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# Recognition of Sulfamethoxazole and Its Reactive Metabolites by Drug-Specific CD4<sup>+</sup> T Cells from Allergic Individuals<sup>1</sup>

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The recognition of the antibiotic sulfamethoxazole (SMX) by T cells is usually explained with the hapten-carrier model. However, recent investigations have revealed a MHC-restricted but processing- and metabolism-independent pathway of drug presentation. This suggested a labile, low-affinity binding of SMX to MHC-peptide complexes on APC. To study the role of covalent vs noncovalent drug presentation in SMX allergy, we analyzed the proliferative response of PBMC and T cell clones from patients with SMX allergy to SMX and its reactive oxidative metabolites SMX-hydroxylamine and nitroso-SMX. Although the great majority of T cell clones were specific for noncovalently bound SMX, PBMC and a small fraction of clones responded to nitroso-SMX-modified cells or were cross-reactive. Rapid down-regulation of TCR expression in T cell clones upon stimulation indicated a processing-independent activation irrespective of specificity for covalently or noncovalently presented Ag. In conclusion, our data show that recognition of SMX presented in covalent and noncovalent bound form is possible by the same TCR but that the former is the exception rather than the rule. The scarcity of cross-reactivity between covalently and noncovalently bound SMX suggests that the primary stimulation may be directed to the noncovalently bound SMX. *The Journal of Immunology*, 2000, 164: 6647–6654.

The majority of hypersensitivity reactions to sulfamethoxazole (SMX)<sup>5</sup> comprise morbilliform, cutaneous eruptions (1, 2) and are not thought to be mediated by Abs (3). Immunohistological findings (4) as well as drug-specific T cell proliferation and T cell cytotoxicity in vitro (5, 6) suggest that T lymphocytes are directly involved in these allergic reactions.

Like most drugs, SMX is thought to be too small to represent a complete Ag. It is not chemically reactive and thus requires metabolism to form a hapten-carrier complex. In vivo it is metabolized predominantly in the liver by *N*-acetyltransferases and *N*-glucuronyltransferase. These biotransformations lead to the formation of nontoxic metabolites. To a limited extent, it is also converted in a cytochrome P-450- and/or myeloperoxidase-catalyzed reaction to a hydroxylamine metabolite (SMX-hydroxylamine (SMX-NHOH)) that can be further oxidized to a nitroso

compound (nitroso-SMX (SMX-NO)) (7, 8). The nitroso compound can bind to thiol groups of proteins (9) and is therefore able to covalently modify self-proteins, which in turn might be recognized as neo-Ags by the immune system. In rats the in vivo administration of SMX-NO but not of SMX itself resulted in production of anti-SMX IgG Abs (10). Thus, it is usually assumed that SMX gains its immunogenicity after oxidative metabolism.

However, recent investigations have revealed that drugs such as lidocaine and SMX, which are considered to be chemically inert, can be recognized by drug-specific T cell clones (TCC) (11–15). This recognition required the continuous presence of the drug and was MHC-restricted and very rapid. It could be best explained by an unstable and direct presentation of SMX or lidocaine without requirement for processing or drug metabolism. The relevance of this form of Ag recognition by preactivated T cells for the sensitization of resting naive T cells to a drug remains unclear. In the case of SMX, it seems feasible that the primary sensitization to SMX occurs to the chemically reactive compound (i.e., SMX-NO) and that the generated TCC cross-react with low-affinity, noncovalently bound SMX. This hypothesis would be compatible with the assumption of a crucial role for drug metabolism in most allergic reactions to drugs (16). Unfortunately, suitable animal models are not available as yet to study the initial provoking and re-stimulation of antigenic forms in SMX-induced hypersensitivity.

Therefore, in this study we addressed this issue by three approaches: 1) we analyzed whether T cells from allergic and non-allergic individuals react with SMX and the metabolites SMX-NO or SMX-NHOH, 2) we generated TCC to SMX and the chemically reactive metabolites and investigated the cross-reactivity in detail, and 3) we assessed the kinetics of TCR down-regulation in TCC cross-reactive with SMX/SMX-NO. Our data indicate that cross-reactive T cells can be detected in and isolated from the peripheral T cell pool of drug-allergic individuals but that their appearance is a rare event. The majority of drug-reactive T cells from allergic individuals recognize the noncovalently bound SMX directly and do not respond to SMX-NO-modified APC. This pattern of Ag

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<sup>5</sup> Abbreviations used in this paper: SMX, sulfamethoxazole; SMX-NHOH, SMX-hydroxylamine; SMX-NO, nitroso-SMX; TCC, T cell clone; B-LCL, EBV-transformed B-lymphoblastoid cell line; LTT, lymphocyte transformation test; GSH, reduced glutathione.

reactivity suggests that in SMX hypersensitivity the major part of the primary stimulation may be directed to the noncovalently bound SMX.

## Materials and Methods

### Donor characteristics

PBMC were obtained from four HIV-negative donors. Two of them (KV and KS) had been on SMX without adverse effects, whereas donor UNO experienced symptoms of drug allergy for the first time 10 years ago. He developed an erythematous exanthem after therapy with co-trimoxazole (SMX plus trimethoprim). Two years later, he suffered a generalized exanthem within 1 day of re-exposure to co-trimoxazole that persisted for several days. No specific IgE and IgG Abs against SMX or trimethoprim were detected, but the lymphocyte transformation test demonstrated strong T cell proliferation to SMX (6, 11). The second allergic donor (KG) was a young pharmacist who developed allergic symptoms (exanthem) after being treated for a urinary tract infection with an unknown sulfonamide. Five years later, she developed a macular exanthem and dyspnea after working with sulfamethoxazole in the laboratory.

