

Determination of interleukin-5 secretion from drug-specific activated *ex vivo* peripheral blood mononuclear cells as a test system for the *in vitro* detection of drug sensitization

B. Sachs, S. Erdmann, J. Malte Baron, M. Neis, T. al Masaoudi and H. F. Merk

Department of Dermatology and Allergology, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Germany

Summary

Background *In vitro* detection of drug sensitization is still limited. The lymphocyte transformation test, which determines drug-specific proliferation, is the only *in vitro* test for detecting drug sensitization at the cellular level irrespective of the reaction's clinical phenotype. Accumulation of eosinophils following IL-5 secretion from drug-specific stimulated T cells is a characteristic histological feature of drug-induced skin eruptions.

Objective We determined whether *in vitro* drug-specific activation of *ex vivo* peripheral blood mononuclear cells from 10 patients with drug-induced maculopapular exanthems and three patients with severe skin reactions results in secretion of IL-5, IL-10 or IFN- γ and assessed the sensitivity and specificity of drug-specific IL-5 secretion as a test system compared with the lymphocyte transformation test and patch tests. Furthermore, the subsets of CD4⁺ and CD8⁺ T cells involved in drug-specific proliferation, IL-5 secretion and mRNA expression were examined in three patients.

Methods Drug-specific proliferation of peripheral blood mononuclear cells in the lymphocyte transformation test was investigated by ³H-thymidine uptake, and culture supernatants taken after 5 days were analysed for IL-5, IL-10 and IFN- γ concentrations by ELISA technique. IL-5 mRNA expression was determined by RT-PCR.

Results Drug-specific activation of peripheral blood mononuclear cells consistently resulted in IL-5 and to a lesser extent in IL-10 and IFN- γ secretion. The sensitivities of the patch test, lymphocyte transformation test and assessment of drug-specific IL-5 secretion for the detection of drug sensitization were 55%, 75% and 92%, respectively.

Conclusion These data suggest a role for the determination of drug-specific IL-5 secretion by *ex vivo* peripheral blood mononuclear cells for the *in vitro* detection of drug-sensitization in drug-induced maculopapular exanthems.

Keywords drug-allergy, interferon- γ , interleukin-10, interleukin-5, lymphocyte transformation test, T cells

Submitted 12 July 2001; revised 24 September 2001; accepted 30 November 2001

Introduction

In this paper we focus on allergic drug-reactions, which account for about one-seventh of all adverse drug reactions [1] but represent a major problem of drug safety because of their potential severity and unpredictability [2–4]. Due to the clinical and histological similarities between drug-allergic skin eruptions and graft-vs.-host disease it has been hypothesized that cutaneous drug-induced reactions represent specific immune responses of T cells against antigens (drugs) that are bonded to the surface proteins of keratinocytes [5–8]. The involvement of T cells in drug-allergy has been confirmed by positive patch

tests to drugs of individuals with a history of a drug-allergic reaction [9], by the demonstration of drug-specific peripheral lymphocytes from such patients in the lymphocyte transformation test (LTT) [3,10–12], and by the demonstration of lymphocytes in the lesions of patients with drug-allergic skin reactions [10, 13].

Conclusive diagnosis of drug allergy still remains a major problem in daily clinical practice. *In vivo* cutaneous tests like patch, prick and intracutaneous tests often do not yield positive reactions, even in well-documented histories of drug-allergic reactions. Final challenge tests, which are considered to be the 'gold standard', bear the risk of severe reactions, are frequently not tolerated by the patients, and do not differentiate between allergic and pseudo-allergic reactions. *In vitro* tests, on the other hand, predominantly refer to the effector phase of immediate-type drug-allergic reactions, encompassing methods which directly or indirectly are based on the presence of specific IgE antibodies or the activation of basophils like CAST-ELISA®

Correspondence: Dr Bernhardt Sachs, Department of Dermatology and Allergology, Rheinisch-Westfälische Technische Hochschule Aachen, Pauwelsstraße 30, 52074 Aachen, Germany. E-mail: Bernhardt.Sachs@post.rwth-aachen.de

[14] and BASO-Test® [15], respectively. Therefore, many drug-allergic reactions, including the common maculopapular exanthems, which are not mediated by specific IgE antibodies, cannot be detected by means of these tests.

Currently the lymphocyte transformation test (LTT) is the only available *in vitro* test for detecting drug sensitization at the cellular level, irrespective of the effector mechanisms and the clinical phenotype of the reaction, as it detects drug-specific proliferation of sensitized T cells [11]. These T cells, circulating in the body, emerge from the clonal expansion of naïve T cells having encountered their specific antigens [16]. However, the LTT includes working with radioactive substances and has its limitations, such as test-immanent difficulties, and therefore should be performed in experienced laboratories [10, 11].

Accumulation of eosinophils promoted by IL-5 secretion from drug-specific activated T cells is a characteristic histological feature in biopsy specimens of drug-induced skin eruptions [17]. These lesional drug-specific T cells expressing the cutaneous lymphocyte antigen (CLA) descend from the peripheral blood and migrate into the skin [7, 17]. Furthermore, drug-allergic reactions are often accompanied by blood eosinophilia following IL-5 secretion from drug-specific activated T cells [18–20]. This prompted us to assess the IL-5 secretion of *in vitro* drug-specific activated *ex vivo* peripheral blood mononuclear cells (PBMC) from patients with drug-induced maculopapular exanthems representing delayed-type reactions and to determine the sensitivity and specificity of drug-specific IL-5 secretion as a test system compared with the LTT and patch tests. In addition *in vitro* IL-10 and IFN- γ secretions of the PBMC were determined in order to investigate whether these cytokines could also serve as indicating parameters for drug-specific T cell activation.

Materials and methods

Patients and controls

During the study period patients that presented to our Department of Dermatology and Allergology were included in the study only when the following criteria were fulfilled: (a) the patient presented with a drug-induced maculopapular exanthem representing a delayed-type reaction; (b) drug-allergy was confirmed by stringent drug-allergy history-category A according to Nyfeler et al. [11]; and (c) the patient gave informed consent, agreeing to participate in the investigations. Ten such patients with well-documented histories of a maculopapular exanthem were included in the present study (Table 1). Patient 6 developed a maculopapular exanthem, additionally expressing some target-like lesions similar to an erythema exudativum multiforme (E.e.m.)-like exanthem. Patient 10 presented with confluent macules depicting a macular, erythrodermic-like exanthem following intake of amoxicillin. Although the phenotypes of these two patients differed slightly from the classical maculopapular exanthem, with regard to the brevity, the skin eruptions of all 10 patients are designated as maculopapular exanthem in the manuscript. Anticonvulsant hypersensitivity syndrome is defined in addition to the occurrence of skin rash by organ manifestations [20, 21], which were apparent in patients 8 and 9.

