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The *in vitro* amplification of β-Lactam-specific memory T cells improves the diagnostic performance of IgE detection and lymphocyte transformation test

L. FILÌ[1], A. MATUCCI[2], A. VULTAGGIO[2], M.G. SEVERINO[3], S. TESTI[3], S. ROMAGNANI[1], E. MAGGI[1], P. PARRONCHI[1]

[1] Department of Experimental and Clinical Medicine, Unit of Internal Medicine, University of Florence, Florence, Italy; [2] Immunoallergology Unit, Azienda Ospedaliero-Universitaria Careggi, Florence, Italy; [3] Allergy and Clinical Immunology Unit, Nuovo Ospedale San Giovanni di Dio, Florence, Italy

Summary

β-Lactams (β-Ls) represent the most frequent cause of adverse drug reactions (ADRs) due to immunological mechanisms. The diagnostic platform is actually based on in vivo assays, whereas in vitro assays do not seem to appropriately answer the requirements of clinical practice and they are mainly field of research or still matter of debate. Both specific IgE determination and lymphocyte transformation test (LTT) exhibit modest (if not low) sensitivity whereas flow-cytometric basophil activation test is still under investigation and limited to immediate reactions. Although short-term T-cell lines (TCLs) have only been used so far to characterize the immunological mechanisms of ADRs, taking advantage from a large number of selected patients with history of β-Lactam sensitization we demonstrate here that hapten-specific TCLs can be induced from the peripheral blood of high proportions of ADR+ patients independently of circulating IgE and LTT, type (immediate vs delayed) or extension (local vs systemic) of the reaction. We also show that wide cross-reactivity between β-L-haptens is exhibited by short-term T-cell lines and detection of specificity can be performed independently of processing-dependent presentation. On the basis of these results, IgE detection, LTT and hapten-specific TCL induction altogether exhibit high specificity (95.5%) and sensitivity (80.5%) thus significantly improving the diagnostic performance of singularly available in vitro tests in β-Lactam sensitization.

Introduction

In most epidemiological studies antibiotics are commonly involved in drug allergy. In particular, β-Lactams (β-Ls) are the most frequent cause of adverse reactions (ADRs) mediated by immunological defined mechanisms and, along with this, amoxicillin has now replaced benzylpenicillin as causative hapten due to its widespread use [1 2]. According to the time interval between drug administration and the onset of symptoms, ADRs are classified as immediate- (within 1 hr) or nonimmediate-(or delayed-) type reactions (from 1 hr on) [3 4] and clinically characterized by skin-limited pictures (urticaria/angioedema and rashes, respectively) or systemic involvement (anaphylaxis and DRESS/AGEP/SJS, respectively) or various combination of them [3]. Traditionally, immediate ADRs are mediated by specific IgE whereas delayed reactions are more heterogeneous [3 5 6] even if the role of T cells (type IV hypersensitivity), recently categorized in at least four different phenotypes, is well-stated in most of them [3 5 7].

However, the role of T cells is not limited to non-immediate reactions as cooperation between helper T cell able to produce T_H2 cytokines and antibody-forming B cells is necessary for IgE-switch [8].

Besides the improvements in understanding the molecular mechanisms of ADRs, the diagnostic management still offers difficulties in clinical practice [4 9 10]. A throughout history is necessary to indentify the culprit drug together with timing of clinical manifestations whereas *in vivo* tests (skin prick, intradermal and patch tests) still represent the basic platform for diagnosis even if potentially risky in patients with severe anaphylaxis [9-11] and not adequately sensitive in non-immediate reactions [10 12]. The *in vitro* assays are still field of research and matter of debate. The detection of specific IgE for years represented the way to ascertain hapten-sensitization with substantial limitation to immediate reactions [10 13 14], major concerns about sensitivity and the influence of total serum IgE levels [15]. In immediate reactions, the possible diagnostic extension to flow-cytometric basophil activation test is under investigation [16]. In cell-mediated reactions, the activation of drug-specific circulating T cells has been investigated by means of proliferation assay (lymphocyte transformation test, LTT) [17-20] with the use of dendritic cells to potentiate hapten-recognition [21] or CD69 expression by drug-activated T cells in cytofluorometric analysis [22] as attempts to improve sensitivity and detect the few circulating memory cells by-passing radionuclide use, respectively. Actually, the frequency of circulating drug-specific T cells has been calculated by few Authors [23 24] and it may be higher than expected and ranging from 1/1,250 to 1/10,000, thus not far from the ordinary antigen-specific T cells [25 26]. These results are at variance with the low sensitivity of LTT (reported between 40 and 70%) [17 27] suggesting a low frequency of circulating precursors eventually influenced by the type of hapten and/or reactions (immediate versus delayed) and the cut-off points of mitogenic index [17 27].

