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Original article

Detection and quantification of drug-specific T cells in penicillin allergy

Drug allergic reactions presenting as maculo-papular exanthema (MPE) are mediated by drug-specific T cells. In this study, the frequency of circulating specific T cells was analyzed by interferon-γ (IFN-γ) enzyme-linked immunospot assay in 22 patients with an allergic MPE to amoxicillin (amox). Amox-specific circulating T cells were detected in 20/22 patients with frequencies ranging from 1 : 8000 to 1 : 30 000 circulating leucocytes. No reactivity was observed in 46 control patients, including 15 patients with immunoglobulin E-mediated allergy to amoxicillin, 11 patients with a history of drug-induced MPE but tolerant to amoxicillin and 20 healthy individuals. Furthermore, amox-specific T cells were still detectable several years after the occurrence of the allergic reaction even after strict drug avoidance. Finally, analysis of drug-specific T cells in one patient allergic to ticarcillin (a penicillin antibiotic distinct from amox) revealed the presence of IFN-γ-producing T cells reactive to ticarcillin and several other betalactam antibiotics, suggesting that the IFN-γ ELISPOT assay is able to detect T cell cross-reactivity against chemically related drugs. These findings confirm that drug-induced MPE is associated with the presence of specific T cells in blood and further suggest that the IFN-γ ELISPOT is a sensitive assay which could improve the diagnosis of betalactam allergy.

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Cutaneous reactions are usual manifestations of drug allergy and are considered as immediate hypersensitivity (IHS) or delayed hypersensitivity (DTH) reactions involving specific immunoglobulin E or T cells, respectively (1, 2). Betalactam antibiotics are frequently responsible for drug allergy. They comprise more than 50 different molecules grouped into two major families, namely penicillins and cephalosporins, which share a betalactam ring and may have identical side chains. Amoxicillin, one of the most frequently prescribed penicillin antibiotics, is responsible for a large number of betalactam DTH allergic reactions, ranging from the frequent maculo-papular exanthema (MPE) to the rare Lyell's syndrome and acute generalized exanthematous pustulosis (3).

Maculo-papular exanthema (MPE) is one of the most frequent clinical forms of drug-induced cutaneous adverse reactions, occurring in the hours or days following the onset of treatment. The name MPE refers to different clinical presentations which include localized or generalized: (i) erythema; (ii) macular eruption; (iii) limited sized papules or large infiltrated plaques; (iv) eczema-like dermatitis. Although there is *in vivo* and *in vitro* evidence for the contribution of T cells in the pathogenesis of MPE (1, 4), the underlying immune mechanism is still unclear. It is postulated that drug MPE patients harbour circulating specific T cells which become activated in the skin upon exposure to the drug and induce MPE via both the release of pro-inflammatory Th1 and Th2 cytokines and cytotoxicity (3). Interferon γ, a type 1 cytokine, the expression of which is restricted to activated T cells, appears crucial in the pathophysiology of MPE (5), inasmuch as it is produced in patients' skin and blood (3).

However, the occurrence of MPE during a drug treatment is not always mediated by a drug-specific DTH reaction involving T cells but can result from nonspecific activation of innate immunity by the pro-inflammatory properties of drugs (6–8). Maculo-papular

Abbreviations: amox, amoxicillin; ceft, ceftriaxon; LTT, lymphocyte transformation test; MPE, maculo-papular exanthema; IHS, immediate hypersensitivity; DTH, delayed hypersensitivity; SFC, spot forming cell.

exanthema may even develop independently of drug administration such as in infectious MPE (9, 10). Therefore, the formal diagnosis of drug allergic MPE requires evidence for the presence of drug-specific T cells in the patient's skin and/or blood. Skin patch tests with the relevant drug can reproduce the local skin inflammation in patients, therefore allowing the diagnosis of drug-induced DTH reaction (11–13). Detection of drug-reactive T cells in the patient's blood is carried out by cell proliferation assays (usually determined by 3H-thymidine incorporation into DNA) such as in the lymphocyte transformation test (LTT) or by ELISA titration of Th1/Th2 cytokines into culture supernatant (14, 15). Several studies have shown that the LTT assay is useful for the diagnosis of penicillin DTH allergy with a sensitivity ranging from 60% to 75% (1, 14). However, a positive LTT assay does not necessarily prove the involvement of T cells, since B cells present in peripheral blood mononuclear cells (PBMC) may also proliferate in response to the drug. Moreover, assessment of the drug specificity of the proliferative response is required to exclude bystander nonspecific activation.

The enzyme-linked immunospot (ELISPOT) assay is a rapid, sensitive and reproducible technique allowing for analysis of the frequency of antigen-specific, cytokine-producing cells (16). The interferon-γ (IFN-γ) ELISPOT assay allows enumeration of low-frequency antigen-specific T-cells with a sensitivity comparable to that of peptide/MHC multimer staining (17). Recent studies have confirmed the usefulness of the IFN-γ ELISPOT for direct *ex-vivo* quantification of peptide- or protein-reactive circulating T lymphocytes in infectious diseases, in patients receiving immunotherapy for cancer and in some forms of drug DTH allergies (18–25).

Because IFN-γ is a key cytokine in the pathophysiology of drug allergy, we tested the ability of the IFN-γ ELISPOT assay to detect drug-specific T cells in 22 patients with a diagnosis of amoxicillin DTH allergy expressing as MPE. Results showed that penicillin-specific T cells were detected in the blood of a vast majority of the patients (20/22) suggesting that MPE is mediated by IFN-γ-producing T cells and that the IFN-γ ELISPOT assay could represent a useful test for *in vitro* diagnosis of drug DTH allergy.

