

ORIGINAL ARTICLES

Immunopathogenesis of hypersensitivity syndrome reactions to sulfonamides

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Cytokines play a role in the immunopathological and molecular mechanisms of sulfonamide-induced hypersensitivity reactions (HSRs). The objective of this study was to analyze the reliability and correlation between the clinical symptoms observed in affected patients ($n = 86$) because of a sulfonamide-induced HSR and their lymphocyte toxicity assay (LTA) values. Another goal was to determine the cytokine secretion in the patient's sera and their expression in the peripheral blood mononuclear cells (PBMCs) and to explore whether a correlation exists among positive LTA score, cytokine levels, and HSR occurrence. The final goal is to determine whether these measures could be used to predict the likelihood of a patient to experience an HSR during sulfonamide treatment. Such a predictive ability would be valuable to the clinician to know whether the patient would tolerate sulfonamides or whether an alternative antibiotic should be prescribed. The LTA showed a good correlation with the clinical involvement of patients with hypersensitivity syndromes. In addition, the pro-inflammatory cytokines presented significant differences in patients that had rash, fever, and organ involvement than in control patients or any of the other patient groups. Expression of tumor necrosis factor alpha (TNF- α) is significantly higher in patients presenting rash, fever, and organ involvement versus all other groups. It is concluded that a positive LTA is a predictor for sulfonamide-induced true HSR. In addition, T-helper cell 1 cytokines (TNF- α , interleukins (ILs) 1 and 2) as well as the chemokine regulated upon activation, normal T-cell expressed and secreted (RANTES) control the pathogenesis of sulfonamide-induced HSR and may be used in early diagnosis of the syndrome. (Translational Research 2007;149:243-253)

Abbreviations: ELISA = enzyme-linked immunosorbent assay; G3PDH = glyceraldehyde-3-phosphate dehydrogenase; GSH = mitochondrial glutathione; GST = glutathione S transferase; HSR = hypersensitivity syndrome reaction; IL = interleukin; IFN- γ = interferon-gamma; LTA = lymphocyte toxicity assay; MT = mitochondrial toxicity; NADP = nicotinamide adenine dinucleotide phosphate; PBMC = peripheral blood mononuclear cell; PCR = polymerase chain reaction; RANTES = regulated upon activation, normal T-cell expressed and secreted; ROS = reactive oxygen species; RT-PCR = reverse transcriptase polymerase chain reaction; SDH = succinic dehydrogenase; SJS = Stevens-Johnson syndrome; SMX = sulfamethoxazole; TEN = toxic epidermal necrolysis; TNF- α = tumor necrosis factor-alpha

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Adverse drug reactions account for 5% of all hospital admissions and occur in approximately 15% of hospitalized patients.¹ The majority of these reactions are common and predictable, being primarily based on the pharmacological properties of the drug given. Unpredictable or idiosyncratic, type B reactions pose a major concern in clinical practice and drug development primarily because they are not dose-dependent.² However, type B reactions are known to occur at certain doses in susceptible individuals.³ One of the most common and potentially fatal adverse drug reactions are hypersensitivity syndromes associated with sulfonamides.^{1,4} The hypersensitivity syndrome reaction (HSR) is a host-dependent drug reaction that is idiosyncratic in nature.

The true HSR is a systemic disease defined by the triad of fever, rash, and internal organ involvement that starts 8–12 weeks after the initiation of therapy.⁵ An incidence of 1/1000 to 1/10,000 exposures is inaccurate because many reactions are common yet underreported. Clinical conditions range from simple skin reactions and general manifestations, such as morbilliform rash, urticaria, angioedema, fever, malaise, anaphylaxis, bronchospasm, and erythema multiforme, to skin complications with the highest mortality rates exhibited by Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), which involve multiorgan function impairment⁵ such as lymphadenopathy, hepatic dysfunction, hematologic dysfunction, renal dysfunction, myocarditis, or myositis.^{6–8}

Sulfonamides are commonly used antibiotic drugs. The sulfonamide family includes a wide spectrum of different products, including sulfacetamide, sulfadiazine, sulfamethoxazole (SMX), sulfisoxazole, sulfamethizole, sulfadoxine/pyrimethamine (Fansidar), sulfapyridine, silver sulfadiazine, sulfamerazine, sulfamethazine, and the most widely used sulfa preparation sulfamethoxazole/trimethoprim (Septra, Bactrim).^{4,9,10}

As a high incidence of HSRs to sulfonamides exists, in approximately 5% of the general population and 10 times greater in HIV-infected patients, significant research has been conducted into finding a safe and reliable method to predict these reactions.^{4,11–15} The drug challenge can be complicated and dangerous because of the potential for cross-reactivity or life-threatening adverse reactions. The use of the lymphocyte toxicity assay (LTA) has been in practice since 1988^{6,16–18} and has recently been validated for SMX in immuno-competent and immuno-compromised patients.^{18,19} The test is based on reactive metabolites that are generated using murine¹⁸ or canine^{16,17} hepatic microsomes as a source of cytochrome P450 (P450). Human lymphocytes from patients with suspected HSRs are used as surrogate target cells for safe *in vitro* re-challenge.¹⁸

Lymphocytes are chosen as target cells for 2 main reasons. First, they do not produce reactive metabolites and do not contain enzymes, which may interfere with microsomal bioactivation of drugs added to culture. Second, they possess various detoxification enzymes, like epoxide hydroxylase and glutathione S transferases (GSTs), and phenotypically express individual variability in these enzymes, thus modeling the potential source of hypersensitivity observed *in vivo*.