All patients were investigated for atopic status by reference to their histories, physical examinations and laboratory results for the 'Erlander score for atopy' [22]. In addition to the 10 patients with maculopapular exanthem, three patients with severe skin reactions (bullous maculopapular exanthem, E.e.m., toxic-epidermal necrolysis, respectively) were investigated, probably

Table 1. Sensitivity and specificity of the different diagnostic procedures patch test, LTT, and detection of drug-specific IL-5, IL-10 and IFN- γ secretion

	Gender	Age	Atopy	Phenotype of delayed-type drug reaction	Causal drugs*	Patch test result	LTT	Drug-specific IL-5 secretion	Drug-specific IFN- γ secretion	Drug-specific IL-10 secretion
1	F	35	no	Maculopapular exanthem	Amoxicillin	pos.	pos.	pos.	pos.	neg.
2	F	44	yes	Maculopapular exanthem	Amoxicillin	neg.	pos.	pos.	neg.	pos.
3	F	25	no	Maculopapular exanthem	Amoxicillin	pos.	pos.	pos.	pos.	pos.
4	F	56	no	Maculopapular exanthem	Ampicillin	pos.	pos.	pos.	neg.	pos.
5	F	35	no	Maculopapular exanthem	Chlorazepate	pos.	pos.	pos.	neg.	neg.
6	F	39	no	Maculopapular exanthem	Fenoterol	pos.	pos.	pos.	neg.	neg.
7†	F	26	no	Maculopapular exanthem	Amoxicillin	neg.	neg.	neg.	neg.	neg.
8	M	24	no	Maculopapular exanthem‡	Carbamazepine§	n.d.	pos.	pos.	pos.	pos.
					Phenytoin§	n.d.	neg.	pos.	neg.	neg.
					Phenobarbital§	n.d.	neg.	pos.	neg.	neg.
9	M	9	no	Maculopapular exanthem‡	Carbamazepine	neg.	pos.	pos.	n.d.	pos.
10	M	30	yes	Macular-erythrodermic exanthem	Amoxicillin	neg.	pos.	pos.	pos.	pos.
n: 10	F: 7	Average	Atopic: 2;	Maculopapular exanthem: 10	Aminopenicillins: 6	Sensitivity: 55%	Sensitivity: 75%	Sensitivity: 92%	Sensitivity: 36%	Sensitivity: 50%
	M: 3	Age: 32	non-atopic: 8		Various drugs: 4	Specificity: n.d.	Specificity: 100%	Specificity: 100%	Specificity¶: 60%	Specificity¶: 100%

*With regard to the stringent history of the drug-induced reaction. †Because no lymphocyte reactivity could be demonstrated *in vivo* or *in vitro* the patient was offered an oral provocation test with amoxicillin, which she denied. ‡Patients 8 and 9 presented with a maculopapular exanthem as one part of an anticonvulsant hypersensitivity syndrome. §Because the patient developed exanthems after intake of carbamazepine, phenytoin and phenobarbital all three drug were taken into account for the calculation of the sensitivity of the different parameters. ¶Only PBMC from the five control persons of the second control group (with exposure to amoxicillin) but not of the first control group were investigated for drug-induced IFN- γ and IL-10 secretion.

representing a different clinical phenotype and entity. No atopy was investigated and no patch tests were performed in the patient with toxic-epidermal necrolysis (TEN) due to fatal course of the disease.

Two control groups were included in the investigation. A healthy and, where possible, age- and sex-matched control person without known allergy to the tested drug and without any intake ever or without intake of the drug in the last 12 months was matched to every patient. PBMC of these control persons were investigated for drug-induced proliferation and IL-5 secretion to rule out unspecific stimulating effects of the drugs. In the second control group PBMC of a further five individuals who were currently or in the last 3 months exposed to amoxicillin without clinical signs of a drug-allergic reaction were investigated for drug-induced proliferation and IL-5, IL-10 and IFN- γ secretion. The present study was approved by the local ethic committee (EK 824) and all patients gave informed consent prior to participation.

Skin tests

The patients were patch tested on the upper part of their back using Haye's test chambers[®] (Haye's Service B.V., the Netherlands), employing the drugs of interest and the German standard series. Results were read after 48, 72 and 168 h according to the International Contact Dermatitis Group scale. The drugs were tested in concentrations according to the recommendations of the European Network for Drug Allergy.

Cultures and reagents

1640 RPMI culture medium (Life Technologies, Paisley, UK) containing 5% autologous heat-inactivated plasma supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), non-essential amino-acids (1X) and penicillin-streptomycin (50 IU/mL) (all Gibco, Paisley, UK) was used. Penicillin-free culture medium was used in the cases of patients with penicillin allergy. Amoxicillin, carbenicillin, phenoxymethyl-penicillin (penicillin V), carbamazepine, doxycycline and fenoterol were purchased from Sigma-Aldrich Chemicals (Steinheim, Germany). Ampicillin (Binotal[®]) and benzyl-penicillin (penicillin G) (Penicillin Grünenthal[®]) were purchased from Grünenthal (Stolberg, Germany). Propranolol (Dociton[®]) was bought from Astra-Zeneca (Wedel, Germany). Phenytoin (Phenhydant[®]) and phenobarbital (Luminal[®]) were purchased from Desitin (Hamburg, Germany). Terbutaline (Bricanyl[®]) was bought from Pharma-stern (Wedel, Germany) and chlorazepate (Tranxilium[®]) from Sanofi Winthrop (München, Germany). Flucloxacillin (Staphylex[®]) was purchased from SmithKline Beecham (Bühl, Germany) and celecoxib was provided by Pharmacia (Erlangen, Germany).

All drugs were dissolved in phosphate buffered saline (PBS) and diluted to the concentrations used prior to employment in the LTT. Except for the penicillins, the drugs were tested in up to six different concentrations in order to cover the range of antigen-responsiveness and to identify toxic concentrations. The penicillins, except for flucloxacillin (0.01, 0.1, 0.5 mg/mL), were included in concentrations of 0.1, 0.5 and 1 mg/mL, which have been proved to be optimal for the stimulation of PBMC in the LTT in preceding experiments.

LTT

The LTT was performed according to a standard protocol, as described elsewhere [11, 23]. Briefly, PBMC from patients and controls were isolated from heparinized whole blood by gradient centrifugation over Ficoll-Hypaque solution (Pharmacia, Uppsala, Sweden) and cultured for 6 days in 96-well round-bottom plates (Becton Dickinson, Le Pont de Claix, France) as triplicate or sixfold cultures (200 μ L/well containing 2×10^5 PBMC) with various concentrations of the indicated drug dissolved in PBS or with PBS alone. Incubations of the PBMC with the mitogen phytohemagglutinin and tetanus toxoid served as positive controls for mitogen-induced and memory cell antigen-specific proliferative and secretory lymphocyte reactivity. In certain experiments with prohaptens (chlorazepate, carbamazepine, phenobarbital, phenytoin), PBMC and the drugs were pre-incubated with pooled human liver microsomes (20 mg/mL protein; NatuTec, Frankfurt, Germany) in parallel cultures in order to generate the ultimate neoantigen by drug metabolism, as described elsewhere [12]. For the last 18 h, 0.6 μ Ci ^{3} H-thymidine (NEN, Vilvoorde, Belgium) was added to each well. Cells were then harvested and incorporated radioactivity reflecting T-cell proliferation was measured in a scintillation counter (1214 Rackbeta, LKB Wallac, Düsseldorf, Germany) as counts per minute (cpm) and presented as mean plus standard deviation. Stimulation index (SI) represents the ratio of average cpm in cultures with PBMC from one individual with and without antigen.

