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ORIGINAL ARTICLE

Allergen-specific T-cell Response in Patients with Phenytoin Hypersensitivity; Simultaneous Analysis of Proliferation and Cytokine Production by Carboxyfluorescein Succinimidyl Ester (CFSE) Dilution Assay

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ABSTRACT

Background: Phenytoin can induce diversified adverse reactions including generalized eruptions and the hypersensitivity syndrome. Delayed-type allergic mechanisms have been postulated to underlie these reactions. The tests most widely used to detect T-cell sensitization to drugs are the patch test and the lymphocyte transformation test (LTT), but their sensitivity is not sufficient. Simultaneous assessment of both the frequencies and the cytokine-producing phenotypes of allergen-specific T cells has become possible with the recently introduced carboxyfluorescein succinimidyl ester (CFSE) assay.
Methods: Seven patients who presented with phenytoin-induced maculopapular exanthema with and without fever were included in this study. Peripheral blood mononuclear cells (PBMCs) were labeled with CFSE and cultured with phenytoin for seven days. The cells were stained with anti-CD4 and cytokine-specific monoclonal antibodies (MoAbs), and analyzed with FACSCalibur.
Results: The phenytoin-specific proliferation of $CD4^+$ cells in patients was significantly higher than in the four controls exposed to phenytoin, and in seven healthy children with no previous phenytoin intake. A significant difference in the percentages of $CD4^+$ IFN-γ^+ cells between patients and the seven healthy children was observed. The sensitivity and specificity of proliferation were 100% and 90.9%, and those of IFN-γ secretion were 71.4% and 100%, respectively.
Conclusions: Phenytoin-specific proliferation may be detected with greater sensitivity by the CFSE dilution assay than the conventional LTT. The assay revealed that both $CD4^+$ and $CD4^-$ T cells proliferated and produced IFN-γ and TNF-α after stimulation with phenytoin. The CFSE dilution assay might be useful for the diagnosis and understanding of drug hypersensitivity.

KEY WORDS
allergen-specific T cell, CFSE dilution assay, IFN-γ, lymphocyte transformation test, phenytoin hypersensitivity

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Table 1 Characteristics of Patients

Case	Age (years)	Sex	Neurological Abnormalities	Hypersensitivity to PHT	
				Symptoms	Interval between Reaction and Analysis (month)
1	2	M	lissencephaly, mental retardation, symptomatic epilepsy	fever, exanthema	19
2	3	F	symptomatic epilepsy	exanthema	1
3	4	M	lissencephaly, mental retardation, symptomatic epilepsy	exanthema	40
4	5	F	frontal lobe epilepsy	exanthema	6
5	11	F	mental retardation, symptomatic epilepsy	exanthema	51
6	11	M	frontal lobe epilepsy	exanthema	50
7	13	M	frontal lobe epilepsy	fever, exanthema	12

INTRODUCTION

Phenytoin is an effective and widely prescribed anticonvulsant agent used in the treatment of focal and generalized tonic clonic seizures.[1] Phenytoin can induce eruptions that include maculopapular exanthema, the Stevens-Johnson syndrome, generalized exfoliative dermatitis, toxic epidermal necrolysis, vasculitis and fixed drug eruptions. Phenytoin is also linked to a hypersensitivity syndrome that manifests with fever, rash and lymphadenopathy.[2] Allergic mechanisms have been postulated to underlie these diversified adverse reactions.[3]

The clinical signs and symptoms of allergic reactions to drugs are extremely heterogeneous[4] and include different allergic reactions such as immediate-type reactions including anaphylaxis, type II and III reactions such as purpura and hypersensitivity vasculitis, as well as delayed-type reactions.[5,6] For all types of allergic reactions, recognition of the antigen by specifically sensitized T-lymphocytes is a prerequisite. In the case of delayed-type hypersensitivity reactions to aromatic anticonvulsant drugs such as phenytoin, the detection of allergen-specific T cells is especially important for diagnosis because of the absence of allergen-specific IgE.[7]