A clear understanding of the processes involved in HSRs will greatly aid in its diagnosis; however, factors such as an unclear immunological mechanism, an unknown epitope that causes a reaction, and whether the presence of an immunological recognition is predictive of a clinical reaction hinders understanding of hypersensitivity reactions.^{5,10,20–23}

Depending on the time course of the reaction, the mechanism of sulfonamide-mediated adverse drug reactions falls into 1 of 2 possible pathways. Immediate-type immune-mediated reactions are presented with symptoms of urticarial rash that is typically without fever, occurring approximately 3 days into therapy. IgE antibodies raised against the drug are usually present. The 5-methyl-3-isoxazolyl group on SMX seem to be very involved in this immune response.⁴ Delayed-type immune-mediated reactions are typically presented with rash and fever, and they may include organ involvement that manifests 1–2 weeks after sulfonamide therapy is initiated.²⁴ As drug-specific activated T-cell clones have been found in these patients; this pathway is considered an immune-mediated HSR. These reactions have limited the therapeutic use of sulfonamides.

HSRs caused by sulfonamide antimicrobials are likely a result of a combination of metabolic and immunologic factors.^{10,20,25,26} The dose and duration of therapy may play a role, as well as defects in the metabolic pathways, the degree of immunodeficiency, bacterial and viral infections, the degree of reactive oxygen species (ROS) produced, if adduct formation occurs, and mitochondrial toxicity (MT) is present.^{1,19,23,27} Cellular immune-mediated components, such as the T cells and cytokine/chemokine mediation, which can exacerbate cellular responses, create complex pathways that lead to a variety of clinical manifestations.

Significant evidence exists implicating the hapten and danger hypotheses in the occurrence of sulfonamide-induced HSRs.²³ Only a small fraction of SMX undergoes oxidation to a reactive metabolite, which occurs through CYP2C9, generating a hydroxylamine derivative (SMX-NOH) capable of being acetylated or reduced (CYP3A4) back to SMX (Fig 1). Further oxidation of SMX-NOH produces a nitroso-metabolite (nitroso-SMX) capable of generating ROS through a redox cycle and binding cellu-

Table I. Mean and standard deviation for the positive and negative LTA values

	Patient LTA result (n)	LTA for SMX mean (SD)	PPV	NPV	Sensitivity	Specificity
R (27)	Positive (4)	18.40 (8.45)	44.60%	53.40%	40.70%	78.20%
	Negative (23)	6.63 (3.26)				
R + F (43)	Positive (24)	20.31 (4.93)	70.50%	81.50%	62.20%	79.10%
	Negative (19)	7.84 (3.36)				
R + F + OI (16)	Positive (15)	22.30 (8.34)	86.70%	99.50%	91.90%	99.10%
	Negative (1)	3.07 (0.00)				

Abbreviations: n, number of patients in the group; R, rash; F, fever; OI, organ involvement; LTA values: positive, MIT $\geq$ 12.5%; negative, MIT $<$ 12.5%; SMX, value indicates cytotoxicity; PPV, positive predictive value; NPV, negative predictive value.

Note: LTA has an excellent positive predictive value for the patients that presented with rash, fever, and organ involvement.

lar proteins, thereby completing the process of haptenization.⁴ Redox cycling alone can be detrimental not only to cellular proteins but to the protective balance of detoxifying molecules as well. Acetylation of SMX-NOH, primarily with N-acetyltransferase 2, helps decrease the available nitroso-SMX for covalent binding.⁴ In a study on rats exposed to nitroso-SMX metabolite, Naisbitt et al²² assessed the cytokine synthesis by responding to T lymphocytes through a quantification of interleukin-4 (IL-4) and interferon-gamma (IFN- γ) levels. In a previous study with the same rat model, they demonstrated that administration of nitroso-SMX resulted in the formation of anti-drug antibodies.²¹ Therefore, the haptenization of the nitroso-SMX might lead to a cellular immune response.

The authors believe that the mitochondrion is critically damaged when it is exposed to ROS, whereas the levels of mitochondrial glutathione (GSH) are low (Fig 1). When SMX is metabolized via the cytochrome P450 pathway, the production of ROS is maintained by detoxifying agents such as superoxide dismutase (E.C.1.15.1.1) and GST (E.C.2.5.1.18). While ROS are being generated, the redox cycling of SMX-NOH and nitroso-SMX depletes the levels of cytosolic GSH, which would lead to a drop in the detoxifying capability of the mitochondrion and damage from ROS. If apoptosis ensues, then the recruitment of chemokines would also follow. This effect adds to the inflammatory process and accelerates the cellular necrosis, leading to an HSR.

The objective of this study is to understand the immunopathological mechanism and molecular mechanism of HSRs. The specific aims of the study were to analyze the reliability and correlation between clinical evidence in immune-competent patients who developed hypersensitivity locally with systemic symptoms caused by SMX/TMP and the results of their lymphocyte toxicity assays.

