RESEARCH ARTICLE

In vitro T lymphocyte proliferation by carboxyfluorescein diacetate succinimidyl ester method is helpful in diagnosing and managing primary immunodeficiencies

Elif Azarsiz | Neslihan Karaca | Birgul Ergun | Mehmet Durmuscan | Necil Kutukculer | Guzide Aksu

Department of Pediatric Immunology, Ege University, Izmir, Turkey

Correspondence

Elif Azarsiz, Department of Pediatric Immunology, Ege University Medical School, Izmir, Turkey.
Email: elif.azarsiz@ege.edu.tr

Background: Functional studies besides routine laboratory tests for the definitive diagnosis of T lymphocyte disorders with isolated T or combined T/B-cell immunodeficiencies are important. We hereby summarized our experience with a carboxyfluorescein diacetate succinimidyl ester (CFSE)-based assay for the assessment of mitogenic T-cell proliferation responses in primary immunodeficiency (PID) patients who have not been diagnosed yet or genetically analyzed, but classified as probably having T-cell defects.

Methods: Unclassified patients (n=46) and controls (n=25) were evaluated for T-cell disorders with CFSE-based assay.

Results: CD3⁺ blast cells after PHA-L stimulation were significantly lower in patients (31.1±28.8) than controls (67.9±8.79; $P<.001$). Nine patients with low and four patients with normal CD3 values had severely decreased blastic transformation. The proliferation response decreased mostly in combined immunodeficiency group. Sixteen of them had impaired proliferation responses. Appropriate molecular genetical analyses were planned after thorough evaluation of each patient.

Conclusions: In vitro lymphocyte cell proliferation analysis by CFSE method is a reliable and practical choice for the assessment of mitogenic T lymphocyte responses in yet unclassified PID patients for targeting further genetical analyses.

KEYWORDS

carboxyfluorescein diacetate succinimidyl ester, immunodeficiency, T lymphocyte proliferation

1 | INTRODUCTION

Primary immunodeficiencies (PID), practically, are a group of 300 rare, chronic disorders characteristically present in childhood with infections that persist with multiple recurrences. T cells are essential for the control of viral and fungal disease. However, they also provide helper function to B cells for antibody responses. Thereby, T-cell disorders present as combined T- and B-cell immunodeficiency with susceptibility to bacterial, viral, and fungal pathogens.¹

In previous years, the diagnosis of certain immunodeficiency diseases could be supported by an absent or decreased proliferation response to mitogenic substances. PID now can be divided as B-cell deficiency, T-cell deficiency, or combined deficiencies by quantification of lymphocyte subsets in peripheral blood by flow cytometry.²

Flow cytometry studies currently present new insights for assessing T-cell functions. The necessity of detailed measurements of T-cell function such as proliferative ability in response to mitogens accompanied with the use of fluorescent nucleoside analogs that incorporate into the DNA of proliferating cells or fluorescent tracking dyes that are incorporated into the cell. Since 1994,³ carboxyfluorescein diacetate

The study was approved by the hospital's ethics committee.

succinimidyl ester (CFSE) has been widely used for investigating lymphocyte division and mitotic activity.^{4,5}

Carboxyfluorescein diacetate succinimidyl ester passively diffuses into cells. It is colorless and nonfluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates. So, CFSE stains intracellular proteins, and is equally partitioned between daughter cells and generates a fluorescent signal that progressively halves with each mitosis. Each new generation in a population of proliferating cells is marked by twofold decrements in cellular fluorescence intensity that are readily discriminated by flow cytometry. So, the combination of flow cytometry and T-cell proliferation testing is sufficient to define severe defects in T-cell immunity. These assays involve a mitogen (eg, phytohemagglutinin, PHA) stimulation and a 72-hour culture of both unstimulated and stimulated cells.⁶

Proliferation assay based on mitogen stimulation using DNA incorporation of the radioactive nucleotide [3H]-thymidine is gold standard test for the functional evaluation of lymphocytes⁴ and the usage of nonradioactive DNA approaches such as incorporation of the nucleoside analog 5-bromodeoxyuridine (BrDU), methyl-thiazolyl-tetrazolium (MTT) colorimetric assay, or flow cytometric assay for specific cell-mediated immune response in activated whole blood (FASCIA) is also other available option.^{4,7,8}