Short-term T-cell lines (TCLs) from the peripheral blood of patients with drug sensitivity were actually used to better characterize the immunological mechanisms of adverse reactions [6 28 29] whereas their application for diagnostic purposes has never been envisaged though it may represent a tool to expand hapten-responsive T cells.

In the present study, using a large number of selected patients with history of β-Lactam sensitization, we demonstrate how the induction of hapten-specific T cell lines with their wide cross-reactivity between β-L-haptens significantly improves the diagnostic performance of the available *in vitro* tests (IgE detection and LTT) independently of the type (immediate *vs.* delayed) or the extension (local *vs.* systemic) of adverse drug reactions.

Materials and methods

SUBJECTS

A total number of 204 consecutive patients who were referred to the Immunoallergology Departments of Azienda Ospedaliero-Universitaria Careggi and Ospedale Nuovo San Giovanni di Dio in Florence with a history of ADRs to β-Ls, were enrolled in the study. All the patients were evaluated for the atopic phenotype on the basis of history, clinical symptoms and skin prick tests (SPT) for common allergens. ADRs were categorized as immediate (within 1 h from the exposure with clinical symptoms characterized by orticaria and/or angioedema or anaphylaxis-related symptoms) or delayed (from 1h to 14 days from the exposure with clinical symptoms characterized by cutaneous rash or other skin involvement or systemic involvement as DRESS, AGEP or SJS/TEN) as stated by ENDA [1].

One hundred thirty five patients showed immediate ADRs whereas 69 delayed ADRs to β-Ls (ADR+ donors). A group of 88 subjects with negative history of adverse reactions (53 atopic and 35 non-atopic) was also enrolled (ADR- donors) with reported tolerance to β-Ls in the last year and negative skin testing. Blood samples were obtained from informed ADR+ and ADR- subjects in accordance with the ethical standards of the responsible regional committee on human experimentation.

β-L hypersensitivity was proven by history and the presence of serum specific IgE. All the ADR+ patients were checked within one year from the reaction, with the exception of 3 of them reporting ADRs more than 10 years before.

The majority (n = 162) of ADR+ patients developed reaction to amoxicillin (Ax), 12 to penicillin (Pe) and 30 to ampicillin (Am). Few patients had positive history of reac-

Table IA. Characteristics of the study population.

	Subjects	
	ADR +	ADR-
N°	204	88
Age (range, yrs)	18-69	18-61
Gender (F/M)	98/106	45/43
Atopy (%)	54	51
Total serum IgE (mean ± SE, KU/L)	164±23	152±36

Table IB. Clinical features of the patients with positive history of adverse drug reaction.

	Patients with history of ADR		
	Any type	*Immediate-type*	*Delayed-type*
	204	135	69
Anaphylaxis		43	
Urticaria-angioedema		92	
Skin-limited			64
DRESS			1
AGEP			3
SJS/TEN			1
Culprit drug (%)			
Amoxicillin	162 (79.4)	112 (83)	50 (72)
Ampicillin	30 (14.7)	14 (10)	17 (25)
Penicillin	12 (5.9)	9 (7)	2 (3)
Time interval since ADR (mean ± SE, yrs)	0.9±0.1		

tions to two different β-Ls: 6 patients to Ax and Am, 3 to Ax and Pe and 1 to Am and Pe.