Isolation and depletion of CD4⁺ and CD8⁺ T cells

Prior to incubation with the drugs in the LTT, CD4⁺ or CD8⁺ T cells were isolated or depleted from PBMC with anti-CD4 or anti-CD8 monoclonal antibody-coated magnetic beads (Dynal, Hamburg, Germany) in three patients, according to the manufacturer's recommendations, as described elsewhere [19, 24]. Depletion of CD4⁺ and CD8⁺ T cells was approved by flow cytometry analysis (FACSscan, Becton Dickinson, Heidelberg, Germany), resulting in a depletion of $\geq 98\%$ using FITC- or PE-conjugated anti-CD3⁺, -CD4⁺, -CD8⁺ and IgG1 and IgG2a isotype control antibodies (SimulTest[™] IMK-Lymphocyte, Becton Dickinson). After depletion, cell numbers were adjusted prior to employment of the PBMC fractions in the LTTs.

Determination of IL-5, IL-10 and IFN- γ in the culture supernatants

After 5 days, or at days 1 to 6, for investigation of the time course, 100 μ L culture supernatant of triplicate or sixfold cultures with PBMC from patients and controls were collected and stored at -20°C until use. After thawing, supernatants were diluted when high cytokine concentrations were expected. Concentrations of IL-5, IL-10 and IFN- γ were determined using commercially available specific sandwich enzyme-linked ELISAs (Immunotech, Marseille, France) with a sensitivity of 1 pg/mL, 5 pg/mL and 0.08 IU/mL, respectively, according to the manufacturer's recommendations. The mean concentration of each sample duplicate was calculated from the standard curve and the standard deviation of the duplicates was determined. Mean value and standard deviation were then multiplied by the dilution factor. Mathematically negative values were regarded as negative results.

IL-5 mRNA preparation and RT-PCR

mRNA was extracted from at least 2×10^6 PBMC with the Oligotex Direct mRNA-purification kit (Qiagen, Hilden, Germany) using a mRNA-enrichment protocol. mRNA concentration of each sample was measured using the Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany) and equal amounts of mRNA were used for reverse transcription. Drug-stimulated and PBS-incubated PBMC were referred to mRNA extraction after 1 to 5 days for investigation of the time course. Reverse transcription and PCR was performed with the GeneAmp RNA PCR kit (Perkin Elmer, Weiterstadt, Germany) according to the manufacturer's instructions. Primers used for IL-5 amplification were 5'-GCTTCTGCATTGAGTTTGCTAGCT-3' (sense) and 5'-TGGCCGTCAATGTATTTCTTTATTAAG-3' (antisense), which resulted in a 294-bp product. β -Actin was used as an internal standard as described before. Amplification was carried out with 35 cycles of 1 min denaturation at 93°C, 1 min annealing at 54°C and 1 min extension at 72°C. Amplification was terminated with an extension step of 5 min duration after the last cycle. PCR products were separated on 1.0% agarose gels (1 x TBE) and stained with ethidium bromide. With regard to the detection of IL-5 mRNA expression in CD4⁺ and CD8⁺ T cells, PBMC were incubated for 3 days with PBS or amoxicillin prior to isolation of CD4⁺ and CD8⁺ T cells from PBMC. In addition to the isolated CD4⁺ and CD8⁺ T cell fractions the PBMC fractions depleted of CD4⁺ and CD8⁺ T cells were referred to mRNA extraction for IL-5 RT-PCR.

Statistical analysis

With regard to the LTTs, a SI exceeding 2.5 (cut-off) was considered a significant positive result, in accordance with the international literature [25,26]. In contrast, no consensus exists regarding which cytokine ratio (average cytokine concentration in cultures with and without antigen (background)) can be designated as positive. We therefore decided arbitrarily for the present investigation that a ratio exceeding 3 should be regarded as a positive result. As with the LTT, the ratio was also referred to as SI. The sensitivity of drug-specific proliferation in the LTT, drug-specific cytokine secretion and patch tests were determined with regard to a stringent history of a drug-allergic skin reaction described as category A according to Nyfeler et al. [11]. Considering the potential risk of an iatrogen sensitization, the healthy controls were not patch tested with the drugs and hence the specificity of the patch-tests could not be determined. PBMC from patients of the first control group were not investigated for IL-10 and IFN- γ secretion.

Results

IL-5, IL-10 and IFN- γ secretion from drug-specific stimulated PBMC is time-dependent, peaking on days 4 to 6

The preceding experiments were carried out in order to assess the IL-5, IL-10 and IFN- γ secretion time course of *in vitro* drug-specific activated PBMC in primary cultures. The supernatants of LTTs from three patients (1,3,10) were taken at various time points between days 1 and 6. We found that drug-specific IL-5, IL-10 and IFN- γ secretion from PBMC gradually increased from day 2 to day 3, until reaching maximal

concentrations on days 4 to 6, whereas no significant cytokine concentrations could be measured in cultures without antigen (data not shown). In the subsequent experiments the supernatants were collected on day 5, as this time-point was considered to allow reliable detection of considerable amounts of the three cytokines. The cytokine secretion time course was paralleled by the drug-specific proliferation time course, exhibiting strongest proliferations between days 5 to 7 (data not shown). In analogy to the determination of IL-5 on the protein level, drug-specific IL-5 mRNA expression was assessed on days 1 to 5 by RT-PCR in patient 3 sensitized to amoxicillin. Signals detected on days 1, 2 and 4 were weaker than on day 3, whereas no signal was seen on day 5 (data not shown).

Drug-specific activation of PBMC consistently resulted in drug-specific proliferation and IL-5 secretion

Ten patients with maculopapular exanthems were included in the investigation. In each LTT, up to six concentrations of one drug were used, resulting in different amounts of drug-specific proliferations and IL-5 secretions per drug. Some patients showed proliferative and secretory lymphocyte reactivity to chemically related drugs (e.g. amoxicillin and ampicillin), resulting in a multitude of drug-specific proliferations and IL-5 secretions. Hence, a total of over 60 drug-specific proliferations and IL-5 secretions were observed. With regard to the SI of the LTTs, mainly moderate responses, rarely exceeding 10, were observed. In general, when drug-specific proliferation of PBMC was detected to a drug in different concentrations, secretion of IL-5 could also be demonstrated, often paralleling the proliferation. However, interindividual or drug-specific differences with regard to the total amount of IL-5 secreted were observed (Figs 1a and b).

Drug-specific proliferation and IL-5 secretion of PBMC from the patients were antigen-specific

The observed *in vitro* proliferative and secretory (IL-5) lymphocyte reactivity to the drugs was antigen-specific. In case of side chain-specific drug-sensitization to aminopenicillins [27] (patient 1,3,4) different penicillins were included in the *in vitro* tests and drug-specific proliferation and IL-5 secretion were only seen with the aminopenicillins (Figs 2a and b), confirming the specificity of both *in vitro* tests. In patient 6, incubation of the PBMC with the culprit drug fenoterol, but not with the chemically related propranolol, resulted in proliferative and secretory lymphocyte reactivity.

