

Original article

Drug-specific *in vitro* release of IL-2, IL-5, IL-13 and IFN- γ in patients with delayed-type drug hypersensitivity

Background: The most prevalent drug hypersensitivity reactions are T-cell mediated. The only established *in vitro* test for detecting T-cell sensitization to drugs is the lymphocyte transformation test, which is of limited practicability. To find an alternative *in vitro* method to detect drug-sensitized T cells, we screened the *in vitro* secretion of 17 cytokines/chemokines by peripheral blood mononuclear cells (PBMC) of patients with well-documented drug allergies, in order to identify the most promising cytokines/chemokines for detection of T-cell sensitization to drugs.

Methods: Peripheral blood mononuclear cell of 10 patients, five allergic to β -lactams and five to sulfanilamides, and of five healthy controls were incubated for 3 days with the drug antigen. Cytokine concentrations were measured in the supernatants using commercially available 17-plex bead-based immunoassay kits.

Results: Among the 17 cytokines/chemokines analysed, interleukin-2 (IL-2), IL-5, IL-13 and interferon- γ (IFN- γ) secretion in response to the drugs were significantly increased in patients when compared with healthy controls. No difference in cytokine secretion patterns between sulfonamide- and β -lactam-reactive PBMC could be observed. The secretion of other cytokines/chemokines showed a high variability among patients.

Conclusion: The measurement of IL-2, IL-5, IL-13 or IFN- γ or a combination thereof might be a useful *in vitro* tool for detection of T-cell sensitization to drugs. Secretion of these cytokines seems independent of the type of drug antigen and the phenotype of the drug reaction. A study including a higher number of patients and controls will be needed to determine the exact sensitivity and specificity of this test.

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Key words: cytokine secretion; drug hypersensitivity; sulfanilamide; T cells; β -lactam.

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Accepted for publication 18 November 2008

Adverse drug-induced reactions are one of the major causes of iatrogenic disease in public health (1). About 15–20% of adverse drug reactions are caused by immunological mechanisms, and can be mediated by IgE, occasionally IgG and T cells (2). The clinical picture of T-cell mediated, delayed-type drug hypersensitivity reactions (DTH) is very heterogeneous and different T-cell subsets are involved (3).

The diagnosis of T-cell mediated drug hypersensitivity reactions is a major challenge in daily clinical practice as patch-, prick- and intracutaneous tests often do not yield

positive results even in patients with a clear history of DTH (4, 5). Challenge tests, which are considered to be the gold standard, are frequently not tolerated by patients, bear the risk of severe reactions and do not differentiate between allergic and nonallergic reactions (6).

The aim of drug hypersensitivity diagnosis is to confirm that the symptoms were caused by a drug hypersensitivity reaction, and to identify the culprit drug. Our group and others have previously shown that *in vitro* T-cell proliferation and activation tests can be applied to the diagnosis of drug hypersensitivities (7, 8). Furthermore, studies by other groups have shown that measurements of cytokine secretion, namely of interleukin-5 (IL-5) and interferon- γ (IFN- γ), are useful for drug hypersensitivity diagnosis (9–15).

Nevertheless, a systematic comparison of different cytokines produced *in vitro* after drug stimulation, which

Abbreviations: AMP, ampicillin; AMX, amoxicillin; DTH, delayed-type drug hypersensitivity reaction; IFN, interferon; IL, interleukin; LTT, lymphocyte transformation test; MPE, maculopapular exanthem; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; SMX, sulfamethoxazole; SPD, sulfapyridine; TT, tetanus toxoid.

would identify the most suitable cytokine or combination of cytokines for diagnosis of drug hypersensitivity, has not yet been reported. In this study, we screened the supernatants of *in vitro* drug-stimulated peripheral blood mononuclear cell (PBMC) of 10 drug-allergic patients for 17 cytokines and chemokines, representing a broad spectrum of T-cell- and monocyte-derived cytokines/chemokines. Although DTHs are T-cell mediated, we could not exclude a T-cell-mediated activation or a direct activation of monocytes from allergic patients by drugs. The goal of our study was to determine (i) such of those cytokines, which are secreted predominantly upon exposure to the relevant drug *in vitro*; (ii) the shortest stimulation time of PBMC with drugs resulting in detectable concentrations of cytokines in supernatants; (iii) whether stimulation by cross-reactive drugs leads to an identical cytokine pattern; (iv) whether β -lactams and sulfanilamides induce different cytokine patterns and (v) whether there are characteristic cytokine secretion patterns for different forms of DTH. Our data show that IL-5, IFN- γ , IL-13 and IL-2 are specifically induced in response to sulfamethoxazole (SMX), sulfapyridine (SPD), ampicillin (AMP) or amoxicillin (AMX) stimulation in PBMC from β -lactams- or sulfanilamide-allergic patients.

Material and methods

Donor characteristics

A summary of the patients' clinical characteristics is given in Table 1. The patients were analysed in clinical remission and were healthy at the time of analysis. Skin tests, lymphocyte transformation tests (LTT) and CD69 measurements were performed as described (7, 8, 16). Five healthy individuals without a history of adverse drug reactions but responsive to tetanus toxoid (TT) served as controls. All patients gave informed consent, and the study was approved by the local ethical committee.