Material and methods

Patients

Thirty-eight patients with a history of immediate (IHS) or delayed (DTH) allergy to penicillin (amoxicillin: 37 patients and ticarcillin: one patient) were included in this study. The patients were analyzed in clinical remission of their drug allergy and were healthy at the time of analysis. The interval between acute allergy and immuno-allergological analysis ranged from 6 months to 32 years. The main clinical data are summarized in Table 1.

Group A (amox-DTH) comprised 23 patients with well-documented history of T cell-mediated (DTH) allergy to amoxicillin (22) and ticarcillin (1) based on the following criteria: (i) maculo-papular exanthema during treatment with amox (developing more than 6 h, but generally within 24–48 h, after penicillin administration), except

Table 1. Clinical characteristics of patients

Patient no.	Type of reaction	Year of reaction	Skin patch-test		Skin prick or intradermal test	
			Amox	Ceft	Amox	Ceft
Amox DTH allergy (group A)						
1	Maculo-papular exanthema	1983	+	–	NT	NT
2	Maculo-papular exanthema	2002	+	–	NT	NT
3	Maculo-papular exanthema	1975	+	–	NT	NT
4	Maculo-papular exanthema	2003	+	–	NT	NT
5	Maculo-papular exanthema	2002	+	–	NT	NT
6	Maculo-papular exanthema	2000	+	–	NT	NT
7	Maculo-papular exanthema	1995	+	–	NT	NT
8	Maculo-papular exanthema	2001	+	–	NT	NT
9	Maculo-papular exanthema	2000	+	–	NT	NT
10	Maculo-papular exanthema	2001	+	–	NT	NT
11	Maculo-papular exanthema	1998	+	–	NT	NT
12	Maculo-papular exanthema	2002	+	–	NT	NT
13	Maculo-papular exanthema	1983	+	–	NT	NT
14	Lyell's syndrome	1979	+	–	NT	NT
15	Maculo-papular exanthema	1990	+	–	NT	NT
16	Maculo-papular exanthema	1975	+	–	NT	NT
17	Maculo-papular exanthema	2003	+	–	NT	NT
18	Maculo-papular exanthema	1997	+	–	NT	NT
19	Maculo-papular exanthema	1990	+	–	NT	NT
20	Maculo-papular exanthema	1998	+	–	NT	NT
21	Maculo-papular exanthema	1995	+	–	NT	NT
22	Maculo-papular exanthema	1991	+	–	NT	NT
23	Maculo-papular exanthema	2004	–	–	NT	NT
Amox tolerant-patients (group B)						
1	Maculo-papular exanthema	1985	–	–	–	–
2	Maculo-papular exanthema	2001	–	–	–	–
3	Maculo-papular exanthema	1999	–	–	–	–
4	Maculo-papular exanthema	1998	–	–	–	–
5	Maculo-papular exanthema	2003	–	–	–	–
6	Maculo-papular exanthema	2005	–	–	–	–
7	Maculo-papular exanthema	2001	–	–	–	–
8	Maculo-papular exanthema	1989	–	–	–	–
9	Maculo-papular exanthema	2006	–	–	–	–
10	Maculo-papular exanthema	2006	–	–	–	–
11	Maculo-papular exanthema	2005	–	–	–	–
Amox IHS allergy (group C)						
1	Anaphylaxis reaction	2004	NT	NT	+	–
2	Anaphylaxis reaction	1996	NT	NT	+	–
3	Anaphylaxis reaction	2002	NT	NT	+	–
4	Anaphylaxis reaction	2003	NT	NT	+	–
5	Anaphylaxis reaction	2004	NT	NT	+	–
6	Anaphylaxis reaction	2004	NT	NT	+	–
7	Anaphylaxis reaction	2003	NT	NT	+	–
8	Anaphylaxis reaction	2005	NT	NT	+	–
9	Anaphylaxis reaction	1996	NT	NT	+	–
10	Anaphylaxis reaction	2005	NT	NT	+	–
11	Anaphylaxis reaction	2004	NT	NT	+	–
12	Anaphylaxis reaction	2004	NT	NT	+	–
13	Anaphylaxis reaction	2005	NT	NT	+	–
14	Anaphylaxis reaction	2003	NT	NT	+	–
15	Anaphylaxis reaction	2003	NT	NT	+	–

NT, no tested.

for one patient (#14) who developed a Lyell syndrome; (ii) highly probable imputability criteria for the responsibility of amox in the development of exanthema; (iii) positive delayed skin patch test to amox.

Group B (amox-tolerant) comprised 11 patients who developed an exanthema in the course of a treatment with amox. However, these patients were not diagnosed as DTH allergic since: (i) patch tests with amox were negative; (ii) controlled administration of oral amoxicillin did not induce any reaction.

Group C (amox-IHS) comprised 15 patients with IHS to amox as evidenced by: (i) anaphylactic reactions developing within 30 min following amox administration; (ii) positive immediate skin prick tests to amox; (iii) negative delayed skin patch tests to amox.

