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Covalent Binding of the Nitroso Metabolite of Sulfamethoxazole Leads to Toxicity and Major Histocompatibility Complex-Restricted Antigen Presentation

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ABSTRACT

Treatment with sulfamethoxazole (SMX) can lead to hypersensitivity reactions. T cells from hypersensitive patients recognize either the parent drug and/or the reactive nitroso (SMX-NO) metabolite. In this study, using a novel in vitro rat splenocyte assay, we have investigated the toxicological and immunological consequences of cell surface haptenation by SMX-NO. SMX-NO was found to be unstable in solution; spontaneous transformation yielded appreciable amounts of SMX-hydroxylamine, nitro-SMX, and the previously unknown azoxy and azo dimers within 15 min. Irreversible binding of SMX-NO to cellular protein was demonstrated by flow cytometry, with haptenation being greater on the surface of antigen-presenting cells than on T cells. The consequences of irreversible binding of SMX-NO were examined in two ways. First, haptenation above a threshold level led to a proportionate increase in cell death (both apoptosis and necrosis). Indeed, the cells that became haptenated were the same as those that underwent necrotic cell death. Second, sensitized splenocytes proliferated in the presence of major histocompatibility complex (MHC)-restricted antigen derived from both viable and dead cells haptenated with low and high levels of SMX-NO, respectively. However, direct modification of MHC by SMX-NO was not the mechanism of antigen presentation. The antigenic threshold of SMX-NO for T-cell proliferation and toxicity was estimated to be between 0.5 and 1 μM and 5 to 10 μM, respectively. The potential of SMX-NO to generate a potent antigen and cause cytotoxicity may in combination provide the signals necessary to induce a hypersensitivity reaction to SMX.

Administration of sulfamethoxazole (SMX) to patients is associated with a high incidence of allergic reactions. Skin rashes are the most common manifestation, and reactions vary in severity from mild IgE-mediated urticarial rashes to the more severe and potentially life-threatening Stevens Johnson syndrome and toxic epidermal necrolysis (Barranco and Lopez-Serrano, 1998). There has been a resurgence of interest in the mechanism(s) of SMX allergy because it was observed that approximately 30% of patients with the human immunodeficiency virus who were administered co-trimoxazole, a combination of SMX and trimethoprim, developed a cutaneous reaction, even when the drug is used at low doses for prophylaxis (Pirmohamed and Park, 2001). Identification and characterization of drug-specific, perforin-secreting T-lymphocytes from peripheral blood (Schnyder et al., 1998) and blister fluid in the acute phase of the reaction (Nassif et al., 2001) provide convincing evidence that drug-induced allergic tissue damage requires activation of an individual's immune system.

The onset of an allergic reaction to SMX (and drugs in general) is thought to involve drug bioactivation and covalent binding, followed by antigen processing and T-cell proliferation (Park et al., 1998; Uetrecht, 1999). To generate an antigenic signal, cytochrome P450 enzymes in the liver metabolize SMX to SMX hydroxylamine (Cribb and Spielberg, 1992). Approximately 2% of an oral dose of SMX is excreted as the hydroxylamine in human urine (Gill et al., 1996; Mitra et al., 1996). SMX-hydroxylamine undergoes rapid autoxidation to nitroso sulfamethoxazole (SMX-NO) (Cribb et al., 1991; Naisbitt et al., 1996), which is chemically reactive and has been shown to haptenate cellular proteins, including the surface of

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J.F. and S.F.G. contributed equally to this manuscript.

ABBREVIATIONS: SMX, sulfamethoxazole; SMX-NO, nitroso sulfamethoxazole; LC-MS, liquid chromatography-mass spectrometry; CFA, complete Freund's adjuvant; DMSO, dimethyl sulfoxide; HBSS, Hanks' balanced salt solution; MHC, major histocompatibility complex; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

viable lymphocytes and keratinocytes (Naisbitt et al., 1999, 2001; Reilly et al., 2000). The balance between bioactivation and bioinactivation by glutathione, cysteine, and detoxification enzymes determine the extent of tissue exposure to SMX-NO (Gill et al., 1997; Naisbitt et al., 2000). Dermal T cells from lesional skin and T cells cloned from peripheral blood of allergic patients have been shown to proliferate in the presence of covalently bound SMX-NO but not the parent drug (Hertl et al., 1995; Schnyder et al., 2000; Burkhart et al., 2001). Several groups have also shown that SMX-NO is directly toxic to cells in vitro (Rieder et al., 1995; Reilly et al., 1998; Hess et. al., 1999); however, the relationship between covalent binding, toxicity, and stimulation of a cellular immune response remains unclear.

The seminal observation that the majority of T cells from SMX-allergic patients recognize the parent drug in the absence of metabolism and antigen processing questions the application of the hapten hypothesis to all cutaneous reactions associated with the administration of SMX (Mauri-Hellweg et al., 1995; Schnyder et al., 1997). To further delineate the role of drug metabolism in SMX hypersensitivity, we developed an in vivo model of SMX immunogenicity. Administration of SMX-NO but not SMX to male Wistar rats resulted in 1) haptenated lymphocytes and epidermal keratinocytes, 2) production of hapten-inhibitable anti-drug antibodies, and 3) formation of drug-metabolite–specific CD4+ and CD8+ T cells (Gill et al., 1997; Naisbitt et al., 2001). In contrast to T cells from naive animals, T cells from rats administered SMX in complete Freund's adjuvant (CFA) proliferated on restimulation in vitro but only in response to the nitroso metabolite. These data provide unequivocal evidence that SMX-NO is formed in vivo after administration of the parent drug. In this study, using a novel proliferation assay that incorporates splenocytes from two syngeneic animals (one sensitized and the other a naive donor), we investigate the relationship between drug metabolite concentration, covalent binding, toxicity, and stimulation of a cellular immune response.

Materials and Methods

Chemicals. CFA, dimethyl sulfoxide (DMSO), glutathione, Hank's balanced salt solution (HBSS), L-glutamine, HEPES, *N*-acetyl cysteine, *N*-acetyl lysine, penicillin, SMX, streptomycin, [^{3}H]thymidine, and trypsin were obtained from Sigma-Aldrich (Poole, UK). Anti-SMX IgG antibody was donated by Dr. A. E. Cribb (University of Prince Edward Island, Charlottetown, Canada). The annexin-V apoptosis detection kit, fluorescent-labeled mouse anti-rat CD3+, CD4+, and CD8+ antibodies, and mouse anti-rat MHC-blocking antibodies were obtained from Beckman Coulter, Inc. (Luton, UK). MACS magnetic microbeads conjugated to mouse anti-rat CD4+ and CD8+ antibodies for cell sorting were from Miltenyi Biotec Ltd. (Surrey, UK). Lymphoprep (1.077 g/ml) was obtained from Nycomed (Birmingham, UK). SMX-hydroxylamine and SMX-NO were synthesized according to the method of Naisbitt et al. (1996) and found to be >99% pure by LC-MS and NMR.