Table 1. Patients' characteristics

Patient's	ID Age (years)/sex	Culprit drug	Disease	Skin test	LTT*	CD69†	Time interval‡
32	64/m	AMX	MPE	Negative	Positive	Positive	19 months
249	26/f	AMX	MPE	Positive	Positive	n.d.	2 months
31	35/m	AMX	MPE	Negative	Positive	Positive	8 months
244	35/f	AMX	MPE	Negative	Positive	n.d.	5 month
358	41/f	AMX	EEM	Positive	Positive	Positive	6 month
11	45/m	SMX	MPE/Malaise	Positive	Positive	Positive	12 years
38	48/m	SPD	DRESS	Negative	Positive	Positive	13 months
245	71/m	SMX	SJS	Negative	Positive	Positive	2 months
32	64/m	SMX	Erythrodermia	Negative	Positive	Positive	19 months
295	41/f	SPD	DRESS	Negative	Positive	Positive	6 month

MPE, maculopapular exanthema; DRESS, drug reaction with eosinophilia and systemic symptoms; EEM, erythema exsudativum multiforme; SJS, Stevens–Johnson syndrome; n.d., not determined.

*LTT: a stimulation index of more than 2 is interpreted as positive.

†CD69 upregulation on T cells: a stimulation factor of more than 2 is interpreted as positive.

‡Time interval between acute allergy and testing.

Isolation of PBMC from blood samples

Peripheral blood mononuclear cells were isolated from heparinized peripheral blood as described previously (17). Cells were cultured in RPMI-1640 (GIBCO®; Invitrogen, Basel, Switzerland) supplemented with 10% heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 2mM L-glutamine (Biochrom, Berlin, Germany) and 25 μ g/ml transferrin (Biotest, Dreieich, Germany). For some experiments, CD3+ cells were depleted by using human CD3 selection kits (Easy Sep®; StemCell Technologies, Grenoble, France) according to the manufacturer's protocol.

Drugs and antigens

Tetanus toxoid (Serum Statens Institutet, Copenhagen, DK) was used at a final concentration of 5 μ g/ml. Appropriate drug concentrations were evaluated previously in toxicity tests as described (7). The following nontoxic drug concentrations were used: AMX – 200, 500 μ g/ml; AMP– 200, 500 μ g/ml; SMX – 100, 200 μ g/ml and SPD – 100, 200 μ g/ml (all from Sigma, Basel, Switzerland). Lipopolysaccharide (LPS)-free drugs were from sterile containers.

Stimulation of PBMC and CD3– cells by drug antigens

A quantity of 2×10^5 PBMC were incubated for the time period indicated (mostly 72 h) with or without the drug, or with TT as a positive control in 100 μ l cell culture medium. Supernatants were stored at -80°C . Lymphocyte transformation test was performed as described previously (18). ^3H -thymidine incorporation was measured by a Trace 96 counter (Inotech, Wohlen, Switzerland).

Cytokine/chemokine measurements

Seventeen cytokines/chemokines, namely IL1- β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN- γ , CCL4, CCL2 and TNF- α , were measured in the supernatants by using commercially available 17-plex bead-based immunoassay kits (Bio-Rad Laboratories, Reinach, Switzerland) according to the manufacturer's protocol. The detection range was

approximately from 1 to 32 000 pg/ml. Cytokine/chemokine concentrations are either shown as pg/ml or as a 'fold increase'. The 'fold increase' is calculated by dividing the cytokine/chemokine concentration of the drug- or TT-stimulated PBMC cultures by the cytokine/chemokine concentration of the unstimulated PBMC culture.

Determination of assay conditions

In order to determine the minimal time interval yielding a robust detection of cytokines, PBMC of patients 11, 244 and 38 were stimulated with the eliciting drugs SMX, AMX and SPD for 24, 48 and 72 h respectively, and the concentrations of the 17 cytokines/chemokines were measured. Figure 1 presents the time kinetics for the five most relevant cytokines, IL-13, IFN- γ , IL-5, IL-2 and TNF- α (patient 38 not shown). An incubation time of 72 h resulted in the clearest increase, and this time interval was consequently used for all experiments.

Data analysis

All experiments were performed twice. If the cytokine concentration was under/over the detectable range, the lowest/highest value of the standard curve was used for calculations. Statistical significance was determined by the two-sided Mann-Whitney test for unpaired data, and the Wilcoxon signed-rank test for paired data. A *P*-value < 0.05 was considered to be statistically significant. Cut-off values were calculated as mean +2 SD as reported elsewhere (19, 20).

Results

Characterization of patients

Ten drug-allergic patients with well-documented and typical drug-related histories and symptoms, positive skin tests in three of 10, positive LTT in 10/10 and CD69 upregulation in eight of eight patients in response to either AMX, SMX or SPD were included in this study (Table 1). Four of five AMX-allergic patients had a maculopapular exanthem (MPE), and one an erythema exsudativum multiforme. Two of the sulfanilamide-allergic patients showed a drug rash with eosinophilia and systemic symptoms, one had a Stevens-Johnson syndrome, one an erythrodermia and one a severe MPE. Of note, patient 32 reacted with MPE to AMX and with erythrodermia to SMX. All five healthy donors had no history of drug allergy, had previously tolerated AMX and were negative in the LTT to all tested drugs.