Table I (A and B) summarizes the characteristics of the donors used in the study.

REAGENTS

The medium used throughout was RPMI 1640 (VLE, Biochrom AG) supplemented with 2 mM endotoxin-free L-glutamine, 1% non-essential aminoacids, 1% sodium pyruvate (Sigma Chemical Co) and $2x10^{-5}$ M 2-ME (Merck) (complete medium). Amoxicillin, Ampicillin and Penicillin were provided from Sigma. rIL-2 was purchased by Novartis (Proleukin®).

β-L-SPECIFIC IGE

Detection of serum β-L-specific immunoglobulin E antibodies was based on the ImmunoCAP System FEIA (Thermo Fisher Scientific Inc.) towards haptens c1 (penicilloyl G), c5 (ampicilloyl) and c6 (amoxicilloyl) according to the manufacturer's instructions with a cut-off value of positivity 0.10 KUA/L.

LYMPHOCYTE TRANSFORMATION TEST (LTT)

PBMC from ADR+ and ADR- subjects were isolated by Ficoll-Hypaque (Pharmacia) density gradient followed by two washings with PBS pH 7.2. $1x10^6$/ml cells were cultured in triplicate in U-round bottomed well plates (Nunc)

in complete medium plus 5% heat-inactivated autologous serum in 0.2 ml final volume for 5 days in presence of three doses of the haptens (2.5-0.5-0.1 mg/ml). After 16 h pulsing with 0.5 mCi ^{3}HTdR per well (Perkin Elmer), cultures were harvested and radionuclide uptake measured by scintillation counting. T-cell proliferation were considered as specific when mitogenic index in response of a single dose of hapten was ≥ 5 or above 3 in two consecutive doses.

GENERATION OF SHORT-TERM HAPTENS-SPECIFIC T-CELL LINES AND CLONES

Short-term drug-specific CD4+ T-cell lines were generated as previously described [29]. Briefly, PBMC from ADR+ and ADR- subjects were stimulated with haptens (Ax, Am or Pe, all at 0.5 mg/ml) for six days and activated T cells were expanded for further 8 days by the addition of rIL-2 (25 U/ml) every three days.

T cell clones were generated from T cell lines derived from PBMC of ADR+ and ADR- subjects and the clonal efficiency evaluated as previously reported [29 30]. The specificity of short-term T-cell lines and T cell clones was assessed as already described [29]. T-cell lines or clones were considered as specific when mitogenic index of a single dose was ≥ 3.

STATISTICAL ANALYSIS

Sensitivity and specificity data were obtained from the cross-tabulation of positive and negative test results in the whole sample, and in subgroups of variables of interest. Statistical analysis of the results was performed by Student's t-test and Mann Whitney test; a p value < 0.05 was considered as significant.

Results

DETECTION OF β-L-SPECIFIC IGE AND MEMORY T CELLS IN ADR+ PATIENTS WITH IMMEDIATE- OR DELAYED-TYPE REACTIONS

We firstly evaluated serum IgE and memory T cells by means of LTT assay specific for the culprit hapten (and the other related β-Ls) in ADR+ patients and ADR- controls.

Fourty six out of 194 (23,7%) ADR+ patients showed detectable IgE levels (> 0.1 KUA/L) towards β-Ls with expected statistically significant prevalence in patients with history of immediate type of reactions (38/126, 30,1%) compared to 8/68 (11,7%) patients with delayed reactions ($p < 0,0001$), whereas only 3/86 ADR- subjects (3.5%, $p < 0.0001$) showed IgE levels above the cut-off limit (Tab. II). Seventy out of 194 (36.1 %) patients (56 with immediate- and 14 with delayed-type reactions) showed β-Ls-specific IgE towards one or more haptens, and, possibly, irrespectively of the culprit drug (data not shown). On the whole, the IgE determination in the serum showed high specificity (95.9%), low sensitivity (40.2%) (Tab. III).