Animals and Immunizing Protocols. Male Lewis rats between 8 and 12 weeks old (175–225 g) were purchased from Charles River (Margate, Kent, UK). For sensitization (n = 4 for each group), SMX-NO (1 mg/kg) was administered in DMSO (100 μl, i.p.) four times weekly for 2 weeks. In separate experiments, rats were administered SMX (50 mg/kg) by a single i.p. injection in CFA (200 μl). On completion of the dosing regimen, animals were sacrificed, and the spleen was removed using aseptic technique. Single-cell splenocyte suspensions were prepared by perfusion with HBSS, and red blood cells were removed by density centrifugation with Lymphoprep (500g for 30 min). Splenocytes were resuspended in culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 25 mM HEPES, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin) and used as described. Naive red cell-depleted splenocytes were isolated from weight-matched male Lewis rats by the same procedure.

Determination of the Chemical Fate of Nitroso Sulfamethoxazole. SMX-NO (40 μM) was incubated with cell culture medium at 37°C for 5 to 90 min. The reaction mixture was analyzed by LC-MS. The eluent was delivered by Jasco PU980 pumps (Great Dunmow, Essex, UK). Analytes in the eluate were monitored with a Jasco UV-975 spectrophotometer (λ = 254 nm). The split flow rate of eluate to the mass spectrometer was ~50 μl/min. Selected-ion monitoring data were acquired in either negative or positive ion mode with a Quattro II tandem quadrupole instrument (Micromass UK Ltd., Manchester, UK). The source temperature was 70°C, the capillary voltage was 3.9×10^3, and the standard cone voltage was 30 V. Analytes were monitored as anions ($[M-1]^-$) for peak integration. The dwell time was 200 ms per channel, and the interchannel delay was 20 ms. Data were processed via MassLynx 2.0 software (Micromass). Aliquots of the solution (20–70 μl) were eluted without treatment from a Prodigy 5-μm ODS (2) column (150 × 4.6-mm i.d.; Phenomenex, Macclesfield, Cheshire, UK) at room temperature with a gradient of acetonitrile (30–60% over 15 min) in formic acid (0.1%); the flow rate was 0.9 ml/min.

The same conditions were used to investigate the chemical fate of SMX-NO in the presence of rat splenocytes. Briefly, SMX-NO (40 μM) was incubated with freshly isolated rat splenocytes (1.5×10^5/well) in a 96-well culture plate at 37°C (5% CO_2) for 0.1 to 24 h. The supernatants of eight wells were combined for LC-MS analysis.

Flow Cytometric Determination of the Extent of Covalent Binding of Nitroso Sulfamethoxazole to CD4+ and CD8+ T Cells and Antigen-Presenting Cells. Red cell-depleted splenocytes (20×10^6) were incubated with SMX-NO (50–250 μM) in culture media (20 ml) in the presence or absence of glutathione (1 mM), *N*-acetyl cysteine (1 mM), or *N*-acetyl lysine (1 mM) at 37°C. After 1 h, cells were centrifuged (500g) and washed in drug-free media (2 × 20 ml) to remove unbound drug. Either CD4+ or CD8+ T cells were depleted using MACS columns (type AS; Miltenyi Biotec Ltd.) and magnetic microbeads conjugated to monoclonal mouse anti-rat CD4+ and CD8+ antibodies. The purity of the separated cells was determined by flow cytometry (Coulter Epics XL software; Beckman Coulter, Inc.) using mouse anti-rat CD4+ and CD8+ antibodies conjugated to FITC and PE, respectively.

Drug metabolite antigen formation on the surface of either CD4+- or CD8+-depleted splenocytes was determined using a hapten-inhibitable rabbit anti-SMX IgG antibody (1: 500, v/v; 40 μl) and a FITC- or PE-conjugated anti-IgG secondary antibody (Naisbitt et al., 1999). Antigen-presenting cells were distinguished from CD4+ or CD8+ T cells on the flow cytometer using FITC- or PE-conjugated anti-rat CD4+ and CD8+ antibodies. The number of cells staining positive for covalently bound SMX was taken to be equivalent to the difference in fluorescence intensity between drug-treated cells and cells incubated with DMSO alone. Irreversible binding of SMX-NO to CD4+ and CD8+ T cells and antigen-presenting cells was also determined after a single i.p. injection of SMX-NO (100 mg/kg) to male Lewis rats. After 1 h, animals were sacrificed, splenocytes were isolated and resuspended in drug-free HBSS, CD4+ and CD8+ T cells were depleted, and covalent binding was determined as described.

Determination of Nitroso Sulfamethoxazole-Mediated Cell Death in Purified CD4+ and CD8+ T Cells and Antigen-Presenting Cells. Red cell-depleted splenocytes were isolated from naive male Lewis rats as described. Splenocytes (20×10^6) were incubated with SMX-NO (10–250 μM) in HBSS (pH 7.4; 20 ml) at 37°C. After 2 h, the cells were washed (2 × 10 ml of HBSS; 500g for

5 min), resuspended in drug-free cell culture medium, and incubated at 37°C for the remainder of the incubation period. After a further 4 and 14 h, an aliquot of cells was washed (2 × 1 ml of HBSS; 500*g* for 5 min), and CD4+ and CD8+ T cells and antigen-presenting cells were isolated using methods essentially the same as those described above. Cell death was determined by flow cytometry using the annexin-V/propidium iodide apoptosis detection kit. SMX-NO–treated purified cells were resuspended in binding buffer, and FITC-labeled annexin-V (1 μg/ml) and propidium iodide (35 μM) were added. The mixture was incubated in the dark for 15 min at 4°C. A minimum of 5000 cells were then analyzed by flow cytometry. Annexin-V binds to phosphatidylserine residues expressed on the surface of apoptotic cells, whereas propidium iodide is a membrane-impermeable fluorescent dye that binds to DNA of cells killed by necrosis or secondary (late-stage) apoptosis (Vermes et al., 1995). A combination of these two characteristics permits simultaneous detection of viable (annexin-V–negative/propidium iodide–negative), apoptotic (annexin-V–positive/propidium iodide–negative), and necrotic (annexin-V–positive/propidium iodide–positive) cells.

Simultaneous Determination of Cell Surface Binding of Nitroso Sulfamethoxazole and Cell Death. Red cell-depleted splenocytes (1×10^6) were incubated with SMX-NO (10–250 μM) in HBSS (pH 7.4; 1 ml) at 37°C. After 2 h, the cells were washed (2 × 10 ml HBSS; 500*g* for 5 min), resuspended in drug-free cell culture medium, and incubated at 37°C for a further 4 h. The cells were washed, pelleted, and the anti-SMX antibody (40 μl; 1:500, v/v) was added; samples were incubated at 4°C. After 20 min, cells were washed in HBSS (2 × 1 ml) and incubated with a FITC-conjugated anti-rabbit secondary antibody. Propidium iodide (35 μM) was then added to the SMX-stained cells. Samples were analyzed for covalent binding and cell death by flow cytometry.