The HLA type of the donor UNO is A2/26, B44/60, DRB1\*01/10, and of the donor KG is A2/68, B50/65, DRB1\*07/13. The HLA types of the nonallergic donors are A1/2, B8/37, DRB1\*03/10 for KV and A28/32, B17/44, DRB1\*07/11 for KS.

### Culture media

The cell culture medium used was RPMI 1640 supplemented with 10% pooled, heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 25 mM HEPES buffer, 2 mM L-glutamine (Seromed, Fakola, Basel, Switzerland), 25  $\mu$ g/ml transferrin (Biotest, Dreieich, Germany), 100  $\mu$ g/ml streptomycin, and 100 U/ml penicillin. TCC were cultured using medium additionally enriched with 20 U/ml rIL-2 (Dr. A. Cerny, Inselspital, Bern, Switzerland). EBV-transformed B-lymphoblastoid cell lines (B-LCL) were grown in RPMI 1640 supplemented with 10% FCS (Life Technologies, Paisley, U.K.), 25 mM HEPES buffer, 100  $\mu$ g/ml streptomycin, and 100 U/ml penicillin.

### Drugs used for T cell stimulation

SMX was obtained from Hoffmann La Roche (Basel, Switzerland), and stock solutions of 10 mg/ml were freshly prepared before use in RPMI 1640 containing 5% 1 N NaOH. SMX acetate, SMX-NHOH, and SMX-NO were synthesized as described by Naisbitt et al. (17) and were >95% pure as assessed by nuclear magnetic resonance and elemental analysis. SMX-acetate was prepared by a standard synthesis with two equivalents of acetic anhydride under reflux. Stock solutions (10 mM) were freshly prepared before use in a mixture of 80% RPMI 1640 and 20% DMSO. To facilitate dissolution, 5% 1 N NaOH was added to the mixture.

### Covalent modification of B-LCL by drug and drug metabolites

Surface modification of APC by drug and drug metabolites was analyzed by indirect immunofluorescence. B-LCL ( $1 \times 10^6$ /ml) were cultured in 0.5 ml serum-containing medium or in 0.5 ml HBSS in the presence of various concentrations of SMX or SMX-NO at 37°C. After 8 h, cells were washed with PBS containing 1% FCS and 0.1% sodium azide (FACS buffer). Then cells were pelleted by centrifugation and incubated with a rabbit anti-SMX Ab (1/50) for 30 min at 4°C. The Ab was kindly provided by Dr. E. A. Cribb (Merck Research Laboratories, West Point, PA). Cells were washed with FACS buffer and then incubated with FITC-conjugated goat anti-rabbit Ig Ab (1/50; Coulter Immunotech, Zurich, Switzerland) for another 30 min at 4°C. After another wash step, the cells were taken up in 500  $\mu$ l FACS buffer, and fluorescence was analyzed on a Coulter XL flow cytometer using System II software version 3.0 (Coulter, Hialeah, FL).

### Lymphocyte transformation test (LTT)

As described earlier, SMX is bound to the MHC-peptide complexes with low affinity and is removed by simple washing procedure (11). In contrast, covalently associated drug cannot be removed by washing. Taking advantage of this characteristic, two stimulation procedures were chosen that differed in the stability of drug binding. 1) Stimulation with "soluble Ag." Freshly isolated PBMC ( $1 \times 10^6$  cells/well) were cultured in 1 ml medium in 24-well plates in the continuous presence of 1000  $\mu$ M SMX, 100  $\mu$ M SMX-NHOH, or 100  $\mu$ M SMX-NO. Ag provided in coculture were designated as SMX-s, SMX-NHOH-s, and SMX-NO-s, respectively. Coincubation with Ag allowed noncovalent, weak association of the drug with MHC-peptide complexes as well as covalent modification by reactive com-

pounds. 2) Alternatively, PBMC were stimulated with the same number of drug-modified, autologous PBMC ("Ag-pulsed APC"). Modification of APC was achieved by incubation of APC with 1000  $\mu$ M SMX or 100  $\mu$ M SMX metabolites for 2–8 h. Then APC were washed twice with HBSS and irradiated (6000 rad). Ag-pulsed stimulator cells were designated as SMX-p, SMX-NHOH-p, and SMX-NO-p, respectively, and were added to  $1 \times 10^6$  cells/well of the responding PBMC. After 5 days, cells were resuspended, and a 200- $\mu$ l aliquot was transferred into 96-well U-bottom microtiter plates. Proliferation of these cells was determined by overnight incubation with 0.5  $\mu$ Ci of [ $^3$ H]thymidine. Cells were harvested, and incorporated radioactivity was measured on a scintillation liquid-free beta counter (Trace 96; Inotech, Wohlen, Switzerland).

### Generation and characterization of specific human CD4<sup>+</sup> TCC

Bulk cultures were generated by stimulation of freshly isolated PBMC with SMX or SMX metabolites continuously present in the cultures or with added drug-modified (pulsed) APC as described above. Reduced glutathione (GSH) was added to some of the cultures at a concentration of 1 mM. After 14 days, part of the cultures were restimulated with either autologous PBMC ( $1 \times 10^6$ /well) and Ag or irradiated PBMC and PHA (1  $\mu$ g/ml) for an additional 14 days. After either one or two stimulations in vitro, T cells were cloned by limiting dilution as described previously (5). In brief, cells from each individual bulk culture were seeded at a concentration of 1–5 cells/well into 96-well round-bottom microtiter plates and restimulated with  $2.5 \times 10^4$  allogeneic irradiated PBMC and PHA (1  $\mu$ g/ml). Two weeks later, well-growing TCC were harvested, propagated, and tested for Ag specificity.

MHC restriction of established TCC was assessed by proliferation assays with partially matched heterologous B-LCL as described (2). The phenotype and monoclonality of TCC was confirmed by immunofluorescence and PCR-based TCR V $\beta$  analysis (5).