We then assayed lymphocyte transformation test (LTT) by stimulating PBMC of 204 ADR+ patients using three different concentrations of each hapten. Seventy-seven out of 204 (37.7%) ADR+ patients showed positive LTT response to the culprit drug (58 to Ax, 13 to Am and 6 to Pe). Among them 47/135 (34.8%) patients had history of immediate- and 30/69 (43.5%) of delayed-type reactions, the difference between the two groups being not significant. Moreover, 54/77 LTT+ ADR+ patients (70.1%)

Table II. Assessment of β-L-specifc IgE levels to the culprit drug in the study population.

History	*Type of reaction*	*N° of patients*	*Patients with β-L-specific IgE (%)*	
			> 0.1 KU/L	*< 0.1 KU/L*
ADR+		194	46 (23.7)[a]	148 (76.3)
	Immediate-type	126	38 (30.1)[b]	88 (69.9)
	Delayed-type	68	8 (11.6)[c]	60 (88.3)
ADR-		86	3 (3.5)[d]	83 (96.5)

p values: a *vs.* d < 0.0001, b *vs.* c < 0.0001

Table III. Diagnostic performance of IgE detection, LTT and TCL-induction in patients with history of ADRs to β-Ls.

Assay (s)	*Performance*	*Type of adverse reaction*		
		Immediate	*Delayed*	*Any*
IgE detection				
	Specificity	95.0	83.2	95.9
	Sensitivity	54.6	60.5	40.2
LTT				
	Specificity	86.5	80.0	91.2
	Sensitivity	51.9	70.5	42.7
IgE detection + LTT				
	Specificity	89.2	80.7	92.5
	Sensitivity	69.7	76.8	57.6
IgE detection + LTT+ TCL induction				
	Specificity	92.5	87.5	95.5
	Sensitivity	85.5	93.2	80.5

also exhibited T cell recognition towards additional one (32 patients) or two (22) β-Ls (Tab. IVB). Additional 13 patients (9 with immediate- and 4 with delayed-type reactions) showed LTT positivity to one (12) or two (1) haptens different from the culprit drug (data not shown). On the other hand, 9 out of 88 (10.2%) ADR- donors exhibited a positive LTT when the maximal concentration of Ax was used, whereas none of them exhibited positive T-cell response towards Pe or Am (Tab. IVA). On the basis of the results, LTT exhibited good specificity (91.2%) but low sensitivity (42.7%) (Tab. III) whereas when both IgE detection and LTT were performed together, a slight increase in sensitivity (57.6%) was observed (Tab. III). When LTT and IgE levels in the same patients were compared, no correlation between the two assays was seen indipendently of the immediate (Fig. 1A) or delayed (Fig. 1B) type of the reactions or the kind of hapten (data not shown) suggesting that, at least in some patients, circulating β-L-specific memory T cells may be too few to be detected by ordinary LTT assay, even though IgE are still detectable in the serum.

HAPTEN-SPECIFICITY OF β-L-SPECIFIC T-CELL LINES CAN BE DETECTED BY EITHER PROCESSING-DEPENDENT OR INDEPENDENT PRESENTATION

In the view that the proportion of circulating β-L-specific T cells may be too low to be detected by the use of LTT, we then used an *in vitro* amplification system generating short-term T-cell lines (TCLs) from PBMC of 128 selected ADR+ patients (78 with immediate and 50 with delayed-type reaction) stimulated with β-L-haptens (Ax, Am and Pe) followed by expansion with rIL-2. To assess hapten-specificity of TCLs, T cell blasts were stimulated in the presence of irradiated autologous antigen-presenting cells plus hapten (processing-dependent presentation) when PBMNC were available, or hapten alone (antigen processing-independent or T-T presentation). In 43 ADR+ patients the specificity of 118 TCLs could be assessed by both systems and compared with the finding that not only antigen-processing dependent and T-T presentation were strictly and significantly correlated ($p = 0.0005$) (data not shown) but also mitogenic indexes (MI) ($p < 0.0001$, Fig. 1A) as demonstrated by Fisher's exact test. To provide further evidence that memory T cells equally well recognized drug haptens after processing-dependent or independent fashion, 85 T-cell clones specific for Pe derived from a Pe-specific TCL of one ADR+ patient (FF) were also studied confirming a strict and significant correlation between the two kind of responses ($p < 0.001$) (Fig. 1B). These findings suggested that, at least for diagnostic purposes, the methods to detect TCL specificity offer similar results and can be equally used.