Development of An Ex Vivo Proliferation Assay to Investigate the Mechanism of Presentation of Nitroso Sulfamethoxazole to T Cells. Freshly isolated splenocytes from a SMX-NO–sensitized animal were suspended in culture medium and dispensed (1.5×10^5/well; 100 μl) into a U-bottomed 96-well plate. Splenocytes from a naive donor animal (10×10^6/tube) were suspended in culture medium (total volume 10 ml) and incubated with SMX-NO (10–250 μM) at 37°C (5% CO_2). After 0.1, 1, 4, and 16 h, the splenocytes were washed with drug-free medium (3 × 10 ml) to remove unbound SMX-NO and antigen-presenting cells, and CD4+ and CD8+ T cells were separated using immunomagnetic microbeads. The protocol was essentially the same as that described above with the exception that two magnetic microbead-conjugated antibodies were used to isolate the purified cells. CD4+ and CD8+ T cells and antigen-presenting cells were irradiated (4500 rads), added (0.5×10^4/well) to the sensitized splenocytes, and incubated at 37°C (5% CO_2) for 72 h. To measure proliferation, [^{3}H]thymidine (0.5 μCi) was added to the cultures for 8 h. Cells were harvested, and [^{3}H]thymidine incorporation was measured as counts per min with a beta counter (PerkinElmer Life Sciences, Cambridge, UK). Proliferative responses were represented with the stimulation index (counts per min in drug-treated cultures/counts per min in cultures with DMSO alone). In separate experiments, SMX-NO–modified naive splenocytes were lysed by repeated freezing and thawing before irradiation. Figure 1 provides an overview of the described methods. As an internal control in each experiment, splenocytes (1.5×10^5/well) from sensitized animals were incubated with soluble SMX-NO (5–250 μM), and proliferation was determined as described.

Determination of the Mechanism of Presentation of Nitroso Sulfamethoxazole to T Cells. MHC dependency of the T-cell response to SMX-NO was determined by the addition of soluble SMX-NO to sensitized splenocytes pretreated with specific anti-MHC class I and/or anti-MHC class II antibodies. The anti-MHC class I and class II antibodies inhibit stimulation of CD8+ and CD4+ T cells, respectively (Schnyder et al., 1997). The antibodies were incubated with sensitized splenocytes at the manufacturer's indicated dilution for 30 min at 4°C. Cells were washed to remove excess antibody and incubated with soluble SMX-NO (10–250 μM) in cell culture media for 72 h at 37°C. Proliferation was determined by [^{3}H]thymidine incorporation.

Human T cell clones recognize SMX-NO bound directly to MHC in the absence of antigen processing (Schnyder et al., 2000). To investigate the possibility that SMX-NO may bind directly to MHC expressed on antigen-presenting cells or activated T cells, anti-MHC class I- and/or II-blocking antibodies were added to naive splenocytes before the addition of SMX-NO (10–250 μM). MHC-blocked, SMX-NO–modified splenocytes were added to the proliferation assay with sensitized splenocytes as described. Splenocytes from the sensitized animals were not exposed to the blocking antibodies or soluble SMX-NO. The amount of SMX-NO covalently bound to naive splenocytes treated with the blocking antibodies was determined by flow cytometry.

Statistical Analysis. All data represent the mean ± S.D. of four experiments carried out in triplicate (unless stated otherwise). Values to be compared were analyzed for nonnormality (Shapiro-Wilk test). Values were often found to be nonnormally distributed, and therefore, the Mann-Whitney test was used for comparison of the two groups, accepting $P < 0.05$ as significant.

Results

Nitroso Sulfamethoxazole Is Rapidly Degraded in Culture. LC-MS analysis in anion mode (SMX-NO was not detectable as $[M + 1]^+$) revealed that SMX-NO (*m*/*z* 266; R_t 11 min), dissolved in cell culture medium at a concentration of 40 μM, yielded appreciable amounts of SMX-hydroxylamine (*m*/*z* 268; R_t 5 min), nitro-SMX (*m*/*z* 282; R_t 10.5 min), and a previously unobserved product apparently corresponding to an azoxy dimer (*m*/*z* 517; R_t 15 min) within 0.1 h; lesser amounts of the putative azo dimer (*m*/*z* 501; R_t 15.5 min) were also found. The hydroxylamine and nitro metabolites were identified by chromatographic comparison with synthetic standards; the peaks of selected-ion current

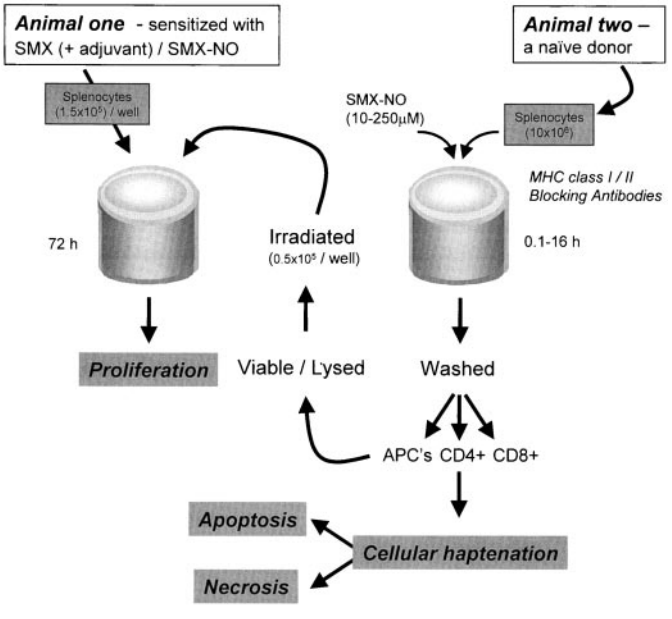

Fig. 1. Scheme illustrating the methods used to investigate the direct toxicological and cellular immunological consequences of hapten formation by SMX-NO. Proliferation was determined by overnight incubation with [^{3}H]thymidine. Covalent binding was determined by flow cytometry with a specific anti-SMX antibody. Apoptotic and necrotic cell death were measured by flow cytometry with the annexin-V/propidium iodide staining procedure.

corresponding to these compounds were absent from mass chromatograms of control incubations. Assignment of the azo and azoxy derivatives was based on the presence in selected ion mass chromatograms of relevant and prominent $[M - 1]^-$ and $[M + 1]^+$ coincident peaks that were absent from incubations of culture media alone. The relative proportions of SMX-NO and its derivatives after 30 min were 0.3:1.2:0.6:1.0:0.2, respectively, when equated with areas of peaks in the selected anion mass chromatograms.

SMX-NO was also dissolved in cell culture medium and incubated with freshly isolated rat splenocytes. Rapid transformation of SMX-NO was observed, and no more than trace amounts of the compound remained after 5 min; after 30 min, the relative portions of hydroxylamine, nitro, azoxy, and azo products determined by negative-ion LC-MS were 1.3:0.6:1.0:2.8.

Nitroso Sulfamethoxazole Binds Preferentially to the Surface of Antigen-Presenting Cells. The purity of separated antigen-presenting cells and CD4+ and CD8+ T cells determined by flow cytometry was consistently greater than 98%. Cell viability after separation was greater than 90%. Incubation of SMX-NO with freshly isolated rat splenocytes revealed that antigen-presenting cells were particularly susceptible to cell surface hapten formation, with a small but significant population of CD8+ T cells also showing haptenated SMX-NO on the cell surface. Less than 5% of CD4+ T cells were haptenated with SMX-NO (Fig. 2). Binding of SMX-NO was concentration-dependent, with the rank order of binding being maintained with the change in concentration (Fig. 2d). Addition of glutathione or *N*-acetyl cysteine, but not *N*-acetyl lysine, reduced cell surface binding of SMX-NO to background levels (results not shown). The same rank order of binding (i.e., antigen-presenting cells > CD8+ T cells > CD4+ T cells) was observed after a single i.p. administration of SMX-NO (100 mg/kg) to male Lewis rats.

