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Poor relevance of a lymphocyte proliferation assay in lamotrigine-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

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Summary

Background Prior use of ‘lymphocyte transformation test’ (LTT) in Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) provided conflicting results, possibly dependent on sampling dates (acute vs. late).

Objective Evaluation of LTT in patients with SJS or TEN who reacted to lamotrigine (LTG). In a small subgroup we explored the possible role of regulatory T cells (T-reg).

Methods Acute phase samples (9) and post-recovery samples (14) from cases of SJS or TEN to LTG were provided by the RegiSCAR-study group. Controls were persons never exposed to LTG (12), patients exposed without reaction (6), and patients who developed a mild eruption to LTG (6). LTT was performed by measuring ³H-thymidine incorporation after 3 days of incubation with phytohemmagglutinin, LTG (10 µg/mL) or medium. Stimulation index ≥ 2 was considered positive. In 16 cases LTT was redone after depletion of T-reg by fluorescence activated cell sorting.

Results Positive LTT was observed in 3/6 cases of mild eruptions, 1/9 SJS/TEN-cases tested during the acute phase and 3/14 SJS/TEN-cases tested after recovery. We noted a very mild and nonsignificant trend for an increased response after depletion of T-reg in late samples from SJS or TEN patients.

Conclusions and Clinical Relevance With the largest number of LTT performed in patients with SJS or TEN to a single drug, we confirmed that reactive cells are rarely detected in these reactions. Poor reactivity did not seem related to T-reg. Other *in vitro* assays than those testing proliferation should be evaluated, before raising the hypothesis that specific cells disappeared by undergoing apoptosis during the reaction.

Keywords adverse drug reaction, lamotrigine, lymphocyte proliferation assay, regulatory T cells, Stevens–Johnson syndrome, toxic epidermal necrolysis

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Introduction

Lamotrigine (LTG) is an anti-epileptic medication that has been associated with rather high rates of cutaneous adverse reactions, including the most severe forms, i.e.

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) [1].

Many hypersensitivity reactions are related to activation of drug-specific immune response and especially T cells in delayed hypersensitivity [2]. The presence of medication specific T cells can often be demonstrated after recovery by positive skin tests or *in vitro* tests, e.g. proliferation of lymphocytes in the so called ‘lymphocyte transformation test’ (LTT). In most types of delayed drug eruptions, skin tests and LTT were shown to be positive in up to 50–70% of patients, depending on the drug involved [3–4]. Concerning LTG, its ability to induce an immune response in humans has been

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demonstrated by patch-testing individuals who previously developed a skin eruption when treated with LTG [5]. Blood lymphocytes from such patients proliferated *in vitro* in presence of LTG [6, 7], and human LTG-specific clones of T cells were obtained [7].

According to most recent advances, the mechanisms of SJS and TEN appear to involve drug-specific cytotoxic CD8 T cells [8, 9] and amplification of the apoptosis of epidermal cells through release of several soluble death mediators, such as perforin, granzyme and TNF-related apoptosis-inducing ligand [10] but especially granulysin [9].

In apparent contrast to the intensity of damage induced by drug-specific T cells in the acute phase, *in vivo* or *in vitro* immunologic tests to medications performed in patients after recovery from SJS or TEN often did not demonstrate drug-specific immune response when tested. Years ago it had been observed that patch tests to suspected medications were significantly less frequently positive after SJS or TEN than after mild drug eruptions [11] and that *in vitro* LTT was most often negative [12], but at that time no cases attributed to LTG had been tested.

More recent publications anyhow challenged these ancient negative results by finding that patients with SJS or TEN (none being related to LTG) had positive LTT at the acute phase of the disease (1 week) that became negative later [13]. The same group of investigators attributed these results to a transitory impairment of regulatory T cell functions during the acute stage of TEN and to their restoration upon recovery [14]. In addition an experimental model of TEN in transgenic mice has also strongly suggested that regulatory T cells played an important role in preventing the phenotypic expression of epidermal necrolysis [15].