Baseline cytokine secretion is comparable for patients in remission and healthy controls

All experiments were performed after 2 months–13 years of occurrence of the DTH. At the time of blood sample collection, all symptoms of DTH had subsided. Therefore, we expected the baseline cytokine/chemokine secretion in

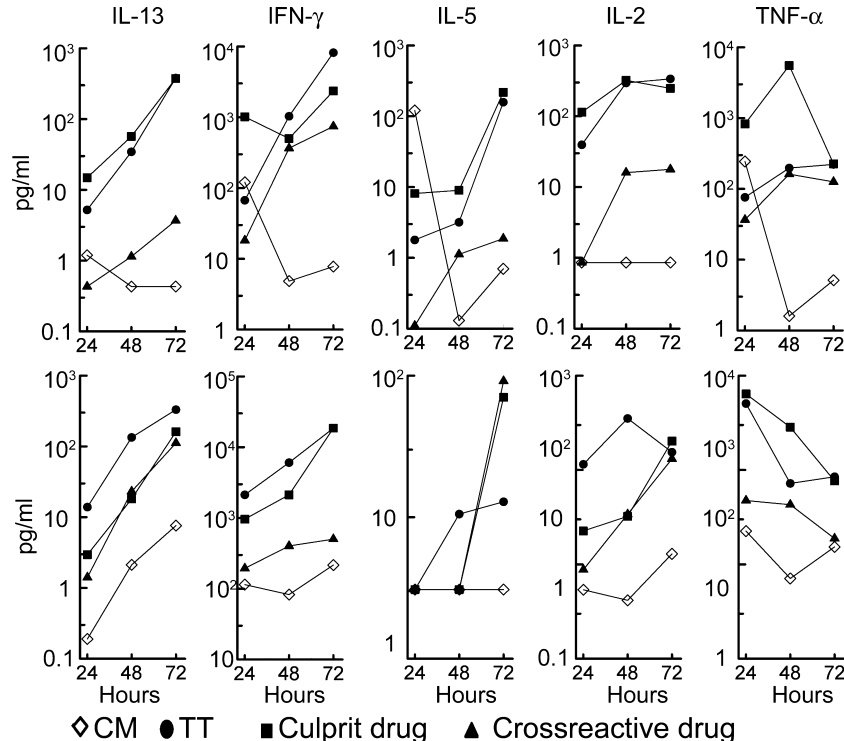


Figure 1. Time kinetics of drug-specific cytokine secretion of PBMC from allergic patients. PBMC from SMX-allergic patient 11 (top panel), and AMX-allergic patient 244 (bottom panel) were stimulated with the culprit and cross-reactive drugs (100 μ g/ml SMX and SPD, or 500 μ g/ml AMX and AMP, respectively), and with culture medium (CM) as negative or 5 μ g/ml tetanus toxoid (TT) as positive control, respectively. Cytokine secretion was determined after 24, 48 and 72 h of stimulation.

healthy controls and patients to be comparable. Cytokine release (pg/ml) from both patients' and healthy donors' PBMC incubated for 72 h without any drug was low for the cytokines (mostly <500 pg/ml), but high for the chemokines (mostly 500–20 000 pg/ml) (Table 2).

In the group of SMX/SPD-allergic patients, only the baseline levels of the chemokines CCL2 and CCL4 were significantly elevated when compared with healthy controls ($P < 0.05$). The other 15 cytokines and chemokines were not elevated (Table 2A,C). Comparing the group of AMX-allergic patients to the healthy control group, the baseline levels of the chemokines CCL2 and CCL4 and additionally IL-6 were significantly increased ($P < 0.05$). The other 14 cytokines and chemokines were not elevated ($P > 0.05$) (Table 2B,C).

Interestingly, we could not observe a consistently higher baseline cytokine secretion in patients in remission for shorter periods (2–8 months) when compared with patients in remission for 1 year or longer.

Drugs can induce a marginal increase in cytokine secretion by PBMC of healthy individuals

To determine the 'unspecific' background cytokine secretion of PBMC in response to the drugs, we measured the cytokine release from PBMC of healthy donors after a 72-h stimulation with all relevant drugs. We observed that the unspecific secretion of cytokines/chemokines of healthy donors was dependent on the drug antigen used for stimulation (Table 3). In the group of sulfanilamides, SMX generally did not induce any cytokine/chemokine secretion in healthy controls (Table 3A). In contrast, SPD increased the median secretion of IFN- γ , IL-2, IL-13, IL-17, CCL4, IL-10, TNF- α , G-CSF and IL-1 β by two- to 13-fold (Table 3B). However, statistical analysis revealed that only IL-1 β secretion was significantly increased after stimulation by SPD when compared with nonstimulated PBMC cultures of healthy individuals ($P < 0.05$).

Table 2. Baseline cytokine secretion of patients in remission and healthy controls in pg/ml