### T cell proliferation assay

To determine the responses to noncovalently MHC-presented drugs, TCC ( $5 \times 10^4$  cells/well) were incubated in 96-well U-bottom plates together with  $5$ – $10 \times 10^4$  B-LCL in 0.2 ml medium in the presence of indicated concentrations of Ag. After 48 h, 0.5  $\mu$ Ci [ $^3$ H]thymidine was added. Cells were harvested 12 h later, and incorporated radioactivity was determined as described above.

To evaluate responses to covalently presented drugs, autologous B-LCL were incubated with indicated amounts of SMX or SMX metabolites in culture medium for 2 or 8 h. Ag-pulsed stimulator cells were then washed twice with HBSS and irradiated (3000 rad), and  $1 \times 10^4$  cells were added to TCC. Proliferation was determined after 48 h as described.

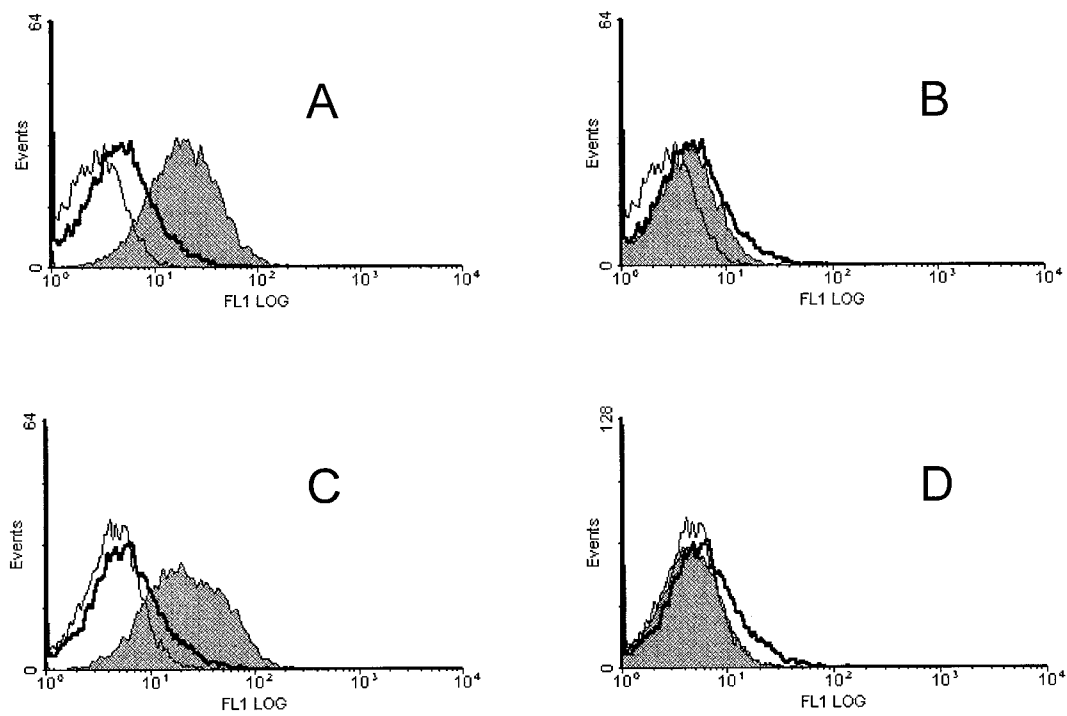
### Determination of TCR down-regulation

Cloned T cells ( $2.5 \times 10^4$ ) were added to  $5 \times 10^4$  autologous B-LCL and incubated in 0.2 ml of medium in U-bottom plates in triplicate in the continuous presence of 100  $\mu$ M SMX, SMX-NHOH, or SMX-NO. Alternatively, the same number of T cells was incubated with B-LCL previously pulsed for 15 min with Ag as described above. The plates were centrifuged for 2 min and incubated at 37°C. At various time points, cells were harvested, washed with PBS containing 0.5 mM EDTA, and stained for 30 min at 4°C with FITC-labeled anti-CD3 (UCHT-1; Dako, Zug, Switzerland). The CD3 fluorescence was measured on a Coulter XL flow cytometer, and the mean CD3 fluorescence of TCC conjugated with APC without Ag was taken as 100% value.

## Results

### Covalent modification of APC by drugs and drug metabolites

To monitor the degree of haptentation of APC by SMX and its reactive metabolites, we incubated B-LCL for 8 h in protein-free buffer with different concentrations of SMX and SMX-NO. Covalent binding of drug was then visualized using a SMX-specific Ab that had been raised in rabbits immunized with SMX-NO. As Fig. 1A shows, SMX-NO at 100  $\mu$ M concentration was able to covalently modify the cell surface of APC. Haptentation of APC was not diminished by the presence of serum protein. Both the amount of positively stained cells as well as the intensity of the staining was comparable irrespective of whether B-LCL had been incubated with SMX-NO in protein-free buffer or in medium containing 10% human AB serum (compare Fig. 1, A and C). SMX at 1000- $\mu$ M concentration did not lead to detectable cell surface



**FIGURE 1.** Haptentation of APC by reactive drug metabolites SMX-NO but not by the parent drug SMX. B-LCL ( $1 \times 10^6/\text{ml}$ ) were cultured in 0.5 ml HBSS (A and B) or in 0.5 ml serum-containing medium (C and D) in the presence of 100  $\mu\text{M}$  SMX-NO (A and C) or 1000  $\mu\text{M}$  SMX (B and D) at 37°C for 8 h. Then cells were incubated with a rabbit anti-SMX Ab that was detected by a FITC-conjugated secondary Ab. Fluorescence of viable cells was analyzed on a Coulter XL flow cytometer. Histograms show specific staining of Ag-pulsed cells (gray), of controls incubated without Ag (bold lines), and of controls incubated with Ag and secondary Ab only (thin lines). One representative of three independent experiments is shown.

modification (Fig. 1, B and D). Thus, SMX is not metabolized in the cell culture to a reactive compound in sufficient amounts to be detected as neoantigen on the cell surface.