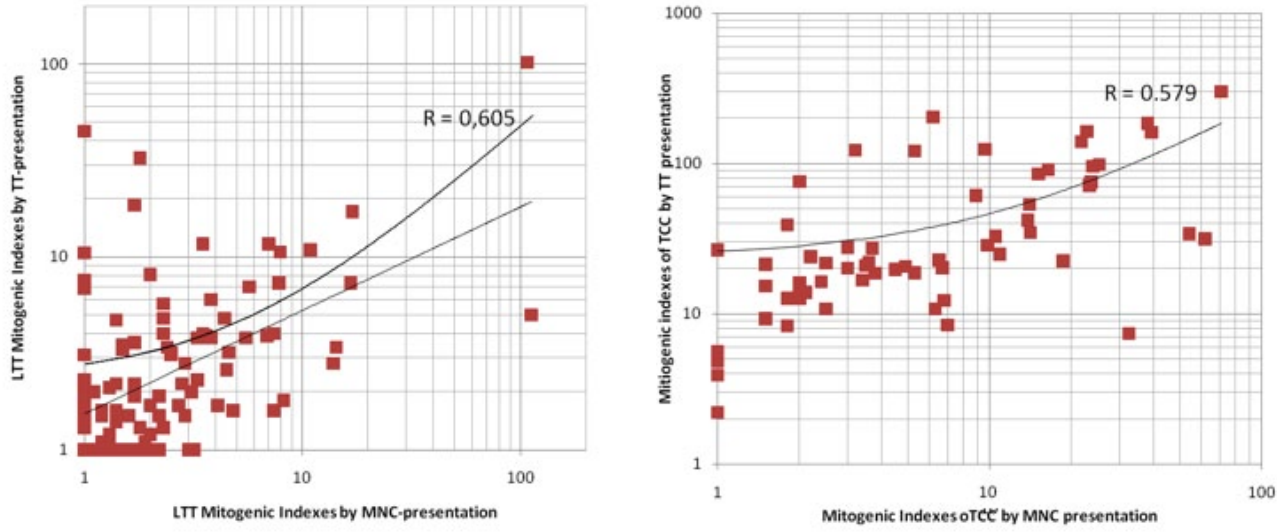

Figure 1. Specificity of T cell lines and clones evaluated by TT- or MNC-presentation of β-Lactams.
A: correlation between the mitogenic indexes of the proliferative response of 118 β-L-specific T cell lines after TT- or autologous MNC-presentation of haptens. The protocol to derive T cell lines and the methods to assess their specificity are described in materials and methods. B: correlation between the mitogenic indexes of the proliferative response of 85 Pe-specific T cell clones after TT- or autologous MNC-presentation of haptens. The protocol to obtain β-L-specific T cell clones and the methods to assess their specificity are described in materials and methods.

β-L-SPECIFIC T-CELL LINES CAN BE INDUCED IN HIGH PROPORTIONS OF ADR+ PATIENTS INDEPENDENTLY OF CIRCULATING IGE AND LTT

Hapten-specific TCLs derived from 128 ADR+ patients were then studied in relation to the time interval between drug intake and clinical symptoms. Similar numbers of specific TCLs could be derived without any significant difference when immediate or delayed reactions were considered (Ax 34/78 *vs.* 24/50 [43.5% *vs.* 48%]; Am 27/78 *vs.* 16/50 [34.6% *vs.* 32%]; Pe 36/78 *vs.* 23/50 [46.1% *vs.* 46%], respectively) (data not shown). As shown in Figure 2, a high degree of cross-reactivity between the three haptens was observed and a considerable proportion of TCLs equally well recognized all the β-Ls independently of the drug used for the induction. The proportion of cross-reacting TCLs was indeed similarly distributed in patients with immediate or delayed reactions (Fig. 2A). Further, and more interestingly, we could derive hapten-specific TCLs in 21 out of 36 patients (58.3%) with history of immediate-type adverse reactions but undetectable serum IgE and negative LTT (IgE- LTT-) with single specificity in 8 patients, recognition of two haptens in 8 and broad reactivity in 5. Analogously, from 19 IgE-LTT- patients with delayed reactions, hapten-specific TCLs could be generated from 13 (68.4%) (single positivity in 7 patients, recognition of two haptens in 4 and broad reactivity in 2) (Tab. V). Of