Currently, both *in vivo* and *in vitro* tests are used for the detection of sensitized T cells. Although an *in vivo* patch test with the suspected compound has been reported to be helpful in determining the cause of drug allergy, the reported sensitivity is relatively low (31.7−50%).[8,9] The test most widely used to detect T-cell sensitization to drugs *in vitro* is a lymphocyte transformation test (LTT),[10,11] which measures ^{3}H-thymidine uptake of dividing cells. Although it has been in use for more than three decades, many laboratories do not achieve sufficient sensitivity with this test.[10] An alternative for the measurement of T-cell proliferation as a read out of LTT is the determination of antigen-dependent-expressed proteins such as cytokines. IFN-γ and IL-5 have been reported to be useful indicators of drug-specific T-cell activation.[11,12]

Recently, Turcanu *et al*. introduced a carboxyfluorescein succinimidyl ester (CFSE) assay to identify food antigen-specific T cells.[13] CFSE is a membrane-permeating dye that binds the amino groups of cytoplasmic proteins with its succinimidyl-reactive group. When cells divide, CFSE-labeled proteins are equally distributed between the daughter cells, thus halving cell fluorescence with each division. Consequently, antigen-specific T cells lose their fluorescence after culture in the presence of the respective antigen ($CFSE^{low}$) and are distinguishable from other cells in culture ($CFSE^{high}$). In this report, we applied this experimental approach to examine both the frequencies and cytokine-producing phenotypes of these phenytoin-specific CD4+ and CD4− T cells simultaneously.

METHODS

PATIENTS AND CONTROLS

Seven patients presenting with phenytoin-induced maculopapular exanthema with and without fever were included in this study (Table 1). Informed consent was obtained from all patients participating in this study. The study was approved by the local medical ethics committee.

Two control groups were included in the investigation. In the first control group (control A), four individuals exposed to phenytoin without clinical signs of a drug-allergic reaction were investigated. In the second control group (control B), seven healthy children with no previous phenytoin intake were examined.

CFSE DILUTION ASSAY

The frequencies and cytokine-producing phenotypes of phenytoin-specific T cells were examined simultaneously using a CFSE dilution assay according to Turcanu *et al*. with some modifications.[13] Briefly, peripheral blood mononuclear cells (PBMCs) isolated from heparinized venous blood by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) were in-

