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Characterisation of Sulfamethoxazole and Sulfamethoxazole Metabolite-Specific T-cell Responses in Animals and Man

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Running Title:
T-cell responses to sulfamethoxazole

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Abbreviations: SMX, sulfamethoxazole; SMX-NO, nitroso sulfamethoxazole; DMSO, dimethyl sulfoxide; HBSS, hanks balanced salt solution.

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Abstract

Sulfamethoxazole (SMX) is associated with hypersensitivity reactions. Identification of drug-specific lymphocytes from hypersensitive patients suggests involvement of the immune system. Lymphocytes from humans recognise SMX and nitroso-SMX (SMX-NO), whereas cells from sensitised rats recognise only SMX-NO. In these investigations we study the nature of SMX-specific T-cells in 4 species. Male rats, mice and rabbits were immunised with SMX (50mg kg^{-1}) or SMX-NO (1mg kg^{-1}). Lymphocytes and/or splenocytes were isolated and incubated with SMX, SMX-hydroxylamine or SMX-NO and proliferation was measured. Lymphocytes were also isolated from SMX hypersensitive patients (n=3) and drug-specific proliferation was measured. In addition, rabbits were bled fortnightly for 4 months to determine whether SMX-NO-specific T-cells cross react with SMX. To confirm that SMX-NO responses were due to covalent binding and not cross reactivity, cells were pulsed with SMX-NO and/or co-incubated glutathione. Splenocytes from mice, rats and rabbits proliferated when stimulated with SMX-NO, but not SMX. A 2h pulse with SMX-NO was sufficient for proliferation, while cells co-incubated with SMX-NO and glutathione did not proliferate. Rabbit lymphocytes proliferated in the presence of SMX-NO and SMX-hydroxylamine, but not SMX. SMX-hydroxylamine was converted to SMX-NO in culture. The SMX-NO-specific response of rabbit lymphocytes was maintained for at least 4 months and the cells did not cross-react with SMX. Human lymphocytes from hypersensitive patients proliferated in the presence of SMX and both metabolites. These results highlight important differences in T-cell recognition of drug(metabolite)

antigens in animals that have been sensitised against a drug metabolite and patients with hypersensitivity to the drug.

Administration of sulfamethoxazole (SMX) is associated with hypersensitivity reactions, the most common of which are cutaneous eruptions. These reactions range in severity from mild antibody-mediated urticarial reactions to the potentially fatal toxic epidermal necrolysis, which is T-cell mediated (Pichler et al., 2002). SMX is used in combination with trimethoprim, as co-trimoxazole, for the treatment of opportunistic infections associated with HIV-infection. In these patients, hypersensitivity reactions are seen in 30% of individuals administered low dose SMX for prophylaxis and 50% given SMX for treatment (Pirmohamed and Park, 2001). Due to the high incidence of SMX hypersensitivity in patients with HIV infection there has been a resurgence of interest in the chemical and immunological mechanisms underlying these reactions.

Low molecular weight substances, including most allergenic drugs, are thought to become immunogenic by binding irreversibly to protein (Park et al., 1998). In the case of SMX this involves CYP and myeloperoxidase catalysed metabolism (Cribb et al., 1990; Cribb et al., 1995). The resultant hydroxylamine, which is not protein reactive (Cribb et al., 1991; Naisbitt et al., 1996), circulates in the periphery (Gill et al., 1997). Further auto oxidation, under conditions of oxidative stress, generates the protein-reactive intermediate nitroso SMX (SMX-NO; Naisbitt et al., 1999, 2001; Reilly et al., 2000; Summan et al., 2002; Manchanda et al., 2002; Figure 1). In solution, SMX-NO is extremely unstable; degradation yields products of oxidation (nitro SMX), reduction (SMX, SMX hydroxylamine) and dimerisation (azo and azoxy adducts) (Naisbitt et al., 2002). Patients with HIV infection have low thiol levels and a decreased capacity to reduce SMX metabolites back to the parent drug (Walmsley et al., 1997; Naisbitt et al.,

2000), which in turn is thought to contribute to the increased susceptibility to SMX hypersensitivity.

To investigate the role of oxidative drug metabolism in SMX hypersensitivity we developed a rat model of drug immunogenicity. Adminstration of SMX-NO, but not the parent drug, led to the production of metabolite-specific antibodies and T-cells (Gill et al., 1997; Naisbitt et al., 2001). The T-cell receptor of SMX-NO specific T-cells was stimulated by an MHC-restricted, processed peptide derived from cellular protein haptenated with SMX-NO (Naisbitt et al., 2002). Chemical (thiol depleting agents) and immunological (adjuvants) modulation, prior to SMX administration, confirmed that oxidative metabolism of SMX was required to stimulate a primary drug antigen-specific immune response *in vivo*. These observations are consistent with the demonstration of SMX metabolite-specific, but not SMX-specific, delayed-type hypersensitivity in mice (Choquet-Kastylevsky et al., 2001) and the original hapten hypothesis.

The belief that low molecular weight chemicals require covalent binding to be immunogenic has recently been challenged (Mauri-Hellweg et al., 1995; Schnyder et al., 1997; von Greyerz et al., 2001). T-cell clones isolated from hypersensitive patients have shown that SMX can be presented directly in the apparent absence of drug metabolism. This form of drug recognition by T-cells is MHC-restricted, but does not require antigen processing (Schnyder et al., 1997). Further studies of SMX hypersensitive patients with maculopapular- and bullous-type skin eruptions has confirmed that SMX can indeed be presented to T-cells directly; however, it must be noted that T-cells, which proliferate in the presence of SMX-NO can also be isolated from most hypersensitive individuals

(Schnyder et al., 2000; Burkhart et al., 2001; Nassif et al., 2002). Thus, the signal that stimulates the immune system is not known.

The aim of this project was to further address the nature of the SMX antigen recognised by T-cells. To fulfil this aim, SMX and SMX-NO sensitised splenocytes from 3 animal species (mouse, rat and rabbit) and lymphocytes from sensitised rabbits and hypersensitive humans were used. To delineate the potential of SMX-NO-specific T-cells to cross-react with SMX with time, lymphocytes from SMX-NO sensitised rabbits were collected fortnightly for 4 months and stimulated *ex vivo* with SMX and SMX-NO.

Materials and Methods

Chemicals

Dimethyl sulphoxide (DMSO), Hanks balanced salt solution (HBSS), L-glutamine, HEPES, penicillin, streptomycin, RPMI 1640, Human AB serum, transferrin, [^{3}H] thymidine, fetal calf serum and SMX were obtained from Sigma Aldrich (Poole, UK). Lymphoprep was obtained from Nycomed (Birmingham, UK). SMX hydroxylamine and SMX-NO were synthesised according to the method of Naisbitt et al. (1996).

Cell culture media

Basal media consisted of RPMI 1640 supplemented with L-glutamine (2mM) HEPES (25mM) streptomycin (100μg ml^{-1}) and penicillin (100μg ml^{-1}). For experiments with human cells this solution was supplemented with 10% human AB serum and transferrin (25mg). In animal experiments the basal media was supplemented with 10% fetal calf serum. All culture media was passed through a 0.45 μM filter before use.