Patient	T-cell-derived																
											Monocyte/macrophage-derived						
	IL-5	IL-13	IFN- γ	IL-2	IL-4	IL-17	IL-6	IL-10	TNF- α	GM-CSF	CCL4†	G-CSF	CXCL8	CCL2†	IL-1 β	IL-12	IL-7*
(A) SMX/SPD-allergic patients																	
11	4	0	15	1	7	10	24	2	5	5	122	2	4510	17840	3	2	2
38	2	1	90	1	3	6	1698	2	50	5	1196	36	22456	18484	49	3	1
32	3	1	2	1	2	4	10	3	10	3	415	1	4108	1621	2	4	4
245	3	1	407	13	5	25	487	24	387	1	1197	711	17355	10409	268	3	3
295	7	10	428	29	5	35	463	58	311	48	221	848	1080	751	768	5	2
Median	3	1	90	1	5	10	463	3	50	5	415	36	4510	10409	49	3	3
(B) AMX-allergic patients																	
244	1	2	394	7	11	15	2030	12	326	17	785	269	5346	5588	144	4	4
31	2	4	48	7	3	2	1634	3	51	1	401	35	5346	3385	75	2	1
32	2	1	5	1	4	43	3	3	19	4	357	1	6768	1431	3	3	2
358	16	46	69	16	2	12	203	5	31	6	94	24	13307	1057	6	3	3
249	2	1	243	6	9	6	17168	5	199	2	4791	190	5346	4675	62	3	3
Median	2	2	69	7	4	12	1634	5	51	4	401	35	5346	3385	62	3	3
(C) Healthy controls																	
380	15	154	216	243	3	19	361	6	41	16	100	59	1663	1081	17	3	3
61	2	1	87	9	1	10	1	2	11	9	115	33	5127	1442	7	3	3
325	2	2	54	8	1	6	35	3	10	6	21	20	5127	1114	6	3	3
326	3	8	50	15	1	4	140	2	20	6	29	29	3962	560	9	3	3
60	3	1	108	12	2	7	91	3	31	11	35	39	8768	1104	20	3	3
Median	3	2	87	12	1	7	91	3	20	9	35	33	5127	1104	9	3	3

SMX, sulfamethoxazole; SPD, sulfapyridine; AMX, amoxicillin.

Data represent the mean of 2 independent experiments. Cytokine values in the two independent experiments were comparable.

*Produced by stromal cells in many tissues.

†Significantly increased when compared with healthy controls. $P < 0.05$, Wilcoxon signed-rank test.

Table 3. Healthy donors, increase in cytokine secretion after PBMC stimulation with AMX, AMP, SMX and SPD (fold increase)

Antigen	Donor	IL-5	IL-13	IFN- γ	IL-2	IL-4	IL-17	IL-6	IL-10	TNF- α	GM-CSF	CCL4	G-CSF	CXCL8	CCL2	IL-1 β	IL-12	IL-7
(A)																		
SMX	380	1	0	1	0	1	1	1	1	2	1	3	3	1	1	2	1	1
SMX	61	1	2	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
SMX	325	1	0	1	1	1	1	6	1	1	1	1	1	1	1	1	1	1
SMX	326	1	1	2	1	1	1	2	1	1	1	1	1	1	2	1	1	1
SMX	60	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1
Median		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Antigen	Donor	IL-5	IL-13	IFN- γ	IL-2	IL-4	IL-17	IL-6	IL-10	TNF- α	GM-CSF	CCL4	G-CSF	CXCL8	CCL2	IL-1 β *	IL-12	IL-7
(B)																		
SPD	380	1	7	1	2	1	2	1	3	5	1	3	13	1	1	6	1	1
SPD	61	1	8	4	2	2	2	1	22	12	2	3	13	1	1	21	2	1
SPD	325	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
SPD	326	1	3	6	1	4	10	3	6	7	3	10	23	1	2	14	2	1
SPD	60	1	3	3	3	1	1	2	1	1	1	1	1	1	1	8	1	1
Median		1	3	2	2	1	2	1	3	5	1	3	13	1	1	8	1	1
Antigen	Donor	IL-5	IL-13	IFN- γ	IL-2	IL-4	IL-17	IL-6*	IL-10	TNF- α	GM-CSF	CCL4	G-CSF	CXCL8	CCL2	IL-1 β	IL-12	IL-7
(C)																		
AMX	380	1	0	2	1	1	1	34	1	2	1	1	1	6	25	9	1	1
AMX	61	1	2	10	3	1	1	50	1	2	1	2	1	2	2	1	1	1
AMX	325	1	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1
AMX	326	1	1	2	2	1	1	6	1	0	1	1	1	2	3	1	1	1
AMX	60	1	2	3	2	1	1	5	2	1	1	2	1	1	1	3	1	1
Median		1	2	2	2	1	1	5	1	1	1	1	1	2	2	3	1	1
Antigen	Donor	IL-5	IL-13	IFN- γ	IL-2	IL-4	IL-17	IL-6	IL-10	TNF- α	GM-CSF	CCL4	G-CSF	CXCL8	CCL2	IL-1 β	IL-12	IL-7
(D)																		
AMP	380	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AMP	61	1	1	1	1	4	1	19	1	2	1	13	19	1	1	4	1	1
AMP	325	1	0	1	1	1	1	2	1	1	1	2	1	1	1	1	1	1
AMP	326	1	1	2	1	2	2	3	1	2	1	1	2	1	3	3	1	1
AMP	60	1	2	2	0	1	1	1	1	1	1	1	1	1	1	1	1	1
Median		1	1	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1

PBMC, peripheral blood mononuclear cell; AMX, amoxicillin; AMP, ampicillin; SMX, sulfamethoxazole; SPD, sulfapyridine.

Fold increase in cytokine/chemokine secretion (concentration of stimulated/concentration of nonstimulated) from healthy donors after stimulation by (A) 100 μ g/ml SMX, (B) 100 μ g/ml SPD, (C) 500 μ g/ml AMX and (D) 500 μ g/ml AMP. Data represent the mean of 2 independent experiments. Cytokine values in the two independent experiments were comparable.

*Significantly increased when compared with unstimulated control. $P < 0.05$, Wilcoxon signed rank test.

