and the drugs SMX, SD, and SP

Low-lying energy conformations of nitrosobenzene, SMX-NO, and the drugs SMX, SD, and SP were located by using the MMFF94aq force field, as implemented in Spartan08, with default settings (http://www.wavefun.com). Pharmacophores were identified by using the Liquid algorithm with cutoffs of 2 Å for all bins.[E3] Estimation of the binding energies associated with nitrosobenzene, SMX-NO, SMX, SD, and SP was achieved by using simple summation of the typical binding values that noncovalent interactions can provide. The values associated with the following features were as follows: hydrogen bond, approximately 20 kJ/mol; hydrophobic interaction, approximately 10 kJ/mol; a methyl hydrophobic interaction, approximately 3 kJ/mol; and van der Waals, approximately 5 kJ/mol. Molecular structures were displayed by using PyMol (http://www.pymol.org), with the molecular surface rendered as dots, pharmacophores rendered as spheres, and molecules depicted in sticks, with carbon in gray, hydrogen in white, nitrogen in blue, and oxygen in red.

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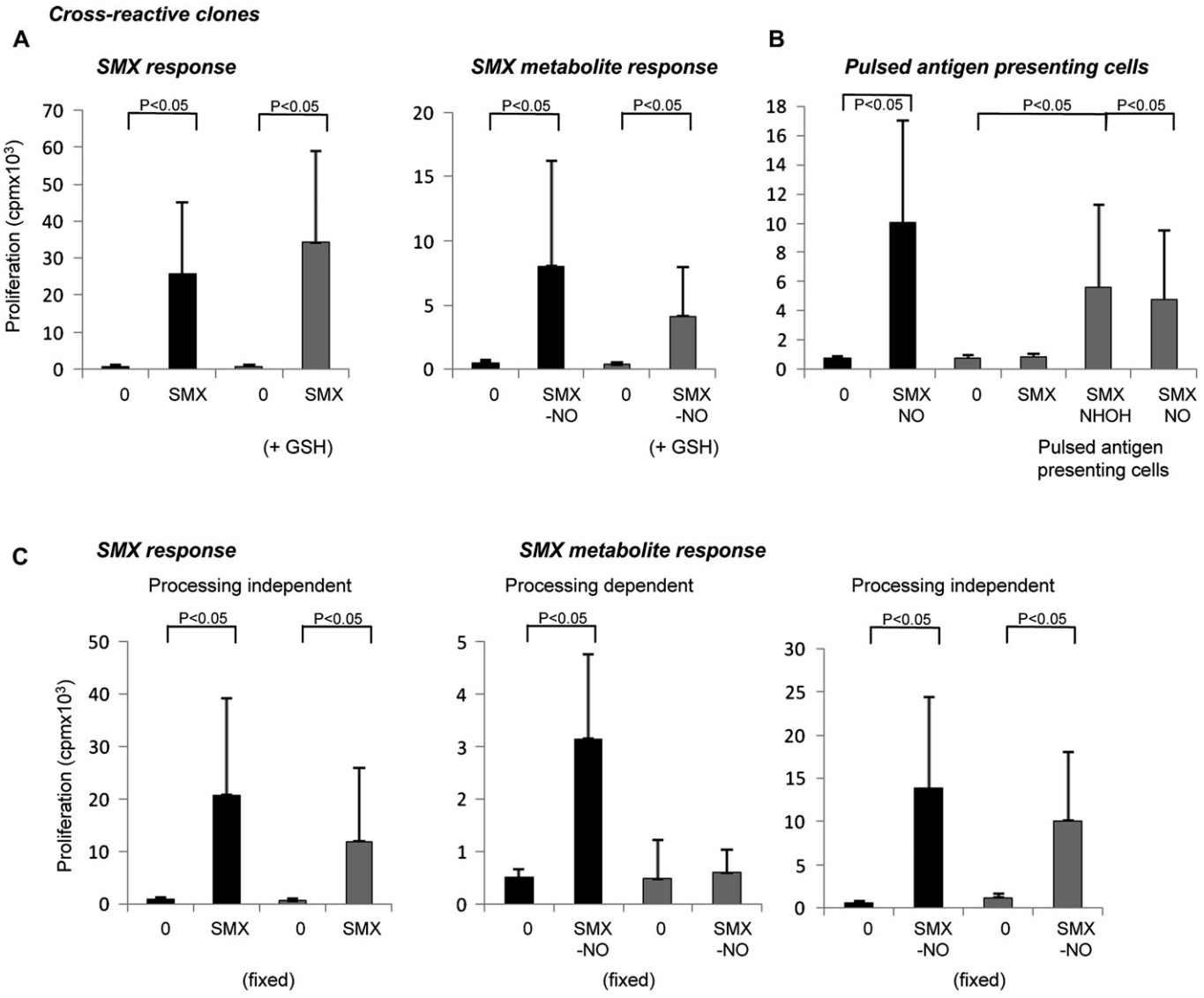