Patient details

Blood lymphocytes were obtained from 3 SMX hypersensitive patients, 3 patients administered SMX without visible adverse effects and 3 unexposed individuals. Of the SMX-hypersensitive patients, 2 were HIV-negative and developed maculopapular rashes after treatment with co-trimoxazole. The third patient, who was HIV-positive and was being treated with co-trimoxazole for prophylaxis for *Pneumocystis carinii* pneumonia, developed a rash and fever. All the patients had a positive rechallenge as part of their clinical care, and their

symptoms improved after discontinuation of co-trimoxazole. Approval for the study was obtained from the local ethics committee and informed consent was obtained from each participant. Lymphocytes were isolated from venous blood by density centrifugation using Lymphoprep. Purified cells were washed with culture media and the yield was assessed using an improved Neubauer haemocytometer (Weber Scientific Int., UK). Viability, which was consistently greater than 95%, was monitored by trypan blue dye exclusion.

Immunising protocols

The rat was chosen as a model since SMX metabolism has been studied previously and been shown to be similar to man (Gill et al., 1997). The immunogenicity of SMX and SMX-NO was studied in mice because of their decreased capacity to metabolise SMX to SMX-NHOH (Naisbitt – unpublished). Metabolism of SMX in rabbits has not been investigated. However, experiments with rabbits allowed us to study the kinetics of the SMX-NO-specific proliferative response and investigate whether SMX-NO-specific T-cells cross react with SMX with time. Male Wistar rats (8 - 12 weeks, 175 - 225g), male CD1 mice (6 – 9 weeks, 20 - 30g) and New Zealand White rabbits (10 - 12 weeks, 2 – 2.5 kg) were purchased from Charles River UK Ltd (Kent, UK). All animals were immunised with SMX (50 mg ml^{-1}) or SMX-NO (1 mg kg^{-1}) in DMSO ip (rats and mice100µl, rabbits, 200µl per injection) 4 times weekly for 2 weeks, using established methodology (Naisbitt et al., 2001). Control animals were administered DMSO alone. Seven days after completion of the immunisation protocol, animals were sacrificed and the spleen was removed using aseptic technique. In separate experiments, blood (7 ml) was taken from the ear of

SMX-NO sensitised rabbits. This procedure was repeated every 2 weeks for 16 weeks. After the 2-week immunisation protocol, the rabbits were not exposed to SMX-NO.

Red cell depleted splenocytes and / or lymphocytes were isolated by density centrifugation using Lymphoprep. Purified cells were washed with culture media and the yield was assessed using an improved Neubauer haemocytometer (Weber Scientific Int., UK). Viability, which was consistently greater than 95%, was monitored by trypan blue dye exclusion.

Determination of the ex-vivo proliferative response of blood lymphocytes and splenocytes to sulfamethoxazole and its metabolites

Isolated human and rabbit lymphocytes and animal splenocytes (mouse, rat and rabbit) were incubated (1.5 X 10^5) in 96-well U-bottomed cell culture plates with SMX (10 – 250 μg ml^{-1}) or SMX-NO (1 – 25 μg ml^{-1}) at 37°C, 5% CO_2. In separate experiments rabbit lymphocytes were incubated with SMX (1 – 250 μg ml^{-1}), SMX-hydroxylamine (0.5 – 100 μg ml^{-1}) or SMX-NO (0.5 – 100 μg ml^{-1}). SMX hydroxylamine was added in the presence and absence of glutathione (1 mM), which is thought to prevent the oxidation of SMX hydroxylamine to SMX-NO. After 3 days (for animal experiments) or 5 days (for human experiments), proliferation was measured by the addition of [^{3}H] thymidine (0.5μ Ci) for the final 8 hours of culture. 3 and 5 days represent optimal proliferation for animal and human cells, respectively (results not shown). Cells were harvested and incorporated radioactivity was measured as counts per minute on a β-counter (Perkin-Elmer Life Sciences, Cambridge UK). Proliferative responses were

calculated as stimulation indices (SI; CPM in drug treated cultures / CPM in cultures containing DMSO alone).

Determination of the nature of the drug antigen presented to T-cells

To determine whether the response to SMX or SMX metabolites was due to covalent binding of the drug (metabolite) to protein, human and animal lymphocytes and splenocytes were pulsed with SMX (200 μg ml^{-1}) or SMX-NO (10 μg ml^{-1}) for 2h using a previously described protocol (Schnyder et al., 2000; Naisbitt et al., 2001). Drug(metabolite) pulsed cells were washed 3 times to remove unbound drug and consequently any response to parent drug is inhibited. In contrast, the response to covalently bound SMX-NO is unaffected. Pulsed cells were transferred to 96 well cell culture plates at 1.5 x 10^5 cells / well and incubated for 3 (animal experiments) or 5 (human experiments) days at 37^oC. Proliferation was assessed by the addition of [^3H] thymidine as described above. In separate experiments, cells were incubated with SMX-NO (10 μg ml^{-1}) and glutathione (1 mM). Glutathione binds covalently to SMX-NO (Naisbitt et al., 1996) and inhibits SMX-NO-specific lymphocyte proliferation (Burkhart et al., 2001; Naisbitt et al., 2001). SMX-specific proliferation is not affected by the addition of glutathione.

Determination of the chemical fate of sulfamethoxazole hydroxylamine in culture in the presence and absence of glutathione

SMX-NO has been shown to be rapidly degraded in culture (Naisbitt et al., 1996; Naisbitt et al., 2002). Major products were SMX hydroxylamine, nitro SMX and azo and azoxy dimers. Covalently bound SMX-glutathione adducts are also

formed when SMX-NO is incubated with glutathione (Cribb et al., 1991; Naisbitt et al., 1996). In contrast, the fate of SMX hydroxylamine in culture is not known. To investigate whether the proliferative response to SMX-NHOH was due to auto-oxidation to SMX-NO and covalent binding to protein, cells were incubated with SMX-NHOH (50 μg ml^{-1}) in the presence or absence of glutathione (1mM). Proliferation was measured by assessment of [^{3}H] thymidine incorporation. The extent of covalent binding was measured by flow cytometry using a previously described protocol (Naisbitt et al., 1999). Briefly, drug-treated cells were stained with a hapten-inhibitable anti-SMX IgG antibody (1:500, v/v; 40 μl) and a PE-conjugated and IgG secondary antibody. 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.

Statistics

All data are expressed as mean ± s.d. The Mann-Whitney test was used for comparison of control and test values, accepting $P < 0.05$ as significant.