The estimated cumulative prevalence rate for all forms of PID has been documented as 30.5/100 000 in Turkey, and our prevalence rate is 18.8/100 000 in Ege University.⁹ Functional studies in PID contrary to routine laboratory methods are not performed in most of the laboratories in our country. Here, we summarize our experience on evaluation of mitogenic T-cell proliferation responses by a CFSE-based assay in primary immunodeficiency patients who have not yet been diagnosed or genetically analyzed, but classified as "probable PID" according to International Union of Immunological Societies (IUIS) diagnosis criteria.¹⁰

2 | PATIENTS AND METHODS

Forty-six patients (22 male and 24 female) from outpatient and inpatient clinics of Ege University Faculty of Medicine, Department of Pediatric Immunology, Izmir, Turkey, were included in this prospective study of 5-year duration (2010-2015). The "written informed consent forms" were obtained from parents and the study was performed after the approval of local ethics committee.

The following criteria were used to select patients: (i) IgG, IgA, or IgM serum levels less than two standard deviations of age-related normal values on admission; (ii) reduced CD3⁺ or CD4⁺ or CD8⁺ T cells; (iii) presence of recurrent infections; and (iv) exclusion of known causes of hypogammaglobulinemia such as drugs, genetic disorders, chromosomal abnormalities, infectious diseases, neoplasias, systemic disorders.

An evaluation sheet was used to summarize the patients' demographic information and laboratory data. Clinical information was obtained from their medical records. Information was collected on

gender, date of birth, age at onset of symptoms, age at diagnosis, age on admission, familial history of primary immunodeficiencies, consanguinity, and history of infections and autoimmune diseases.

A total of 25 healthy volunteers (11 male and 14 female) with no present and past history of autoimmune or any systemic disease and any acute infection in the month prior to blood collection were selected as "control group."

Serum immunoglobulins (IgG, IgA, IgM) were analyzed quantitatively in comparison with age-related normal levels by Dade Behring BNII Nephelometer, Siemens, Germany.¹¹ Specific antibody responses were investigated with commercial ELISA kits as antitetanus IgG, anti-Haemophilus influenzae type b IgG (IBL, Hamburg, Germany). All flow cytometric analyses were performed using FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA, and data were prepared according to standardized publishing rules.¹²⁻¹⁴ The percentages and absolute counts of lymphocyte subsets in comparison with age-related normal levels (CD3⁺ T, CD19⁺ B, CD3⁺CD4⁺ T helper, CD3⁺CD8⁺ T cytotoxic, CD3⁺HLA⁺DR⁺ activated T cell, and CD3⁺CD16⁺CD56⁺ natural killer cell) were investigated by flow cytometry using peripheral blood anticoagulated with EDTA.¹⁵ Also, CD3⁺CD4⁺CD45RA⁺-naive, CD3⁺CD4⁺CD45RO⁺ memory, CD3⁺TCR $\alpha\beta$ ⁺, and CD3⁺TCR $\gamma\delta$ ⁺ cells were investigated by flow cytometry (BD Biosciences, San Jose, CA, USA).

For in vitro T lymphocyte proliferation testing, we modified CFSE labeling method that was designated by Ouah and Parish.¹⁶ Peripheral blood mononuclear cells were isolated by Ficoll-Paque (Biochrom, Berlin, Germany) gradient centrifugation. Equally mixed sodium-heparinized blood and Ficoll solution were centrifuged at 600 g for 20 minutes at 20°C. Mononuclear cells were collected from the interphase into RPMI (RPMI 1640 medium containing 2 mm L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, Invitrogen, Carlsbad, CA, USA) and washed and recentrifuged again at 400 g for 15 minutes at 20°C. Cells in the pellet were counted using trypan blue with Countess cell counter (Thermo Fisher Scientific, Waltham, MA, USA).

A stock solution of CFSE was prepared by dissolving CFSE (Molecular Probes, Eugene, OR, USA) in DMSO at a concentration of 5 mmol/L. This stock was frozen in small aliquots to avoid excessive freeze and thaw cycles. In labeling process, 1 mL cell suspension was added to the bottom of the falcon tube. 1:10 PBS diluted CFSE stock solution was added at transverse tube position, and then, the tube was rapidly turned and vortexed to ensure homogeneous dispersal. At high concentrations, the CFSE load becomes toxic to cells (giving very bright, but very dead cells). Titration of CFSE concentration was performed to attain optimal fluorescence and test results. A final CFSE concentration of 5 µmol/L was preferred if cell counts were lower than 50.10⁶ cells/mL. After labeling, cells were incubated for 5 minutes in dark at 20°C, and then, 10× volume of RPMI with 5% FBS (fetal bovine serum, Biochrom, Germany) was added and centrifuged again at 300 g for 5 minutes at 20°C. Cells were washed three times and 1 mL RPMI per 5% FBS was added. Labeled cells were placed directly in culture media under conditions where they were stimulated to divide. Cells were incubated at 37°C in a 5% CO₂ incubator for 72 hours in 96-well flat-bottom plates with either medium alone and stimulated with 1 µg per 100 µL PHA-L (phytohemagglutinin-L, Sigma-Aldrich, Hamburg, Germany).