*Stimulation of PBMC by noncovalently and covalently presented drugs in a LTT*

To study the role of covalent vs noncovalent drug presentation in SMX hypersensitivity, we analyzed the reactivity of PBMC from two SMX-allergic and two nonallergic individuals. Ag was provided either in coculture (indicated by -s) or covalently bound on prepulsed APC (indicated by -p) in a 5-day LTT. As Table I shows, the continuous presence of soluble SMX, SMX-NHOH, and SMX-NO resulted in a strong proliferation to each of these compounds. Stimulation with preincubated and then washed PBMC led to a reproducible weak (stimulation index, 1.3–2.8) to

moderate (stimulation index, 4.7) proliferation to the SMX metabolites SMX-NHOH and SMX-NO but not to SMX itself. In contrast, PBMC from two nonallergic donors did not respond to either Ag stimulation.

*Generation of TCC by stimulation with covalently and noncovalently presented drug*

Different protocols were used to generate SMX-specific TCC with the aim to mimic different forms of sensitization and T cell reactivation upon Ag encounter in vivo. Coincubation with Ag would lead to noncovalent, weak association of drug and MHC-peptide complexes as well as to covalent modification by reactive compounds. Therefore, it will be similar to the initial/primary encounter of T lymphocytes with drugs. However, preincubation of APC with SMX-NHOH and SMX-NO and subsequent washing will

Table I. Stimulation of PBMC by noncovalently and covalently presented drugs

Form of Ag	Proliferative Response (SI) <sup>a</sup>							
	Allergic donor KG		Allergic donor UNO		Nonallergic donor KV		Nonallergic donor KS	
	100 $\mu\text{M}$	1000 $\mu\text{M}$	100 $\mu\text{M}$	1000 $\mu\text{M}$	100 $\mu\text{M}$	1000 $\mu\text{M}$	100 $\mu\text{M}$	1000 $\mu\text{M}$
SMX-s	17.2	8.2	20.2	4.8	ND <sup>b</sup>	1.0	ND	0.8
SMX-NO-s	6.8	Tox <sup>c</sup>	21.5	Tox	0.7	Tox	0.6	Tox
SMX-NHOH-s	6.0	Tox	24.7	Tox	0.4	Tox	0.8	Tox
SMX-p	1.0	1.0	1.1	1.0	ND	0.9	ND	1.0
SMX-NO-p	1.3	Tox	2.8	Tox	ND	Tox	0.9	Tox
SMX-NHOH-p	2.2	Tox	4.7	Tox	0.1	Tox	0.3	Tox

<sup>a</sup> Freshly isolated PBMC from four donors were incubated for 5 days in a standard LTT assay as described in *Materials and Methods*. Cells were cultured either in the continuous presence of 100  $\mu\text{M}$  or 1000  $\mu\text{M}$  of the indicated Ag (-s) or together with autologous PBMC, which had been preincubated with the same dose of the respective Ag, washed, and irradiated (-p). Proliferative responses are expressed as stimulation index: [(cpm culture with drug)/(cpm culture without drug)]. Proliferation without drug was regularly less than 500 cpm.

<sup>b</sup> ND, not done.

<sup>c</sup> Tox, the compound was toxic at this concentration.



Table II. Summary of TCC generated by stimulation with noncovalently and covalently presented drugs and their metabolites

Primary Stimulation in Vitro	Secondary Stimulation in Vitro	Number of TCC Specific for <sup>a</sup>		
		SMX-s only	SMX-NO-p only	SMX-NO-p and SMX-s
UNO cloning 1				
SMX-s	—	15 <sup>b</sup> (8.15,Z 1.1) <sup>c</sup>	0	0
SMX-p	—	1	0	0
SMX-NHOH-p	—	5	0	1 (N1)
SMX-NO-s	—	13	1 (N3)	1 (N2)
SMX-NO-p	—	6	0	0
UNO cloning 2				
SMX-NHOH/GSH-s	SMX-NHOH/GSH-s	31	0	1 (NO3)
SMX-NHOH/GSH-s	Allo/PHA	30 (SMX3)	0	2 (NO5, NO6)
SMX-NO-s	SMX-NO-s	37 (SMX2)	0	0
SMX-NO-s	Allo/PHA	41 (SMX5)	0	1 (NO2)
KG cloning 1				
SMX-s	SMX-s	1	0	0
SMX-s	Allo/PHA	1	0	0
SMX-NHOH-s	SMX-NHOH	0	0	0
SMX-NHOH-s	Allo/PHA	1 (KG1)	1 (KG4)	0
SMX-NO-s	SMX-NO-s	0	0	0
SMX-NO-s	Allo/PHA	0	0	2 (KG2, KG3)
KG cloning 2				
No Ag	—	0	0	0
SMX-s	—	17 (KG2.1)	0	0
SMX-p	—	0	0	0
SMX-NHOH-s	—	1	0	0
SMX-NHOH-p	—	0	2 (KGNO1)	1 (KGX1)
SMX-NO-s	—	6	0	1
SMX-NO-p	—	1	0	1

<sup>a</sup> TCC were generated by limiting dilution from bulk cultures after the indicated stimulation as described in *Materials and Methods* (s, noncovalently bound drugs; p, covalently presented compounds). TCC were then tested for their proliferative response to SMX-s and SMX-NO-p.

<sup>b</sup> Numbers of clones tested positive at least twice for a given Ag.