Results

Administration of nitroso sulfamethoxazole to mice, rat and rabbits stimulates a potent drug-metabolite-specific cellular immune response

Splenocytes from SMX-NO sensitised mice, rats and rabbits proliferated following *ex vivo* stimulation with SMX-NO (Figure 2). Proliferation was observed at SMX metabolites concentrations that are seen in humans following high doses of SMX (Mitra et al., 1996; Gill et al., 1996). The strength of the SMX-NO-specific proliferative response was greater in rabbits when mice, rats and rabbits were compared (mouse, 4.3 ± 1.6; rat, 14.5 ± 2.2, rabbit 32.8 ± 3.0; $P < 0.05$: 10 μg ml^{-1} SMX-NO). Concentrations of SMX-NO above 5 μg ml^{-1}, inhibited mouse splenocyte proliferation. Splenocytes from rat and rabbit were less sensitive to the toxic effects of SMX-NO. In these species, SMX-NO concentrations above 25 μg ml^{-1} inhibited proliferation (results not shown). Splenocytes from animals administered SMX or SMX-NO did not proliferate following *ex vivo* exposure to SMX. Spelocytes from control mice, rats and rabbits did not proliferate following *ex vivo* exposure to SMX or SMX-NO.

To show that formation of a SMX-NO cellular hapten was a prerequisite for SMX-NO-specific proliferation of mouse, rat and rabbit splenocytes, cells from SMX-NO sensitised animals were pulsed with SMX-NO, washed and cultured for the remainder of the incubation period in the absence of soluble drug. We have previously shown that covalent binding of SMX-NO to cellular protein is rapid; cellular conjugates can be detected after as little as 5 minutes (Naisbitt et al., 2001). In addition, in separate experiments, SMX-NO sensitised splenocytes were cultured with SMX-NO and glutathione. Mouse, rat and rabbit splenocytes proliferated following a 2 h pulse with SMX-NO (Figure 3); the extent of

proliferation was similar to that seen with soluble SMX-NO. In contrast, no proliferation was seen when splenocytes were co-incubated with SMX-NO and glutathione (Figure 3).

Proliferation of lymphocytes from sulfamethoxazole hypersensitive humans and sulfamethoxazole (metabolite) sensitised rabbits

Lymphocytes from sulfamethoxazole hypersensitive patients, with and without HIV infection, proliferated in the presence of SMX, SMX hydroxylamine and SMX-NO (n = 3; Table 1). Figure 4 shows SMX, SMX hydroxylamine and SMX-NO specific proliferation of lymphocytes from HIV-positive hypersensitive patient 1. The response to SMX and SMX hydroxylamine was concentration-dependent, whereas SMX-NO-specific proliferation was detectable at each concentration tested (1 - 25μg ml^{-1}; Figure 4). Lymphocytes from patients exposed to SMX without adverse effects and unexposed individuals did not proliferate with SMX or SMX-NO.

SMX-NO sensitised rabbit lymphocytes proliferated strongly in the presence of SMX-NO and SMX hydroxylamine, but not the parent drug (Figure 4). The concentrations of SMX hydroxylamine and SMX-NO that caused proliferation were similar to that seen in hypersensitive humans. SMX hydroxylamine-specific proliferation was concentration-dependent, while the response to SMX-NO was seen at each concentration tested. Lymphocytes from untreated rabbits did not proliferate with SMX or SMX-NO.

Lymphocytes from SMX hypersensitive humans and SMX-NO sensitised rabbits proliferated when pulsed with SMX-NO, washed and resuspendend in drug-free media for the remainder of the incubation period. In contrast, lymphocytes

pulsed with SMX did not. The addition of glutathione had no effect on SMX-specific proliferation of human lymphocytes, while SMX-NO-specific proliferation of rabbit and human lymphocytes was inhibited (results not shown).

Proliferation of rabbit lymphocytes to SMX hydroxylamine was inhibited by the addition of glutathione at low concentrations; however, proliferation was seen with higher concentrations of SMX hydroxylamine (Figure 5a). Proliferation of human lymphocytes with SMX hydroxylamine and glutathione was not studied since they proliferate in the presence of covalently and non-covalently bound SMX. The response of rabbit lymphocytes to SMX hydroxylamine is intriguing since the above data seem to suggest that the response can be directed against non-covalently bound drug (metabolite) as well as a drug haptenated protein. To study whether SMX hydroxylamine is converted to SMX-NO in culture, we measured the formation of SMX covalent adducts by flow cytometry. In the absence of glutathione, SMX hydroxylamine bound rapidly to cells (Figure 5b). Covalently bound SMX was detectable on cells after 4 h in culture at 37^0C. In the presence of glutathione, which is thought to prevent oxidation of SMX hydroxylamine (Gill et al., 1996), covalent binding was delayed, but not inhibited. Covalently linked SMX cellular adducts were seen after 48 h (Figure 5c).

Nitroso sulfamethoxazole-specific rabbit lymphocytes are long lasting in vivo and do not cross react with sulfamethoxazole

To study the duration of the SMX-NO-specific immune response *in vivo* and to see whether SMX-NO-specific T-cells cross react with SMX with time, SMX- and SMX-NO-specific proliferation of lymphocytes from rabbits sensitised with

SMX-NO was assessed fortnightly for 4 months. It is important to note that, on completion of the 2-week immunisation protocol, rabbits were not exposed to either SMX or SMX-NO. Data presented in figure 6 shows that although the extent of SMX-NO-specific lymphocyte proliferation declined with time, significant proliferation was seen after 4 months. In addition, SMX-NO-specific lymphocytes did not cross react with SMX.

Discussion

Drug hypersensitivity reactions are a major clinical problem. Identification of drug-specific, T-cells in the peripheral circulation and skin, at the time of the reaction, provides evidence that the immune system is involved in the development of clinical symptoms (Mauri-Hellweg et al., 1995; Schnyder et al., 1997; Yawalkar et al., 2000). Despite this, the nature of the drug antigen that stimulates hypersensitivity reactions is a matter of debate. From the current state of knowledge, there are three possible mechanisms as to how drugs might stimulate T-cells: first, protein reactive drugs such as penicillin bind covalently to protein, which is then recognized to the immune system (Padovan et al., 1996); secondly, many drugs are not chemically reactive *per se* and do not appear to be immunogenic. They may however become chemically reactive after drug metabolism (Naisbitt et al., 2001,2002). Finally, *in vitro* studies have demonstrated that certain, chemically inert drugs can form sufficiently strong non-covalent bonds with MHC molecules to stimulate T-cells directly (Schnyder et al., 1997; Schnyder et al., 2000).

SMX is a dihydropteroate synthetase inhibitor that is effective in the treatment of opportunistic diseases associated with the progression of HIV infection. Unfortunately, SMX administration is associated with the development of hypersensitivity reactions in 30–50% of patients with HIV, some of which can be severe and cause deaths (Pirmohamed and Park, 2001). SMX is not chemically reactive and does not bind covalently to protein (Cribb et al., 1991; Naisbitt et al., 1996). However, CYP2C9-mediated hydroxylation of the terminal amine residue of SMX generates SMX hydroxylamine (Cribb et al., 1995), which under conditions of oxidative stress, is converted to the protein-reactive metabolite

SMX-NO (Naisbitt et al., 1999; Naisbitt et al., 2001; Reilly et al., 2000; Summan et al., 2002; Manchanda et al., 2002). CYP2C9 is expressed in liver, skin and macrophages (Cribb et al., 1995; Baron et al., 1998; Saeki et al., 2002) and indeed Reilly et al. (2000) have recently shown that cultured human keratinocytes metabolise SMX to SMX hydroxylamine. The aim of our study was to investigate the nature of the SMX antigen presented to drug-specific T-cells in four species: mice, rats, rabbits and humans.