Similarly to sulfanilamides, we observed differences in cytokine secretion in response to the β -lactams AMX and AMP from PBMC of healthy donors. Amoxicillin induced a median increase in secretion of IL-1 β , CXCL8, CCL2, IL-2, IL-13, IFN- γ and IL-6 by two- to fivefold, but only the increase in IL-6 secretion was statistically significant ($P < 0.05$) (Table 3C). On the other hand, PBMC from healthy donors reacted to AMP stimulation with increased median secretion of IFN- γ and IL-6 (Table 3D). However, statistical analysis showed that the increase of these cytokines was not significant.

In summary, the tested drugs generally either did not or only weakly increased the cytokine/chemokine secretion in healthy donors, with the exception of IL-6 and IL-1 β ,

which were statistically increased after stimulation by AMX and SPD respectively.

Cytokine secretion pattern of PBMC from drug-allergic patients in response to sulfanilamides and penicillins

We measured the cytokine/chemokine release from PBMC of drug-allergic donors after a 72-h stimulation with the culprit or cross-reactive drug. Fold increase was again calculated as cytokine/chemokine concentration of stimulated PBMC divided by cytokine/chemokine concentration of nonstimulated PBMC (Table 4).

In the group of sulfanilamides, the culprit drug strongly induced IL-13, IFN- γ and IL-2 with a median fold

Table 4. Allergic patients increase in cytokine secretion after PBMC stimulation with culprit and cross-reactive drug, and TT (fold increase)

	Patient	IL-5	IL-13	IFN- γ	IL-2	IL-4	IL-17	IL-6	IL-10	TNF- α	GM-CSF	CCL4	G-CSF	CXCL8	CCL2	IL-1 β	IL-12	IL-7
(A) Sulfanilamide-allergic patients																		
cu																		
SMX	11	236	493	708	195	6	7	19	9	22	713	26	10	1	1	9	3	1
SPD	38	1	42	65	580	1	7	1	1	16	7	3	12	1	1	3	1	1
SMX	32	1	2	3	13	1	1	1	1	1	1	1	1	1	1	1	1	1
SMX	245	5	112	40	475	1	1	5	3	2	1	8	1	1	1	1	1	1
SPD	295	4	23	13	36	9	168	9	5	12	19	15	6	1	1	3	2	1
Median		4	42	40	195	1	7	5	3	12	7	8	6	1	1	3	1	1
cr																		
SPD	11	2	30	68	21	2	2	41	4	117	44	14	252	1	1	79	1	1
SMX	38	1	16	30	71	1	1	1	1	3	1	2	3	1	1	1	1	1
SPD	32	1	1	1	7	1	1	1	1	1	1	1	1	1	1	1	1	1
SPD	245	5	4	2	8	1	1	1	1	1	1	2	0	1	1	1	1	1
SMX	295	0	0	5	3	5	48	24	1	6	12	2	9	1	1	5	1	1
Median		1	4	5	8	1	1	1	1	3	1	2	3	1	1	1	1	1
pos.																		
TT	11	160	1159	196	159	4	12	90	4	133	19	15	727	1	1	90	1	1
TT	38	1	12	127	49	7	7	7	11	32	43	7	140	1	1	17	3	1
TT	32	2	7	7	63	1	1	8	1	3	1	2	1	2	5	2	1	1
TT	245	89	921	154	1796	23	7	32	15	38	63	52	399	1	1	44	1	1
TT	295	2	2	1	6	1	19	2	2	1	2	3	2	1	1	1	1	1
Median		2	12	127	63	4	7	8	4	32	19	7	140	1	1	17	1	1
(B) Amoxicillin-allergic patients																		
cu																		
AMX	244	12	15	50	19	3	1	3	4	7	1	14	14	1	1	4	1	1
AMX	31	75	48	46	531	3	1	1	3	34	1	4	1	1	1	3	1	1
AMX	32	1	3	1	3	1	1	5	1	9	1	1	1	2	1	1	1	1
AMX	358	39	83	13	3	6	6	25	9	80	1	5	15	1	7	13	0	1
AMX	249	1	54	24	5	1	8	1	19	2977	13	1	312	1	1	18	1	1
Median		12	48	24	5	3	1	3	4	34	1	4	14	1	1	4	1	1
cr																		
AMP	244	15	11	3	14	2	1	2	4	2	1	3	3	1	1	2	1	1
AMP	31	521	22	10	187	2	1	1	2	19	1	2	1	1	1	1	1	1
AMP	32	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
AMP	358	156	330	12	5	9	4	24	38	130	1	6	12	1	6	13	1	1
AMP	249	5	7	2	3	1	2	1	5	3	2	1	2	1	1	2	1	1
Median		86	17	7	10	2	2	2	5	11	1	3	3	1	1	2	1	1
pos.																		
TT	244	21	30	103	17	3	7	3	3	8	1	19	5	1	1	3	1	1
TT	31	1005	48	13	64	6	1	2	2	26	1	171	1	1	1	5	1	1
TT	32	1	1	3	26	1	1	9	1	2	1	1	1	2	4	1	1	1
TT	358	37	197	109	8	11	66	56	1	390	6	12	21	3	17	24	0	1
TT	249	12	375	1060	25	3	16	1	28	220	1	67	1	11855	1	1	45	1
Median		21	48	103	25	3	7	3	2	26	1	19	1	2	1	3	1	1

SMX, sulfamethoxazole; SPD, sulfapyridine; TT, tetanus toxoid; AMX, amoxicillin; AMP, ampicillin.