Drugs

The betalactam antibiotics were obtained from the hospital pharmacy: amoxicillin, ticarcillin, ceftazidin, cefuroxim, (Glaxo-SmithKline, les Ulis, France); ampicillin, oxacillin, penicillin V, cefatrizin, cefazolin (Bristol Myers Squibb, New York, NY, USA); cefixim; cefpodoxim (Aventis, Marne-la-Vallée, France); ceftriaxon (Roche, Neuilly sur Seine, France); bacampicillin (Astra Zeneca, Rueil-Malmaison, France); piperacillin (Wyeth, Blois, France); cefalexin (Sciencex, Issy les Moulineaux, France).

Patch tests

All 49 patients received patch tests to amox and ceftriaxon. Skin patch tests were performed according to the standard procedure used for the diagnosis of allergic contact dermatitis (14, 26). Finn chamber tests were applied on the back skin and reading was performed at 48 h. Drugs were as a 1–10% aqueous solution prepared by the hospital pharmacy (amoxicillin, ampicillin, oxacillin, penicillin V, piperacillin, cefalexin, ceftazidin, ceftriaxon: 10%; cefuroxim; cefixim; cefpodoxim: 5%; bacampicillin: 4%; cefatrizin: 2.5%) (27). Patch tests were considered positive if the drug-induced skin inflammation included at least erythema and oedema corresponding to and of the International Contact Dermatitis Research Group (ICORG) guidelines (26). Controls included: (i) one patch test with saline, as a negative control; (ii) two patch tests with sodium lauryl sulfate (SLS) 0.25% and 0.5%, as controls to detect patients with sensitive skin able to develop nonspecific positive skin patch tests.

Analysis of cross-reactivity among betalactam antibiotics

Patients allergic to one betalactam often develop skin DTH reactions to other molecules of the family, suggesting that T cells specific for one molecule may be cross-reactive to others. Analysis of the cross-reactivity with other betalactam antibiotics was tested by patch tests with a panel of 17 different molecules among penicillins and cephalosporins. Since the 45 patients of the present study had negative patch tests to ceftriaxon, a third generation cephalosporin, this drug was used as a negative control in patch testing studies and in *in vitro* immunological assays.

Lymphocyte transformation test (LTT)

Peripheral blood mononuclear cells recovered from patients' blood (10 ml) immediately after patch reading were distributed in triplicate in 96 round-bottom microwell plates (2×10^5/well), in medium supplemented with 10% autologous serum, in the absence or presence of amox (0.2 mg/ml, 0.5 mg/ml and 1 mg/ml) or ceftriaxon (1 mg/ml), as previously described (28, 29). Positive controls included anti-CD3-stimulated cultures (OKT3 mAb, 5 μg/ml). The cultures were incubated for 5 days at 37°C in 5% CO_2, and 1μCi [^{3}H]thymidine was added to each well for the last 12 h. The amox-specific proliferation (LTT) was determined by [^{3}H]thymidine incorporation using a beta counter Top Count (Perkin-Elmer, Boston, MA, USA). The results were expressed as stimulation indices (SI): (cpm in cultures + drug)/(cpm in cultures without drug). An SI > 2.5 was regarded as positive at any concentration. The 2.5 proliferation index was determined statistically using ROC curves derived from results of LTT obtained in DTH allergic patients (true positive) and in IHS allergic patients (false positive).

ELISPOT IFN-γ assay

Peripheral blood mononuclear cells were prepared from patients' blood immediately after patch reading by Ficoll density gradient centrifugation. Cells from the interface were washed in PBS and resuspended at a final concentration of 5×10^6 cells/ml in RPMI-1640 medium supplemented with 2 mM L-glutamine, 25 mM Hepes buffer and 10% heat-inactivated autologous serum. The phenotype of PBMC was carried out by fluorescence activated cell sorter analysis using monoclonal antibodies (mAbs) directed to T (anti-CD3, CD4, CD8), B (anti-CD19) and NK (anti-CD16 and CD56) surface cell markers. Results showed that all patients and controls had normal numbers of T (2000–4000/μl), B and NK cells. The number of IFN-γ-producing PBMC was determined using an ELISPOT assay kit (Human IFNγ ELISPOT PVDF-Enzymatic, Diaclone, Besançon France). Briefly, 96-well nitrocellulose plates (Millipore Multiscreen plates cat MAIPN4510) were coated overnight at 4°C with anti-IFN-γ antibody provided in the kit and blocked with dry skimmed milk for 2 h at 37°C. The plates were washed three times with PBS before use and PBMC (5×10^5 in 100 μl) were incubated overnight at 37°C/5% CO_2 in the presence of amoxicillin (0.1, 0.5, and 1 mg/ml) or ceftriaxon 1 mg/ml. Plates were washed three times with PBS/Tween 0.1% and incubated for 2 h at 37°C with a biotinylated anti-IFN-γ antibody and then extensively washed. Interferon-γ spot-forming cells (SFCs) were developed using streptavidin-alkaline phosphatase, incubated for 2 h at 37°C, and extensively washed before adding the substrate (5-bromo-4-chloro-3-indolyl-phosphate). The number of IFN-γ SFCs present in each well was counted using a Carl Zeiss vision ELISPOT (Zeiss SAS, Le Pecq, France). Results of the IFN-γ ELISPOT assay are expressed as the numbers of IFN-γ SFC/10^6 PBMC cultured with the drug minus the nonspecific background numbers of IFN-γ SFC obtained in PBMC cultures without the drug, which was usually below 20 (Fig. 1A). When the background level was above 20 IFN-γ-SFC/10^6 PBMC the test was not included and was redone > 4 weeks later. In all cases the background level decreased below 20 SFC/10^6 PBMC at the second ELISPOT assay. During the development of the ELISPOT assay to detect circulating IFN-γ-producing cells in amox allergic patients, we observed that the three amox concentrations (0.1, 0.5, and 1 mg/ml) gave dose-dependent positive results (Fig. 1B). Therefore, we chose the 1mg/ml concentration of amox for the entire study.