A secondary objective of this study is an analysis of the cytokine secretion in the sera and their expression in the peripheral blood mononuclear cell (PBMC) of pa-

tients that presented sulfonamide-induced HSRs. The utility of these cytokines [tumor necrosis factor- α (TNF- α), IL, and IFN- γ] as potential noninvasive markers for susceptibility to sulfonamide-induced HSRs has also been evaluated.

MATERIALS AND METHODS

Patients. In the study, 86 individuals presented an HSR to sulfonamides. The patients were referred to the Glaxo Wellcome-Sunnybrook Drug Safety Clinic for diagnosis by drug safety clinicians and dermatologists (L.E.S., N.H.S.) 0.5–4 years after presentation of HSR. A clinical pharmacologist and internal medicine specialist (N.K.) reviewed all clinical data and its correlation with the laboratory data. Some patients who developed SJS or TEN were hospitalized and treated by burn surgeons (J.F., R.C., J.H., and M.G.). A hepatologist (L.C.) was a consultant for the cases that presented liver involvement. The clinical biochemist and pharmacology specialist (M.G.N.) and the laboratory technician (I.M.) were responsible for the laboratory diagnosis. Ethical approval for the study was obtained from the Scientific and Ethics Review Committees of the Sunnybrook and Women's College Health Sciences Centre, and all the patients signed the informed consent to participate. Participants in this study underwent *in vitro* LTA testing with SMX (Table I). The LTA test results were correlated with the patient's clinical data.

The subjects were divided in 2 subgroups as follows:

- Patients: 86 patients who had developed a clinical HSR to a sulfonamide.
- Controls: 220 immunocompetent individuals who received sulfonamides without developing HSR and voluntarily participated in the study.

Patients who were hospitalized during an acute episode of HSR (SJS or TEN) in the burn unit of the hospital visited the Drug Safety Clinic after they recuperated from the active HSR and they agreed to partic-

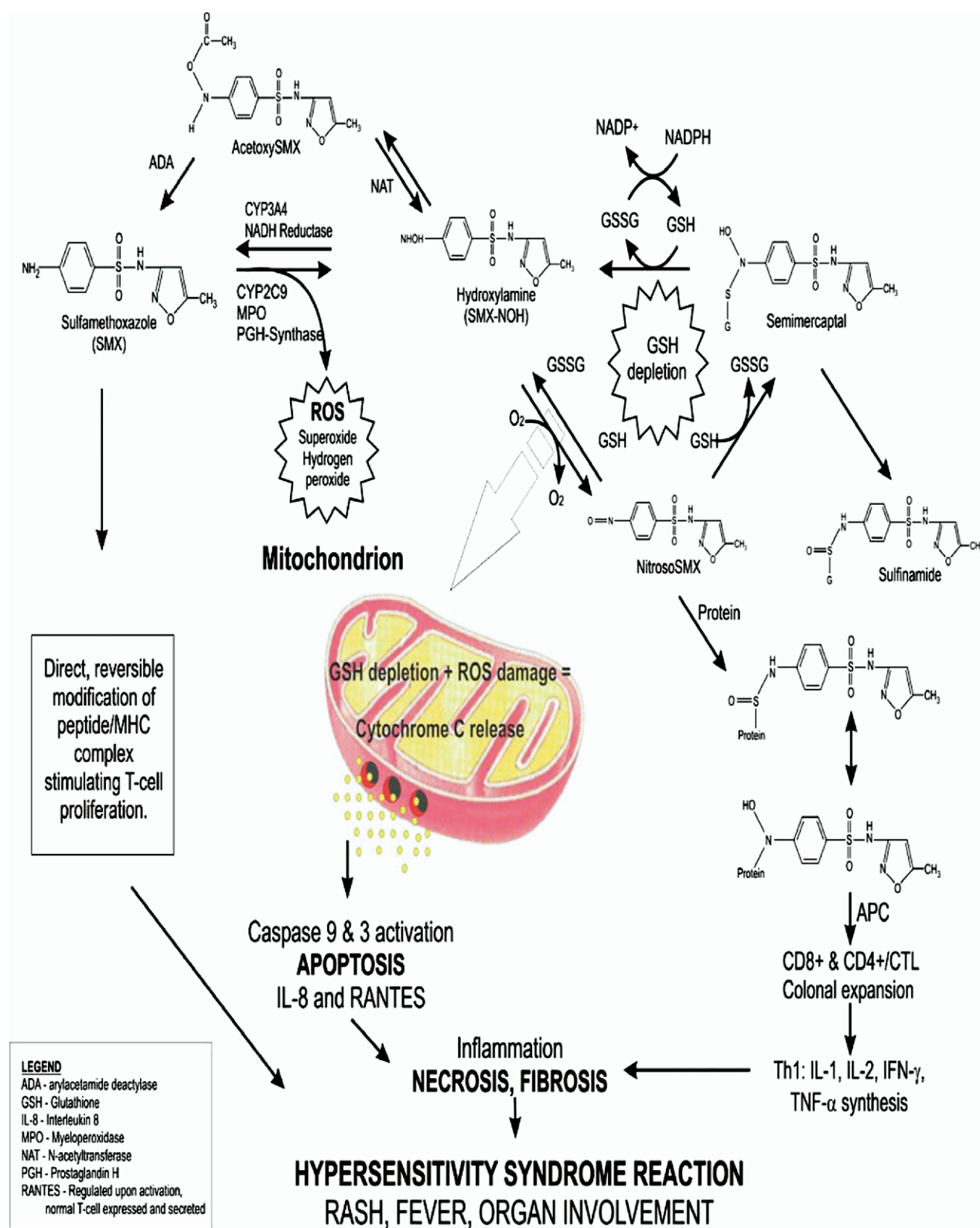


Fig 1. Model of hypersensitivity syndrome reaction.