Fold increase in cytokine/chemokine secretion (concentration of stimulated/concentration of nonstimulated) after stimulation by culprit drug (cu), cross-reactive drug (cr) and positive control TT (pos.) in (A) sulfanilamide-allergic and (B) amoxicillin-allergic patients. Drug concentrations are 100 μ g/ml SMX and SPD, 500 μ g/ml AMX and AMP. Data represent the mean of two independent experiments. Cytokine values in the two independent experiments were comparable.

increase of 40–195 (Table 4A). A weak increase (median increase three- to 12-fold) was observed for IL-5, IL-1 β , IL-10, IL-17, IL-6, TNF- α , GM-CSF, CCL4 and G-CSF (Table 4A). The cross-reactive drugs of the sulfanilamide group induced a weaker cytokine/chemokine secretion. Only IL-13, IFN- γ , IL-2, TNF- α and G-CSF were

increased (median three- to eightfold). The median secretion of these cytokines in drug-allergic patients was higher when compared with cytokines secreted by PBMC from nonallergic patients, however (Table 3).

Upon *in vitro* stimulation with the culprit AMX, a strong secretion of IL-5, IL-13, IFN- γ , TNF- α and G-CSF

(median increase 12- to 48-fold), and a weaker secretion of IL-2, IL-4, IL-6, IL-10, CCL4 and IL-1 β (median increase three- to fivefold) was induced (Table 4B). Similar to AMX, AMP induced a strong secretion of IL-5 and IL-13 (median increase 17- to 86-fold) and a weak median increase (five- to 11-fold) of IL-2, IL-10, CCL4, G-CSF, IFN- γ and TNF- α (Table 4B).

Peripheral blood mononuclear cells stimulated with TT revealed a cytokine/chemokine secretion pattern similar to the drugs. Predominantly secreted cytokines were IFN- γ , IL-2, IL-13, IL-17, TNF- α and CCL4 for both patient groups (median increase seven- to 140-fold). Further secreted were G-CSF, GM-CSF, IL-4, IL-5, IL-6, IL-10 and IL-1 β (Table 4).

In summary, IL-2, IL-5, IFN- γ , IL-13 and TNF- α , induced by both culprit and cross-reactive drugs, were increased most consistently among the 17 cytokines/chemokines in patients, when compared with healthy controls.

Statistical analysis showed that the release of IL-5, IFN- γ , IL-13, IL-2, but not of TNF- α , induced by the culprit drug was significantly higher in SMX/SPD-allergic patients when compared with drug-stimulated healthy donors. In contrast, upon stimulation with the cross-reactive drug, none of the five cytokines except

IL-2 as mentioned above was secreted in statistically significant higher amounts when compared with controls (Fig. 2, top panel). In AMX-allergic patients, the culprit (AMX) drug induced significantly higher amounts of IL-5, IFN- γ , IL-13, IL-2 and TNF- α when compared with nonallergic individuals, whereas the cross-reactive drug (AMP) did not induce a statistically significant increase in TNF- α (Fig. 2, bottom panel). With a cut-off value (calculated as mean + 2 SD of healthy donors) of 10 for IFN- γ and IL-13, two for IL-5, five for IL-2, six of 10 patients were positive for IL-5, eight of 10 for IFN- γ , IL-13 and IL-2.

Cytokine/chemokine secretion is T-cell dependent

Delayed-type drug hypersensitivity reaction have been shown to be T-cell mediated (3). To demonstrate that the secretion of IL-5, IFN- γ , IL-13 and IL-2 was T-cell dependent, we depleted PBMC of SMX-allergic patient 11 and AMX-allergic patient 244 (Fig. S1A,B) from CD3⁺ cells. When CD3⁻ cells were stimulated in parallel to PBMC by the culprit drugs, we observed that the secretion of IL-5, IFN- γ , IL-13 and IL-2 was absent in CD3⁻ cell cultures. Further, we could show that the occasional secretion of monocyte-derived CCL-4 and