The aim of the present study was to revisit the presence of drug-specific T cells by using the LTT and the possible role of regulatory T cells in several groups of patients and controls exposed to the same drug: LTG. We, therefore, performed LTTs to LTG in normal controls and three groups of patients: patients exposed to LTG without any reaction, patients who experienced a mild skin reaction attributed to LTG, patients with SJS or TEN attributed to LTG. In the latter group we compared reactivity of lymphocytes obtained during the acute phase of the disease and that of lymphocytes obtained after recovery.

Patients and methods

Patients

Several groups of patients were evaluated.

Patient group 1 comprised 17 patients who suffered from SJS or TEN in relation to LTG intake.

These patients had been included in the multinational RegiSCAR-cohort [16] during the acute phase of their disease. The study had been approved by relevant ethical committees in each participating country and patients had provided a signed informed consent for extensive interview on medical history, disease characteristics, exposure to medications and blood sampling aimed at *in vitro* research on the disease.

The diagnosis of SJS ($n = 9$), SJS-TEN overlap ($n = 5$) or TEN ($n = 3$) was validated by an expert committee reviewing all clinical data but blinded for drug exposure. The committee also determined the probable index-day, i.e. the day of onset of the adverse reaction. Causality of LTG was considered 'probable' or 'very probable' in all cases included in the present study using ALDEN, a specific algorithm for causality assessment in SJS or TEN elaborated and validated by the RegiSCAR-study group [17].

In most patients a first blood sample was obtained during hospitalization for the acute stage of SJS or TEN and a second 6 to 12 weeks after recovery. These samples will be referred to as 'early' and 'late' samples, respectively, in this manuscript.

Patient group 2 included six patients who had experienced a skin rash that had no clinical feature suggestive of SJS/TEN during the first 2 months following initiation of treatment with LTG. The phenotype of the reaction was less well characterized than that for SJS/TEN. However, the absence of systemic symptoms, absence or short duration of hospitalization and resolution of rash in less than 15 days indicated a 'mild drug eruption'. Causality of LTG had been suspected by the treating physician, usually a neurologist. Blood samples were obtained within 1 year after the reaction. No other test (e.g. patch tests) was performed.

Patient group 3 (exposed controls) included six patients who had been treated with LTG for at least 2 months without any reaction.

Control group (unexposed controls) consisted of 12 persons who had never been treated with LTG.

Cells

From each blood sample drawn on lithium heparinate, mononuclear cells (PBMC) were separated by centrifugation on Ficoll-hypaque diluted in RPMI/dimethyl sulfoxide (DMSO) and frozen in liquid nitrogen.

Culture medium and chemicals

Culture medium consisted of RPMI-1640 supplemented with 10% pooled heat-inactivated human AB serum, penicillin, streptomycin, L-glutamine (200 mM, 100×).

Lamotrigine obtained from Sigma-Aldrich (Lyon, France) was first diluted as indicated in DMSO at a

concentration of 20 µg/mL and second in culture medium (RPMI-1640) for a final concentration of 10 or 20 µg/mL.

Determination of lamotrigine-specific blood mononuclear cell proliferation in vitro

Freshly isolated or most often frozen and thawed PBMCs (1×10^5 ; total volume 0.2 mL per well) were incubated with LTG (10 or 20 µg/mL) in 96-well U-bottomed tissue culture plates for 3 days (37°C, 5% CO₂). Proliferation was measured by the addition of ³H thymidine (0.5 µCi) for the last 18 h of the incubation. Cells were harvested and ³H thymidine incorporation was measured as counts per minute on a β-counter (Top Count). Proliferative responses were calculated as stimulation index (SI): counts per minute in drug-treated cultures/counts per minute in cultures with medium and DMSO alone. The SI in presence of LTG was considered positive when ≥ 2 . As a positive control PBMCs were incubated with phytohaemagglutinin (PHA), 10 µg/mL. Experiments where the SI index observed with PHA was < 5 were discarded.