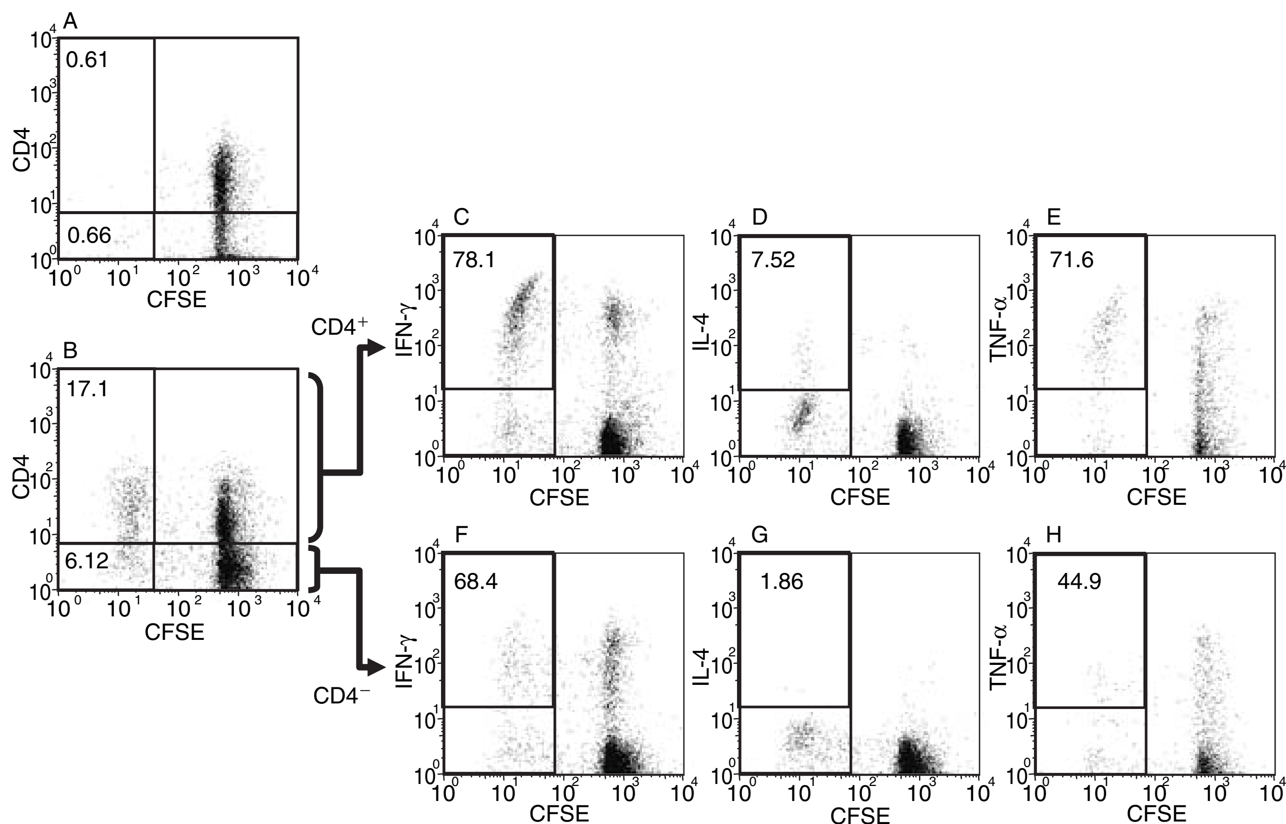

Fig. 1 Representative results from patient 3. PBMCs from patient 3 were isolated, labeled with CFSE, and cultured in the presence of 50 μg/ml of phenytoin. On day 7, PBMCs were restimulated with PMA and ionomycin in the presence of brefeldin A for 6 hours, then the cytokine production of PC-5-labeled CD4+ cells was assessed using intracellular cytokine staining with the PE-labeled MoAbs indicated. **A** represents the background levels of $CFSE^{low}$ cells in $CD4^+$ (left upper quadrant) and $CD4^-$ cells (left lower quadrant) in culture without phenytoin. **B** represents the percentages of $CFSE^{low}$ cells in $CD4^+$ (left upper quadrant) and $CD4^-$ (left lower quadrant) cells in culture with phenytoin. **C** to **E** show the percentage of indicated cytokine-positive cells in phenytoin-specific $CFSE^{low}$ $CD4^+$ cells, **F** to **H** show the percentages of cytokine-positive cells in phenytoin-specific $CFSE^{low}$ $CD4^-$ cells.

cubated with CFSE (Sigma, St. Louis, MO, USA, labeling concentration of 5 μM) for 10 minutes at 37℃, and excess dye was washed away. CFSE-labeled PBMCs were then cultured with 50 μg/ml of phenytoin (Aleviatin, Dainippon-Sumitomo, Japan) in RPMI-1640 supplemented with 5% autologous plasma at a concentration of 1×10^6 cells/ml under humidified conditions and 5% CO2. After seven days of culture, PBMCs were restimulated with PMA (50 ng/ml) (Sigma) and ionomycin (1 μg/ml) (Sigma) in the presence of brefeldin A (10 μg/ml) (Sigma) for six hours. The cells were then stained with PC-5-labeled anti-CD4 antibody, fixed and permeabilized with FIX & PERM cell permeabilization kits (Caltag Laboratories, Burlingame, CA, USA) and stained with PE-labeled IFN-γ, IL-4, TNF-α or IL-10 specific monoclonal antibodies (MoAbs; Immunotech Coulter; Miami, FL, USA). Stained cells were acquired with FACSCalibur (BD Biosciences, Milpitas, CA, USA) and the data were analyzed with CellQuest software (BD Biosciences).