To define the threshold of positivity in the IFN-γ ELISPOT assay, the presence of amoxicillin-induced, IFN-γ-producing cells was tested in PBMC of 20 nonallergic healthy individuals who had received amoxicillin in the past years or months. Results showed that the background frequency of amoxicillin- and ceftriaxon-induced IFN-γ SFC ranged from 5 to 25 SFC/10^6 PBMC (Fig. 1C). Therefore the frequency of penicillin-specific IFN-γ SFC was considered significant if above 30 IFN-γ SFC/10^6 PBMC (mean + 2SD).

Since IFN-γ could be produced by different cell types, including T cells, B cells and NK cells, we performed a series of ELISPOT

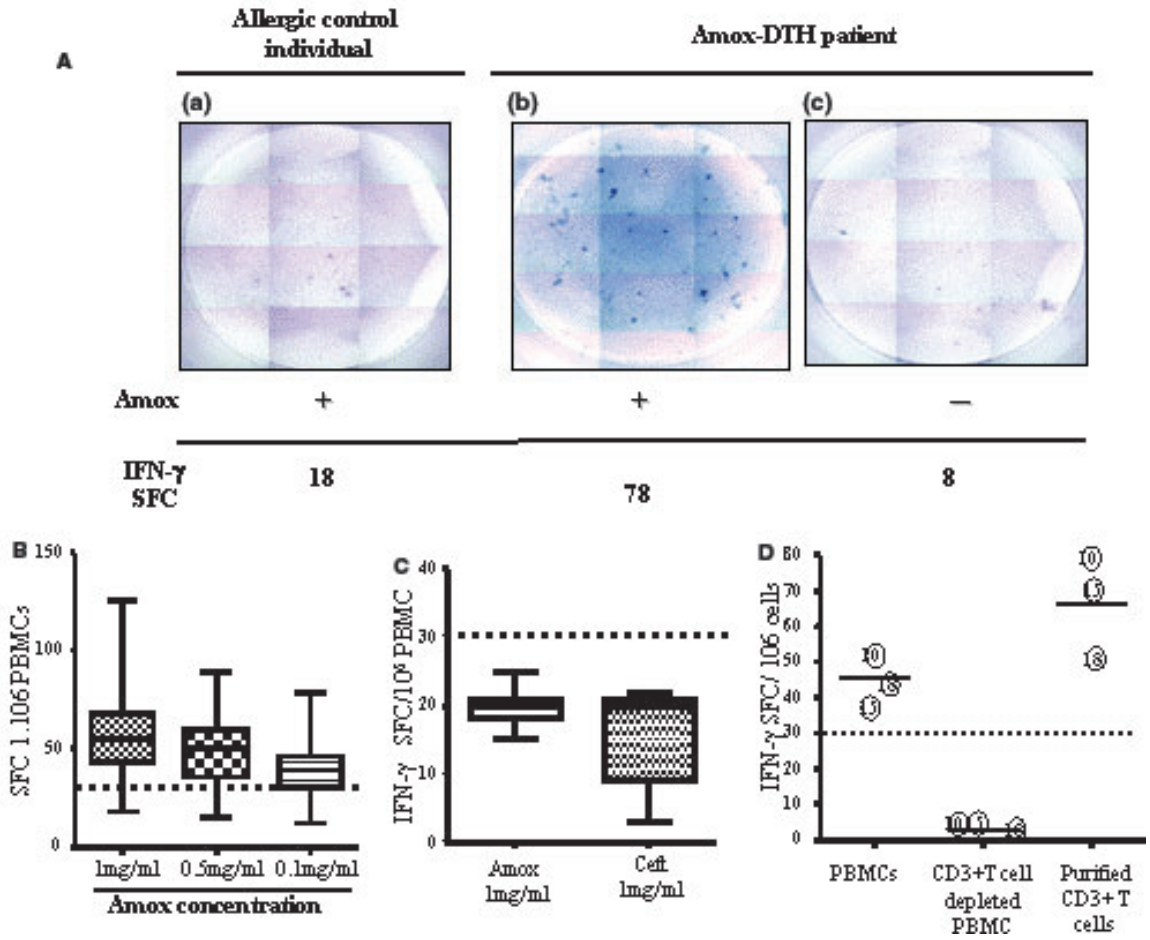

Figure 1. Standardization of the penicillin-specific IFN-γ ELISPOT assay. (A) Purified PBMC (5×10^5 in 100 μl) from a nonallergic control individual (a) and from an amox-DTH patient (b,c) were cultured for 24 h with 1 mg/ml amox (a,b) or medium alone (c) in 96 well ELISPOT plates. The figure shows plate wells in which each spot represents an IFN-γ secreting cell. The results are expressed as the mean number of IFN-γ SFC/10^6 PBMC in triplicate wells. (B) Dose–response studies. PBMC from the 23 amox-DTH patients were cultured with different concentrations of amox in the ELISPOT assay. Results are expressed as numbers of IFN-γ SFC/10^6 PBMC (mean + 2 SD of triplicate wells). (C) Determination of the positivity threshold in the IFN-γ-ELISPOT assay. The frequencies of circulating amox- and ceftriaxon-induced IFN-γ-producing T cells were determined in 20 normal nonallergic individuals. Results are expressed as numbers of IFN-γ SFC/10^6 PBMC (mean + 2 SD of triplicate wells). The value of 30 IFN-γ SFC/10^6 PBMC was defined as the threshold value above which the ELISPOT assay was considered positive. (D). T cells are the IFN-γ-producing cells in the ELISPOT assay. CD3 + T cell-depleted PBMC and purified CD3 + T cells were obtained from PBMC of patients 10, 15 and 18 by magnetic bead isolation technique (Miltenyi Biotech, CD3 Microbeads) and tested for the presence of amox-specific SFCs in the ELISPOT assay. Results are expressed as numbers of IFN-γ SFC/10^6 PBMC (mean + 2 SD of triplicate wells).

experiments in three patients (No. 10, 15, 18) using total PBMC, CD3 + T cell-depleted PBMC and purified CD3 + T cells. As shown in Fig. 1D, IFN-γ-SFCs could be detected in total PBMC and purified T cells whereas depletion of T cells in total PBMC totally abolished the IFN-γ-production. These data demonstrate that T cells are the amox-specific IFN-γ-producing cells in the ELISPOT assay.