Selective Toxicity of Antigen-Presenting Cells by Nitroso Sulfamethoxazole. Recently, Hess et al. (1999) demonstrated that CD8+ T cells isolated from human peripheral blood were particularly susceptible to the direct toxic effects of SMX-hydroxylamine. In this study, we investigated the viability of CD4+ and CD8+ T cells and antigen-presenting cells, purified from rat spleen, in the presence or absence of SMX-NO. Cell death was determined using the annexin-V/propidium iodide staining procedure (Vermes et al., 1995), with similar metabolite concentrations and time points to those described previously (Hess et al., 1999). A concentration-dependent increase in cell death was observed when purified CD4+ and CD8+ T cells and antigen-presenting cells were incubated with SMX-NO (50–250 μM) for 6 h (Fig. 3). Neither apoptosis nor necrosis was observed with concentrations of SMX-NO below 50 μM. Antigen-presenting cells displayed the greatest loss of viability compared with CD4+ and CD8+ T cells ($P < 0.05$; 50 and 100 μM SMX-NO). At lower concentrations of SMX-NO (50–100 μM), the majority of dead antigen-presenting cells stained positive for annexin-V and propidium iodide and were therefore necrotic; however, at higher concentrations, a significant number of apoptotic cells were also observed ($P < 0.05$). The same order of cell death was observed after 16 h (results not shown).

Selective Toxicity of Nitroso Sulfamethoxazole-Modified Cells. The relationship between cellular haptenation with SMX-NO and the induction of cell death was determined by flow cytometry. SMX-NO caused a significant increase in haptenation (10–250 μM; $P < 0.05$) and cell death

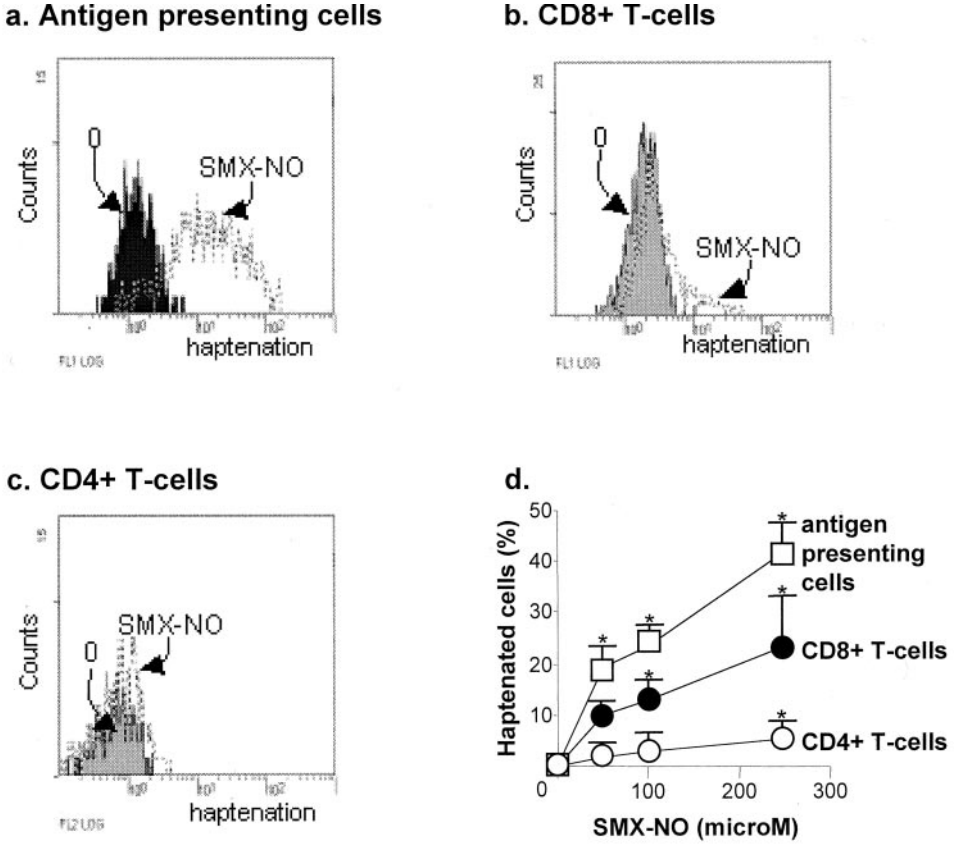

Fig. 2. Cell surface haptenation of SMX-NO to purified rat splenocytes. Splenocytes were incubated with solvent or SMX-NO (250 μM) at 37°C for 1 h. CD4+- and CD8+-deficient splenocytes were obtained using magnetic microbeads conjugated to the respective monoclonal antibody. CD8+ or CD4+ T cells and antigen-presenting cells were further separated by flow cytometry using mouse anti-rat CD8+ and CD4+ antibodies conjugated to PE and FITC, respectively. Cell surface binding of SMX-NO was analyzed using a fluorescent-labeled anti-SMX antibody. Flow cytometric traces show cell surface binding of SMX-NO to antigen-presenting cells (a), CD8+ (b), and CD4+ (c) T cells. d, dose-dependent cell surface binding of SMX-NO (50–250 μM) to the purified cells. The results are presented as the percentage of positively stained cells from four experiments, with incubations carried out in duplicate. Statistical analysis was performed by comparing incubations in the presence of drug with those in the absence of drug ($\star$, $P < 0.05$).

(100–250 μM; $P < 0.05$) (Fig. 4). Although covalently bound SMX-NO was found on the surface of viable and dead cells, above a threshold level of haptenation, the cells that became haptenated were those that underwent necrotic cell death. A discrete population of weakly haptenated viable cells was seen with low concentrations of SMX-NO (10–50 μM; Fig. 4, b and c).

T Cell Proliferation Requires Presentation of Nitroso Sulfamethoxazole-Modified Cellular Protein. Splenocytes from rats administered SMX-NO displayed a concentration-dependent proliferative response on in vitro restimulation with soluble SMX-NO (5–50 μM; Fig. 5a). Concentrations of SMX-NO above 50 μM inhibited proliferation. Splenocytes from sensitized rats also proliferated in the presence of SMX-NO–modified (10–250 μM), irradiated naive cells (Fig. 5). A 5-min pulse with SMX-NO was sufficient for hapten formation and T-cell proliferation (Fig. 5b). Importantly, in these experiments, sensitized splenocytes were not exposed to soluble SMX-NO. In contrast to the traditional proliferation assay with soluble SMX-NO (Fig. 5a), concentrations of SMX-NO that are known to cause toxicity produce a strong proliferative response (Fig. 5, b-e).

To investigate 1) the relationship between the extent of hapten formation, toxicity, and the induction of a proliferative response and 2) whether the observed proliferative response was caused by SMX-NO binding to antigen-presenting cells or T cells, SMX-NO–modified, purified cells were added to the proliferation assay with splenocytes from SMX-NO–sensitized animals. Although antigen-presenting cells were particularly susceptible to haptenation by SMX-NO compared with T cells, there were no discernible differences between the proliferative responses obtained after the addition of purified SMX-NO modified antigen-presenting cells, CD4+, or CD8+ T cells (Fig. 6; open circles). Splenocyte cultures from sensitized animals also responded readily to SMX-NO–modified lysed naive splenocytes (Fig. 6; closed circles). A stronger proliferative response of splenocytes from SMX-NO–sensitized rats was achieved with lysed antigen-presenting cells, but not T cells, when the addition of SMX-NO–modified naive lysed and viable cells were compared.