FIGURE 1 Dot blot graphics of (A) unstimulated CD3⁺ T cells and (B) PHA-L stimulated CD3⁺ T cells and histogram graphic of (C) daughter cells, % blast transformation

Cells were harvested after 72 hours of culture, and pellets were collected to 100 μ L RPMI and were stained with anti-CD3 APC. They were incubated for 30 minutes in the dark, and then, 2.5 mL PBS per 0.1% BSA (bovine serum albumin, Biochrom, Germany) was added and centrifuged at 300 g for 5 minutes and washed. Prior to flow cytometric analysis, 500 μ L PBS per 0.1% BSA was added. Cells were analyzed within one hour on the flow cytometer FACSCalibur (BD Biosciences, USA) under standard conditions for monitoring fluorescein. For each sample, at least 10,000 signals were analyzed. Compensation beads for the fluorochromes were run in each analysis as well as flow set beads for internal control of the cytometer readings.

In the analysis of PHA-stimulated cells with Cell-Quest program (BD Biosciences, USA), light scatter profiles of CD3⁺ cells were used to calculate the percentage of T cells that had moved from the resting (Figure 1) to the blast population (percentage blast transformation, %M). Histograms displaying CFSE were gated by light scatter to include all viable CD3⁺ T lymphocytes. On the other hand, the proliferation index (PI) of mitotic cells was calculated using ModFit cell cycle analysis software (Figure 2).

Statistical analyses were performed using SPSS (Windows version 16.0; SPSS Inc., Chicago, IL, USA) and a two-sided P value $< .05$ was considered to indicate statistical significance. All data were expressed as mean $\pm$ SD except when indicated otherwise. Correlation comparisons

between paired samples were made by Spearman's product-moment correlation coefficient. Mann-Whitney U test and Kruskal-Wallis test were used for comparisons between clinical parameters and variables. Pearson chi-square or Fischer's exact test was used to investigate relations between categorized variables.

3 | RESULTS

A total of 46 patients with mean 64.9 ± 55.2 months of age (min-max: 2-204 months) were included into this study. Twenty-four of them were female (52%) and 22 of them were male (48%).

All demographical and laboratory findings of patient group are summarized in Table 1. As shown in Table 1, the mean age for the initiation of symptoms and on admission was 29.7 ± 38.2 months and 49.9 ± 48.3 months (1-192 months), respectively.

A total of 70.5% of patients had several infections on admission and sinopulmonary infections were the most frequent type (82.8%). Herpetic keratitis was seen in four patients. Family history of primary immunodeficiencies in siblings or first-degree relatives was positive for twelve patients (26.1%) and twenty patients (43.5%) were born to consanguineous parents ($n=10$ first-degree, $n=5$ second-degree, $n=5$ third-degree cousin marriage). Nine patients (19.6%) had hepatosplenomegaly.

(A) Unstimulated cells, PI = 1.04 **(B) PHA-L-stimulated cells, PI = 1.96**

FIGURE 2 An example of cell division graphic indicating CD3⁺ cell proliferation indexes (PI)

As shown in Table 1, the mean baseline immunoglobulin levels were 803.4 ± 440.1 , 67.3 ± 80.9 , and 113.2 ± 114.2 mg/dL for IgG, IgA, and IgM, respectively, in the patient group. IgG, IgA, and IgM values were decreased in comparison with age-related normal reference ranges in 45.7% ($n=21$), 53.3% ($n=24$), and 41.3% ($n=19$) of patients, respectively. A total of 26.1% patients ($n=12$) had low values for all immunoglobulins (IgG, A, M) and 33.3% of patients ($n=15$) had low values for both IgG and IgA. The specific antibody responses were negative in 36% of patients. There was no response against protein antigens measured as antitetanus IgG and carbohydrate antigens measured as anti-Hb IgG in 30% and 28.5% of patients, respectively.