Statistical analysis

The correlation between lymphocyte transformation tests and IFN-γ ELISPOT assay results were made by the Pearson correlation coefficient.

Results

The presence of circulating amox-specific T cells using the IFN-γ ELISPOT assay was analyzed in amox allergic and nonallergic patients and compared to the LTT which is the reference *in vitro* immunological test for penicillin allergy (14, 29, 30). The 49 patients comprised 23 selected patients with a typical delayed drug allergy to amox (amox-DTH, Group A), 11 patients tolerant to amox (amox-tolerant, Group B) and 15 patients with immediate hypersensitivity to amox (amox-IHS, Group C) (Table 1). All patients received patch tests to amox,

which were only positive for the 23 amox-DTH patients, and patch tests to ceftriaxon, which were negative for all 49 patients. Blood was drawn immediately after patch test reading.

Detection of drug-reactive cells in amoxicillin-allergic patients using the lymphocyte transformation test

Fifteen out of the 22 amox-DTH patients had circulating lymphocytes which proliferated after *in vitro* restimulation in the presence of amox (SI > 2.5) (Fig. 2A). No lymphocyte proliferation to the control antibiotic ceftriaxon was detected in any patient. Likewise, none of the 11 amox-tolerant patients exhibited *in vitro* reactivity of PBMC to amox. Most (11/15) amox-IHS patients did not respond to amox, although 4 out of 15 developed a borderline proliferative response (SI = 2.5). Thus, the LTT assay for the detection of amox-DTH patients had a sensitivity of 68% with a confidence interval of (49–88%) and a specificity of 85% (71–98%). These results confirm that the LTT is a useful test for the detection of drug-specific T cells.

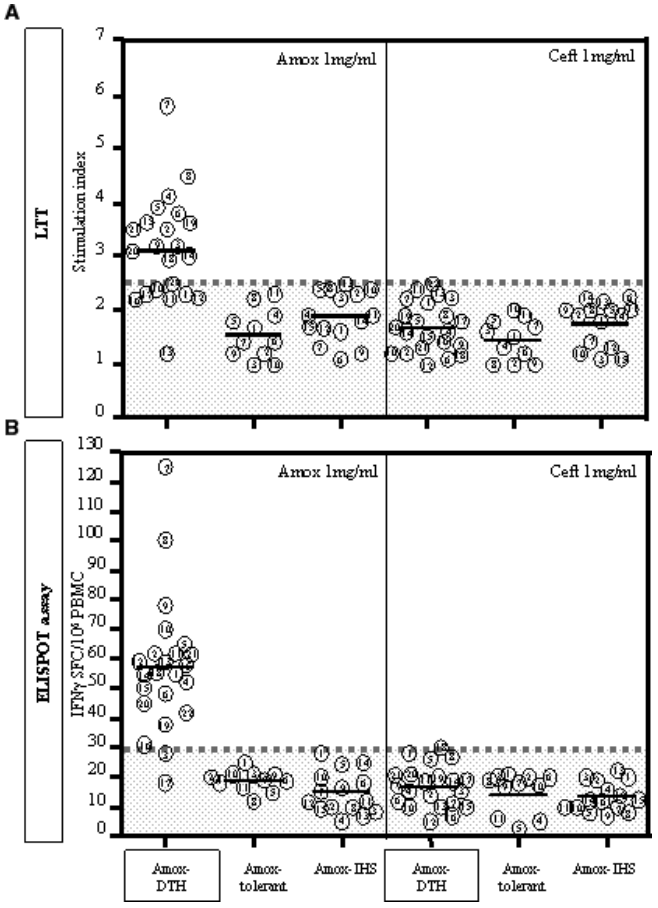

Figure 2. Analysis of amox-specific T cells using the LTT and the IFN-γ ELISPOT assays. Peripheral blood mononuclear cells from amox-DTH, amox-tolerant and amox-IHS patients were cultured in the presence of either amox or the control antibiotic ceftriaxon in two parallel experiments for LTT (A) and ELISPOT (B) assays. Each number represents an individual patient. (A) Drug-specific proliferation and SI of patients PBMC in the LTT. (B) Number of IFN-γ-producing cells per 10^6 PBMC in the ELISPOT assay after 24 h of culture. The dotted lines in panels A and B represent the background levels of nonspecific stimulation index (A) and penicillin-induced IFN-γ SFC (B) in nonallergic patients. Values above the dotted lines are considered positive.