Presentation of Nitroso Sulfamethoxazole Is MHC-Restricted. Antibody blocking experiments were performed with specific anti-MHC class I and class II antibodies to investigate whether the response to soluble SMX-NO was MHC restricted. Splenocyte cultures from rats administered SMX-NO, treated with anti-MHC class I or anti-MHC class II antibodies, or both, did not proliferate or proliferated to a lesser extent in the presence of soluble SMX-NO compared with splenocytes in the presence of SMX-NO alone (Fig. 7a).

Direct Modification of MHC by Nitroso Sulfamethoxazole Is Not the Mechanism of Antigen Presentation to T Cells. To investigate whether presentation of SMX-NO–modified cellular protein to T cells requires antigen processing, naive splenocytes were pretreated with anti-MHC class I or anti-MHC class II antibodies, or both, before the addition of SMX-NO. Addition of specific anti-MHC antibodies blocks direct, processing-independent presentation, whereas processing-dependent presentation is not inhibited, or proliferation is inhibited to a lesser extent. Splenocytes from SMX-NO–sensitized animals displayed only a minimal decrease in

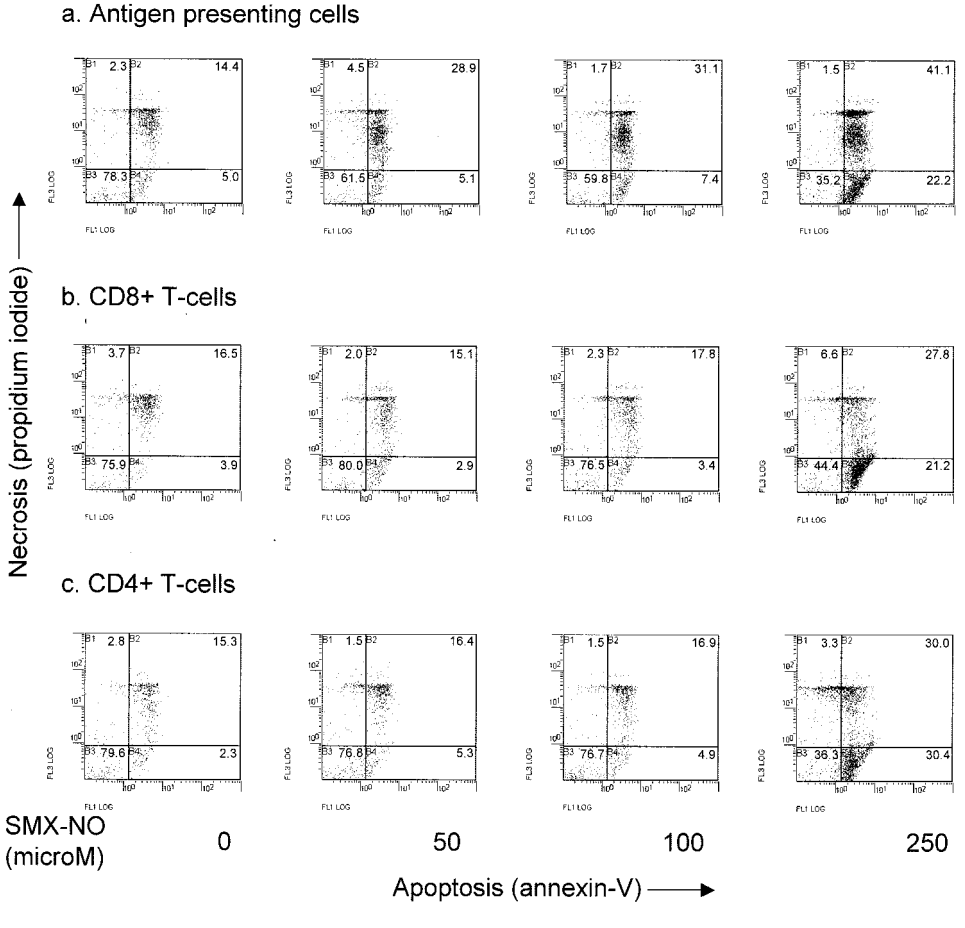

Fig. 3. Dose-dependent toxicity of purified antigen-presenting cells (a), CD8+ (b), and CD4+ (c) T cells cultured with SMX-NO (50–250 μM) in HBSS at 37°C. After 2 h, the cells were washed, resuspended in drug-free cell culture medium, and incubated at 37°C for a further 4 h. Cell were stained with annexin-V (1 μg/ml) and propidium iodide (35 μM). A combination of annexin-V and propidium iodide permits simultaneous detection of viable (annexin-V–negative/propidium iodide–negative), apoptotic (annexin-V–positive/propidium iodide–positive), and necrotic (annexin-V–positive/propidium iodide–positive) cells. Flow cytometry traces from one of three representative experiments are shown.

proliferation when naive splenocytes that were subsequently incubated with SMX-NO were treated with antibodies against MHC class I and II (Fig. 7 b). Similarly, flow cytometric analysis demonstrated that binding of SMX-NO to MHC represents only a small portion of the total amount of SMX-NO expressed on the cell surface (Fig. 7c).

Discussion

Allergic reactions associated with administration of SMX involve the activation of specific T cells that release perforin and cause tissue damage in the patient (Schnyder et al., 1998; Nassif et al., 2001). The clinical characteristics of such reactions are well defined; however, despite intensive research, the nature of the drug antigen presented to T cells remains ill defined. In these studies, using T cells from animals sensitized with SMX or SMX-NO in vivo, we detail: first, the toxicological consequences of cellular haptenation by SMX-NO; second, the nature of the chemical antigen recognized by the immune system; and third, the mechanism of presentation of SMX-NO.

SMX-NO was found to be extremely unstable in culture medium, being transformed spontaneously into nitro-SMX and SMX-hydroxylamine (Cribb et al., 1991; Naisbitt et al., 1996). Other products were identified as azo- and azoxy-SMX. Dimerization of aromatic nitroso compounds has been