STATISTICAL ANALYSIS

Statistical significance was measured by the two-tailed Kruskal-Wallis one-way ANOVA for ranks followed by the Mann-Whiney *U* test with Bonferroni corrections for individual groups using SPSS software (SPSS Inc., Chicago, IL, USA).

RESULTS

A SIGNIFICANTLY HIGH NUMBER OF $CFSE^{low}$ $CD4^+$ AND $CD4^-$ T LYMPHOCYTES APPEARED IN PATIENTS AFTER PHENYTOIN STIMULATION

PBMCs from patients were labeled with CFSE and then cultured for seven days in the presence of phenytoin. In Figure 1, representative results from patient 3 are shown. In phenytoin-treated cultures, considerable populations of $CFSE^{low}$ $CD4^+$ and $CD4^-$ T lymphocytes emerged (17.1% and 6.12% of $CD4^+$ and $CD4^-$ cells, respectively) (Fig. 1B); however, the

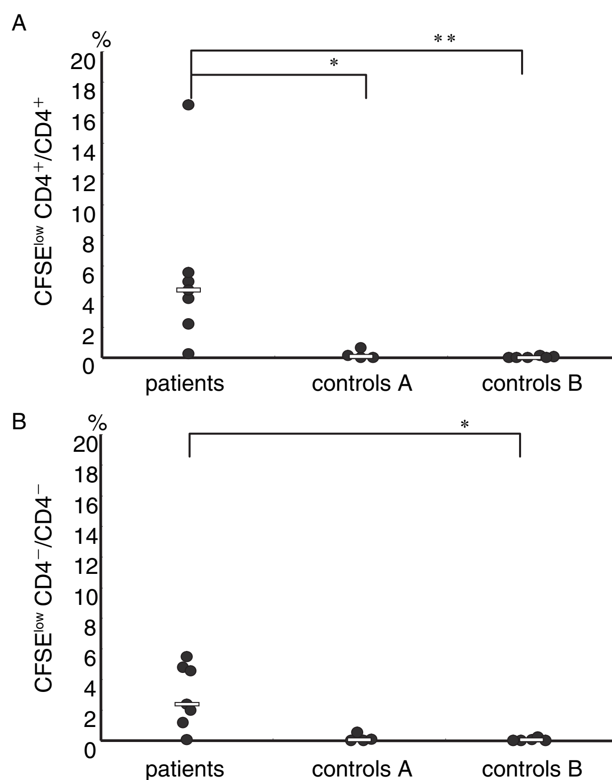

Fig. 2 Proliferation of phenytoin-specific T lymphocytes. PBMCs were isolated, CFSE-labeled, and cultured in the presence of phenytoin for 7 days, then the percentage of antigen-specific CFSE^{low} CD4$^+$ cells in CD4$^+$ cells (**A**) and CFSE^{low} CD4$^-$ cells in CD4$^-$ cells (**B**) was assessed by flow cytometry. Points represent data from different individuals, while bars show the median values. Statistical significance was measured by the Kruskal-Wallis one-way ANOVA for ranks followed by the Mann-Whiney *U* test with Bonferroni corrections for individual groups (** $p < 0.01$, * $p < 0.05$).

proportions of CFSE^{low} cells were small when PBMCs were cultured without phenytoin (0.61% and 0.65% of CD4$^+$ and CD4$^-$ cells, respectively) (Fig. 1A).

Phenytoin-specific proliferation was calculated by subtracting the proportion of CFSE^{low} cells without stimulation from the proportion after phenytoin stimulation, and these results are presented in Figure 2. Phenytoin-specific proliferation of CD4$^+$ cells in patients was significantly higher than in Control A exposed to phenytoin (median 4.41%, range 0.26 to 17.1% *vs*. median 0.07%, range 0.0 to 1.83%, $p < 0.05$) (Fig. 2A), and in seven healthy children with no previous phenytoin intake (Control B) (median 4.41%, range 0.26 to 17.1% *vs*. median 0.0%, range 0.0 to 0.12%, $p < 0.01$) (Fig. 2A).