Table IVA. Assessment of β-L-specifc T-cell proliferation to the culprit drug in the study population.

History	*Type of reaction*	*N° of patients*	*Patients with positive LTT to hapten (%)*			
			Any	*Ax*	*Am*	*Pe*
ADR+	All	204	77 (37.7)	58	13	6
	Immediate	135	47 (34.8)[a]			
	Delayed	69	30 (43.5)[b]			
ADR-		88	9 (10.2)	9	0	0

p value: a *vs.* b NS

Table IVB. Assessment of β-L-specific T-cell recognition towards the different haptens in patients with history of ADRs.

Recognition of β-Lactams	*N° of patients exhibiting positive LTT (%)*		
	Immediate-type (135 pts)	*p value*	*Delayed-type (69 pts)*
One hapten	28 (20.7)	< 0.001	7 (10.1)
Two haptens	15 (11.1)	< 0.02	17 (24.6)
Three haptens	12 (8.8)	NS	10 (14.5)
Amoxicillin	46 (34.1)	NS	33 (47.8)
Ampicillin	27 (20.0)	<0.02	25 (36.2)
Penicillin	22 (16.3)	NS	13 (18.8)

note, TCLs derived from IgE-LTT- patients (irrespective of immediate or delayed reactions), also exhibited a high degree of crossreactivity to the three different β-Ls, even if at a lower extent than in IgE (+/-) LTT+ patients (Fig. 2B). On the basis of these results, when the three different assays (IgE detection, LTT and hapten-specific TCL induction) were considered together, a further increase in specificity (95.5%) and a dramatic amelioration of sensitivity (80.5%) was observed (Tab. III).

Discussion

The diagnosis of drug hypersensitivity is a major challenge in clinical practice as skin tests may result in nega-