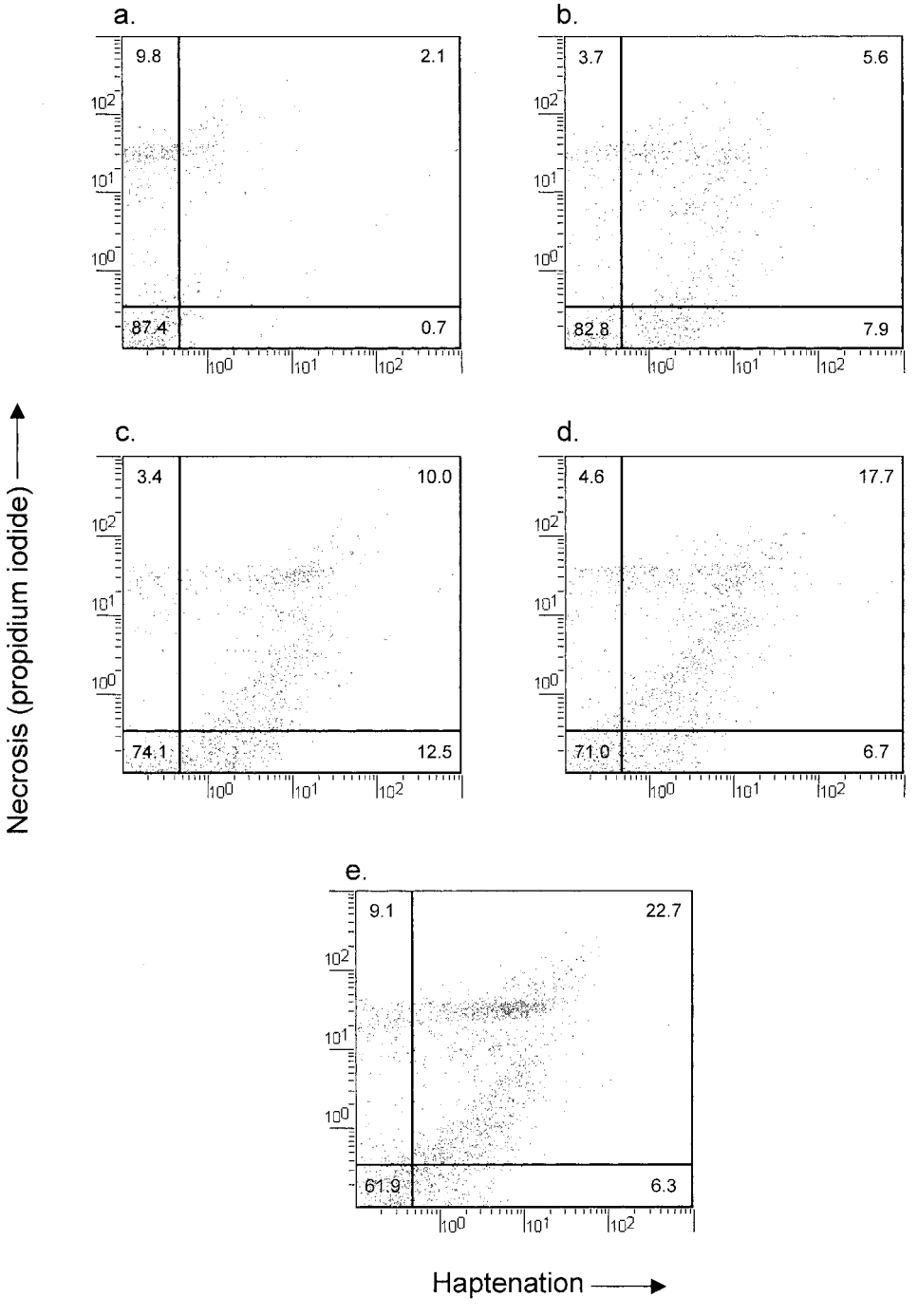

Fig. 4. Flow cytometric assessment of the relationship between cell surface binding of SMX-NO and cell death. Cells were incubated with 0 (a), 10 (b), 50 (c), 100 (d), and 250 μM (e) SMX-NO in HBSS at 37°C. After 2 h, the cells were washed, resuspended in drug-free cell culture medium, and incubated at 37°C for a further 4 h. Cells were then stained with a FITC-conjugated anti-SMX antibody to measure haptenation and with propidium iodide to measure cell death. Shown are representative traces of one experiment carried out in triplicate.

described previously (Tocher et al., 1994); the reaction is preceded by reduction of one molecule of SMX-NO to the hydroxylamine. The rate of disappearance of SMX-NO was increased in the presence of rat splenocytes; SMX-NO was not detected after 0.1 h. Enhanced disappearance of SMX-NO was associated with an increased production of azo-SMX. These data can be explained if an intracellular enzyme, possibly related to the aldehyde dehydrogenase, which has been shown to catalyze the reduction of SMX-NO to SMX-hydroxylamine in human blood mononuclear cells (Naisbitt et al., 1999), catalyzes the reduction by splenocytes. Increased levels of SMX-hydroxylamine react with SMX-NO to yield larger quantities of azo-SMX.

SMX-NO is a cytotoxic metabolite that haptenates cysteine residues in proteins, including the surface of viable lymphocytes and keratinocytes (Rieder et al., 1995; Reilly et al., 1998; Hess et. al., 1999; Naisbitt et al., 1999; Reilly et al., 2000). We recently demonstrated that haptenation by SMX-NO was seen at concentrations that were not associated with toxicity (Naisbitt et al., 1999); however, the relationship between cellular haptenation, cell death, and the induction of an immune response remains ill defined. In these studies, SMX-NO bound irreversibly to splenocytes, and a greater number of hapten epitopes were found on the surface of antigen-presenting cells compared with T cells (Fig. 2). The amount of covalent binding corresponded to the number of sulfhydryl groups expressed on the surface of antigen-presenting cells and T cells (Lawrence et al., 1996). In addition, SMX-NO killed antigen-presenting cells preferentially (Fig. 3). Although the viability of cells conjugated with low levels of SMX-NO was not significantly diminished ($P > 0.05$; SMX-NO 10–50 μM; Fig. 4, b and c, bottom right quadrant), above a threshold level of haptenation (approximately 10% of maximal binding), the amount of covalent binding was directly proportional to the extent of cell death (SMX-NO 100–250 μM; Fig. 4, d and e, top right quadrant). These data agree with our previous report showing that haptenation can occur in the absence of toxicity; however, cells conjugated with high levels of SMX-NO go on to die by apoptosis followed by secondary necrosis.

We have previously demonstrated that splenocytes from animals administered SMX (in complete Freund's adjuvant) proliferated after in vitro restimulation with SMX-NO (Naisbitt et al., 2001). In these studies, a concentration-dependent proliferative response of sensitized splenocytes was observed with low concentrations of SMX-NO (5–50 μM); these concentrations caused low levels of cellular haptenation, and viability was not reduced. Higher concentrations of SMX-NO (100–250 μM) caused toxicity, and proliferation was inhibited (Figs. 4 and 5a). To investigate whether SMX-NO–specific T cells also recognize haptenated dead cells and/or cellular protein, we developed a novel proliferation assay that incorporates cells from two rats, one sensitized and the other a naive donor (Fig. 1). Splenocytes from the naive animal were incubated with SMX-NO in vitro, washed repeatedly (to remove unbound drug), irradiated (to prevent nonspecific proliferation), and added to the proliferation assay containing cells from the sensitized animal. Splenocytes from the sensitized animal were not exposed to soluble SMX-NO. Sensitized splenocytes proliferated in the presence of SMX-NO modified naive cells; a 5-min exposure of the naive cells to SMX-NO was sufficient to stimulate a strong proliferative response (Fig. 5). Maximum proliferation was seen when highly conjugated splenocytes with a reduced viability were added to the proliferation assay. Nevertheless, it can be seen that haptenated viable cells are antigenic in this system (Fig. 4, b and c and Fig. 5). These data agree with previous studies showing that T cells recognize antigen on lightly conjugated viable cells and highly conjugated cells with diminished viability (Soeberg et al., 1978). It is therefore possible that viable cells haptenated with low levels of SMX-NO will internalize and present the SMX-NO–modified membrane protein, whereas highly conjugated dead cells (which cannot function