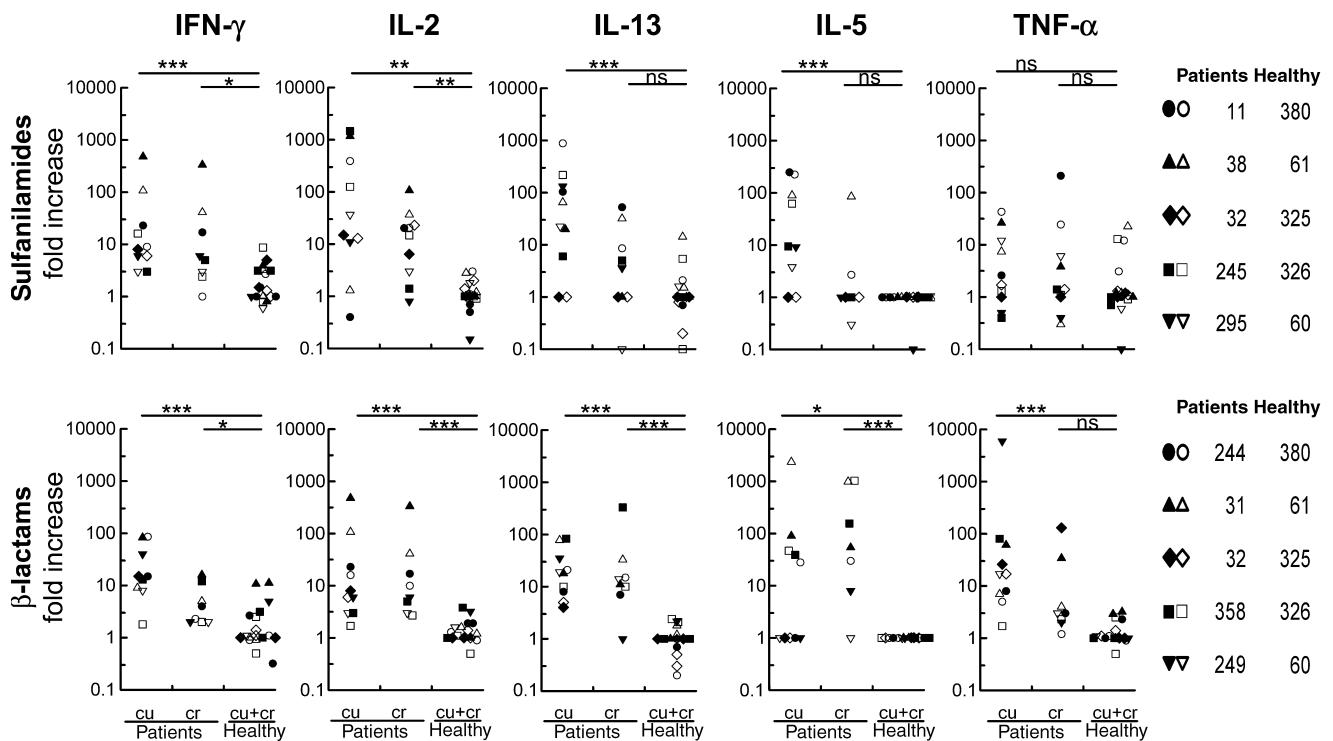


Figure 2. Drug-induced secretion of IFN- γ , IL-2, IL-13, IL-5 and TNF- α from drug-allergic patients and healthy controls. Top panels show cytokine secretion in response to 100 μ g/ml SMX and SPD, and bottom panels to 500 μ g/ml AMX and AMP. PBMC of patients and healthy donors were stimulated either with culprit drug (cu), cross-reactive drug (cr), culture medium (cm) or tetanus toxoid (not shown). Results are shown as fold increase of negative control. Open and filled symbols represent two independent experiments, respectively. * P < 0.05; ** P < 0.01; *** P < 0.001.

TNF- α in patient 11 was abolished if CD3+ cells were depleted from PBMC (Fig. S1C).

Discussion

In the present study, we show a drug-specific increase in secretion of IL-2, IL-13, IFN- γ and IL-5 in PBMC cultures of 10 drug-allergic patients. Cells were stimulated for 72 h as this was the minimal time interval still resulting in robust secretion of IL-2, IL-13, IL-5 and IFN- γ , all of which were significantly increased in response to both culprit sulfanilamides and β -lactams. TNF- α secretion reached statistical significance only in response to culprit β -lactams. Cross-reactive drugs-induced secretion patterns that were comparable to the culprit drug, but quantitatively diminished.

Assaying cytokine secretion patterns has previously been postulated as a promising *in vitro* read-out system of drug hypersensitivity (21), and several studies have suggested to measure IL-5 or IFN- γ in the supernatant of drug-stimulated PBMC (10, 14, 22). The reported patterns of cytokine secretion were proposed to be more sensitive than proliferation assays and to be better correlated with the pathogenetic events. However, a systematic comparison of different cytokines produced *in vitro* after drug stimulation to identify the most suitable cytokine/chemokine for diagnosis of drug hypersensitivity, has not yet been reported. We addressed this question by screening the release of 17 cytokines/chemokines from PBMC of either drug-allergic patients in remission or healthy controls after stimulation with the relevant drugs. The baseline cytokine/chemokine secretion of patients in remission and of healthy controls was comparable.

To determine the most suitable cytokines/chemokines for diagnosing drug sensitization, it is important to determine the unspecific background cytokine secretion induced by the respective drugs. We observed either absence of or only weak drug-induced secretion of cytokines/chemokines in nonallergic individuals. However, not all drugs had the same stimulatory effect: while SMX- and AMP-induced cytokine/chemokine secretion did not induce any significant unspecific cytokine secretion, AMX induced a statistically significant increase of IL-6 in nonallergic individuals. Sulfapyridine-induced cytokine/chemokine secretion in healthy donors more frequently than any of the other drugs, even though only IL-1 β was significantly increased. Overall, we could not observe any difference in induction of cytokine secretion by sulfanilamides when compared with penicillins. Rather, differences in cytokine secretion induced by any one drug seemed to be dependent on each of the healthy individuals. Of note, no cellular proliferation was observed in LTT with cells from any of the healthy donors. The relevance and potential role of this stimulatory effect on healthy donors' PBMC,

notably observed at nontoxic drug concentrations, remain unclear at present. However, this drug-dependent cytokine secretion in healthy individuals needs to be considered in the interpretation of cytokine release from drug-allergic patients when applied as a potential diagnostic tool.