FIG E1. The response of SMX- and SMX metabolite–responsive T-cell clones toward antigen-presenting cells incubated with drug (metabolites) in the presence and absence of glutathione **(A)**, drug (metabolite)–pulsed antigen-presenting cells **(B)**, and glutaraldehyde-fixed antigen-presenting cells (**C**). Results are presented as the mean ± SD tritiated thymidine incorporation.

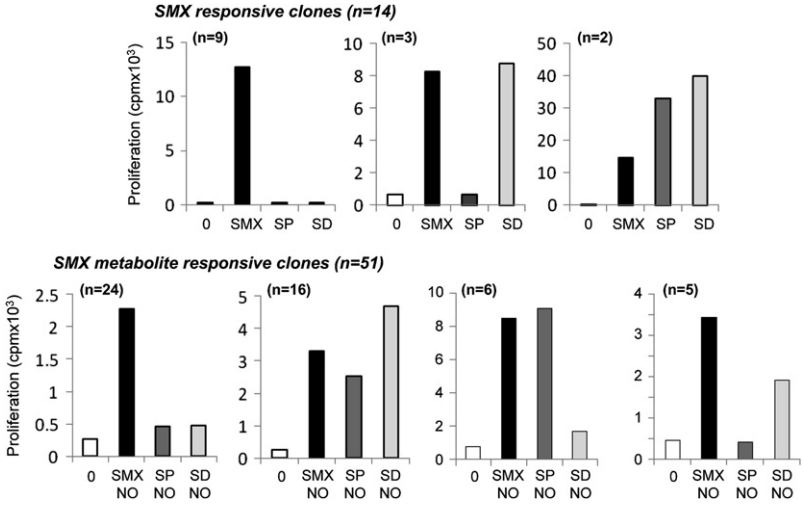

FIG E2. The response of T-cell clones to SMX, SD, and SP and their hydroxylamine and nitroso metabolites. Results are presented as the mean tritiated thymidine incorporation of representative T-cell clones.

TABLE E1. Clinical details of hypersensitive patients and lymphocyte proliferation

Patients' details			Lymphocyte transformation test (SI)								
			Parent drug: SMX, SD, SP (100μg/mL)			NHOH: SMX, SD, SP (40 μmol/L)			NO: SMX, SD, SP (40 μmol/L)		
	Details of the reaction	Culprit drug	SMX	SD	SP	SMX	SD	SP	SMX	SD	SP
Patient 1	Exanthema and malaise	SMX	8.7	12.2	8.1	6.1	4.6	5.3	4.9	2.1	3.2
Patient 2	DRESS and erythroderma	SMX	15.3	8.3	3.9	3.5	<2	<2	2.1	<2	<2
Patient 3	SJS (exanthema, conjunctivitis, and bullae)	SMX and SP	11.2	<2	3.3	16.0	5.2	4.2	24.8	2.4	2.3

DRESS, Drug rash with eosinophilia and systemic symptoms; *SI*, stimulation index; *SJS*, Stevens-Johnson syndrome.