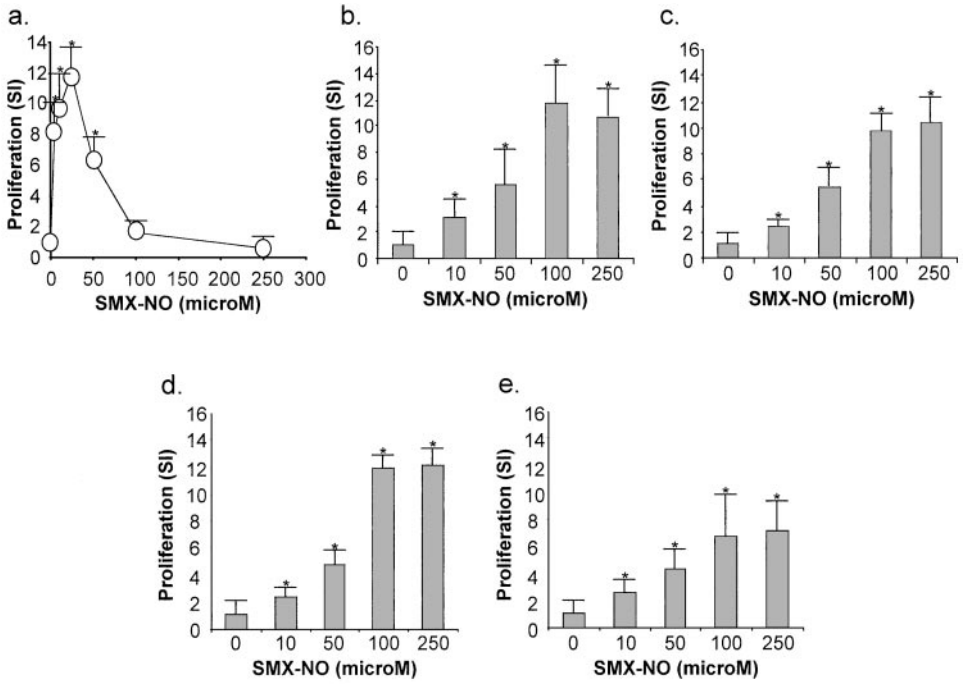

Fig. 5. Dose-dependent proliferative response of splenocytes from SMX-NO–treated rats (1 mg/kg) cultured with SMX-NO (a) or SMX-NO–modified (b-e) naive, irradiated splenocytes. Naive splenocytes had been incubated with SMX-NO for 0.1 (b), 1 (c), 4 (d), or 16 h (e). Sensitized splenocytes were not exposed to soluble SMX-NO. After 72 h, proliferation was determined by incorporation of [^{3}H]thymidine over an additional 8 h. The cpm in the control cultures (DMSO alone) did not exceed 600. Results are presented as mean ± S.D. from four rats, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitized splenocytes after the addition of drug and solvent-treated naive splenocytes (⋆, $P < 0.05$).

as antigen-presenting cells) will be taken up and presented by adjacent viable cells.

To investigate the propensity of various cell types to act as a source antigen, purified SMX-NO–modified CD4+ and CD8+ T cells and antigen-presenting cells were added to the proliferation assay. Although antigen-presenting cells were particularly susceptible to cell surface hapten formation and toxicity, SMX-NO modification of antigen-presenting cells and T cells generated an equipotent antigenic signal (Fig. 6, open circles). These data indicate that a low concentration of SMX-NO bound covalently to various cell types can generate a maximal proliferative response in the assay.

Our understanding of the mechanism(s) of T-cell recognition of drug antigens derive largely from in vitro studies with T cell clones isolated from the peripheral blood of allergic individuals (Schnyder et al., 1998; Zanni et al., 1998). T cell clones from SMX-sensitive patients recognize MHC-restricted SMX and SMX-NO in the absence of antigen processing (Schnyder et al., 1997, 2000; Burkhart et al., 2001). Presentation of SMX requires the formation of a labile complex between MHC, the T-cell receptor, and the drug itself, whereas SMX-NO presentation requires the formation of a stable covalent bond. The former mechanism contradicts the

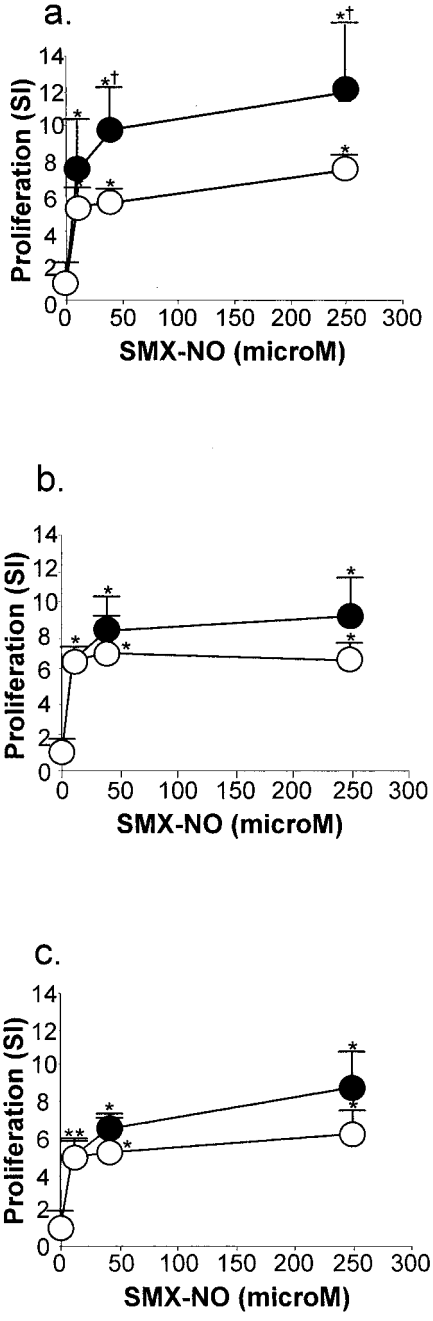

Fig. 6. Dose-dependent proliferative response of splenocytes from SMX-NO–treated rats (1 mg/kg) cultured with lysed (●) or viable (○) SMX-NO–modified naive, irradiated antigen-presenting cells (a), CD4+ T cells (b), or CD8+ T cells (c). Naive splenocytes were incubated with SMX-NO for 1 h. Sensitized splenocytes were not exposed to soluble SMX-NO. After 72 h, proliferation was determined by incorporation of [^{3}H]thymidine over an additional 8 h. The cpm in the control cultures (DMSO alone) did not exceed 600. Results are presented as mean ± S.D. from four rats, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitized splenocytes after the addition of drug- and solvent-treated naive splenocytes (⋆, $P < 0.05$) and lysed and viable drug-treated naive splenocytes (†, $P < 0.05$).