Similar tendencies were seen in the phenytoin-specific proliferation of CD4$^-$ cells, although statistical significance was weaker. Phenytoin-specific proliferation of CD4$^-$ cells in patients was significantly higher than in Control B (median 2.36%, range 0.04 to 5.46% *vs*. median 0.04%, range 0.0 to 0.25%, $p < 0.05$) (Fig. 2B), but was not significantly different from that in Control A (median 2.36%, range 0.04 to 5.46% *vs*. median 0.04%, range 0.0 to 0.52%).

PHENYTOIN-SPECIFIC T CELLS DISPLAY TH1 DOMINANCE

Figures 1C to 1H demonstrate representative results of cytokine production. We expressed the results as a percentage of cytokine-positive cells among CFSE^{low} (antigen-specific) lymphocytes: 78.1%, 7.52% and 71.6% of CFSE^{low} CD4$^+$ cells and 68.4%, 1.86% and 44.9% of CFSE^{low} CD4$^-$ cells were stained with PE-labeled antibody to IFN-γ, IL-4 and TNF-α, respectively.

We compared the cytokine production of phenytoin-specific CD4$^+$ cells for all study groups. A significant difference in the percentages of IFN-γ$^+$ cells between patients and Control B (median 59.5%, range 0 to 78.1% *vs*. median 0%, range 0 to 5.65; $p < 0.05$) was observed (Fig. 3A), but the percentage in patients was not significantly different from that in Control A (median 59.5%, range 0 to 78.1% *vs*. median 0%, range 0 to 24.3%). A similar tendency was observed in percentages of IFN-γ$^+$ cells among CD4$^-$ cells, but the differences were not statistically significant.

Phenytoin-specific T cells did not seem to secrete considerable levels of IL-4, and there was no significant difference in the percentages of IL-4$^+$ CFSE^{low} cells from patients and controls (Figs. 3C, 3D). TNF-α-positive cells tended to correlate with INF-γ-positive cells (data not shown).

DETECTION OF PHENYTOIN-SPECIFIC PROLIFERATION AND IFN-γ SECRETION OF CD4+ CELLS PROVED SENSITIVE PARAMETERS

The detection of drug-specific proliferation and IFN-γ secretion from the CD4$^+$ cells of the seven patients proved reliable sensitive parameters for the detection of drug sensitization (Table 2). The sensitivity, specificity, positive and negative predictive values (PPV and NPV) were calculated and the best cut-off points (0.26% for proliferation and 24.3% for IFN-γ production) were selected as those that maximized the sum of the specificity and sensitivity. Differences in the calculated sensitivity of proliferation (100%) and IFN-γ secretion (71.4%) resulted only from a control subject who showed a high percentage of IFN-γ-secreted cells. The specificity of the proliferation was relatively low (90.9%) because the low proliferative response of PBMC from a patient decreased the cut off point. This patient had a history of maculopapular exanthema following phenytoin intake six months before this examination.