The IFN-γ ELISPOT assay allows for optimal detection of amox-specific T cells in DTH allergic patients

The 22 amox-DTH patients presented amox-specific, IFN-γ SFC ranging from 20 to 130 SFC/10^6 PBMC (mean: 59 ± 21) (Fig. 2B). Twenty out of 22 patients (91%) had numbers of IFN-γ producing cells above the threshold value of 30 SFC/10^6 PBMC and were therefore considered as harbouring circulating amox-specific T cells. The ELISPOT assay gave reproducible results since it detected similar numbers of amox-specific T cells in three patients (3, 12 and 21) tested twice at a 3-month interval (data not shown). None of the 22 amox-DTH patients presented a response to the control drug ceftriaxon (10–30 IFN-γ SFC/10^6 PBMC). Amox-specific IFN-γ secreting T cells were undetectable in PBMC from either amox-IHS or amox-tolerant patients (< 30 IFN-γ SFC/10^6 PBMC). The sensitivity and specificity of the IFN-γ ELISPOT assay for diagnosis of amox-DTH allergy were 91% (79–100%) and 95% (82–98%), respectively (Fig. 2B). Thus the ELISPOT assay appeared as a sensitive test for the diagnosis of amox-DTH allergy. Of note, patients 1, 10, 11, 12, 13 and 16 were not detected in the LTT but displayed significant numbers of amox-specific T cells in the ELISPOT assay.

Comparison of the data obtained for individual patients using the LTT and the ELISPOT assays showed a partial but significant correlation ($R = 0.54$; $P < 0.05$) between the amox-induced stimulation index in the LTT assay and the number of amox-induced IFN-γ SFC in the ELISPOT assay (Fig. 3). Interestingly, the patients with the highest specific proliferative responses (SI) in the LTT appeared as those with the highest frequency of amox-specific T cells in the ELISPOT assay.

Long term persistence of specific T cells in amox-allergic patients

Circulating drug-specific T cells were assessed at various time (years) points after MPE diagnosis. As shown in Fig. 4, the frequency of amox-specific IFN-γ SFC in blood (as well as the intensity of the proliferative responses in the LTT) was inversely correlated to the time after diagnosis for MPE. Thus, higher numbers of amox-specific SFCs were observed in patients with the most recent episode of MPE reaction. In addition, amox-specific T cells were detectable both by ELISPOT (Fig. 4B; patients 1, 3, 13, 14, 16) and LTT assays (Fig4A; patients 3 and 14) for as

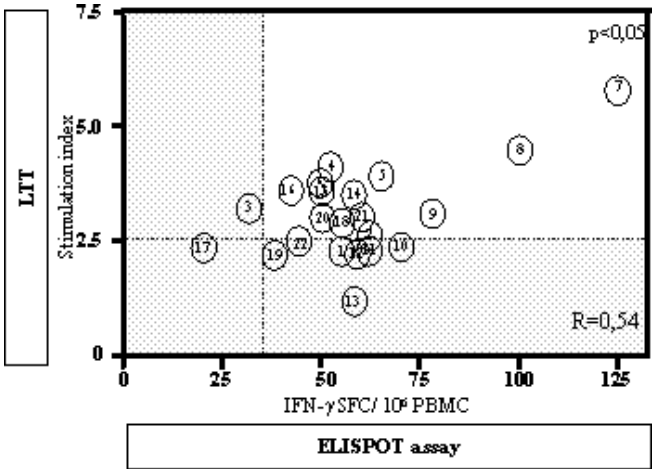

Figure 3. Correlation between the proliferative response of PBMC (LTT) and the numbers of amox-specific T cells (ELISPOT). Comparison of the stimulation index in the LTT assay to the frequency of IFN-γ SFC in the ELISPOT assay showed a partial but positive correlation ($R = 0.54$; Pearson linear correlation coefficient) with a two-tailed P-value of 0.008. Each number represents a patient. The dotted lines represent the background level of nonspecific penicillin-induced proliferation (LTT) and IFN-γ SFC (ELISPOT) in nonallergic patients.

long as 20 years after the occurrence of the MPE reaction, confirming previously reported long term T cell memory in drug allergy (25). Interestingly, the ELISPOT was clearly more sensitive than the LTT for the detection of circulating amox-specific T cells in patients with ancient MPE allergy. Indeed, 9 out of 10 patients with > 10 years interval between MPE and immunological analysis were found positive in the ELISPOT while only 2 were positive in the LTT. Alternatively, some patients with recent MPE allergy (patients 10, 11, 17, 22 for LTT and patients 3 and 17 for ELISPOT) did not have amox-specific T cells detected at the time of analysis.