Evaluation of regulatory mechanisms

In early and late mononuclear cells from some patients (selected only on availability of enough mononuclear cells), the proportion of CD4-CD25^{bright} T cells was determined by fluorescence activated cell sorting (FACS) analysis. Relevant antibodies [IgG2a-FITC (mouse), IgG1-PE (mouse), CD25-FITC, CD4-PE] were purchased from Beckman Coulter (Roissy CDG, France).

In 16 cases (four in each group) a second proliferation assay was performed after PBMCs had been depleted from CD4-CD25^{bright} T cells by Flow Cytometry FACS (Dako Cytomation, Trappes, France) or by MicroBeads (MACS, CD4⁺ CD25⁺ bright Regulatory T Cell Isolation Kit human; Miltenyi Biotec, Paris, France). Proliferation assays of CD4⁺ CD25^{low} in presence of LTG were done the same way as for non-separated PBMC. Statistical comparisons between groups were done using Fisher's exact test for comparing rates of positivity, *P*-values < 0.05 were considered significant.

Results

As defined by a SI of 2 or more, a positive LTT to LTG was observed (Table 1 and Fig. 1) in none of 12 non-exposed controls, in 1 of 6 exposed controls, in 3 of 6 patients who had mild reactions (late samples), and in a few cases of SJS/TEN (1/9 for early samples and 3/14 for late samples). Compared to non-exposed controls only the group of patients with mild reactions had significantly higher proliferation (*P* = 0.02, Fisher's test). The rate of positivity in SJS or TEN was neither significantly

different from that of the control groups nor from the group of patients with mild reactions.

The percentages of CD4-CD25^{bright} cells were evaluated by FACS analysis in 16 cases. The percentages of all lymphocytes that sorted as strongly labelled for both CD4 and CD25 (area R12 of Fig. 2) varied from 0.06% to 4.5% (median 0.64%) without significant differences between any group towards exposed controls.

As shown in Table 2, depletion of CD24-CD25^{bright} cells did not change substantially the proliferation to LTG in any group. The response to stimulation by PHA was slightly decreased after cell sorting but that was not significant (overall means and SD of SI were 119 ± 113 and 195 ± 146 respectively, *P* = 0.14).

Discussion

The more salient result of this study is the low rate of positive LTT to LTG in patients with SJS or TEN related to that drug whether tested in the acute phase or after recovery. That contrasted with a higher rate of positivity in the group of milder erythematous eruptions, even though causality assessment had been much less stringent in this group of patients than in cases of SJS or TEN.

Low rates of positive LTT after recovery from SJS or TEN attributed to other drugs were previously reported by our team [12] and recently by another group [13]. On the other hand our results markedly differed from those of Kano et al. [13] by finding no more drug-specific reactivity in blood samples obtained in the acute stage. Actually in our study early and late samples did not differ significantly as a group, with only a possible trend towards a few more borderline positive cases in late samples.

We do not believe that these negative results were related to inability of frozen PBMC to proliferate, since we always used PHA-driven proliferation as a positive control. In all but two of 98 cultures, one of non-fractionated lymphocytes and one after cell sorting, we had a strong proliferation with PHA (mean stimulation indices in presence of PHA above 150 in all groups). The two samples failing to proliferate in presence of PHA were excluded from analyses.

In addition, the fact that lymphocytes from three of six patients with mild drug eruptions strongly proliferated in presence of LTG, after having been frozen, thawed, eventually separated and finally tested with the same methods than those from patients with SJS/TEN strongly argues against a technical problem.