Splenocytes from mice, rats and rabbits sensitised with SMX-NO were found to proliferate *in vitro* following stimulation with SMX-NO, but not the parent drug (Figure 2, Table 1). Similarly, lymphocytes from SMX hypersensitive patients proliferated with SMX-NO; however, in contrast to the animal studies, lymphocytes from hypersensitive patients also responded to the parent drug (Figure 4; Table 1). Chemicals were administered via ip injection and not orally because SMX-NO is rapidly reduced by liver cells (Gill et al., 1997). Following a single ip injection, SMX-NO is sufficiently stable to circulate in the periphery and bind to epidermal keratinocytes (Naisbitt et al., 2001) – the target cells in SMX hypersensitivity. To confirm that the proliferative response was directed against SMX and/or a SMX-NO modified cellular protein, splenocytes and/or lymphocytes were: first, pre-incubated with glutathione; and secondly, pulsed with SMX-NO, a procedure that removes non-covalently bound drug but does not prevent the presentation of drug-modified cellular protein (Schnyder et el., 2000; Naisbitt et al., 2001).

Importantly, lymphocytes from hypersensitive patients were tested for their ability to proliferate against drug (metabolite) antigens several months or even years after the development of hypersensitivity, while sensitised animals were

tested 4-7 days after drug administration. Thus, to study the nature of the drug antigen in SMX hypersensitive patients and sensitised animals directly, lymphocytes from rabbits sensitised with SMX and SMX-NO were cultured with SMX and SMX metabolites every two weeks for 4 months. Results obtained were essentially the same as that seen with rabbit splenocytes: cells from SMX-NO sensitised rabbits proliferated in the presence of SMX-NO but not SMX, while administration of SMX did not stimulate a cellular response. The response to SMX-NO declined slowly with time, but was still significant 4 months after completion of the sensitisation protocol (which represents approximately 5 years in a human life), and there was no cross reactivity with SMX (Figure 6). These data clearly indicate that the nitroso metabolite of SMX is extremely immunogenic. SMX- and SMX-NO-specific proliferation of human lymphocytes indicates that the T-cell repertoire in humans seems to be significantly different to that seen in animals. There are two possible explanations for the observed results: first, there is a difference in drug presentation by the antigen presenting cells in hypersensitive patients; and secondly, the receptor system is more readily triggered in T-cells from hypersensitive patients thus overriding the need for covalent binding *in vitro.* However, since the number of antigen molecules required to stimulate T-cells is incredibly low – Irvine et al. (2002) estimate that T-cells can be stimulated when as little as 10 ligands are present – they are below the limits of chemical detection at present. In view of the relative lack of sensitivity of currently available analytical techniques it is unknown whether there is on-going metabolism within immune cells. One possibility of overcoming this would be to use transfected autologous cells expressing high levels of P450 isoforms such as CYP2C9 – this is being investigated.

It must be noted that animals sensitised with SMX-NO do not develop SMX hypersensitivity. It is possible that animals administered soluble SMX-NO do not receive the appropriate antigenic signal to cause pathology. In this respect, systemic administration of SMX-NO generates an exogenous antigen by direct cell surface haptenation (Naisbitt et al., 1999; Manchanda et al., 2002), while in patients administered SMX it is possible that SMX-NO would be formed intracellularly (Cribb et al., 1990; Cribb et al., 1995; Reilly et al., 2000). Protein binding at the site of metabolic activation generates an endogenous antigen that might stimulate a more potent cellular immune response.

The mechanism by which the chemically inert, pro-reactive metabolite SMX-hydroxylamine stimulates T-cells is not fully understood. This is of particular importance since approximately 2% of an oral dose of SMX is excreted in urine as the hydroxylamine (Gill et al., 1997). Likewise, the altered redox status in plasma of patients with HIV infection (Walmsley et al., 1997) is known to favour the conversion of SMX hydroxylamine to SMX-NO and the generation of covalently bound drug-protein adducts (Naisbitt et al., 2000). The results of our present study show that oxidation of SMX hydroxylamine stimulates lymphocytes from SMX-NO sensitised animals and hypersensitive patients (Figure 5). Glutathione inhibited SMX hydroxylamine-mediated lymphocyte proliferation at low metabolite concentrations (Figure 5); however, proliferation was still seen with higher, supra-therapeutic concentrations of SMX hydroxylamine. This was intriguing, since glutatione is thought to prevent the oxidation of SMX hydroxylamine to SMX-NO (Naisbitt et al., 1999). We initially considered the possibility that the proliferative response might have been due to a non-covalent interaction between SMX hydroxylamine and the T-cell receptor.

However, flow cytometric analysis of cell culture incubations revealed that although oxidation of the hydroxylamine was delayed, oxidation and covalent binding of SMX hydroxylamine was seen after 48 hours (Figure 5). These data highlight the fact that maintenance of levels of sulfhydryl containing compounds such as glutathione plays a critical role in inhibiting the conversion of SMX hydroxylamine to SMX-NO. In patients with a disturbed redox balance as is seen with HIV infection (van der Ven et al., 1995), increased generation of SMX-NO may to be one factor that contributes to the increased incidence of drug hypersensitivity.

The reasons why all SMX hypersensitive patients have T-cells that proliferate in the presence of both SMX and SMX-NO are not known. It is possible that oral exposure to a drug antigen may lead to the generation of T-cells that proliferate in the presence of the parent drug; however, there is no obvious scientific rationale for this. Factors that determine the nature of individual susceptibility will be also important (Figure 7). To this end, studies have shown that NAT2 and GST polymorphisms are not determinants of individual susceptibility (Pirmohamed et al., 2000; O'Neil et al., 2002). Polymorphisms in other drug metabolising enzymes such as myeloperoxidase may be important but have not been investigated. It seems that all patients administered SMX will be exposed to both the parent drug and SMX-NO. The factors determining whether antigen formation results in a hypersensitivity reaction are unknown, but could include the following. First, it is possible that T-cell receptor engagement *per se* is insufficient to lead to tissue damage and in the absence of co-stimulation tolerance or immunological ignorance may supersede; these phenomena have been reported to occur in patients exposed to the contact allergen nickel

(Cavani et al., 1998). Secondly, the ability of a drug (metabolite) to stimulate an immune response that leads to tissue damage might be directly related to the presence of complementary bi-directional drug binding domains within MHC and the T-cell receptor. The expression of these binding domains is under genetic control and therefore differs from individual to individual. Recent studies using the HIV-1 nucleoside-analogue reverse transcriptase inhibitor abacavir as a paradigm have shown that HLA-B57 was present in 78% (Mallal et al., 2002) and 46% (Hetherington et al., 2002) of abacavir hypersensitive patients versus 2% and 4% of abacavir exposed controls. Similarly, in SMX hypersensitivity, Ozkaya-Bayazit and Akar (2001) have reported a link between the HLA-B22 haplotype and susceptibility to SMX hypersensitivity. The relationship between the expression of specific T-cell receptors and drug hypersensitivity has not been studied; however, most drug-specific T-cells isolated from the blood of individuals with hypersensitivity to the drugs carbamazepine (Naisbitt et al., in press a), lamotrigine (Naisbitt et al., in press b) and phenobarbital (Hashizume et al., 2002) have been shown to express the V beta chain 5.1. Although these data are preliminary, there appears to be a correlation between the expression of immunological receptors and the phenotypic features of drug hypersensitivity.