For IL-5 we could not observe a sensitivity as high as previously reported (12) as only six of 10 patients were clearly positive for IL-5 in our assay. Drug-specific secretion of IL-2, IL-13 and IFN- γ was observed in eight of 10 patients. Thus IL-2, IL-13 and IFN- γ seem to be more sensitive parameters for drug sensitization than IL-5. The specificity of these three cytokines seems lower, however, as a four- to 10-fold increase of IL-2, IL-13 and IFN- γ was observed in one to two healthy controls. Therefore, a combination of two cytokines, namely IL-5 as a very specific marker, and either IL-2, IL-13 or IFN- γ as more sensitive parameter(s) would presumably be sufficient to detect sensitizations to drugs. By depleting CD3+ cells, we could further show that the cytokines that characterize patients with DTH *in vitro* are T-cell dependent, and that this assay would therefore detect drug-reacting T cells.

All other cytokines/chemokines, were only occasionally increased after drug stimulation in allergic patients, and do not seem suitable for diagnostic purposes. It is noteworthy that monocyte-derived cytokines such as TNF- α , G-CSF, IL-1 β , CCL-4 and IL-12 were occasionally increased in some patients and healthy donors. This secretion was not consistent over repeated experiments. However, we could show in patient 11 that CCL4 and TNF- α secretion were abolished if T cells were depleted. This suggests that the secretion of non-T-cell-derived cytokines is T-cell dependent and probably not a direct effect of the drug.

According to our data, the cross-reactive drugs cause a similar cytokine/chemokine pattern as the culprit drugs, even though quantitatively diminished especially after sulfanilamide stimulation. The very similar cytokine secretion pattern between AMX and AMP is most probably attributable to their chemical similarity, the difference being one aryl-hydroxy group in the side-chain only.

The *in vitro* drug-stimulated PBMC showed a Th₀ pattern as reported previously (17, 23), which was similar to TT. The control antigen TT generally induced a stronger cytokine secretion than any drug and was similar to the culprit drug-induced pattern, even though it was varying between individual patients. It seems that the cytokine secretion depends more on an 'inherent' reactivity of *in vitro* stimulated PBMC than on the type of antigen or clinical phenotype (17). This implies that it is difficult to associate the *in vitro* secretion of a certain cytokine with a clinical picture.

In conclusion, the measurement of IL-5 as a very specific marker in combination with either IFN- γ , IL-13 or IL-2 as a more sensitive marker might be a useful tool for the *in vitro* detection of T-cell sensitization to drugs. For β -lactam sensitization, TNF- α might be suitable as

well. The secretion of IL-5, IFN- γ , IL-13 and IL-2 seems to be independent of the type of drug and the phenotype of the drug reaction. A prospective study with a larger number of suspected drug-allergic patients and healthy individuals will be needed to provide an accurate threshold for positivity and quantitative data on the sensitivity and specificity of these tests for drug hypersensitivity diagnosis.

Acknowledgments

This work was supported by Swiss National Science Foundation grant no. 310000-113744/1 to W.J. Pichler, and no. 3100A0-116113/1 to B.O. Gerber, by the Kamillo Eisner Foundation, and an unrestricted grant by Pfizer.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

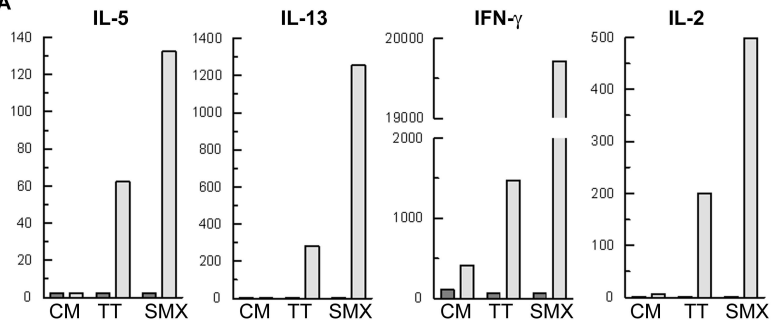
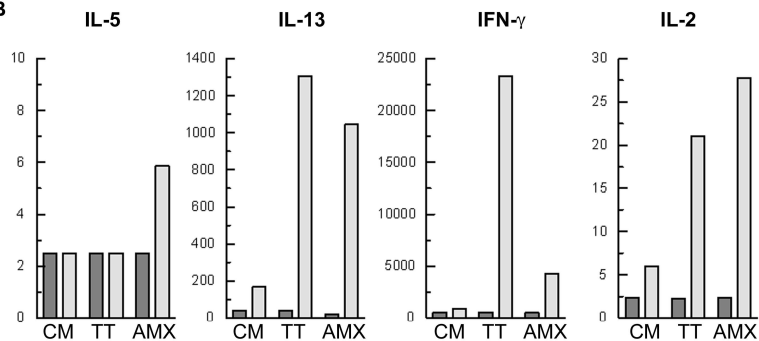
Figure S1. Secretion of IFN- γ , IL-2, IL-13, IL-5, TNF- α and CCL-4 from drug-allergic patients is T-cell-dependent. (A) Patient 11, secretion of IL-5, IL-13, IFN- γ and IL-2 from PBMC or CD3-depleted PBMC (CD3-) stimulated by SMX (100 μ g/ml), TT (5 μ g/ml, positive control) or CM only (negative control). (B) Patient 244, secretion of IL-5, IL-13, IFN- γ and IL-2 from PBMC or CD3-depleted PBMC (CD3-) stimulated by AMX (500 μ g/ml), TT (5 μ g/ml, positive control) or CM only (negative control). (C) Patient 11, secretion of TNF- α and CCL4 from PBMC or CD3-depleted PBMC (CD3-) stimulated by SMX (100 μ g/ml), TT (5 μ g/ml, positive control) or CM only (negative control).

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