Detection of drug-specific IL-5 secretion of PBMC proved a sensitive parameter

Detection of drug-specific proliferation in the LTT and IL-5 secretion from the PBMC of the 10 patients proved reliable sensitive parameters for the detection of drug-sensitization as compared with patch tests (Table 1). Differences in the calculated sensitivity of the LTT (75%) and the IL-5 secretion (92%) resulted only from the diverse proliferative and secretory (IL-5) lymphocyte reactivity of the PBMC from patient 8. This patient had a history of a maculopapular exanthem as part of an anticonvulsive hypersensitivity syndrome following intake of carbamazepine, phenobarbital and phenytoin, which is in accordance with the definition of this syndrome [20, 21]. Hence, with regard to the calculation of the sensitivity all three anamnestic events

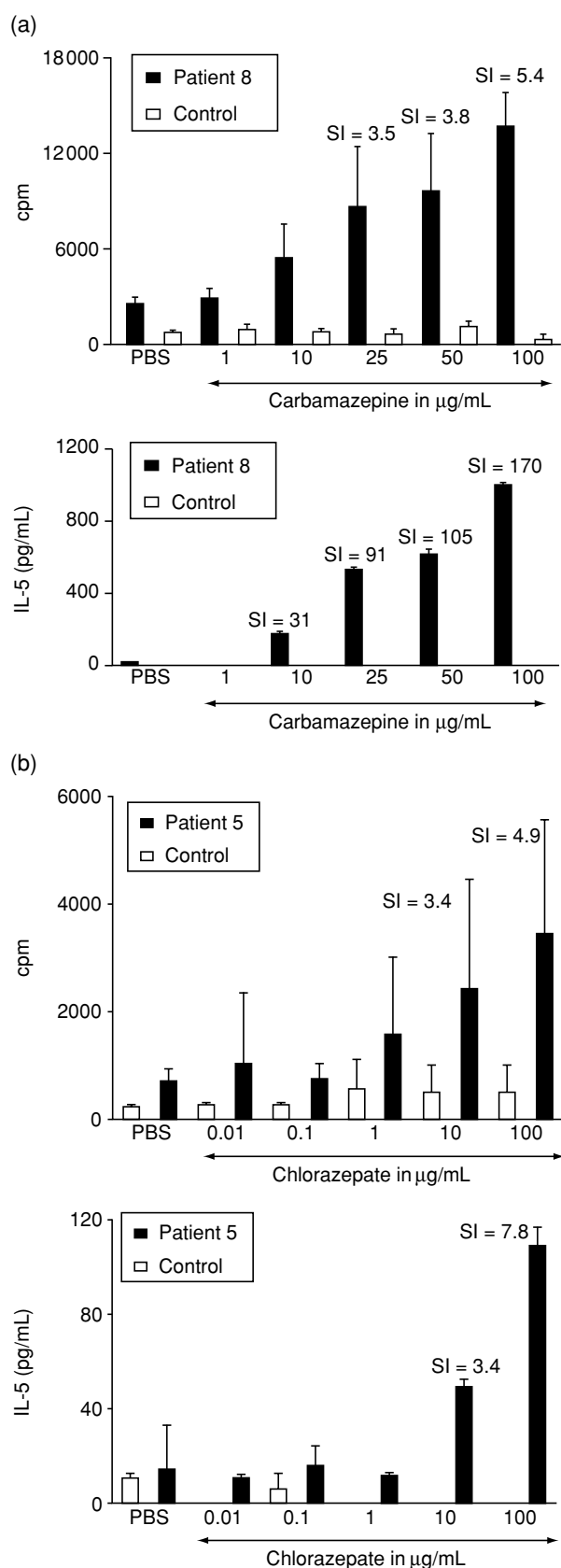


Fig. 1. *In vitro* drug-specific proliferation is associated with IL-5 secretion. PBMC from patient 8 with history of an anticonvulsant hypersensitivity syndrome after intake of carbamazepine, phenytoin and phenobarbital (a) and PBMC from patient 5 with chlorazepate-induced

were taken into account. In the LTT only positive reactions to carbamazepine were detected, whereas drug-specific IL-5 secretion was additionally observed to phenytoin and phenobarbital, although only at a single concentration, respectively. If the sensitivity was calculated only with regard to the carbamazepine-induced exanthem, excluding the phenytoin and phenobarbital-induced skin eruptions, the sensitivity would be 90% for both methods.

Neither PBMC from control persons of the first (without exposure to the drug) nor of the second (with exposure to the drug without clinical symptoms) control group proliferated or secreted IL-5 when incubated with the various drugs. However, proliferation and IL-5 secretion was detectable following stimulation with tetanus toxoid or the mitogen phytohemagglutinin. Hence, the specificity of drug-specific proliferation (LTT) and IL-5 secretion was 100% for both test systems (Table 1).

No consistent secretion of IL-10 and IFN- γ by drug-specific activated PBMC

In contrast to drug-specific proliferation and IL-5 secretion, drug-induced activation of PBMC from the 10 patients with maculopapular exanthems did not consistently lead to the secretion of significant amounts of IL-10 and IFN- γ with regard to a positive SI, resulting in a poor sensitivity of these parameters (50%, 36%, respectively). Considering the calculation of the specificity, only PBMC from persons of the second control group (with exposure to the drug) were investigated. Whereas no drug-specific secretion of IL-10 was observed following incubation of the PBMC from the control persons with the drugs, significant amounts of IFN- γ were secreted from PBMC of two control persons, resulting in a specificity of 100% and 60% for these parameters, respectively.

Demonstration of drug-specific lymphocyte reactivity failed in three patients with severe drug reactions

In the three additional patients with severe drug-reactions differing from the clinical entity of maculopapular exanthems no drug-specific lymphocyte reactivity could be demonstrated either *in vivo* by patch tests or *in vitro* by LTT or detection of drug-specific IL-5 secretion (data not shown). As two patients suffered from a bullous drug-induced skin reaction suggesting involvement of cytotoxic lymphocytes their PBMC were also investigated for *in vitro* drug-specific IFN- γ secretion, which could not be detected.

Drug-specific proliferation can be attributed to CD4⁺ or CD8⁺ T cells

It has been shown that in primary cultures both CD4⁺ and CD8⁺ T lymphocytes are activated by drugs [28]. However, drug-specific proliferating T cell clones generated *in vitro* were reported to either express a CD4⁺ or CD8⁺ phenotype [25,29,30]. We found that depletion of CD8⁺ cells of the PBMC from two patients (1, 3) with sensitization to amoxicillin did not reduce amoxicillin-specific proliferation, whereas depletion of

maculopapular exanthem (b), were incubated with carbamazepine or chlorazepate, respectively, and drug-specific proliferation and IL-5 secretion were determined. Carbamazepine-stimulated PBMC of patient 8 secreted about 10 times more IL-5 than the chlorazepate-stimulated PBMC of patient 5, suggesting interindividual or drug-specific differences.