<sup>c</sup> Designation of representative clones.

lead to exclusive presentation of covalently bound Ag as visualized by Ab staining. Therefore, it will resemble the situation after generation of reactive SMX metabolites and modification of self-proteins. The results obtained with individual protocols are summarized in Table II.

In the first set of cloning procedures, PBMC were stimulated once either with cocultured Ag or with Ag-pulsed APC. A total of 43 drug-specific TCC could be generated from the SMX-allergic donor UNO by limiting dilution, and the results of the specificity analysis are summarized in Table II (UNO cloning 1). Noncovalently presented SMX or cocultured SMX-NHOH and SMX-NO were recognized by 40 (93%) of these TCC. Two TCC (clone N1 and N2) showed specific proliferation to covalently presented SMX-NHOH/NO additional to recognition of SMX. One TCC (clone N3) was specific for the bound oxidative SMX metabolites only. The response of the TCC N2 and N3 to SMX was HLA DR10-restricted. The TCR V $\beta$  elements used by these clones were V $\beta$ 20 for clone N2 and V $\beta$ 7 for clone N3. Some 30 drug-specific CD4<sup>+</sup> TCC could be generated from donor KG (KG cloning 2). Of these, 25 (83.3%) were specific for SMX-s, two recognized SMX-NO-p, and three were cross-reactive between these forms of Ag.

In the second set of cloning procedures, PBMC were stimulated twice (UNO cloning 2 and KG cloning 1). Initially, cells were stimulated in vitro by addition of either SMX-NO or SMX-NHOH to the bulk culture. To prevent spontaneous conversion of SMX-NHOH to the nitroso compound, the antioxidant GSH was added to some of the cultures at a concentration of 1 mM (17). After 14 days of culture, cells were restimulated in two different ways. 1) Specific T cells were boosted by addition of autologous PBMC and

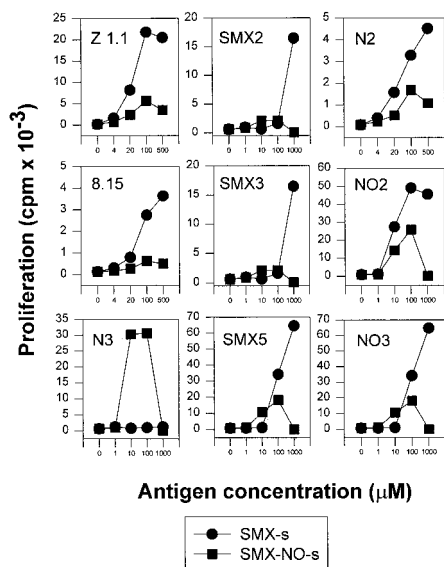
the same Ag (SMX-NO or SMX-NHOH/GSH, respectively) as was used for the first stimulation. 2) Specific T cells were preserved by restimulation with allogeneic PBMC and PHA. A fortnight after secondary stimulation in vitro, TCC were obtained by limiting dilution.

As shown in Table II, the vast majority (97%) of clones from UNO cloning 2 recognized exclusively the chemically inert parent compound SMX. Only four (NO2, NO3, NO5, and NO6) clones responded to both low-affinity associated as well as covalently bound SMX. These findings were in agreement with a high precursor frequency of T cells specific for SMX-s (1:3,000 PBMC) compared with the frequency of SMX-NHOH-p- or SMX-NO-p-specific cells (less than 1 in 100,000 PBMC) as determined by limiting dilution analysis (data not shown).

In KG cloning 1, the following panel of TCC was obtained: three clones recognized SMX-s only, and one clone (KG4) recognized exclusively SMX-NO-modified APC, whereas two others (KG2 and KG3) responded to both noncovalently associated as well as covalently bound SMX metabolites.

#### Response of TCC to continuously present SMX or SMX metabolites

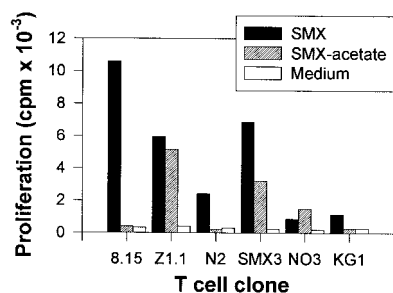
The TCC obtained from the different cloning protocols were then analyzed for their response to SMX and SMX-NO. To this end, B-LCL were used as APC. The Ag was added to the culture and remained there for the full length of the assay. Fig. 2 shows the results of a representative panel of clones. With the exception of clone N3, all TCC responded well to SMX in a dose-dependent



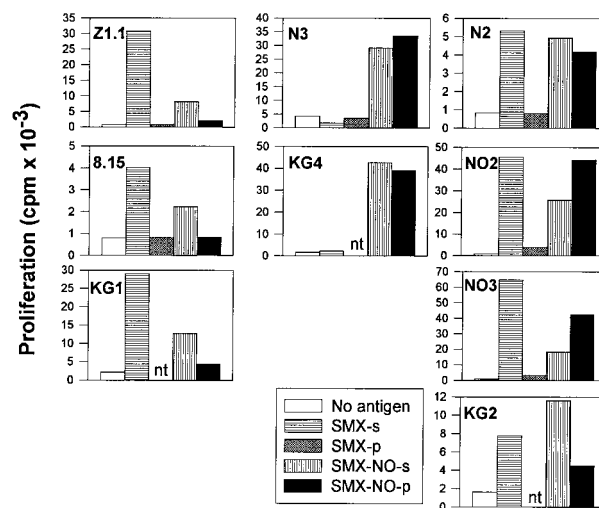
**FIGURE 2.** Dose-dependent proliferative response of TCC to cocultured SMX and SMX-NO. TCC were incubated with B-LCL as APC in the presence of indicated concentrations of SMX or SMX-NO. After 48 h, proliferation was determined by incorporation of [ $^3$ H]thymidine over an additional 8 h. Results are given as mean cpm for triplicate cultures.