ipate in the study. They accepted to have their laboratory diagnostic performed at 2 different times: (1) during the acute phase and (2) when they no longer presented symptoms.

Drugs and chemicals. The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl) 2, 5 diophenyl-tetrazolium bromide (MTT) was obtained from Sigma Chemical Company (St. Louis, Mo). All remaining reagents were of analytical grade obtained from the usual commercial sources.

Laboratory methods. Cytotoxicity assay. The study used an *in vitro* LTA test.¹⁸

Microsome preparation. Swiss mice were obtained from the National Institutes of Health and were treated intraperitoneally with phenobarbital, 10 mg/kg of body weight per day for 3 days, to induce cytochrome P450 enzymes. Animals were sacrificed in conformity with protocol approved by the animal ethics committee. Livers were removed aseptically and homogenized in 3 volumes (w/v) of 0.15-M KCl. The homogenate was spun for 10 min at $500 \times g$. The supernatant was spun for 15 min at $9000 \times g$, and the resulting supernatant was then spun for 1 h at $100,000 \times g$. The pellet was resuspended in EDTA/KCl buffer at pH 7.4. The cytochrome P450 activity was verified in the microsomal fraction. Minimal concentration of cytochrome P450 in the induced microsomes was 1-mol P450/mg protein. Induced microsomes from phenobarbital-treated mice showed greater P450 activity than those from mice that had not been pretreated with the drug.

Lymphocyte preparation. Fresh, heparinized blood was diluted 3-fold with minimal essential medium and was layered on a Ficoll–Paque density gradient. PBMCs containing 22% monocytes and 78% lymphocytes were collected by centrifugation and suspended in a HEPES-buffered medium. Lymphocytes were then counted using a microscope by a technician specialized in hematological analysis and resuspended at a density of 2×10^6 cells/mL.

Incubation of lymphocytes. Lymphocytes were incubated at 37°C for 24 h, along with 0.6-mM nicotinamide adenine dinucleotide phosphate (NADP), 2.24-mM glucose-6-phosphate, and 2- μ M glucose-6-phosphate dehydrogenase as a control (primary control). The treatments were as follows: lymphocytes plus parent drug only (secondary control), lymphocytes plus microsomes only, and finally lymphocytes plus parent drug plus microsomes (test group). Microsomal activation of the parent drug has been previously shown to be NADP-dependent; thus, the addition of NADP and glucose-6-phosphate to the culture is crucial to the production of the reactive metabolites.

Each reaction involves the incubation of patient lymphocytes (viability ~95%) at 37°C with 0.28-mg mi-

croosomal protein, 0.6-mM NADP, 2.24-mM glucose-6-phosphate, 2 U of glucose-6-phosphate dehydrogenase, and the causative drug. Cytotoxicity was assessed using the tetrazolium dye MTT (3-[4,5-dimethyl thiazol-2-yl]2,5 diphenyl-tetrazolium bromide). Viable, intact cells take up the yellow dye MTT and can convert it to a purple product using the mitochondrial enzyme succinic dehydrogenase (SDH) (E.C. 1.3.99.1). The principle behind the MTT dye conversion test is that lymphocytes harmed by reactive drug metabolites undergo mitochondrial damage and lose the ability to convert the dye from MTT to formazan. This purple product can be quantitated spectrophotometrically at an endpoint mode using 2 wavelengths: Absorbance is measured at 560 nm, whereas absorbance at 690 nm is used as a reference.

At the end of the 24-h incubation period, MTT (100 μ L) was added for 1 h. The cells were lysed, and the dye was dissolved with 100- μ L isopropyl alcohol. The cytotoxicity was measured using an enzyme-linked immunosorbent assay (ELISA) reader. An LTA result equal to or higher than 12.5% of cytotoxicity was considered to be positive for immunocompetent subjects.¹⁸ Each experimental condition using the conventional methodology was studied in triplicate sample, and the mean percent of dead lymphocytes was determined. The change from baseline values (calculated as percent dead cells in the presence of drug and murine hepatic microsomes minus the percent of dead cells in the absence of drug) was used as the measure of toxicity.

The Maxline Microplate Reader (Molecular Device Corporation, Menlo Park, Calif) was used to determine the release of cytokines in media. The reader was connected to a computer using SOFT MAX software 2.3 for Windows to program the template, the temperature, and the kinetic or endpoint-mode of reading according to specific experimental needs for each endpoint.