At variance with other studies, we tested the proliferation of PBMCs after 3 days *in vitro* culture when most others use 5 or 6 days. This choice had been based on the theoretical assumption that we were looking for a secondary rather than primary immune response, and

Table 1. Overall results of lymphocyte transformation test using non-separated lymphocytes from controls and patients

	Stimulation indexes (SI) with LTG		<i>P</i> -value for positive SI vs. non-exposed controls	SI with PHA Mean $\pm$ SD
	Individual values	Mean $\pm$ SD		
Non-exposed controls (<i>n</i> = 12)	1.00 1.86 1.26 1.16 1.37 1.49 1.47 1.52 1.07 1.22 1.55 1.17	1.35 $\pm$ 0.24		166 $\pm$ 203
Exposed controls (<i>n</i> = 6)				
# 1	1.26	1.42 $\pm$ 0.37	0.33	236 $\pm$ 119
# 2	1.31			
# 3	2.09			
# 4	1.58			
# 5	1.08			
# 6	1.20			
Mild reactions (<i>n</i> = 6)				
Pt BEI	0.48	2.89 $\pm$ 2.97	0.02	297 $\pm$ 125
Pt CI	4.29			
Pt MAC	7.49			
Pt MAH	1.22			
Pt YO	2.86			
Pt BEL	0.98			
SJS/TEN early samples (<i>n</i> = 9)				
Pt AJ...SJS/TEN-overlap	1.25	1.21 $\pm$ 0.42	0.88	307 $\pm$ 261
3310233 SJS/TEN-overlap	1.13			
3350332 SJS/TEN-overlap	0.74			
3350271 TEN	1.03			
9140010 SJS/TEN-overlap	1.35			
9150095 SJS	1.09			
9160282 SJS	0.68			
9150026 SJS	1.59			
7210017 SJS/TEN-overlap	2.06			
SJS/TEN late samples (<i>n</i> = 14)				
9140005 SJS	1.48	1.56 $\pm$ 0.48	0.27	262 $\pm$ 196
3310017 TEN	1.35			
3310209 SJS	1.89			
9140010 SJS/TEN-overlap	0.93			
9150026 SJS	1.72			
9150095 SJS	0.90			
9140042 SJS	1.48			
9140119 SJS	1.13			
9150179 SJS	2.30			
7210016 TEN	1.75			
9140127 SJS	0.87			
9160282 SJS	2.04			
7210017 SJS/TEN-overlap	1.72			
3310233 SJS/TEN-overlap	2.27			

Patients are identified either by a RegiSCAR anonymous 7 digit number or by a combination of initials of first and last names when not included in RegiSCAR. **Bold:** patients tested both early and late.

LTG, lamotrigine; PHA, phytohaemagglutinin; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

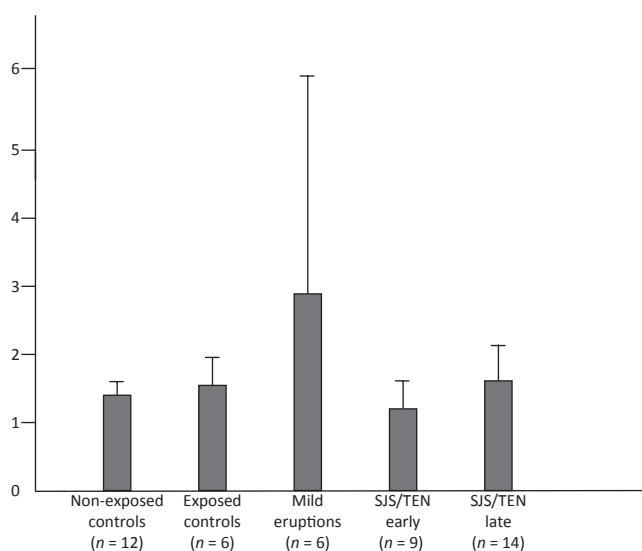


Fig. 1. Mean (+SD) stimulation indices of lymphocytes exposed to lamotrigine (10 µg/mL) by groups of controls and patients.