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Footnotes

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Table 1 Proliferation of lymphocytes and splenocytes from hypersensitive humans and animals sensitised with SMX-NO.

	Splenocytes		Lymphocytes	
	SMX (200 μg ml^{-1})	SMX-NO (10 μg ml^{-1})	SMX (200 μg ml^{-1})	SMX-NO (10 μg ml^{-1})
Mouse	1.2[a]	4.3*	np[b]	np
Rat	0.7	14.5*	np	np
Rabbit	1.2	32.8*	0.9	11.9*
Human	np	np	13.1*[c]	4.7*

[a] results of animal studies represent mean SI of at least four separate experiments, carried out in triplicate. *P < 0.05; when proliferation of drug(metabolite) and DMSO-treated cells were compared. Co-efficient of variation was less than 20%.

[b] np, experiment not possible since human splenocytes were not available and the number of lymphocytes isolated from mice and rats was too small.

[c] results of human studies represent mean data from three hypersensitive patients, experiments carried out in triplicate. *P < 0.05; when proliferation of drug(metabolite) and DMSO-treated cells were compared. Co-efficient of variation was less than 20%.

Figure Legends

Figure 1

Scheme depicting our current understanding of the fate of SMX and SMX oxidative metabolites in vivo.

Figure 2

Proliferative response of rabbit, mouse and rat splenocytes from SMX-NO-treated rats (1 mg kg^{-1}) cultured with SMX or SMX-NO for 72 h. Proliferation was measured by incorporation of [^{3}H] thymidine. The cpm of control cultures did not exceed 1000. Results represent the mean ± s.d. from four animals, incubations carried out in triplicate. Statistical analysis compares drug-treated splenocytes with cell incubations containing DMSO alone (*$P < 0.05$).

Figure 3

Splenocytes proliferate in the presence of covalently bound SMX-NO and not free drug (metabolite). Splenocytes from rabbits, mice and rats sensitised with SMX-NO (1 mg kg^{-1}) were cultured with SMX-NO (10 μg ml^{-1}) for 72 h in the presence and absence of glutathione (300 μg ml^{-1}). Splenocytes were also pulsed with SMX-NO (10 μg ml^{-1}, rat and rabbit; 5 μg ml^{-1}, mice; 2 h), washed and resuspended in drug-free media for the remainder of the incubation period. Mouse splenocytes were incubated with a lower concentration of SMX-NO because 10 μg ml^{-1} inhibited splenocyte proliferation (Figure 2). After 72 h, proliferation was measured by incorporation of [^{3}H] thymidine. The cpm of control cultures did not exceed 1000. The results are presented as the mean ± s.d. of one animal out of four for each species. Statistical analysis compares

drug-treated splenocytes with cell incubations containing DMSO alone (*P < 0.05).

Figure 4

Proliferative response of human and rabbit lymphocytes from SMX hypersensitive patients and rabbits sensitised with SMX-NO (1 mg kg^{-1}), respectively, cultured with SMX, SMX hydroxylamine or SMX-NO for 96 or 72 h. Proliferation was measured by incorporation of [^{3}H] thymidine. The cpm of control cultures did not exceed 2000. The results are presented as the mean ± s.d. of one experiment out of three and four for the human and rabbit experiments, respectively. Statistical analysis compares drug-treated splenocytes with cell incubations containing DMSO alone (*P < 0.05).

Figure 5

The proliferative response to SMX hydroxylamine is directed against an SMX-modified protein and not the free drug. (a) Proliferation of rabbit lymphocytes from animals sensitised with SMX-NO (1 mg kg^{-1}) cultured with SMX hydroxylamine in the presence and absence of glutathione (1 mM) for 72 h. Proliferation was measured by incorporation of [^{3}H] thymidine. The cpm of control cultures did not exceed 1000. The results show data from 1 of 4 experiments carried out in triplicate and are presented as the mean ± s.d. Statistical analysis compares drug-treated splenocytes with cell incubations containing DMSO alone (*P < 0.05). (b, c) Cell surface haptenation of lymphocytes after incubation with SMX hydroxylamine (50 μg ml^{-1}) in the presence or absence of glutathione (1mM) for (b) 4 or (c) 48 h. SMX-NO (10 μg

ml^{-1}) was used as a positive control. Fluorescence intensity of viable cells was analysed by flow cytometry using a hapten-inhibitable anti-SMX antibody and a FITC-conjugated anti-IgG secondary antibody. Hashed lines show cells incubated in the presence of secondary antibody alone.

Figure 6

SMX-NO-specific lymphocytes do not cross react with SMX. Proliferative response of rabbit lymphocytes isolated from animals sensitised with SMX-NO (1 mg kg^{-1}) cultured with SMX or SMX-NO for 72 h. Lympohcytes were isolated 1 – 16 weeks after completion of the sensitisation protocol. Proliferation was measured by incorporation of [^{3}H] thymidine. The cpm of control cultures did not exceed 2000. The results show data from 2 of 4 rabbits and are presented as mean SI. Co-efficient of variation was consistently less than 20 % and has been removed for clarity. Statistical analysis compares drug-treated splenocytes with cell incubations containing DMSO alone (*$P < 0.05$).

Figure 7

Scheme depicting factors involved in the conversion of a SMX (metabolite)-specific immune response into tissue damage.