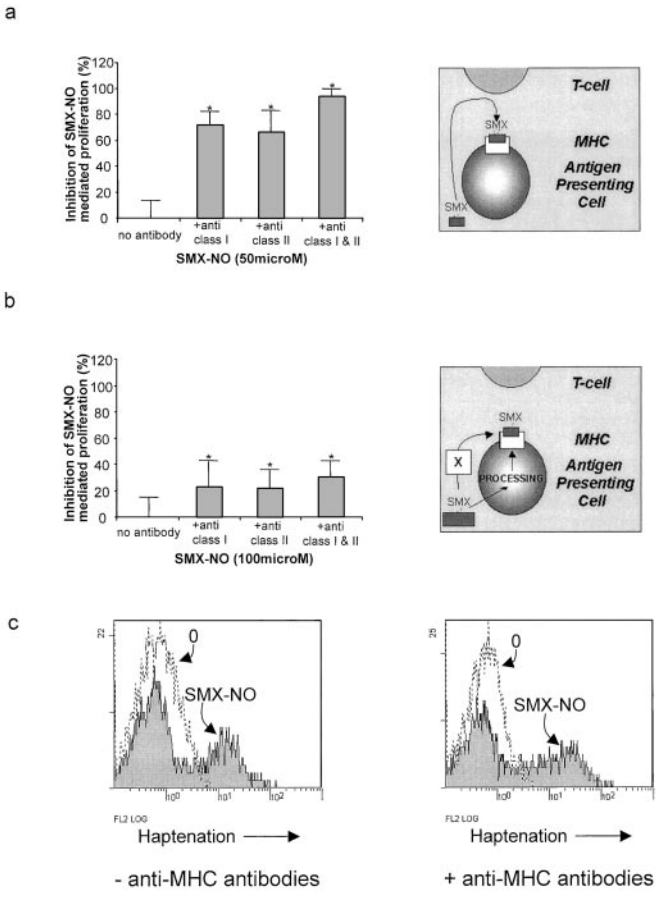

Fig. 7. a, MHC-restricted presentation of SMX-NO. Splenocyte cultures from rats treated with SMX-NO (1 mg/kg) were incubated with soluble SMX-NO (50 μM) in the presence or absence of monoclonal anti-MHC class I- and/or class II-blocking antibodies. Positive control was proliferation without the addition of any antibody. b, T cells do not recognize SMX-NO bound directly to MHC. Anti-MHC class I and/or II antibodies were added to naive splenocytes before the addition of SMX-NO (100 μM). MHC-blocked, SMX-NO–modified splenocytes were then added to the proliferation assay with sensitized splenocytes. Proliferation was determined by the addition of [^{3}H]thymidine for the final 8 h of a 72-h incubation. c, cell surface haptenation of rat splenocytes after incubation with solvent control or SMX-NO (100 μM) for 1 h in the presence or absence of monoclonal anti-MHC class I and class II antibodies. Cell surface binding was analyzed by flow cytometry using an anti-SMX antibody and a PE-conjugated anti-IgG secondary antibody. One representative of three independent experiments carried out in duplicate is shown.

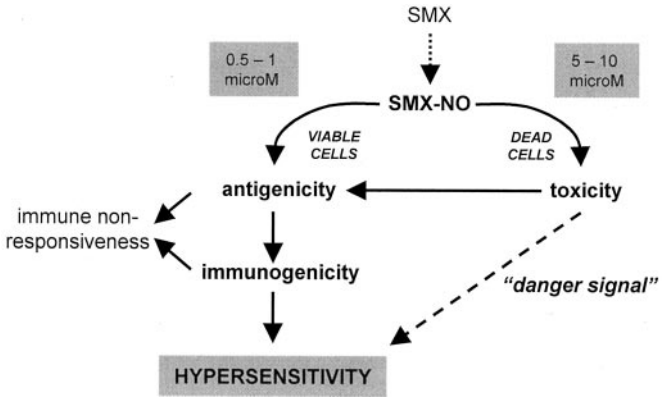

Fig. 8. Scheme illustrating the proposed relationship between hapten formation by SMX-NO, toxicity, and the initiation of hypersensitivity reactions in patients.

traditional view of drug presentation proposed by Landsteiner and Jacobs (1935), in which processing of a drug protein conjugate is a prerequisite for presentation (Kalish and Askenase, 1999). In these studies, using freshly isolated splenocytes from SMX-NO–sensitized animals, we have demonstrated that covalent binding of SMX-NO to cellular protein stimulates T-cell proliferation. The proliferative response was inhibited by anti-MHC antibodies and was therefore MHC and antigen-presenting cell restricted (Fig. 7a). Capping of MHC on naive cells before exposure to SMX-NO did not inhibit proliferation of sensitized splenocytes, indicating that direct modification of MHC was not the mechanism of antigen presentation (Fig. 7b). SMX-NO–modified cellular proteins may therefore undergo processing before antigen presentation (Park et al., 1998; Kalish and Askenase, 1999). Because T cells generated from a primary immune response transform into long-lived memory cells, with a lower threshold to be reactivated, it is possible that with time the SMX-NO–specific T cells generated in these studies may cross-react with SMX. This would explain the different mechanisms of presentation observed here and elsewhere (Schnyder et al., 1997, 2000); however, direct experimental evidence to support this hypothesis has not been forthcoming.

Activation of an effective immune response in vivo involves the presentation of an MHC-restricted antigen in the presence of costimulatory signals (e.g., the interaction of B7 with CD28 and CTLA-4) and cytokines released by antigen-presenting cells (Curtsinger et al., 1999). Antigen-presenting cells are activated by endogenous signals [referred to as "danger signals" by Matzinger (1994)] received from dead cells (Gallucci et al., 1999; Shi et al., 2000; Gallucci and Matzinger, 2001). Keratinocytes can bioactivate SMX; bioactivation results in covalent binding and cell death (Reilly et al., 2000). Therefore, it is possible that hapten-modified dead or dying keratinocytes provide a "danger" signal and a beacon for sensitized T cells. LC-MS analyses of cell culture supernatant and SMX-NO demonstrate that only 10% of the starting material remained after 0.1 h. From these data, we estimate that the antigenic threshold of SMX-NO for T cells was between 0.5 and 1 μM, which is less than the concentration of SMX metabolites found in human plasma after administration of a therapeutic dose of SMX (Gill et al., 1997). The threshold concentration of SMX-NO for toxicity was one order of magnitude higher (i.e., 5–10 μM). These data lead us to hypothesize that the propensity of SMX-NO to cause cell death in patients may also determine individual susceptibility (Fig. 8). These theories derive from animal models of contact sensitization, which have demonstrated that potent chemical haptens are not immunogenic in the absence of an irritant (danger) signal. The irritant signal is dose-dependent, occurs at higher concentrations of the hapten than the antigenic signal, and is the primary determinant of sensitization (McFadden and Basketter, 2000; Zhang and Tinkle, 2000). The increased incidence of SMX allergy with human immunodeficiency virus infection (Pirmohamed and Park, 2001), which is also a known danger signal (Descamps et al., 2001; Sullivan and Shear, 2001), is consistent with this proposal and provides a clinical parallel to our in vitro observations.

In conclusion, we have demonstrated that above a threshold level, there is a direct relationship between cell surface hapten formation and toxicity. Hapten formation is also critical for the development of a cellular immune response; sensitized splenocytes recognize live and dead cells haptenated with low and high levels of SMX-NO, respectively. The critical question that remains to be addressed is why these T cells recognize SMX-NO–modified cellular protein, whereas T cells isolated from the peripheral blood of drug allergic individuals recognize SMX and SMX-NO bound directly to MHC or a peptide embedded within. Studies are underway with naive human peripheral blood to further delineate the mechanism(s) of presentation of SMX to T cells.

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