way. Drug concentration for half-maximal proliferation was between 10 and 50  $\mu$ M. Clone N3 proliferated strongly to SMX-NO-s. The response was still maximal at an Ag dose that for SMX-specific clones was not sufficient to sustain a full response. This indicates an efficient presentation of SMX-NO even in the presence of serum proteins. All other TCC responded weakly but significantly to SMX-NO-s with a half-maximal concentration comparable to the one observed for SMX. A concentration of SMX-NO-s above 500  $\mu$ M appeared to be toxic for the cells (18, 19). Therefore, TCC that recognized SMX-s appeared to recognize SMX-NO-s as well, and clones that responded to both SMX-s and SMX-NO-p did not differ in the way they reacted to coincubated compounds.

N-Acetyl SMX is the major urinary metabolite in both rats and humans, accounting for up to 50% of the dose (10, 20). It is non-toxic and cannot covalently modify proteins. When a representative panel of SMX-specific TCC was tested for recognition of SMX acetate, three of six clones responded significantly to this metabolite (Fig. 3).



**FIGURE 3.** Proliferative response of TCC to SMX acetate. TCC were incubated with B-LCL as APC in the presence of 1000  $\mu$ M SMX, SMX acetate, or medium alone. After 48 h, proliferation was determined by incorporation of [ $^3$ H]thymidine over an additional 8 h. Results are given as mean cpm of duplicate cultures.




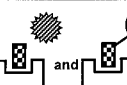

**FIGURE 4.** Distinct specificity patterns of TCC to noncovalently and covalently presented SMX. TCC were incubated with B-LCL as APC in the presence of 100  $\mu$ M SMX (SMX-s) or SMX-NO (SMX-NO-s). Alternatively, TCC were cultured with APC prepulsed for 8 h with 100  $\mu$ M SMX (SMX-p) or SMX-NO (SMX-NO-p). Control cultures had no added Ag. After 48 h, proliferation was determined by incorporation of [ $^3$ H]thymidine over an additional 8 h. TCC are grouped vertically according to their pattern of Ag recognition. Results are given as mean cpm for triplicate cultures and show one representative of the three experiments performed. nt, not tested.

#### Response of TCC to covalently bound SMX metabolites

We further investigated the response of TCC to APC prepulsed with either SMX (SMX-p) or SMX-NO (SMX-NO-p) and compared the results with the proliferation generated by coincubation with the same Ags over the time of the assay (SMX-s and SMX-NO-s, respectively). As already mentioned above, the parent compound SMX is removed by washing because it is not able to covalently modify APC. The results for representative clones are shown in Fig. 4. Three patterns of responses could be delineated; the vast majority of clones proliferated to SMX-s (and also to SMX-NO-s). Data are shown for clones 8.15, KG1, and Z1.1. A small group of clones (N2, NO2, NO3, and KG2) recognized noncovalently bound SMX, SMX-NO-s, and additionally covalently bound SMX-NO. A third group (clones N3 and KG4) was specific for covalently bound SMX-NO but could not respond to SMX-s. A summary of these recognition patterns is shown in Fig. 5.

#### Presentation of SMX-NO does not require processing

Presentation of covalently bound SMX-NO could require uptake of the hapten-carrier compound and Ag processing. Alternatively, the covalent modification of proteins could be processing-independent and could occur on the surface of the APC. We addressed this question by measuring the kinetics of Ag recognition by specific TCC. The down-regulation of TCR surface expression serves as a sensitive measure for such recognition. We monitored TCR expression of TCC for 6 h after stimulation of either noncovalently or covalently presented SMX. TCC 8.15, N2, and N3 were chosen to represent different patterns of specificity. As shown in Fig. 6, all clones responded to their respective Ags by decreased TCR expression within 15 min. This rapid down-regulation is indicative for a processing-independent Ag presentation. Two further lines of evidence support this view. When we pulsed APC with SMX metabolites for different lengths of time, only 15 min of preincubation of B-LCL with SMX-NO was required for efficient covalent modification of APC. A further increase in the length of the pulse for

	SMX coincubation	SMX-NO coincubation	SMX-NO pulse	
Antigen Presentation				Occurrence
Representative Clones				
Z 1.1, 8.15, SMX3 SMX5, KG1	++	+	-	208/222
N2, NO2, NO3, KG2, KGX1	++	++	++	10/222
N3, KG4, KGNO1	-	++	++	4/222

**FIGURE 5.** Schematic representation of the reactivity patterns of TCC derived from two patients with allergy to SMX.

up to 12 h did not result in a significant increase of the proliferative T cell response (data not shown). Such a short time is generally not considered sufficient to allow efficient uptake, processing, and presentation of Ag (11, 21). Additionally, glutaraldehyde-fixed APC were able to present covalently bound SMX-NO (data not shown).

**Discussion**

In this study, we addressed the question of primary sensitization to drugs by assessing the response of peripheral blood T cells from allergic individuals to SMX and its reactive metabolites. Furthermore, we analyzed the pattern of cross-reactivity of SMX-specific CD4<sup>+</sup> TCC. Finally, we measured the kinetics of TCR down-regulation in representative TCC as a measure of the need for Ag uptake and processing.