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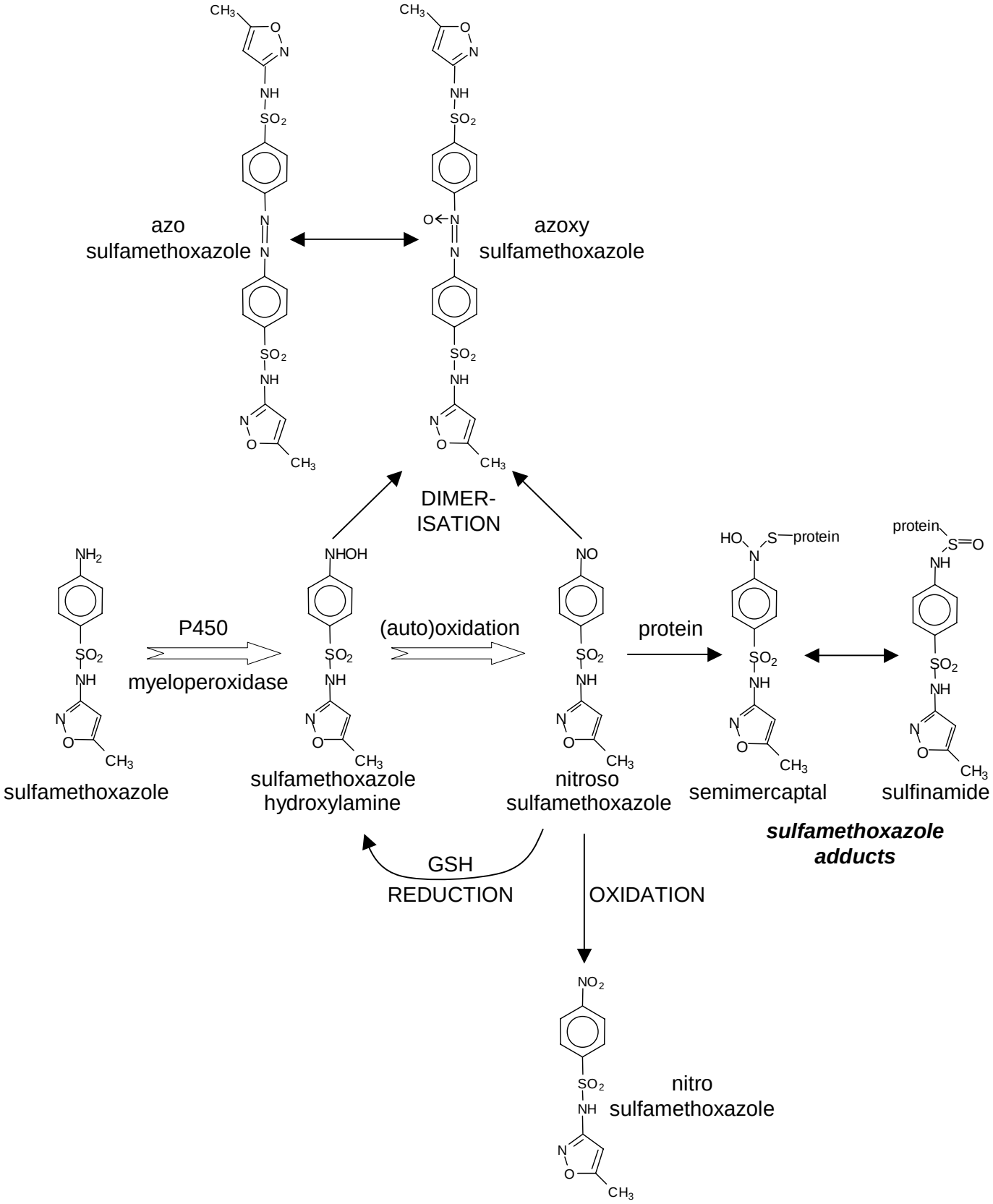

Figure 1

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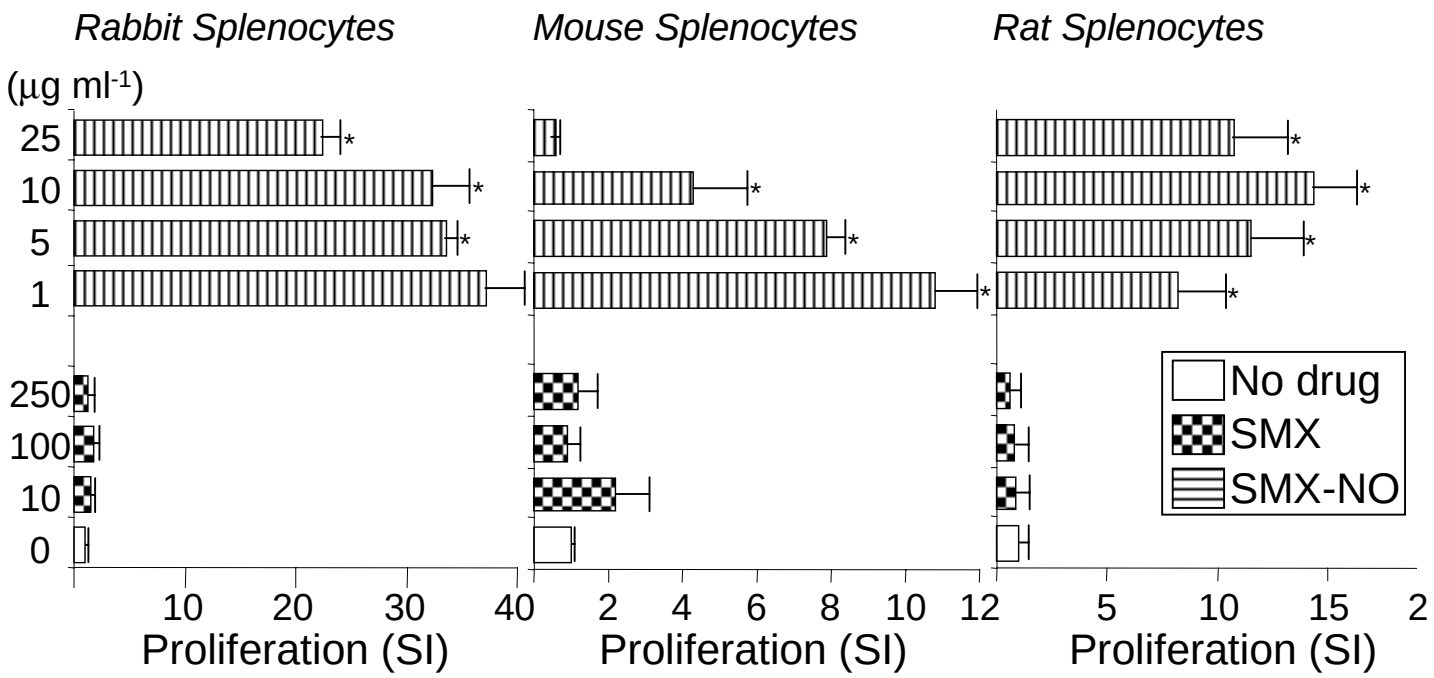

Figure 2

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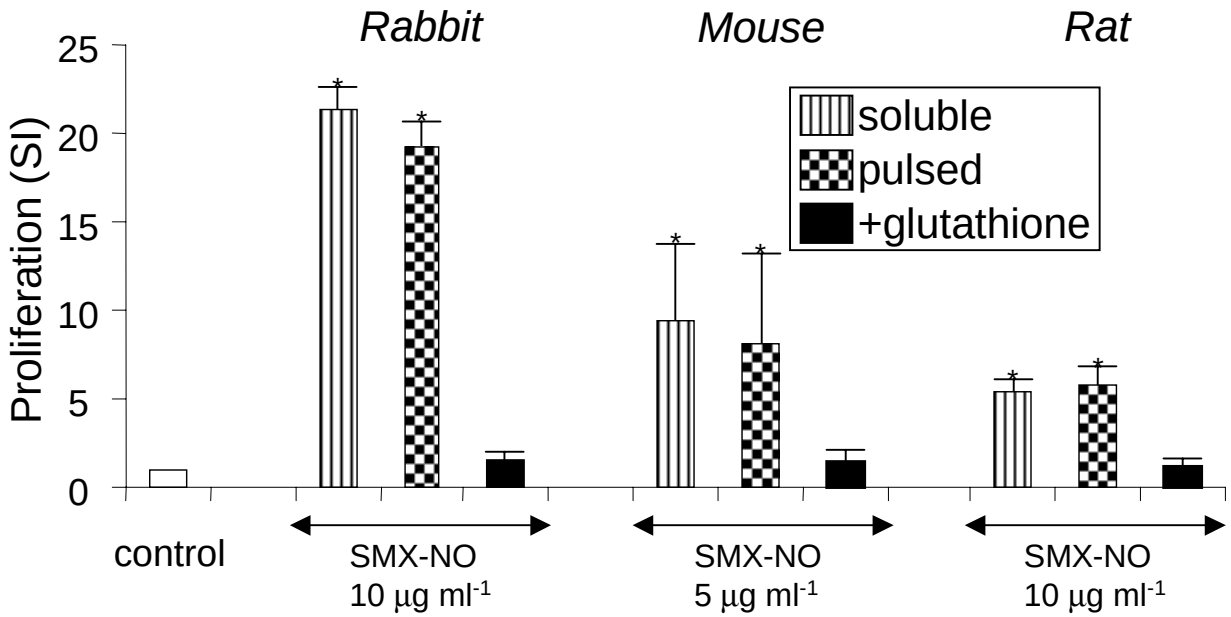