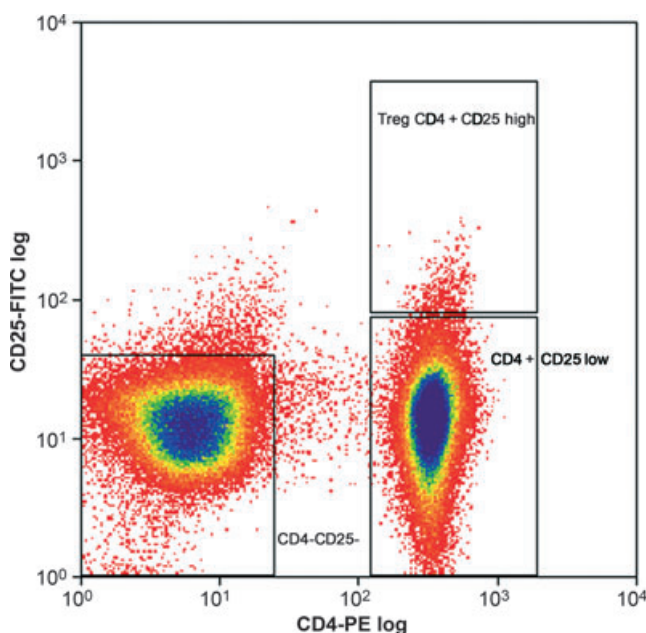


Fig. 2. Example of FACS sorting of lymphocytes obtained from a patient during the acute phase of SJS/toxic epidermal necrolysis.

on the finding (data not shown) that in the few patients with a strong response to LTG, the stimulation indices were similar when tested after 3 or 6 days. We acknowledge that we may have missed a weaker proliferation that could have been detected with more prolonged cultures. Since the same methods were used for testing patients with SJS/TEN and milder drug eruptions we consider anyhow that this limitation cannot explain the differences between the two groups.

The drug concentrations finally used in all experiments (10 µg/mL) had been chosen for being close to the therapeutic plasma level. In a few patients with a strong positive response we had checked that both 10 and 20 µg/mL provided an optimal stimulation, whereas a lower concentration was much less efficient and concentrations above 40 µg/mL had some toxic effect on normal lymphocytes (data not shown).

In the study by Kano et al. [13] the threshold for positive LTT had been fixed to 1.5, instead of 2 in our study. As can be seen in Table 1, re-analysing our data with a 1.5 threshold would result in higher rates of 'positive' results similarly within patients and controls groups, suggesting an increased risk of false positive results.

We, therefore, consider that methodological issues cannot explain the paradox that the patients with the most severe reactions *in vivo* had no detectable or very weak lymphocyte proliferation *in vitro*, either during the acute stage of the disease or later.

Our exploration of a possible role of T-reg was obviously very preliminary because of low numbers. We also acknowledge that the process of cell sorting may have impaired the capacity of cells to proliferate. Actually, the proliferation to PHA was slightly, but not significantly reduced after depletion of CD4-CD25^{bright} cells. But even in cases where a strong response to PHA was still observed, there was no suggestion that depletion of CD4 T-reg increased the response to LTG.

We consider that our study has several areas of strength when compared to prior ones: expert validation of diagnoses and assessment of drug causality, comparison of several groups of controls and patients exposed to the same medication, larger numbers of patients with SJS or TEN than in prior evaluations of LTT in these diseases, substantial proportion of individual cases tested twice during acute phase and after recovery.

The low frequency of proliferation in tested samples should not be interpreted as a constant phenomenon in SJS or TEN, since positive results have occasionally been observed in prior studies. Nevertheless, our results point to the infrequent positivity of LTT in SJS or TEN as compared to the positive results in patients with less severe eruptions. Practically our results not only confirm that after SJS or TEN, LTT cannot be relied on for causality assessment, but they also raise several issues on immunological mechanisms.