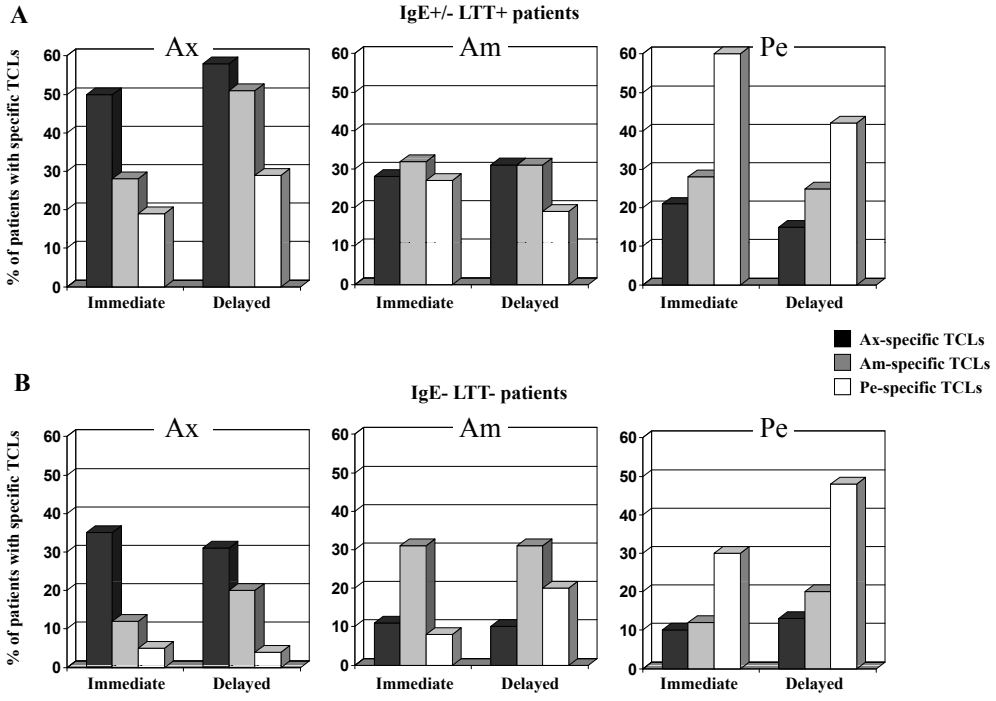

Figure 2. Cross-reactivity of T cell lines derived from patients exhibiting positive or negative LTT. Proportions of T cell lines specific for Ax, Am, and Pe derived from PBMNC of ADR+ patients showing LTT positive with or without IgE (IgE+/-) (panel A) and LTT negative (panel B) assays. The latter group of ADR+ patients showed also no detectable anti-hapten IgE Abs in the serum (IgE-). Each T cell line was generated against one β-L (Ax: black columns, Am: grey columns, Pe: white columns) and also assessed with the other two, as described in materials and methods. The percentages of specific and cross-reacting T cell lines are reported.

Table V. β-L-specificity of T cell lines derived from patients with history of ADRs but negative to both LTT and IgE.

Type of reaction	*N° of patients LTT - IgE (%)*		*N° of T cell lines reactive to*		
	Total	*With hapten-specific T cell lines*	*One hapten*	*Two haptens*	*Three haptens*
Immediate	36	21 (58.3)	8	8	5
Delayed	19	13 (68.4)	7	4	2

tive responses, challenge tests may be risky in systemic reactions and contraindicated in severe reactions and available in vitro methods, though safe, are usually considered as complementary because of concerns about their sensitivity [4 7 13].

In this study, using a large number of patients with selective history of adverse reactions to β-Lactams we analyzed specificity and sensitivity of IgE detection and LTT and showed that induction of hapten-specific T cell lines significantly improves the diagnostic performances thus representing an attractive method to enlarge *in vitro* diagnostic tools.

We first investigated β-L-specific IgE levels towards the commercially available penicilloyl G and V, ampicilloyl and amoxicilloyl in all the patients. The persistence of circulating IgE in the absence of the stimulating agent (in this case, hapten) is still a challenge even if long-living plasma cells, at least specific for conventional antigens, are shown to accumulate in the bone marrow [31]. However, β-L-specific levels are usually lower than allergen-specific IgE, reduce progressively over the time [10 13] and are deeply influenced by total IgE levels [15]. For this reason, patients of our study were selected as referring adverse reactions within one month to one year time-interval from the acute manifestations and showing less than 250 KU/L total IgE. IgE detection exhibited a low overall sensitivity as less than one fourth of patients showed circulating detectable levels of IgE to the culprit β-L with expected statistically significant prevalence in immediate- type of reactions. However, as a confirm of the high degree of cross-reactivity between β-Lactams, the percentage of patients exhibiting detectable levels of drug-specific IgE consistently increased to about one third when it was not limited to the culprit drug but broadly considered towards any of the haptens.