Cytokine measurement. Cytoscreen, Immunoassay Kits, Human IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, regulated upon activation, normal T-cell expressed and secreted (RANTES), and TNF- α , ELISA (Biosource International, Camarillo, Calif) were used for the quantitative determination of cytokines in cell culture media. The assay is designed to recognize both natural human and recombinant human cytokines. The correlation coefficient was linear ($r = 0.989$) in a concentration range between 2 and 500 pg/mL.

Cytokine expression in PBMC. Total cellular RNA was isolated from PBMC by a modification of the method of Chomczynski and Sacchi.¹⁷ Taq DNA Polymerase and amplifier sets for human IL-1, human IL-6, and human TNF- α as well as glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control were purchased from

CLONTECH Laboratories, Inc. (Palo Alto, Calif). Polymerase chain reaction (PCR) was performed according to the method of Kawarski.¹⁸ The cytokine gene expression was evaluated by RT-PCR. Briefly, 50–400-ng total RNA was reverse transcribed, the resulting cDNA was amplified with each gene-specific primer, and the PCR reaction was optimized for cycle number. The IL-1 sequence was as follows: (5') 5' CAA GGA GAG CAT GGT GGT AGT AGC AAC CAA CG 3' and (3') 5' TAG TGC CGT GAG TTT CCC AGA AGA AGA GGA GG 3'. The primers for IL-6 were as follows: upstream: 5' ATG AAC TCC TTC TCC ACA AGC GC 3' and downstream (3'): 5' G AAG AGC CCT CAG GCT GGA CTG 3'. For TNF- α , the primers were as follows: 5' primer- 5' ATG AGC ACT GAA AGC ATG ATC CCG 3' and 3' primer: 5' GCA ATG ATC CCA AAG TAG ACC TGC CC 3'. In subsequent experiments, the RNA concentration and PCR cycle number were chosen from the linear portion of the curve for each cytokine. From the product of reverse transcriptase polymerase chain reaction (RT-PCR), 5 μ L were used for electrophoresis. The amount of DNA was normalized for the amount of the housekeeping G3PDH, which did not change under condition in these experiments. In each sample, both the cytokine and the housekeeping gene, G3PDH, were quantified under the same conditions. G3PDH is preferred to β -actin, because levels of the β -actin mRNA have been found to be regulated by a variety of agents, whereas G3PDH was reported to be refractory to regulatory agents.²⁸ The same RT-PCR technique under the conditions requested by each cytokine was used to measure changes in different samples, which received different treatments. For the PCR amplification, a Perkin-Elmer Cetus DNA Thermal Cycler model 480 (Cetus Corporation, Emeryville, Calif) was used. PCR products were separated in 2% agarose gel. In addition to being the predicted size, the nature of PCR products was confirmed by automated sequencing. For the gel-electrophoresis, a Gel apparatus (Bio-Rad Laboratories, Hercules, Calif) was used. A cDNA synthesis kit was purchased from Bethesda Research Laboratories, Inc. (Bethesda, Md). The electrophoresis was visualized with ethidium bromide, and the image analysis of the expression was quantitated with a Phosphorimager: SI/Densitometer (model PST-486) connected to an analytic, automatic program ImageQuantNewT (Molecular Dynamics, Sunnyvale, Calif). Each determination was performed in triplicate in at least 3 different cell populations. The results of the cytokines cellular expression were presented in percentage with controls at 100% value.

Data analysis. Results are expressed as mean $\pm$ SD. Statistical analysis was performed using 1-way analysis

of variance between the groups. A $P < 0.05$ was considered as significant.

All statistical analysis was done using SPSS 11.5 (SPSS Incorporated, Chicago, Ill). Sensitivity, specificity, positive predictive and negative predictive values, and positive and negative likelihood ratios were calculated using the SPSS 11.5 program.

RESULTS

Patient population. In this study, the following patients were used: 220 controls (160 women and 60 men) that did not present any symptoms of HSRs to sulfonamides and 86 patients that had a previous history of sulfonamide use. The patients with a history of an adverse reaction and symptoms were placed in one of the following categories: (1) rash, (2) rash and fever, or (3) rash, fever, and organ involvement. Patients with rash had a wide range of symptoms: eczema, non-follicular papules, blisters, conjunctivitis, desquamation of skin, pruritic, erythematous, and skin vasculitis. Fever was considered any indication of increased body temperature. Organ involvement included the following: thrombocytopenia, raised liver enzymes, hepatocytotoxicity, leukocytosis, eosinophilia, lymphadenopathy, and pancytopenia. The clinical syndrome appears 1–56 days after the initial therapy.⁵

Of the 86 exposed patients, 14 were men with a mean age of 46.64 ± 28.94 and 72 were women with a mean age of 52.07 ± 14.40 . Of the total 86 patients, 43 had symptoms and had positive LTA results and 43 had clinical symptoms and had negative LTA results. Control population ($n = 220$) was as follows: 60 male volunteers (36.42 ± 8.64) and 160 female volunteers (49.44 ± 21.40). Overall, 2 of 220 control individuals did not present any symptoms but were positive for their LTA, and 218 did not present any symptoms and were negative for their LTA values. The statistical significance is shown in the Table I.²⁹ The statistical analysis for patients with rash, fever, and organ involvement indicated a sensitivity of 92.90%, suggesting a strong true-positive rate, and a specificity of 99.1%, which nicely identifies patients without the disease. Of the group of 16 individuals that had presented rash, fever, and organ, and organ involvement, 12 had been treated in the burn unit for SJS or TEN. Overall, 5 of these 12 patients had LTA performed as a diagnostic test during the SJS/TEN episode, and it was repeated in this study 2–4 years after the episode. The LTA remained positive to the drug even after 2–4 years post-SMX-induced SJS.