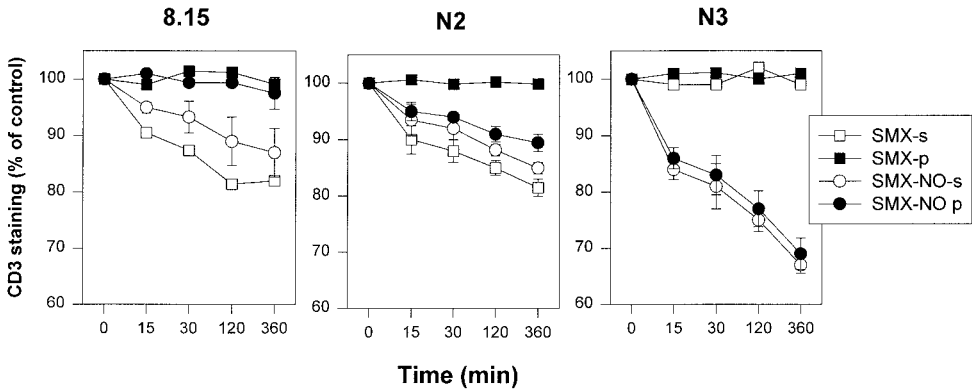
Freshly isolated blood lymphocytes from drug-hypersensitive but not from nonallergic individuals proliferated to SMX and SMX metabolites when the compounds were left in the culture for the entire time of the assay. When autologous PBMC were prepulsed for up to 8 h with SMX and then used as APC, they did not induce proliferation of T cells from drug-allergic patients. However, such pulsing should allow sufficient uptake and metabolism to reactive compounds to achieve presentation of drug-modified MHC-peptide complexes. In contrast, SMX-NO- and SMX-NHOH-pulsed PBMC were recognized significantly.

These data as well as the fast kinetics of TCR down-regulation of TCC upon activation showed that T cells from allergic individuals are able to recognize both labile-associated SMX as well as SMX-NO bound to the outside of the APC. They provide no evidence for an intracellular metabolism of SMX to a reactive com-

pound, which then generates immunogenic drug-modified proteins.

A panel of 222 TCC were generated by stimulation with different forms of the drug to investigate their specificity for labile MHC-presented or covalently associated SMX. The responding T cell repertoire in our patients was highly skewed toward the CD4<sup>+</sup> phenotype. This seems to contradict observations by Hertl et al. (22) in which CD8<sup>+</sup> T cells were predominant in biopsies of a SMX-induced bullous exanthem. The apparent discrepancy might be due to the particular morphology of bullous exanthem in contrast to our patients with maculopapular eruptions, where CD4<sup>+</sup> T cells clearly predominate in vivo (N. Yawalkar, unpublished observation). We have shown in previous studies that it is quite possible to generate CD8<sup>+</sup> TCC specific for SMX from drug-allergic individuals (13). Thus, the predominance of CD4<sup>+</sup> T cells reflects the in vivo situation in our patients rather than a technical limitation.

Although the majority of TCC (UNO, 96%; KG, 77.7%) were specific exclusively for noncovalently bound SMX-s, a small fraction of TCC responded to both noncovalent SMX-MHC-peptide conjugates and nitroso-SMX-modified APC. This clearly demonstrates that the T cell repertoire in SMX-allergic patients is biased toward the recognition of noncovalently-presented drug. One clone from donor UNO was obtained from the bulk cultures in the presence of SMX-pulsed APC. This suggests that the frequency of SMX-specific T cells in some allergic individuals is high enough to allow the rare outgrowth of clones in the presence of IL-2 but no antigenic pressure.



**FIGURE 6.** Rapid down-regulation of TCR upon recognition of specific Ag. TCC were stimulated with APC in the continuous presence of SMX (SMX-s) or SMX-NO (SMX-NO-s). Alternatively, TCC were cultured with APC prepulsed for 8 h with 100  $\mu$ M of SMX (SMX-p) or SMX-NO (SMX-NO-p). The cells were stained at the indicated time points for TCR surface expression as described in *Materials and Methods*. Results indicate the percentage of CD3 mean fluorescence  $\pm$  SD calculated from values without added Ag. Data of three independently performed assays are shown.

In protein-free buffer, SMX-NO but not SMX efficiently haptened the surface of B-LCL. Similar results have been shown previously for neutrophils and lymphocytes (9). Under tissue culture conditions, there might be the possibility that serum proteins compete with cell surface proteins for covalent binding and thereby reduce the number of epitopes generated. However, as staining with a SMX-specific Ab shows, the haptenation of APC cell surfaces was as efficient in the presence as in the absence of serum protein. When we compared the functional response of TCC and PBMC specific for covalently presented SMX to APC haptenated with SMX-NO in the presence or absence of serum, T cells responded equally well to both types of APC (data not shown). This would suggest that both the number of B cell epitopes as well as the number of relevant functional T cell epitopes generated by SMX-NO are not reduced by the presence of serum protein.

Furthermore, there is no indication that the quality or quantity of epitopes generated by coinubation of APC with SMX is greater than the one generated by pulsing of cells with SMX-NO. In contrast, TCC specific for SMX-NO proliferate to SMX-NO-p, usually at concentrations that are 10–100 times lower than those of SMX-s required by SMX-specific TCC (data not shown). Thus, our data indicate that the skewing toward the recognition of noncovalently presented drug reflects the precursor frequency of specific T cells rather than the quantity or quality of available epitopes.