Our study population was also further *in vitro* screened by means of lymphocyte transformation test (LTT). Several reports calculated sensitivity and specificity of this test with concerns about the size of the population, the wide range of sensitizing drugs and clinical manifestations [19 27]. In the study of Luque et al. [17] assessing a robust number of selected patients sensitivity was 62% and specificity 92.8%. Our results confirmed specificity value but were at variance about overall sensitivity. In addition, in our hands, sensitivity was higher in delayed-type than immediate reactions. These differences could be partially due to the cut-off limits of proliferation as we adopted as positive MI (as mean MI from triplicate cultures) > 5 in response to a single concentration of the drug and > 3 in at least two consecutive concentrations in order to maintain optimal specificity. Despite these restricted limits, it was not clear why positive proliferative responses could be also observed in a small number of healthy subjects despite drug tolerance and negative skin prick tests. Similar results have already been observed by others [17 27]. It cannot be excluded that previous exposures of normal donors to β-Ls might have been responsible of the development of few non-pathogenic specific memory T cells. A second explanation could reside into *in vitro* culture conditions as T-cell response only occurred at the highest concentration of amoxicillin. It is thus possible that these conditions, not normally occurring *in vivo*, could be able to activate T cells *in vitro*. A third possibility could be represented by the well-known instability of amoxicillin in aqueous solutions with the production of several degradation products including non-allergenic haptens [32 33]. Even if at-the-moment-prepared solutions of β-Ls from powder stocks were used throughout the study, limited amounts of haptens different from the entire molecule could be assessed by NMR spectral studies (Filì L and

Occhiato E, personal observations). As a fact, however, in β-L-reactive patients the simultaneous detection of IgE and LTT did not significantly improve the diagnostic accuracy of drug sensitization.

The amplification of specific memory T cells inducing drug-specific T cell lines slightly improved the percentage of positive patients, the overall proportion of them still being not over 50%. However and notably, hapten-specific T cell lines could be obtained in about two third of patients negative to both IgE detection and LTT irrespectively of immediate- or delayed-type reactions. This result indicates that ordinary *in vitro* tests may be false negative when the frequency of hapten-specific T cells is low. Even though fluorescent dye 5, 6-carboxylfluorescein diacetate succinimidyl ester (CFDA-SE) analysis would have been of help in disclosing this possibility [22 34], the method would have been unfeasible in a large scale study as the present one.

The induction of hapten-specific T cell lines has been previously limited to research studies [28] and considered as difficult to afford. However, this culture system is an affordable *in vitro* method in laboratories of expertise while simultanously performing LTT. The combination of three different *in vitro* methods to assess β-L-sensitization (IgE detection plus LTT and T cell line induction) maintains specificity and dramatically increases sensitivity and thus could be of particular interests in those patients who suffered from severe life-threatening adverse reactions where skin testing may be risky or contraindicated [9-11]. Further studies are needed to ascertain whether induction of drug-specific T cell lines is able to detect circulating hapten-specific T cells in those ADRs characterized by hematologic or organ-specific involvement. In addition, all the patients of the study (with the exception of three subjects) were selected between one month and one year after the acute drug-related manifestation. This allowed not to underestimate specific IgE levels but, on the other hand, not to clarify the optimal time to perform LTT and/or induction of T cell lines. However, as a confirmation of long-lasting sensitization [25], 3 patients with systemic involvement (2 with anaphylaxis and one with DRESS) produced T cell lines more than 10 years after the drug reaction. Thus, induction of T cell lines, as expanding few memory cells, may represent an *in vitro* diagnostic tool also to ascertain old drug sensitizations and could be restricted to those patients with negative standard *in vitro* tests (circulating IgE and/or LTT).

It is known that haptens can elicit activation of specific T cell after processing and presentation by professional APCs as linked to serum proteins or after direct binding to MHC antigens [35]. In our study, we screened a high number of drug-induced T cell lines and found that β-L-specificity could be either assessed under MHC-restricted conditions when autologous MNC as antigen-presenting cells were available or, with comparable efficiency, directly adding the single haptens into the culture (so called T-T presentation). The correlation between the two systems was confirmed at clonal level. This finding obviously represents a clear advantage as allowing a simple screening system not requiring additional blood sampling from the patients.

On the whole, even though *in vivo* skin tests are accurate methods to assess β-L sensitization, the results of our exclusively culture-focused study demonstrate that expansion of memory cells through the induction of hapten-specific T cell lines significantly improves the accuracy of the standard *in vitro* tests and enlarge the diagnostic tools in the sensitization to β-Lactams.

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• Correspondence: Paola Parronchi, Department of Experimental and Clinical Medicine, viale Pieraccini 6, 50134 Firenze, Italy – Tel. +39-055-4271353 – E-mail: paola.parronchi@unifi.it