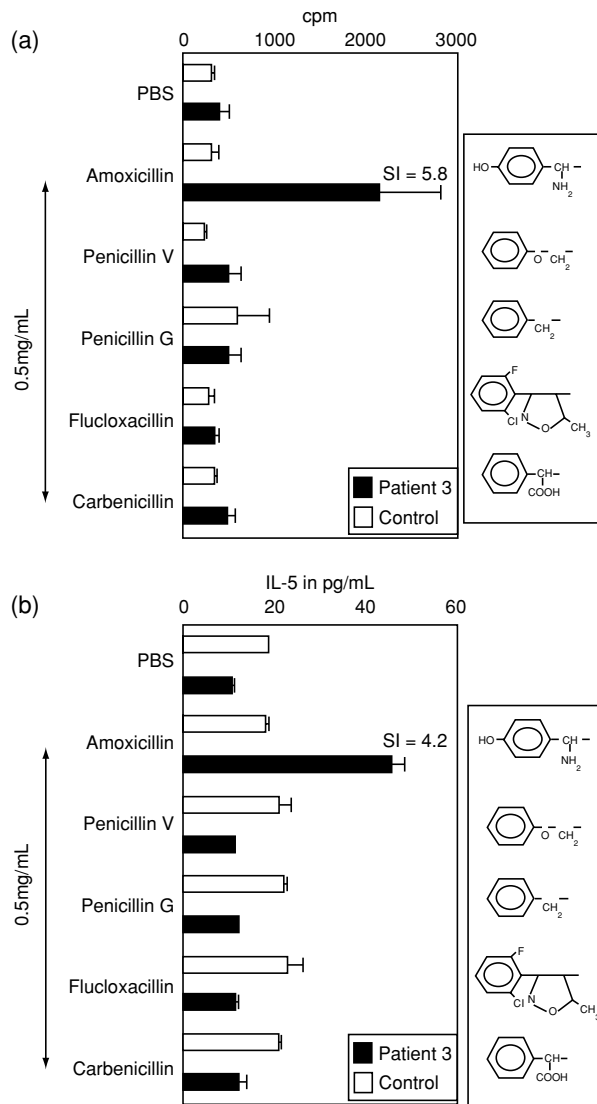


Fig. 2. (a) and (b). Drug-specific proliferation and IL-5 secretion was antigen-specific. PBMC from three patients with side chain-specific sensitization to aminopenicillins were incubated with various penicillins and drug-specific proliferation and IL-5 secretion were determined. Drug-specific proliferation and IL-5 secretion were only seen to aminopenicillins, confirming the specificity of both *in vitro* tests. A representative experiment with PBMC from patient 3 at one penicillin concentration (0.5 mg/mL) is shown.

CD4⁺ cells abrogated drug-specific proliferation, suggesting that drug-specific proliferation was mediated by CD4⁺ T cells (Fig. 3a). In contrast, depletion of CD4⁺ cells from the PBMC of patient 10 obviously enhanced drug-specific proliferation (SI = 26.3), suggesting a suppressive role for the CD4⁺ T cells, whereas depletion of CD8⁺ cells reduced drug-specific proliferation (SI = 2.3), resulting in a negative result with regard to the definition of the SI. Hence, drug-specific proliferation of the PBMC from patient 10 was attributed to CD8⁺ T cells (Fig. 3b).

CD4⁺ and CD8⁺ T cells may contribute to drug-specific IL-5 secretion

In two patients (1,3) with drug-specific proliferation mediated by CD4⁺ T cells, depletion of CD4⁺ T cells abrogated drug-specific IL-5 secretion (Fig. 4a). Depletion of CD8⁺

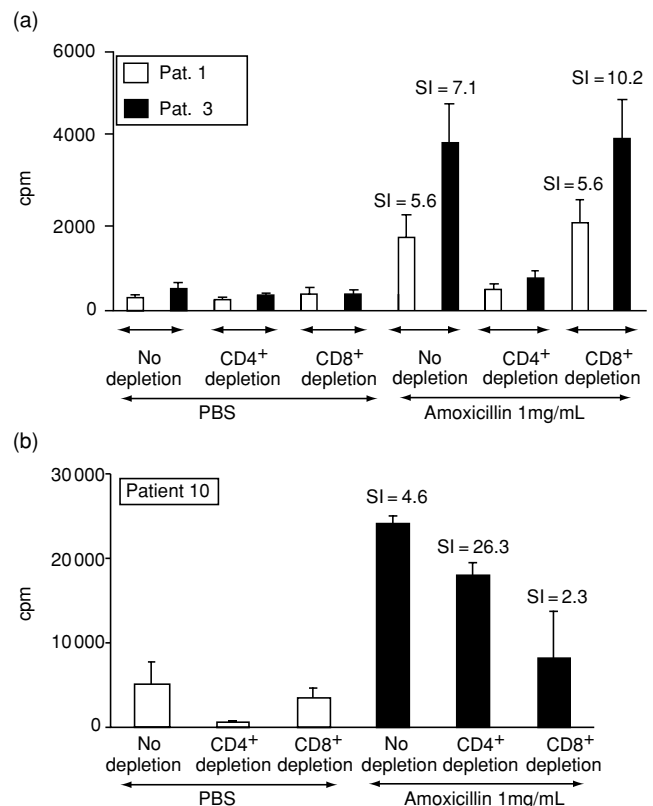


Fig. 3. Drug-specific proliferation can be attributed to CD4⁺ or CD8⁺ T cells. PBMC from three patients with known sensitization to aminopenicillins were either depleted of CD4⁺ or CD8⁺ T cells and incubated with amoxicillin or the solvent PBS. Depletion of CD4⁺ cells from the PBMC of patients 1 and 3 abrogated drug-specific T cell proliferation, whereas depletion of CD8⁺ cells did not suppress drug-specific T cell proliferation significantly (a). In contrast, depletion of CD4⁺ T cells from PBMC of patient 10 enhanced drug-specific proliferation, whereas depletion of CD8⁺ T cells reduced drug-specific proliferation of PBMC (b).

T cells also decreased drug-specific IL-5 secretion to some extent, suggesting that besides the CD4⁺ T cells, CD8⁺ T cells could be involved in drug-specific IL-5 secretion. In patient 1 the IL-5 background secretion of CD8⁺-depleted PBMC was below the detection limit, hence IL-5 secretion in amoxicillin-stimulated CD8⁺-depleted PBMC was divided by the detection limit of the IL-5 specific ELISA (1.5 pg/mL). In patient 10, drug-specific proliferation was attributed to CD8⁺ T cells. Depletion of CD4⁺ T cells from his PBMC resulted in increased drug-specific IL-5 secretion (SI = 6.9), whereas depletion of CD8⁺ T cells reduced drug-specific IL-5 secretion (SI = 3.2). However, it should be noted that the definition of the SI indicated a positive result for the IL-5 secretion (Fig. 4b). In summary, unlike drug-specific proliferation, drug-specific IL-5 secretion could not exclusively be attributed to a distinct T cell subset, suggesting that both CD8⁺ and CD4⁺ T cells may contribute to drug-specific IL-5 secretion.

Drug-specific IL-5 mRNA expression was found in both CD4⁺ and CD8⁺ T cells

PBMC from two patients (3,10) with drug-specific proliferation mediated by CD4⁺ or CD8⁺ T cells, respectively, were investigated for drug-specific IL-5 mRNA expression.