DISCUSSION

In the LTT, allergen-stimulated lymphocytes undergo

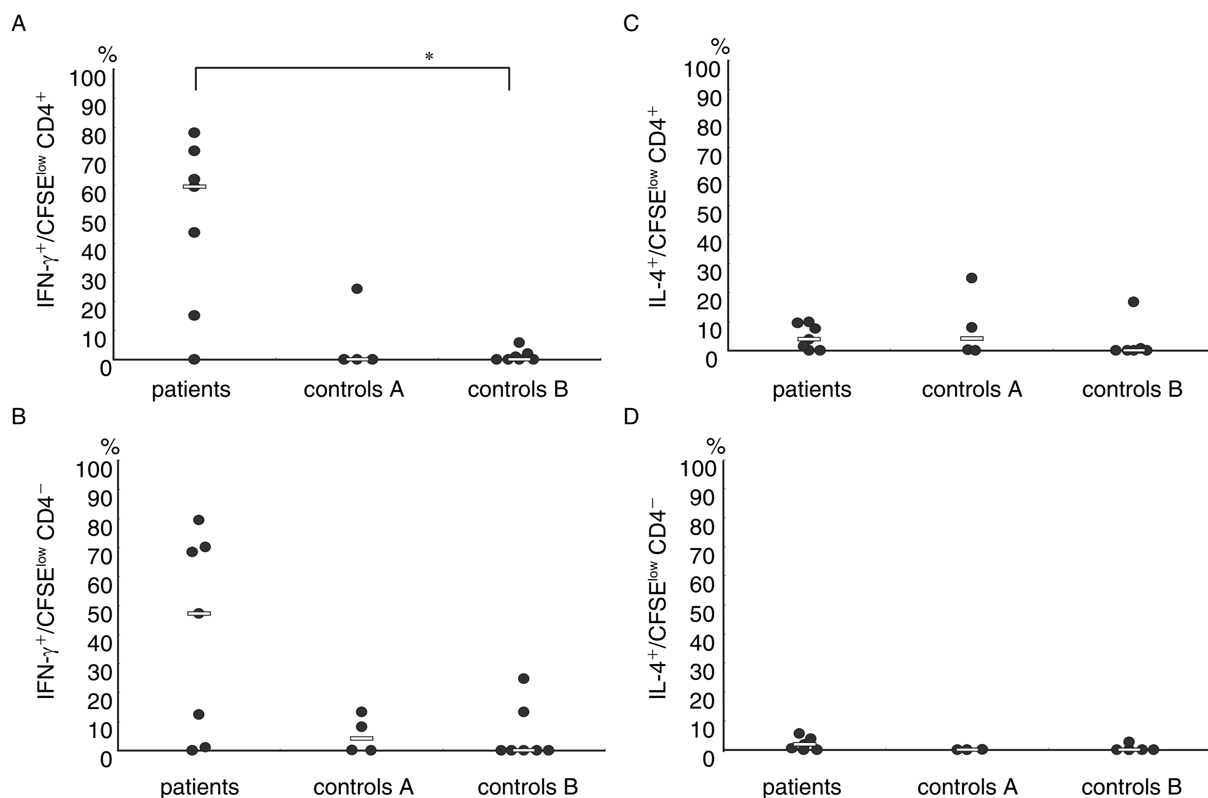

Fig. 3 Intra-cytoplasmic cytokine profiles of antigen-specific T lymphocytes. PBMCs were isolated, CFSE-labeled, and cultured in the presence of phenytoin for 7 days, then the cytokine-producing phenotype of antigen-specific (CFSE^{low}) CD4$^+$ (**A, C**) and CD4$^-$ (**B, D**) cells was assessed by flow cytometry. Intra-cytoplasmic cytokine profiles were presented as percentages of CFSE^{low} cells that produce IFN-γ (**A, B**) and IL-4 (**C, D**). Points represent data from different individuals, while bars show the median values. Statistical significance was measured by the Kruskal-Wallis one-way ANOVA for ranks followed by the Mann-Whiney *U* test with Bonferroni corrections for individual groups ($^* p < 0.05$).

blastogenesis and generate lymphokines such as IL-2, followed by a proliferative response that can be measured by the incorporation of ^{3}H-thymidine during DNA synthesis. Retrospective evaluation of the sensitivity and specificity of the LTT to β-lactam antibiotics revealed a sensitivity of 78% and a specificity of 85%, better than the patch test (64% and 85%, respectively).[14] However, the sensitivity achieved by many laboratories does not achieve such a level, despite the fact that the LTT has been in use for more than three decades.[10] An alternative for the measurement of T-cell proliferation as a read out of the LTT is the determination of antigen-induced proteins such as cytokines. IFN-γ has been reported to present a useful indicator of drug-specific T-cell activation.[15] Sachs *et al.* reported that the specific activation of peripheral blood mononuclear cells consistently resulted in IL-5 secretion, and that the sensitivities of the patch test, the LTT, and the assessment of drug-specific IL-5 secretion for the detection of drug sensitization were 55, 75, and 92%, respectively.[12]