Figure 3

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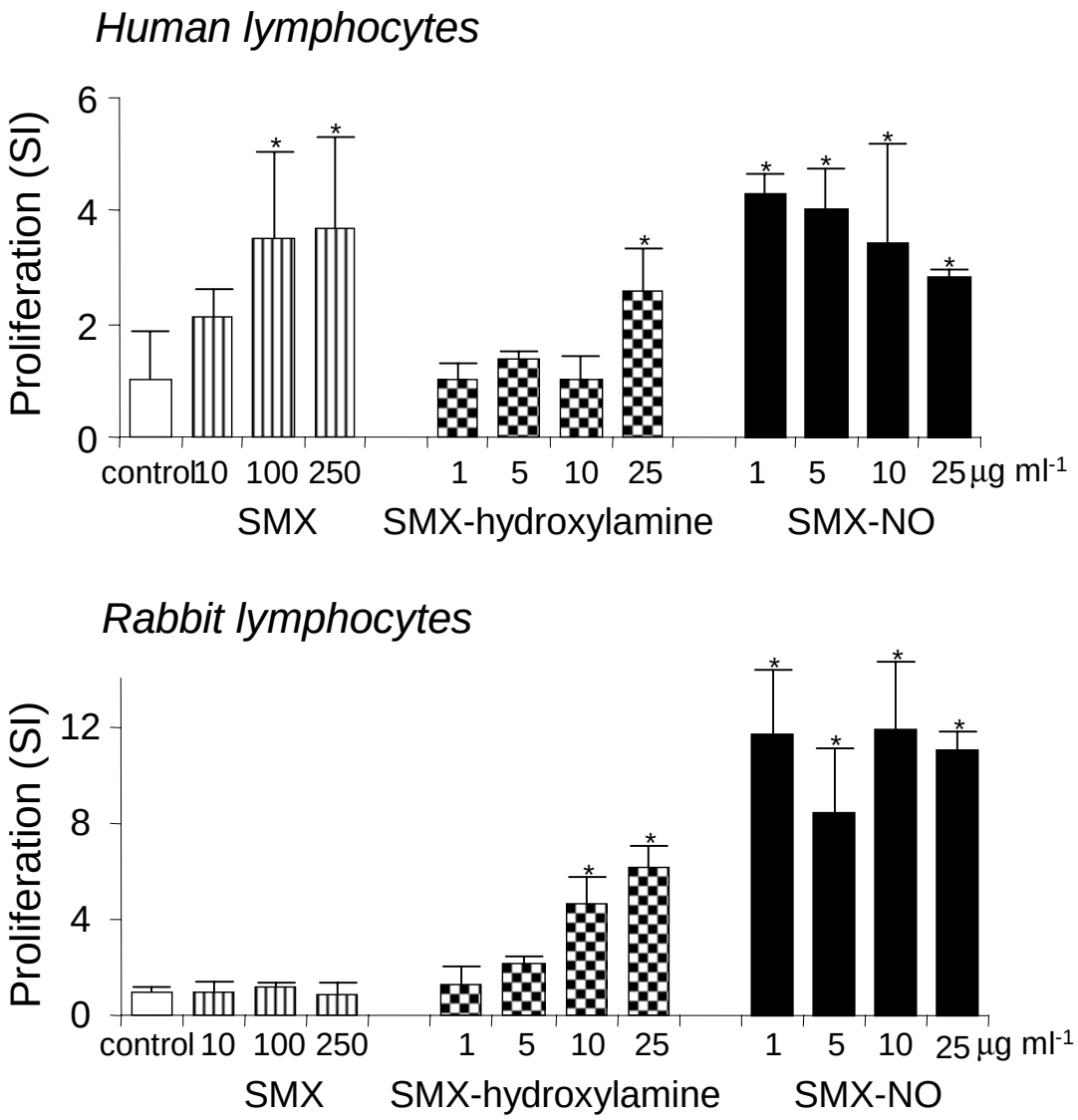

Figure 4

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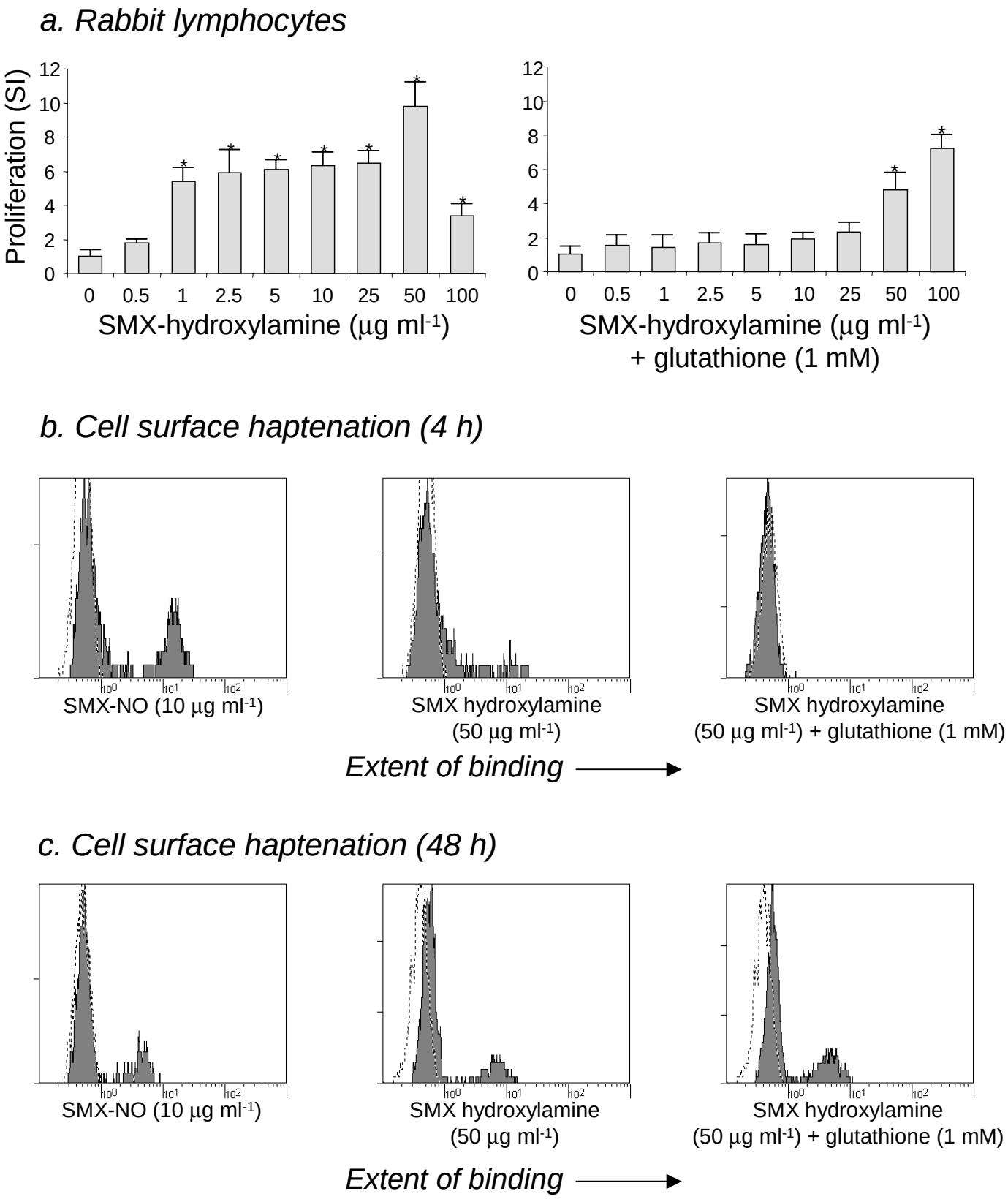