Cytokine results. Cytokines were measured in the sera of patients 2–4 years after the HSR occurred. The level of TNF- α for women with positive LTA values was

Table II. The distribution of cytokine levels among the different groups studied

Interleukines pg/mL (mean $\pm$ SD)	Control (220)	Rash (27)	Rash + fever (43)	Rash + fever + organ involvement (16)
IL-1	50 $\pm$ 15	55 $\pm$ 10	80 $\pm$ 8	180 $\pm$ 20 [§]
IL-2	65 $\pm$ 15	75 $\pm$ 15	90 $\pm$ 15	260 $\pm$ 10 [§]
IL-4	78 $\pm$ 10	46 $\pm$ 10	42 $\pm$ 4	39 $\pm$ 4
IL-5	25 $\pm$ 15	66 $\pm$ 10 [†]	55 $\pm$ 10 [†]	50 $\pm$ 15
IL-6	38 $\pm$ 10	36 $\pm$ 10	52 $\pm$ 8	69 $\pm$ 6 [*]
IL-12	69 $\pm$ 10	58 $\pm$ 10	72 $\pm$ 12	195 $\pm$ 15 [§]
IL-18	130 $\pm$ 5	110 $\pm$ 15	190 $\pm$ 5 [*]	250 $\pm$ 10 [†]
RANTES	50 $\pm$ 10	45 $\pm$ 15	55 $\pm$ 15	150 $\pm$ 15 [§]

Note: Controls were considered volunteer individuals that took sulfonamides and did not develop any reactions to the drug; the group of patients that took sulfonamide developed only a rash.

* $P < 0.05$ higher than levels in patients with rash and controls.

† $P < 0.05$ higher than the levels in patients with rash and fever and $P < 0.001$ higher than patients with rash and controls.

‡ $P < 0.05$ higher than controls.

§ $P < 0.001$ higher than the levels in patients with rash, rash and fever, and controls.

|| $P < 0.001$ higher than the levels of HSRs patients.

statistically higher than in men with positive LTA values. The level of TNF- α in HSR-LTA-negative patients did not differ from the normal population (60 pg/mL). A significant correlation of 0.001 was noted for HSR-LTA-positive and higher TNF- α .

Regarding the distribution of cytokine levels in the sera of different groups (controls, rash, rash + fever, rash + fever + organ involvement) summarized in Table II, the population with only rash presented higher IL-5 levels than controls ($P < 0.05$) and the other groups. In patients presenting rash + fever, IL-18 levels were significantly higher ($P < 0.05$) than controls and subjects with rash only. The patients with true HSRs presented significantly higher values for IL-1, IL-2, and IL-12 ($P < 0.001$) than all other groups. IL-6 was found to be less in the sera of control patients and patients presenting only rash. In addition, IL-18 levels were significantly higher than patients with rash + fever ($P < 0.05$) and patients with just rash ($P < 0.001$) or controls ($P < 0.001$).

Figure 2 presents the values of TNF- α , Fas, and IL-8 in control volunteers, patients presenting rash, and patients presenting rash + fever + organ involvement. TNF- α is 6 times more elevated in patients presenting rash + fever + organ involvement versus the controls and patients presenting the same levels in controls and those with only rash (Fig 2). It is also 3 times more elevated in individuals presenting rash with fever. Fas levels in the individuals presenting rash + fever + organ involvement are 3 times more elevated than in patients presenting rash with fever pointing to the apoptotic processes involved in the immunopathogenesis of true hypersensitivity syndromes. The individuals presenting rash + fever + organ involvement had IL-8 levels 5 times

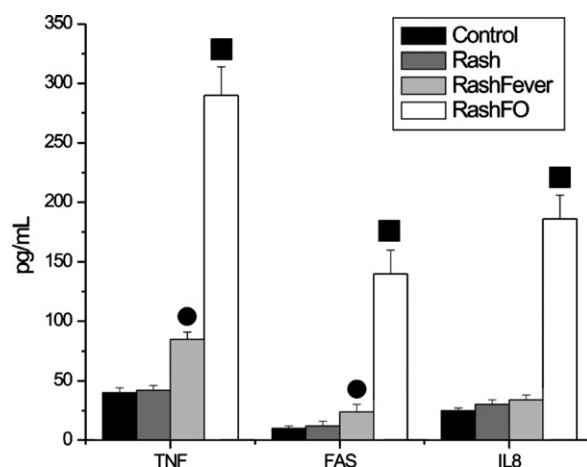


Fig 2. Correlation among serum levels of TNF- α , Fas, and IL-8 and the clinical symptoms in patients with HSRs. The levels of TNF- α , Fas, and IL-8 were significantly ($P < 0.0001$) elevated in patients with rash + fever + organ involvement (RashFO) as compared with controls and patients that presented only rash or rash + fever (Rash-Fever). TNF- α levels and Fas levels in patients presenting rash + fever were significantly higher ($P < 0.001$) versus the control and the patients presenting only rash. No changes have been observed between the levels of IL-8 in control and in patients presenting rash or rash + fever.

higher than the levels in the individuals presenting only rash or rash with fever or controls. Expressions of ILs other than TNF- α were not significantly higher between the patients presenting rash + fever + organ involvement and those presenting only rash. Expression of TNF- α was significantly elevated in patients with rash + fever + organ involvement versus controls or patients with rash or rash + fever (Fig 3).