In this study we demonstrated that phenytoin-specific proliferation and cytokine production of PBMC from seven patients with phenytoin hypersensitivity could be detected by the CFSE dilution assay. The determination of phenytoin-specific CD4$^+$ cell proliferation and INF-γ production by this assay resulted in more sensitive parameters (100% and 71.4%, respectively) for the detection of drug-sensitized T cells than the LTT or patch tests. Although both the CFSE and LTT assays detect lymphocyte proliferation, it is possible to examine proliferations of CD4$^+$ and CD4$^-$ cells separately but simultaneously by the CFSE assay. Furthermore, true antigen specific cells which divided several times in the culture might be distinguished from bystander cells that divided a few times at the late phase of culture. Thus, our results revealed the potential of the CFSE dilution analysis, although, a study involving a larger number of subjects is necessary to establish the superiority of the CFSE assay to the LTT. As use of this method could be applied to other drugs, the CFSE dilution analysis might provide a non-radioactive, sensitive alternative for the *in vitro* detection of drug sensitization.

It has been shown that in primary cultures of PBMCs both CD4$^+$ and CD8$^+$ T lymphocytes are acti-

Table 2 Diagnostic Accuracy of $CD4^+$ cell Proliferation and IFN-γ Secretion

	Results		Sensitivity (%)	Specificity (%)	PPV* (%)	NPV* (%)
	Positive†	Negative†				
Proliferation			100	90.9	87.5	100
Patient	7	0				
Control	1	10				
IFN-γ secretion			71.4	100	100	84.6
Patient	5	2				
Control	0	11				

† Cut-off points were 0.26% for proliferation and 24.3% for IFN-γ production.

* PPV denotes positive predictive value, NPV denotes negative predictive value.

vated by drugs.[16] As T-cell activation with a particular function seems to lead to a specific clinical picture of hypersensitivity, the intensive characterization of drug-specific T cells might contribute to the diagnosis and understanding of drug hypersensitivity. In this respect, the cloning of antigen-specific T cells has proven to be a powerful tool to investigate the immune response to allergens at the T-cell level.[17,18] Nevertheless, several cycles of T cell stimulation in the presence of antigen, feeder cells, and exogenous cytokines are necessary to establish T cell clones. Since this is both time and labor consuming, only a limited number of clones can be studied. Furthermore, human T cells tend to develop a TH2-skewed cytokine-producing phenotype after repeated stimulation *in vitro* under neutral conditions.[19]

In this report, using the CFSE dilution assay we revealed that both $CD4^+$ and $CD4^-$ phenytoin-specific T cells existed and that both mainly produced IFN-γ and TNF-α (Th1 and Tc1). A small population of $CD4^+$ cells secreted IL-4 (Th2). Pichler proposed that further classification of delayed-type (type IV) hypersensitivity reactions through the release of certain cytokines and chemokines led to a better understanding of drug hypersensitivity pathogenesis:[7] Type IVa, Th1 cells activate and recruit monocytes; Type IVb, Th2 cells activate and recruit eosinophils; Type IVc, Tc cells develop cytotoxicity; and Type IVd, IL-8 secreting T cells activate and recruit neutrophils. The CFSE dilution assay combined with cytoplasmic IL-8 or perforin staining besides INF-γ and IL-4 might provide further insight into various drug allergies.

In summary, we analyzed phenytoin-specific T-cell proliferation and cytokine production simultaneously using the CFSE dilution assay. Phenytoin-specific proliferation may be detected with greater sensitivity by the CFSE dilution assay than the conventional LTT. Furthermore, the assay revealed that both $CD4^+$ and $CD4^-$ T cells proliferated and produced IFN-γ and TNF-α after stimulation with phenytoin, suggesting that both Th1 and Tc1 participated in the pathogenesis. The CFSE dilution assay might be useful for the diagnosis and understanding of drug hypersensitivity.

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