It has to be considered that B-LCL may lack the ability to convert SMX to reactive metabolites. Therefore, we cannot formally exclude the possibility that covalent presentation of SMX via metabolism to reactive compounds and subsequent haptenation of intracellular proteins would lead to structurally different T cell epitopes than those generated after binding of SMX-NO from the outside of the cell. In some cases we used PBMC coinubated with SMX to screen for the presence of SMX and SMX-NO-reactive clones within the T cell lines. We did not obtain a different panel of reactivities, i.e., increased frequency of cross-reactive TCC, suggesting that the metabolizing potential of the APC is not crucial for the specificity of our TCC (data not shown). When we compared the responses of TCC to drugs continuously present over the time of the assay, SMX appeared to be more antigenic than SMX-NO (Fig. 2). This was not due to an insufficient presentation of SMX-NO, as the few clones able to react to SMX-s and SMX-NO-s showed similar dose-response curves and required similar concentrations for half-maximal proliferation. All the compounds were prepared as highly (>95%) pure substances; from the data presented, the presence of trace amounts of SMX (~5  $\mu$ M) present in the SMX-NO stock solutions cannot account for the proliferative response obtained with the clones that were cross-reactive and those that responded to SMX-NO only. Responses to SMX-NHOH were similar to those of SMX-NO; this can be explained by the fact that SMX-NO and SMX-NHOH exist in equilibrium in aqueous solution. Half of all SMX-specific TCC tested responded to *N*-acetyl SMX, the major nontoxic metabolite of SMX excreted in human urine. These data are in keeping with our previous observations of a considerable degree of cross-reactivity of SMX-specific TCC with SMX derivatives bearing the same sulfanilamide core structure (15, 16).

Based on the analysis of a panel of TCC, we could outline three patterns of drug recognition. The majority of TCC proliferated in response to noncovalently bound SMX and to various degrees to noncovalently bound SMX metabolites. Similar clones generated independently were broadly cross-reactive with other noncovalently presented sulfonamides (14), which might explain the additional reactivity with SMX-NO-s. Only four TCC reacted with the covalently bound SMX-NO but not with SMX-s. The response

patterns of representative clones N3 and KG4 imply also that SMX-NO-s covalently modifies APC or that both clones are cross-reactive for noncovalently and covalently bound SMX-NO. Ten of 222 TCC generated by different stimulation patterns cross-reacted with SMX presented in a covalently bound form (SMX-NO-p) and with noncovalently bound SMX-NO or SMX.

These findings represent the first direct experimental evidence that T cells recognize and are stimulated by SMX-NO. The presence of exclusively SMX-NO-reactive clones also supports the view that these T cells have encountered metabolite-modified APC at some time point in their life span. This may happen similarly as for trinitrophenyl in the form of haptenated self-peptides (23). It is tempting to speculate that the sole presence of such metabolite-specific and SMX/SMX-NO-cross-reactive T cells indicates that SMX metabolites by themselves might cause primary stimulation of SMX-reactive T cells and thereby initiate drug allergy. However, several lines of evidence argue against SMX-NO or SMX-NHOH as the exclusive and primary sensitizer of an SMX-specific immune response. First, PBMC of both allergic patients responded relatively weakly to SMX-NO-p or SMX-NHOH-p compared with SMX-s. This poor antigenicity could be explained by a low number of antigenic epitopes generated by the reactive SMX metabolites. Alternatively, it may reflect a difference in T cell precursor frequencies specific for noncovalently and covalently presented forms of SMX and SMX metabolites. Second, bulk cultures with Ag in the form of SMX-NO/NHOH-pulsed PBMC gave rise to SMX-s-specific rather than SMX-NO-p-specific clones (Table II, UNO Cloning 1). One could argue that for the generation or detection of SMX-NO-p-specific TCC the APC used (Ficoll-purified PBMC and B-LCL) were not suitable. For example, they might not have the appropriate self-peptide required for covalent drug binding and presentation embedded in their MHC. However, the proliferation of 14 clones in the presence of SMX-NO-pulsed APC demonstrated a sufficient capacity by the chosen APC to present the respective Ag. Moreover, the Ag-presenting capacity of SMX-NO-pulsed APC was confirmed by the kinetics of Ag-specific down-regulation of TCR surface expression. It has to be stressed that this presentation did involve binding of the reactive compound to proteins on the outside of the cell but not Ag uptake and processing. Third, although the ratio between SMX-s and SMX-NO-p or cross-reactive cells varied between individuals, the great majority of TCC generated in this study from PBMC by addition of SMX or oxidative SMX metabolites recognized only noncovalently presented SMX (and to some extent SMX-NO-s). Thus, it might be argued that T cell cross-reactivity between covalently presented SMX metabolites and noncovalently presented SMX is the exception rather than the rule. Only 1.8% of all TCC recognized SMX-NO-p exclusively and only 4.5% were cross-reactive compared with 93.6% responding to SMX-s. If the relevant Ag for the primary stimulation was indeed covalently bound SMX, one would expect a higher incidence of SMX-specific TCC that also react with SMX-NO-p. We are aware that caution is needed in extrapolating directly from specific T cell numbers to T cell function in the pathogenesis of disease. A detailed functional analysis of single-specific and cross-reactive clones will be undertaken to address this question in the future.

Two further arguments support the hypothesis that the soluble, labile-bound SMX might be the relevant Ag even for primary T cell stimulation. First, the kinetics of TCR recognition are identical with those of the recognition of peptide Ags and obey the predictions of the “serial triggering” model (14, 24). This implies that the drug-MHC-TCR interaction is sufficiently strong to trigger T cells and, together with adhesion molecules, may also allow the stimulation of naive T cells. Second, the TCC specific for SMX and



related compounds bear an unbiased array of TCR. Thus, the T cell response is polyclonal and heterogeneous (14). Therefore, it is likely that already in the phase of T cell induction, SMX interacts with the MHC-peptide complex in several ways, generating distinct antigenic determinants each time. Such behavior is better explained by a noncovalent binding of the drug to the MHC-peptide complex than by a covalent MHC binding.

In conclusion, our data show that recognition of covalently and noncovalently bound drugs by the same TCR is possible; however, such cross-reactivity is rather the exception. The dominant presence of SMX-s-specific T cells and the scarcity of cross-reactivity between covalently and noncovalently bound SMX suggests that the bulk of the primary stimulation is directed to noncovalently bound SMX.

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