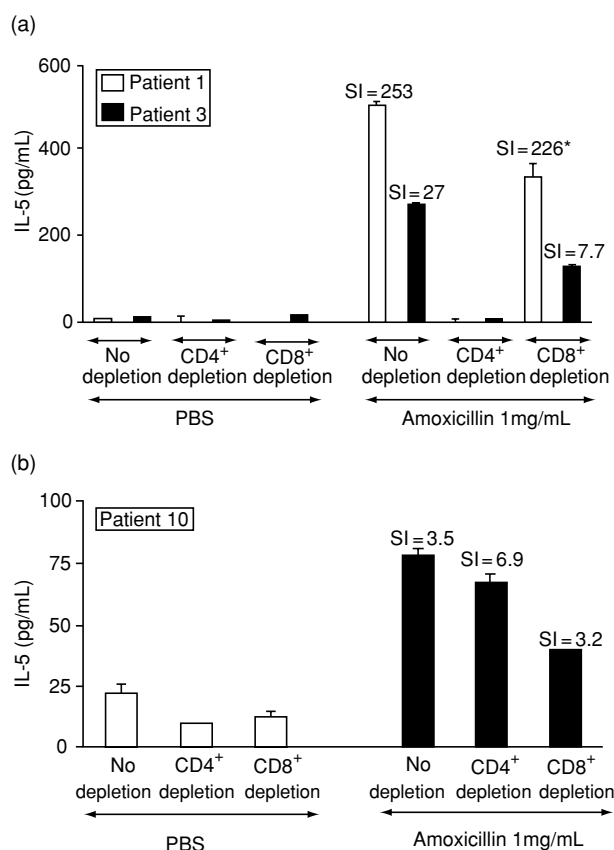


Fig. 4. Drug-specific IL-5 secretion may be attributed to CD4⁺ or CD8⁺ T cells. PBMC from three patients with known sensitization to aminopenicillins were either depleted of CD4⁺ or CD8⁺ T cells and incubated with amoxicillin or the solvent PBS. Depletion of CD4⁺ T cells from the PBMC of patients 1 and 3 resulted in abrogation of IL-5 secretion, whereas depletion of CD8⁺ T cells reduced IL-5 secretion (a). In contrast, depletion of CD4⁺ T cells from patient 10 enhanced drug-specific IL-5 secretion, whereas depletion of CD8⁺ T cells slightly reduced IL-5 secretion resulting in a SI of 3.2 (b). In patient 1 the IL-5 background secretion of CD8⁺-depleted PBMC was below the detection limit, hence IL-5 secretion in amoxicillin-stimulated CD8⁺-depleted PBMC was divided by the detection limit of the IL-5-specific ELISA (1.5 pg/mL)(*).

Isolated CD4⁺ and CD8⁺ T cells of PBMC from both patients showed IL-5 mRNA expression when incubated with amoxicillin but not when incubated with PBS. In addition, IL-5 mRNA expression could also be detected in the CD4⁺ and CD8⁺ depleted PBMC fractions following incubation with amoxicillin but not with PBS, suggesting that drug-specific activation of PBMC results in IL-5 mRNA expression in both T-cell subsets (Fig. 5). No drug-specific IL-5 mRNA expression could be detected in PBMC from a healthy control person (data not shown).

Discussion

In the present study we could demonstrate that *in vitro* drug-specific activation of *ex vivo* PBMC from nine patients with drug-induced maculopapular exanthems consistently results in drug-specific IL-5 secretion by the PBMC. Furthermore, determination of drug-specific IL-5 secretion turned out to be a more sensitive parameter (92%) for the *in vitro* detection of drug-sensitized T cells than the LTT (75%) or patch tests (55%)

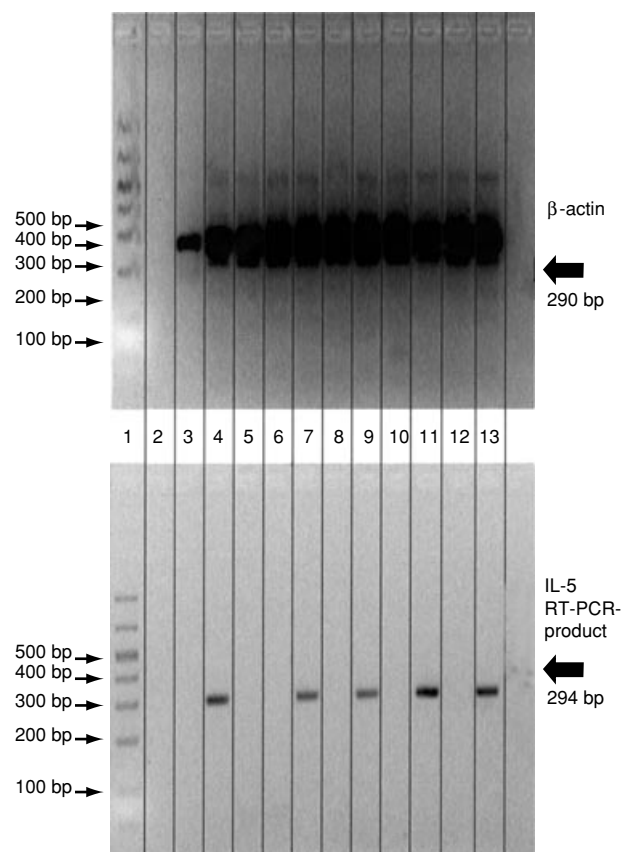


Fig. 5. Drug-specific IL-5 mRNA expression can be attributed to CD4⁺ and CD8⁺ T cells. PBMC from two patients sensitized to penicillins were incubated with amoxicillin, benzyl-penicillin or the solvent PBS. After 3 days one part of the PBMC was frozen directly, whereas CD4⁺ and CD8⁺ T cells were isolated from the other part before both isolated and depleted cell fractions were frozen. With regard to the patients' PBMC, IL-5 mRNA expression could not be detected in PBMC, nor in CD4⁺ or CD8⁺ isolated and CD4⁺ and CD8⁺ depleted cell fractions incubated with PBS. Drug-specific IL-5 mRNA expression was observed in PBMC incubated with amoxicillin, in CD4⁺ and CD8⁺ isolated T cell fractions and in the CD4⁺ and CD8⁺ depleted cell fractions. Lane 1 = molecular weight marker; lane 2 = PCR negative control; lanes 3, 4 and 5 = PBMC incubated with PBS, amoxicillin, benzyl-penicillin; lanes 6 and 7 = CD4⁺ isolated cells incubated with PBS, amoxicillin; lanes 8 and 9 = CD4⁺ depleted PBMC fraction incubated with PBS, amoxicillin; lanes 10 and 11 = CD8⁺ isolated cells incubated with PBS, amoxicillin; lanes 12 and 13 = CD8⁺ depleted PBMC fraction incubated with PBS, amoxicillin. One representative experiment from patient 3 with side chain-specific sensitization to aminopenicillins is shown.

with regard to a stringent history of a drug-allergic reaction. In addition, drug-specific IL-5 mRNA expression could be detected after 3 days, thus offering a saving of time compared with the LTT. Hence, *in vitro* detection of drug-specific IL-5 secretion proved a non-radioactive, sensitive alternative, for the *in vitro* detection of drug-sensitization in patients with maculopapular exanthems in the present investigation. This method was not confined to beta-lactams, which reliably induce *in vitro* activation of sensitized T cells, but also worked with different drugs.