Concerning early samples, the very low rate of positive LTT is unlikely explained by regulatory T cells. As shown earlier [13] the percentage of T-reg was low during the acute stage of SJS/TEN. Given the profound lymphopenia, especially affecting CD4⁺ T cells, observed in these patients [18], the absolute number of

Table 2. Stimulation indices (SI) with lamotrigine (10 µg/mL) or PHA, before and after depletion of CD4-CD25^{bright} T lymphocytes

	Lymphocytes CD4-CD25 ^{bright}	SI of non-separated lymphocytes		SI after depletion of CD4-CD25 ^{bright}		<i>P</i> -value positive SI after vs. before depletion
	% of all Ly	LTG	PHA	LTG	PHA	
Exposed controls						
# 1	1.78	1.08	386	1.10	100	
# 2	4.57	1.30	233	1.10	242	
# 3	0.06	1.39	132	1.07	31	
# 6	0.54	1.32	81	1.31	60	
Mild eruption						
Pt MAC	0.51	6.20	235	2.87	24	0.43
Pt MAH	1.05	1.19	15	0.56	128	
Pt YO	0.66	3.14	133	2.35	330	
Pt BE	1.82	0.98	401	1.06	56	
SJS/TEN early samples						
Pt CH	0.13	1.06	127	1.28	26	1
9150095	0.72	1.09	189	1.00	92	
3310233	0.72	1.30	89	1.21	414	
9150026	0.33	1.93	276	1.28	70	
SJS/TEN late samples						
9140005	0.11	1.48	202	2.94	101	0.14
9150179	0.10	2.30	29	2.07	71	
7210017	0.13	1.71	60	1.97	36	
3310233	0.14	2.27	533	1.43	130	

LTG, lamotrigine; PHA, phytohaemagglutinin; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

T-reg is even more profoundly depressed than suggested by the percentage. It is, therefore, not surprising that the depletion of CD4-CD25^{bright} cells did not result in any substantial change to drug-related proliferation of lymphocytes from the acute stage. One explanation to the low rate of reactivity of blood lymphocytes may be the redirection of specific cells to the target tissues.

We had *a priori* postulated that the depletion of T-reg could induce the restoration of a drug-specific proliferative response in late samples. In none of the experiments performed after depletion of CD4-CD25^{bright} cells, we observed a definite increase in drug-specific response. This is especially noticeable in cases that had a borderline positive proliferation with non-separated PBMC. Since the numbers of samples tested remained relatively low, our negative results do not exclude a possible role of CD4-CD25^{bright} T-reg. Exploring other types of suppressive cells, e.g. myeloid-derived suppressive cells (MDSC) or CD8⁺ T-reg should be also considered in future experiments.

One could also argue that proliferation assays are not optimal for testing drug-specific memory cytotoxic T cells, which were shown to play a key role in SJS/TEN and that other *in vitro* tools such as cytotoxicity assays or Elispot to a variety of cytokines could be more appropriate [19]. This limitation of not having included several different read-outs definitely calls for further studies.

Considerations on the mechanisms of SJS/TEN should integrate prior findings that only 1 of 10 TEN patients re-administered the suspected drug had a mild eruption [20]. Although this practice would nowadays be considered unethical, it suggested that the ‘immunological memory’ of the reaction was probably not so strong. Hence, we speculate that during the acute phase the release of enormous concentrations of death mediators that characterizes SJS/TEN also resulted in apoptosis of most of these specific T cells.

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Contribution of each author: Ya-Hui Tang performed most LTT and drafted the manuscript. Maja Mockenhaupt contributed to design of study, organization of sample collection, validation of cases and critically reviewed the manuscript. Adeline Henry performed FACS analyses and cell separation, contributed to manuscript elaboration and revision. Luigi Naldi contributed to collection of samples, validation of cases and manuscript conception and revision. Malika Bounoua contributed to collection of samples (especially for controls) and to manuscript revision. Sabine Le Gouvello

contributed to performing LTT, critical revision of results and elaboration and revision of manuscript. Armand Bensussan organized and controlled all labora-

tory work, analysis of results, revision of manuscript. Jean-Claude Roujeau designed the study, got funding, wrote the final manuscript.

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