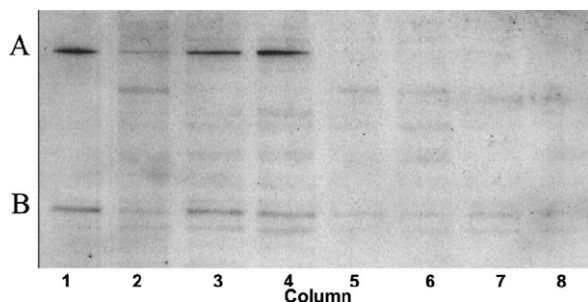


Fig 3. Expression of TNF- α in the PBMCs of patients with hypersensitivity syndromes to sulfonamides (Line A). Line B is the expression of glucose-6-phosphate dehydrogenase, which was taken to act as a housekeeping enzyme/cytokine. Column 1: patient presenting rash + fever + organ involvement after treatment with sulfonamide antibiotics has significant ($P > 0.001$) expression of TNF- α (686 pics) versus control volunteers (0 pics). Column 2: patient presenting rash + fever (88 pics). Column 3 and 4: patient presenting rash + fever + organ involvement (550 pics). Columns 5 and 6: control volunteers. Columns 7 and 8: patients presenting only rash.

DISCUSSION

It is critical to have an understanding of the biochemical processes that occur during HSR. It allows for a better comprehension of predisposing factors, potential cross-reactivity, and the duration of treatment, and it aids clinicians in their assessment of further or continued therapy.⁴ Gender is thought to be a predisposing factor; however, in this study, no significant difference existed between gender-related positive or negative LTA results, and yet women were the majority (83.7%) of subjects. This result could be, in part, because of the higher percentage of female patients referred to the clinic. However, it has been established that gender differences can be based on cytochrome P450 expression, hormonal influence, drug metabolism, or obesity.^{30,31}

Current *in vitro* investigations contribute to understanding of the fundamental biology underlying adverse drug reactions, providing clues to their immunopathogenesis. A positive predictive value of 92.3% has made the *in vitro* LTA test a reliable tool for the diagnosis of the true HSRs caused by sulfonamide antibiotics. It is in this respect that an LTA test is most acknowledged as being a predictor test for true HSR.

Clinical symptoms of rash, fever, and organ involvement that occur in HSRs may be because of the involvement of cells such as neutrophils, monocytes, and macrophages, as well as specialized cells such as Langerhans cells within keratinocytes.^{23,32,33} In this study, the chemoattractant substances such as chemokines (IL-8 and RANTES) were higher in the HSR that presented the organ involvement when compared with the individuals that presented less important symptoms such as fever. In their review, Rubin and Kretz-

Rommel³² suggest that metabolites of SMX may compete with chloride ions for this pathway producing drug-free radicals, which are promptly detoxified with GSH conjugation or acetylation. The system requires controlled levels of GSH to maintain low levels of toxic agents. Depletion of GSH by its use in phase II reactions may leave organelles such as the mitochondria vulnerable to ROS (Fig 1). Based on this idea, the specific mitochondrial enzyme SDH activity (data not shown) that correlates directly to mitochondrial toxicity occurring in HSRs was measured.

Mitochondrial damage may be only part of the complications involved in HSRs, but it is a critical one. Defects in any of the 5 complexes in the respiratory chain would greatly add to mitochondrial toxicity. The LTA is a marker of one such disorder because it employs the use of SDH (E.C. 1.3.99.1), an enzyme part of complex II in the mitochondrial respiratory chain.³⁴ As SDH is very specific to mitochondrial activity, its dysfunction would indicate cell death when the cell is stressed. As is the case with the LTA, cells from susceptible individuals have shown high degrees of mitochondrial apoptosis when exposed to drug metabolites, which would indicate a defective pathway in the respiratory chain that most likely involves SDH. Deficiency in glutathione synthetase (E.C.6.3.2.3) showed increased toxicity to sulfadiazine metabolites generated via the hepatic microsomal system. An inherited deficiency in GST activity within the target cells is responsible for the development of sulfonamide toxicity in susceptible individuals.