In vitro IL-5 production of drug-specific T cells, T cell lines and clones has been described before [12, 28, 31–33] but mainly in conjunction with other cytokines (IFN-γ, IL-4, IL-10) in order to classify T cell responses according to a Th1, Th2 or Th0 pattern. However, the determination of drug-specific IL-5 secretion with regard to the identification of a diagnostic

parameter for the *in vitro* detection of drug sensitization in patients with the distinct phenotype of maculopapular exanthems has not yet been studied.

In addition, IL-5 secretion of polyclonal PBMC was assessed after 5 days following antigen (drug)-specific instead of unspecific stimulation [23, 25, 34, 35], which may explain the lower IL-5 concentrations detected in our system compared with stimulations with mitogen. Beyond that, in the present study, polyclonal peripheral lymphocytes were used, whereas in several studies describing cytokine release oligoclonal T cell lines and monoclonal T cell clones were investigated [23, 25].

The observed drug-induced IL-5 secretion was antigen-specific. Incubations of the patients' PBMC with irrelevant antigens like benzyl-penicillin in case of side chain-specific sensitization to aminopenicillins [27] did not result in proliferation or IL-5 secretion (Fig. 2a, b). Furthermore, PBMC from control persons with and without exposure to drugs did not secrete IL-5 when incubated with the drugs, resulting in a specificity of 100%.

In our experiments drug-specific IL-5 secretion was time dependent. Highest IL-5 concentrations in the supernatants were detected about day 5, as has been reported by others [18, 28, 31]. One reason for the *in vitro* IL-5 concentration reaching maximum levels later than, for example IL-4, which was reported to decrease after 3 days [36], may be that IL-5 is, unlike the *in vivo* situation, not consumed by eosinophils. The observed IL-5 time course following drug-specific proliferation *in vitro* may therefore reflect an IL-5 accumulation.

In contrast to IL-5, no consistent secretion of significant amounts of IL-10 and IFN- γ were detected in the culture supernatants following stimulation with the appropriate drug (Table 1), resulting in a sensitivity of 50% and 36%, respectively. In addition, PBMC from two control persons of the second control group secreted significant amounts of IFN- γ when incubated with amoxicillin. Hence, differing from other findings [37], the Th1-like cytokine IFN- γ did not prove a helpful parameter for the *in vitro* detection of drug sensitization in our test system, although patients with delayed-type drug-allergic reactions were investigated. IL-10 was included in our test battery because it is considered a Th2-like cytokine that can skew the lymphocyte response from a Th1 to a Th2 pattern. However, the observed poor *in vitro* secretion of IFN- γ following challenge of the patients' PBMC with the culprit drug could not be attributed to IL-10 secretion, which occurred infrequently.

In contrast to the 10 patients with maculopapular exanthems, no specific lymphocyte reactivity in the three additional patients with severe drug-induced skin eruptions could be demonstrated by patch tests, LTT and determination of drug-specific IL-5, as reported by other investigators [38]. The reason for this observation remains speculative. Severe bullous drug-eruptions are also regarded as T cell-mediated reactions but may involve different T cell subsets exerting distinct pathogenic mechanisms, which may end up with keratinocyte-induced apoptosis by the up-regulation of Fas and FasL on keratinocytes [39]. Therefore, the appropriate diagnostic tools should be selected with regard to the clinical phenotype of the drug-induced skin reaction, which often represents a distinct pathophysiology.

IL-5 is considered a Th2-like cytokine and *in vitro* IL-5-secretion of PBMC from patients with immediate-type immune reactions could be attributed to Th2-skewed effector

mechanisms. In the present investigation only patients with maculopapular exanthems representing delayed-type drug-allergy were included, which, in contrast to immediate-type reactions, was reported to be associated with a Th1 *in vitro* cytokine profile [24]. However, we observed that PBMC from nine patients with delayed-type drug-allergic reactions irrespective of an atopic status secreted significant amounts of IL-5 upon specific antigen challenge *in vitro*, suggesting that *in vitro* secretion of IL-5 occurs in delayed-type and immediate-type drug-allergic reactions. This, at first sight contradictory finding, suggests that drugs as non-peptide small molecular weight compounds may exert distinct T cell reactions, e.g. secretion of IL-5, which rather reflect a general mechanism following T cell activation by drugs and cannot be attributed to a certain clinical phenotype or an atopic status of the individual.

In the present investigation PBMC from three patients with sensitization to amoxicillin were referred to CD4⁺ and CD8⁺ isolation and depletion experiments. Drug-induced proliferation attributed to CD4⁺ cells was found with PBMC of patients 1 and 3, whereas drug-specific proliferation from PBMC of patient 10 was attributed to CD8⁺ T cells (Figs 3a and b). The results obtained with regard to drug-specific IL-5 secretion and mRNA expression of CD4⁺ or CD8⁺ T cells were more heterogeneous, suggesting that both T cell subsets could be involved. These findings are supported by the data of others describing specific activation of both CD4⁺ and CD8⁺ T cells by drugs depending on the nature of the antigen [28]. Whereas stimulation of drug-specific T cells with drug-protein conjugates induced activation of CD4⁺ T cells only, challenge with the free drug resulted in stimulation of CD4⁺ and CD8⁺ T cells [24, 25]. These results were brought in relation to MHC restriction and processing requirements of free or protein-conjugated drugs [28]. However, the *ex vivo* PBMC employed in our investigation represent a section of the *in vivo* circulating drug-specific T cell clones, which may either be of the CD4⁺ or the CD8⁺ phenotype [25, 29].

With regard to our experimental design, the free drug amoxicillin was added to LTT cultures enriched with 5% autologous plasma, instead of the fetal calf serum used by other investigators [24]. The reactive free drug amoxicillin may rapidly bind to some extent to the autologous plasma proteins contained in the *in vitro* cultures, resulting in immunogenic protein-conjugated antigen and unconjugated free amoxicillin antigen, which could activate both CD4⁺ and CD8⁺ T cells. In summary, it may be speculated that drug-specific proliferation, probably representing a more distal effector mechanism, was attributed to CD4⁺ or CD8⁺ T cells, whereas drug-specific IL-5 mRNA expression and to a lesser extent IL-5 secretion, probably reflecting an earlier stage of T cell activation, could be attributed to both subsets. Hence, drug-specific proliferation and IL-5 secretion could be considered as two independent read-out parameters, both of which – although consistently associated – may not be linked in a causal sense such as drug-specific proliferation and IL-2 secretion.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft grant (SA 866/1–1), by the START-program of the Rheinisch-Westfälische Technische Hochschule Aachen, and by the Interdisciplinary Center for Clinical Research in

Biomaterials and Tissue–Material Interaction in Implants (BMBF project No.01 KS 9503/9).