Monitoring cross-reactivities of specific T cells among betalactam antibiotics

Penicillin-specific T cells may be cross-reactive to different betalactams sharing common T cell epitopes, explaining why patients allergic to one penicillin sometimes develop skin DTH reactions to other betalactams (31, 32). We thus tested whether T cells specific to different penicillins could be detected by ELISPOT assay in patient 23, who developed a ticarcillin-induced MPE and had positive skin patch tests to ticarcillin as well as to ampicillin, bacampicillin and penicillin but negative skin tests to ceftriaxon. As shown in Fig. 5, the ELISPOT and LTT assays confirmed the *in vivo* patch test results and revealed that the ticarcillin-specific T cells were cross-reactive to ampicillin, bacampicillin and penicillin but not to ceftriaxon. Similar results were obtained in 4 other amox-DTH

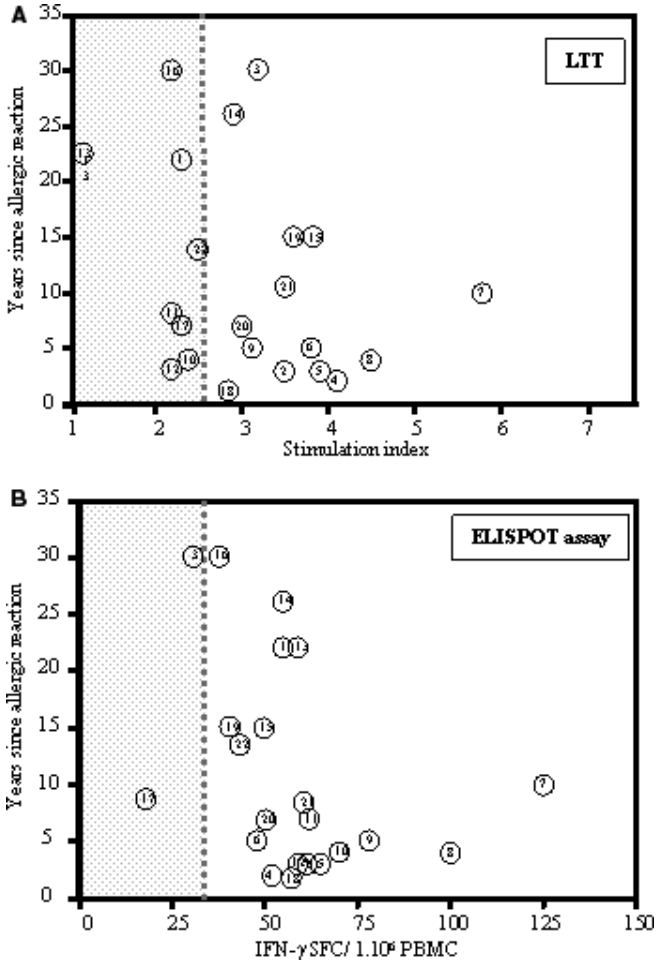

Figure 4. Long-term persistence of amox-specific T cells. Analysis, in amox DTH patients, of amox-specific proliferative responses of PBMC in the LTT (A) and of the frequency of amox specific T cells in the ELISPOT assay (B) as a function of time (years) between the drug reaction and the *in vitro* analysis. Each number represents a patient. The dotted lines represent the background level of nonspecific responses.

patients (patient 2, 9, 20, 22). Patients 2, 9 and 22 had positive skin patch-tests to amoxicillin and bacampicillin but negative tests to ceftriaxon. The ELISPOT assay was positive with a frequency of specific T cells of 62 (#2), 78 (#9) and 41 (#22) SFC/10^6 PBMC for amox, of 46 (#2), 53 (#9) and 38 (#22) SFC/10^6 PBMC for bacampicillin and negative to ceftriaxon. For patient 20, who scored positive skin tests to piperacillin but not to ceftriaxon, the ELISPOT assay was positive for both penicillins (42 and 36 SFC/10^6 PBMC for amox and piperacillin, respectively) and negative for ceftriaxon.

Discussion

In the present study, we analyzed the presence of circulating drug-specific T cells in selected penicillin

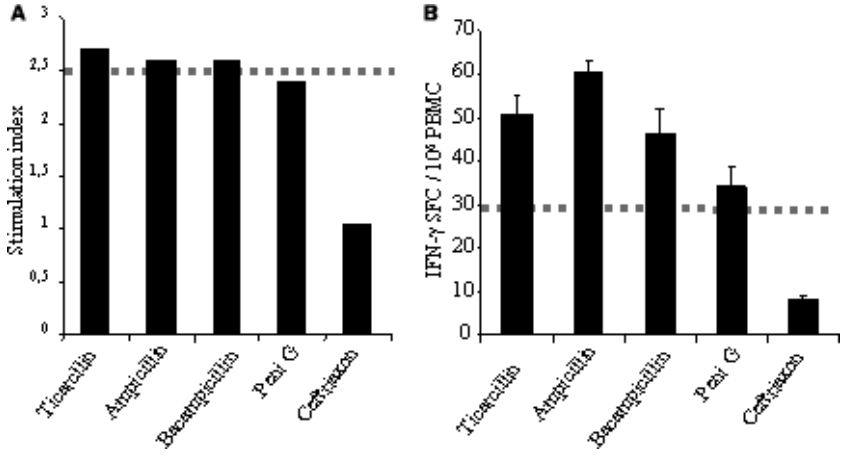

Figure 5. Analysis of T cell cross-reactivity among penicillins in an MPE patient allergic to ticarcillin. Peripheral blood mononuclear cells from a patient with DTH allergy to ticarcillin were cultured in the presence of ticarcillin or other betalactam antibiotics, and tested using the LTT (A) and the IFN-γ ELISPOT (B) assays. Results are expressed as SI (LTT) and as numbers of SFC/10^6 PBMC (ELISPOT). The doted lines represent the background level of nonspecific responses for each assay.