Cytokines are regulatory peptides that can be produced by virtually every cell in the body.³⁵⁻³⁸ The cytokine family consists of several subfamilies: the ILs; the TNF family; IL-6 and related cytokines; interferons; and chemokines such as IL-8, RANTES, colony-stimulating factors, and others. In most tissues, including the liver, constitutive production of cytokines is absent or minimal. However, as physiologic and pathologic stimuli activate cells, the production of these autocrine, paracrine, and endocrine effector molecules increase, and they, in turn, orchestrate the tissue's response to stimuli such as inflammation, apoptosis, and necrosis,³⁹⁻⁴³ but paradoxically they can also mediate the regeneration of liver tissue after injury.⁴⁴⁻⁴⁶ In this study, the levels of IL-5, a Th2-type response, was elevated in patients with only a rash, pointing to an allergic type of reaction in these individuals. Furthermore, patients that had presented a true HSR 2-4 years earlier had significantly elevated levels Fas and TNF- α .

Cytokines involved in immune responses play a myriad of roles. The T-cell response generated because of the presence of haptenized SMX metabolites has been given much attention.^{47,48} It has been found that SMX

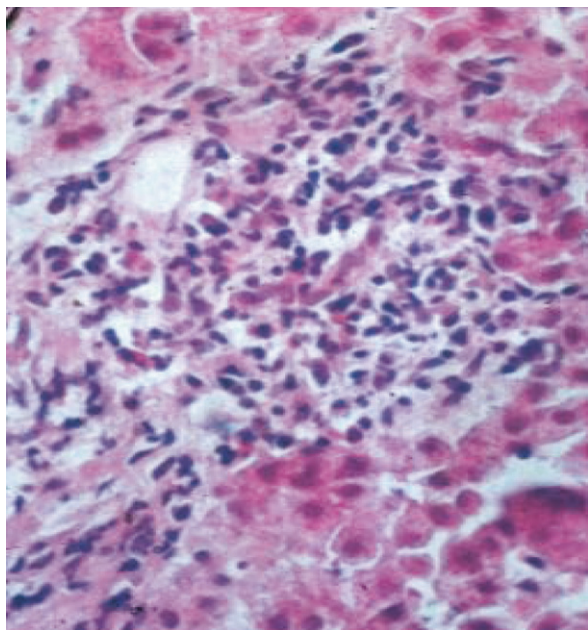


Fig 4. Biopsy of a 43-year-old woman that presented with an acute hepatic injury because of SMX. The type of the injury is hepatocellular with a large number of lymphocytes producing inflammation. There is a non-zonal necrosis; some hepatocytes present a large nucleus. In the upper right corner, a Mallory-like body can be observed, whereas in the bottom left corner, apoptotic cells are detected. H&E $\times$ 40.

adducts can elicit an immune response that can involve $CD4^+$ and $CD8^+$ T-cells. Immunohistochemical studies of T cells in sulfonamide-induced HSRs have shown high levels of perforin and granzyme B, suggesting that $CD8^+$ T-cytotoxic cells are involved.¹

Inflammatory processes that employ cytokine and chemokine factors can have a variety of effects. SMX may cause subtle changes in the homeostasis of inflammatory mediation that may lead to significant effects (Fig 4). The SMX-NOH metabolite is thought to interfere with cell signaling that normally occurs within PBMCs.⁴⁹ SMX-NOH caused an interference of the Janus kinase–signal transducer and activator of transcription pathway, which led to a disruption in the IL-2 receptor signaling²³ and resulted in higher levels of IFN- γ , which meant that there would be an increase in cell-mediated responsiveness. This process was observed as an alteration in the signaling cascade leading to an “immune deviation” changing cytokine production within skin or liver cells. These results showing the high values of Fas in “true hypersensitivity” are in concordance with Abe et al,⁵⁰ indicating that toxic epidermal necrolysis and Stevens–Johnson syndrome are induced by soluble Fas ligand.

LTA is a predictor for true HSR. TNF, IL2, IL8, and

RANTES correlate positively with the LTA and severity of HSR. Validation of the LTA on a larger number of patients who underwent a true HSR to SMX would be beneficial. T1-helper cytokine responses (TNF- α) contributed to Fas-induced apoptotic processes in the liver and in the skin. As the patients presented with levels of TNF- α as high as in TEN with HSR, this study defines a possible common pathway for SJS/TEN. This study finds that both metabolic as well as immunologic factors are thought to be involved in HSR.

CONCLUSION

A correlation exists between the severity of symptoms observed in affected patients by sulfonamide-induced HSR and their LTA values. The patients presenting multiple organ involvement have a high LTA. For this population and their children, LTA determination is very important. Standardization of the LTA may allow this test to become a routine component of diagnosing an adverse reaction. The test may eventually help to elucidate the mechanism of cytotoxicity caused by metabolites and the pattern of inheritance in defects of detoxification pathways in patients with hypersensitivity. The TNF- α secretion in the patient’s sera and its expression in the PBMC are in correlation with corresponding LTA. The patients with rash, fever, and organ involvement have higher levels of TNF- α than in control patients or any of the other patient groups. Expression of TNF- α is significantly higher in patients presenting rash, fever, and organ involvement versus all other groups. Moreover, the clinical symptoms of hypersensitivity include fever, fatigue, headache, and myalgia, all of which are unspecific and may be induced by proinflammatory cytokines. A collaborative result of serum TNF- α , RANTES, Fas, IL8, and LTA may serve in identifying a population at risk for HRS.

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