References

- Hoigné R, Schlumberger HU, Vervloet D, Zoppi M. Epidemiology of allergic reactions. In: Burr M, ed. *Epidemiology of Clinical Allergy. Epidemiology of Allergic Reactions to Drugs*. Monographs in Allergy, Vol. 31. Basel: Karger, 1993:140–70.
- Roujeau J-C, Kelly JP, Naldi L et al. Medication use and the risk of Stevens–Johnson syndrome or toxic epidermal necrolysis. *N Engl J Med* 1995; 333:1600–7.
- Sachs B, Rönna AC, Ruzicka T, Gleichmann E, Schuppe H-C. Lamotrigine and toxic epidermal necrolysis. *Lancet* 1996; 348:1597 (letter).
- Sullivan JR, Shear NH. What are some of the lessons learnt from *in vitro* studies of severe unpredictable drug reactions? *Br J Dermatol* 2000; 142:205–7.
- Reilly TP, Lawrence HL, Doll MA et al. A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. *J Invest Dermatol* 2000; 114:1164–73.
- Wolkenstein P, Tan C, Lecoer S et al. Covalent binding of carbamazepine reactive metabolites to P450 isoforms present in the skin. *Chem Biol Interact* 1998; 113:39–50.
- Blanca M, Posadas S, Torres MJ et al. Expression of the skin-homing receptor in peripheral blood lymphocytes from subjects with nonimmediate cutaneous allergic drug reactions. *Allergy* 2000; 55:998–1004.
- Yawalkar N, Egli F, Hari Y et al. Infiltration of cytotoxic T cells in drug-induced cutaneous eruptions. *Clin Exp Allergy* 2000; 30:847–55.
- Barbaud A, Reichert-Penetrat S, Trechot P et al. The use of skin testing in the investigation of cutaneous adverse drug reactions. *Br J Dermatol* 1998; 139:49–58.
- Romano A, Torres MJ, Quarationo D et al. Diagnostic evaluation of delayed hypersensitivity to systemically administered drugs. *Allergy* 1999; 54:S23–7.
- Nyfel B, Pichler WJ. The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. *Clin Exp Allergy* 1997; 27:175–81.
- Sachs B, Erdmann S, al Masaoudi T, Merk HF. In vitro drug-allergy detection incorporating human liver microsomes in chlorazepate-induced skin rash: drug-specific proliferation associated with interleukin-5 secretion. *Br J Dermatol* 2001; 144:316–20.
- Hertl M, Bohlen H, Jugert F et al. Predominance of epidermal CD8+ T lymphocytes in bullous cutaneous reactions caused by beta-lactam antibiotics. *J Invest Dermatol* 1993; 101:794–9.
- Kubota Y, Imayama S, Toshitani A et al. Sulfidoleukotriene release test (CAST) in hypersensitivity to nonsteroidal anti-inflammatory drugs. *Int Arch Allergy Immunol* 1997; 114:361–6.
- Paris-Köhler A, Demoly P, Persi L et al. In vitro diagnosis of cypress allergy by using cytofluorimetric analysis of basophils (Basotest). *J Allergy Clin Immunol* 2000; 105:339–45.
- Lanzavecchia A, Sallusto F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 2000; 290:92–7.
- Yawalkar N, Shrikhande M, Hari Y et al. Evidence for a role for IL-5 and eotaxin in activating and recruiting eosinophils in drug-induced cutaneous eruptions. *J Allergy Clin Immunol* 2000; 106:1171–6.
- Choquet-Kastylevsky G, Intrator L, Chenal C et al. Increased levels of interleukin 5 are associated with the generation of eosinophilia in drug-induced hypersensitivity syndrome. *Br J Dermatol* 1998; 139:1026–32.
- Till S, Dickason R, Huston D et al. IL-5 secretion by allergen-stimulated CD4+ T cells in primary cultures: relationship to expression of allergic disease. *J Allergy Clin Immunol* 1997; 99:563–9.
- De Shazo R, Kemp SF. Allergic reactions to drugs and biologic agents. *JAMA* 1997; 278:1895–906.
- Shear N, Spielberg S. Anticonvulsant hypersensitivity syndrome. *J Clin Invest* 1988; 82:1826–32.
- Diepgen TL, Fartasch M, Hornstein OP. Kriterien zur Beurteilung der atopischen Hautdiathese. *Dermatosen* 1991; 39:79–83.
- Brugnolo F, Annunziato F, Sampognaro S et al. Highly Th2-skewed cytokine profile of beta-lactam specific T cells from nonatopic subjects with adverse drug reactions. *J Immunol* 1999; 163:1053–9.
- Gaspard I, Guinépain MT, Laurent J et al. IL-4 and IFN- γ mRNA induction in human peripheral lymphocytes specific for β -lactam antibiotics in immediate and delayed hypersensitivity reactions. *J Clin Immunol* 2000; 20:107–16.
- Brander C, Mauri-Hellweg D, Bettens F et al. Heterogeneous T cell response to β -lactam-modified self-structures are observed in penicillin-allergic individuals. *J Immunol* 1995; 155:2670–80.
- Schnyder B, Pichler WJ. Skin and laboratory tests in amoxicillin- and penicillin-induced morbilliform skin eruption. *Clin Exp Allergy* 2000; 30:590–5.
- Patriarca G, D'Ambrosio C, Schivino D et al. Clinical usefulness of patch and challenge tests in the diagnosis of cell mediated allergy to beta lactams. *Ann Allergy Asthma Immunol* 1999; 83:257–66.
- Mauri-Hellweg D, Bettens F, Mauri D et al. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin and carbamazepine. *J Immunol* 1995; 155:462–72.
- Weltzien HU, Padovan E. Molecular features of penicillin allergy. *J Invest Dermatol* 1998; 110:203–6.
- Zanni MP, von Greylz S, Schnyder B et al. HLA-restricted, processing and metabolism-independent pathway of drug recognition by human $\alpha\beta$ T lymphocytes. *J Clin Invest* 1998; 102:1591–8.
- Pichler WJ, Zanni M, von Greylz S et al. High IL-5 production by human drug-specific T cell clones. *Int Arch Allergy Immunol* 1997; 113:177–80.
- Yawalkar N, Reimers A, Hari Y et al. Drug-induced linear IgA bullous dermatosis associated with ceftriaxone- and metronidazole-specific T cells. *Dermatology* 1999; 199:25–30.
- Cederbrant K, Maruccsson-Stahl M, Hultman P. Characterization of primary recall in vitro lymphocyte responses to bacampicillin in allergic subjects. *Clin Exp Allergy* 2000; 30:1450–9.
- Lagging E, van Hage-Hamsten M, Grönneberg R, Elfmann L, Härfast B. Cytokine production in PBMC from allergics and non-allergics following in vitro allergen stimulation. *Immunol Lett* 1998; 60:45–9.
- Mikami C, Ochiai K, Kagami M, Tomioka H, Tanabe E. In vitro interleukin-5 (IL-5) production by peripheral blood mononuclear cells from patients with drug hypersensitivity. *J Dermatol* 1996; 23:379–81.
- Jung T, Wijdenes J, Neumann C, de Vries JE, Yssel H. Interleukin-13 is produced by activated human CD45RA+ and CD45RO+ T cells: modulation by interleukin-4 and interleukin-12. *Eur J Immunol* 1996; 26:571–7.
- Halevy S, Cohen A, Livni E. Acute generalized exanthematous pustulosis associated with polysensitivity to paracetamol and bromhexine: the diagnostic role of in vitro interferon-gamma release test. *Clin Exp Dermatol* 2000; 25:652–4.
- Roujeau JC, Albengres E, Moritz S et al. Lymphocyte transformation test in drug-induced toxic-epidermal necrolysis. *Int Arch Allergy Appl Immunol* 1985; 78:22–4.
- Viard I, Wehrli P, Bullani R et al. Inhibition of toxic epidermal necrolysis by blockade of CD95 with human intravenous immunoglobulin. *Science* 1998; 282:490–3.