allergic patients using the IFN-γ ELISPOT assay. Twenty-two patients had a clear history of delayed-type hypersensitivity to penicillin expressing as MPE and positive cutaneous patch test reactivity after 48/72 h. Results showed that the ELISPOT assay: (i) can detect numbers of amoxicillin-specific T cell precursors as low as 1 : 30 000 blood leucocytes, (ii) is able to discriminate between immediate and delayed HS patients, (iii) allows for the detection of T cells cross reacting with other members of the betalactam family, and (iv) is more sensitive than the classical lymphocyte transformation test for the diagnosis of penicillin DTH allergy. The frequency of specific T cell precursors in amoxicillin allergic patients ranged from 30 to 125 per 10^6 PBMC (mean 6 : 10^5 PBMC) and is in the range of the frequency of antigen-specific T cells reported in sulfonamide-induced MPE (28) and in T cell-mediated contact dermatitis to metals (33).

The IFN-γ ELISPOT assay exhibited high sensitivity and specificity since specific T cells were detected in 20/22 (i.e. 91%) of amox-DTH patients whereas only 1/26 nonDTH allergic patient had an unexpected but weak reactivity to an unrelated control antibiotic. The reason why circulating amox-specific T cells were undetected in 2/22 amox-allergic patients (patients 3 and 17) is unclear. It is possible that the specific T cells responsible for MPE in these 2 patients produced cytokines other than IFN-γ (e.g. IL-5). In this respect, Erdmann et al. reported that PBMC from nine patients with drug-induced MPE consistently produced IL-5 in response to *in vitro* drug-induced T cell activation (34). Furthermore, the recent study of Beeler et al. on an ELISPOT analysis of five patients with severe drug DTH cutaneous allergy showed that all patients displayed circulating drug-specific T cells producing various types of cytokines including IFN-γ, IL-2, IL-4, IL-5 and IL-13. More importantly, the pattern of cytokines produced by specific T cells varied from one patient to another. Whereas IFN-γ producing drug specific T cells were observed in four out of five patients, one patient had circulating specific T cells producing IL-2, IL-4, IL-5 and IL-13 but not IFN-γ. These results suggest that a more extensive analysis of Th1/Th2 cytokine-producing T cells should be performed in drug sensitive patients for optimal detection of specific T cells and diagnosis.

The LTT is the assay currently most used for the *in vitro* diagnosis of penicillin allergy (35, 36). It is based on the proliferative capacities of primed T and B lymphocytes in response to the relevant antigen. Our findings, in line with previous reports (29, 36), confirm that LTT is able to detect amox-specific T cells in most allergic patients. Although LTT has a high (91%) specificity, its sensitivity is weak (68%) as circulating amox-specific T cells could be detected in only 15 of 22 allergic patients. One possible explanation for the relatively poor sensitivity of the LTT might be the nonspecific background proliferation of PBMC cultured without the drug (14), masking antigen-specific T cell proliferative responses. It is thus possible that only patients, whose PBMC contain high number of specific T cells can be detected in the LTT. Our data are in line with this hypothesis since only patients with the highest frequency of circulating amox-specific IFN-γ producing T cells ($>$ 75 SFC/10^6 PBMC) in ELISPOT were detected by the LTT assay (SI $>$ 2.5) (patients 4–10; 14–16; 18; 20–22) (Fig. 4). The threshold of SI at 2.5 to determine positivity by LTT was chosen to exclude both amox-tolerant patients (patients 8 and 11) as well as patients with IgE-mediated hypersensitivity (IHS) (patients 2, 3, 5, 8, 10 and 13) that in this study gave PBMC proliferation with a SI $>$ 2. In this respect, our data, showing that some patients with IHS (patients 2, 3, 5, 8, 10 and 13)

have a borderline proliferative response to amox using the LTT (with SI > 2.5), are in line with the reports of Luque et al. showing that the LTT could give positive results in patients with penicillin HIS (35). In contrast, none of the 15 amox IHS allergic patients in our study exhibited circulating IFN-γ-producing cells upon *in vitro* restimulation with amox, demonstrating that the ELISPOT assay can discriminate between DTH and IHS.

Our results further emphasize that interferon γ is central to the pathophysiology of MPE (5) since most of the amox-allergic patients of our study exhibited circulating IFN-γ-producing T cells upon *ex-vivo* restimulation with the drug. However, the precise mechanisms by which skin-infiltrating, IFN-γ-producing T cells are able to recruit leucocytes and to induce the inflammatory reaction characteristic of MPE remain unknown. Besides MPE, IFN-γ production in blood and/or skin has been associated with the development of several different types of delayed drug allergic reactions, such as fixed drug eruptions, acute exanthematous pustulosis, Stevens–Johnson syndrome and toxic epidermal necrolysis (Lyell syndrome) (3, 15, 37–41), following treatment with penicillins, sulfamethoxazole and anti-convulsants (25). This suggests that the IFN-γ ELISPOT assay could be useful for the diagnosis of a variety of delayed drug allergies.

Of note, the present study was performed on selected patients with proven drug DTH and IHS allergy to amox. Future studies will have to confirm the usefulness of the assay in a higher number of patients presenting with drug-induced hypersensitivity reactions and especially in patients who scored negative after skin patch testing with the drug.

Collectively, our findings demonstrate that the IFN-γ ELISPOT assay appears to be a sensitive and relevant assay for the diagnosis of drug allergy based on detection of drug-specific T cells and has the ability to discriminate patients with immediate from those with delayed-type reactivity to drugs.

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