Figure 5

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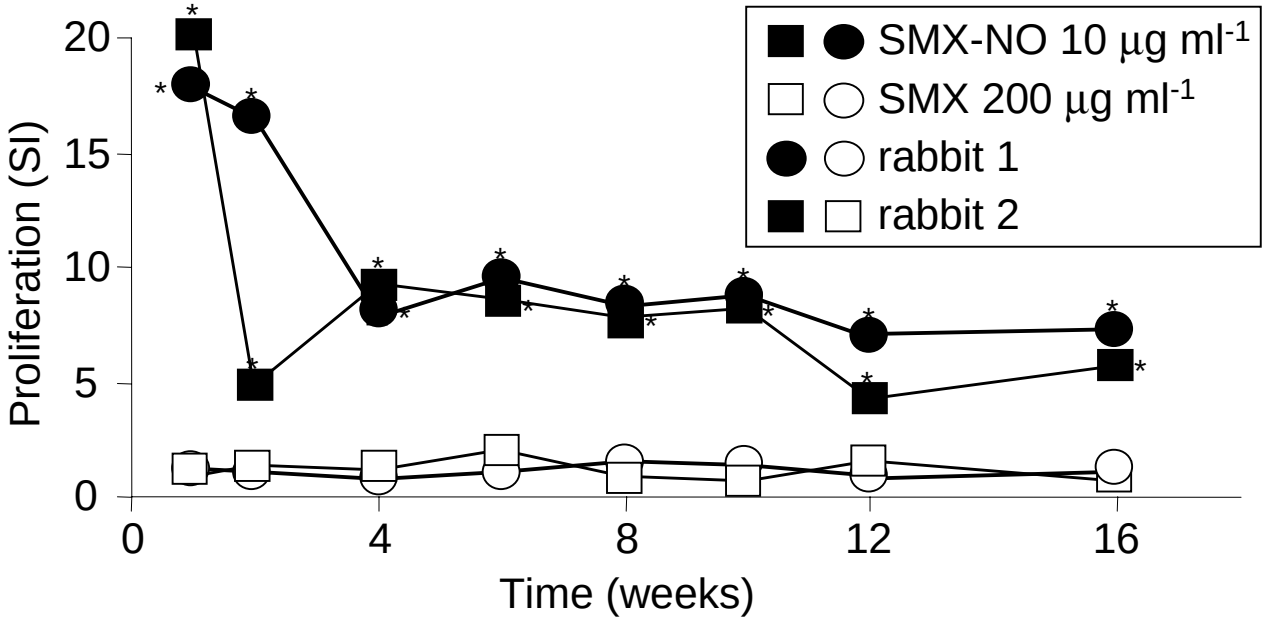

Figure 6

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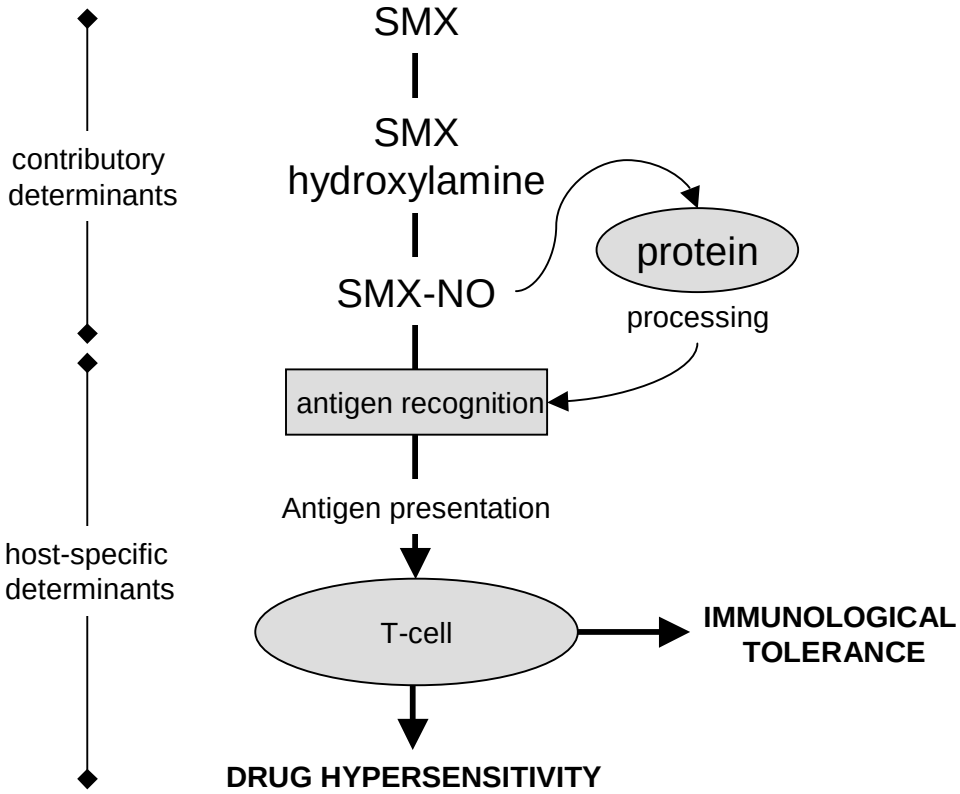

Figure 7