In immunophenotyping, 34.8% patients ($n=16$) had showed low CD3⁺ cell percentages, while CD19⁺ cells were decreased in 41.3% of patients ($n=19$). The mean percentages of CD3⁺CD45RO⁺ naive, CD3⁺CD45RO⁺ memory, CD3⁺TCR $\alpha\beta$ ⁺, and CD3⁺TCR $\gamma\delta$ ⁺ cells in patients were 35.2 ± 21.3 , 20.2 ± 10.7 , 74.8 ± 30.5 , and 11.8 ± 10.0 , respectively.

T lymphocyte proliferation findings in control and patient groups are summarized in Table 2. As shown in Table 2, the percentages of the CD3⁺ blast cells after PHA-L stimulation which was called M% showed

statistically significant difference between patient group ($31.1 \pm 28.8\%$) and control group ($67.9 \pm 8.79\%$; $P < .001$). The proliferation responses in patients were lower than controls. M% also showed positive correlation with CD3 ($P = .032$, $r = .337$), CD4 ($P < .001$, $r = .503$) cell counts, and CD3% ($P = .025$, $r = .330$).

Mean daughter cell proliferation index, PI, of the patient group was increased from 1.03 ± 0.02 to 1.43 ± 0.51 after PHA-L stimulation and this increase was statistically significant ($P < .001$). PI after stimulation showed positive correlation with CD3% ($P = .045$, $r = .297$) and CD4% ($P = .013$, $r = .362$). Control group proliferation index increased from 1.01 ± 0.01 to 1.82 ± 0.49 ($P < .001$).

M% values were evaluated in comparison with control group mean values (mean $\pm$ SD) and were decreased in 76.1% of patients ($n=35$) and showed positive correlation with PI after stimulation ($P = .000$, $r = .698$). Thirteen patients with low CD3 values (81.2%) showed decreased blast transformation, and nine of them had low values below 5%. However, a total of 73.3% (22/30) patients with normal CD3 percentages had showed decreased blast transformation and four of them had low values below 5%. On the other hand, if values were categorized as low and normal, neither M% ($P = .722$) nor PI after stimulation

TABLE 1 Demographical and laboratory findings in patient group (n=46)

	Unit	Mean±SD
Age of symptoms	months	29.7±38.2
Age on admission	months	49.9±48.3
Age in study	months	64.9±55.2
IgG	mg/dL	803.4±440.1
IgA	mg/dL	67.3±80.9
IgM	mg/dL	113.2±114.2
WBC	cells/mm ³	8830±5313
Absolute lymphocyte	cells/mm ³	3123±2056
CD3 ⁺ T cell	cells/mm ³	2055±1498
	%	63.9±17.9
CD3 ⁺ CD4 ⁺ T helper cell	cells/mm ³	786.1±878.5
	%	33.9±15.5
CD3 ⁺ CD8 ⁺ T cytotoxic cell	cells/mm ³	521.6±466.6
	%	25.0±13.6
CD19 ⁺ B cell	cells/mm ³	557.1±545.7
	%	18.2±14.8
CD3 ⁺ CD16 ⁺ 56 ⁺ natural killer cell	cells/mm ³	391.9±466.8
	%	14.1±11.7
CD3 ⁺ HLA DR ⁺ active T cell	cells/mm ³	325.3±569.6
	%	15.9±15.3

TABLE 2 T lymphocyte proliferation indexes with flow cytometric analysis in both control and patient groups

	Control group (n=25)	Patient group (n=46)	P
M%	67.9±8.79	31.1±28.8	.000*
PI before stimulation	1.01±0.01	1.03±0.02	.003*
PI after stimulation	1.82±0.49	1.43±0.51	.000*

*P<.05.

(P=.424) did not show any statistically significant relation to CD3 low or normal percentages.

The patients were evaluated with their clinical and laboratory data. Frequencies of patient groups diagnosed according to IUIS 2015 were as follows: combined immunodeficiencies (n=19, 41.3%); intrinsic and innate immunity defects (n=11, 23.9%); combined immunodeficiencies with associated or syndromic features (n=9, 19.6%); predominantly antibody deficiencies (n=4, 8.7%); diseases of immune dysregulation (n=2, 4.3%); and autoinflammatory syndromes (n=1, 2.2%).

Daughter cell proliferation data of different diagnosis groups are summarized in Table 3. Neither M % (P=.213) nor PI (P=.387) showed statistically significant difference between IUIS diagnosis groups. The percentages of CD3 did not differ between groups (P=.622).

As shown in Table 3, the decrease in T-cell proliferative responses was mostly seen for combined immunodeficiency (CID) group. The mean M% was 22.2±22.9 and proliferation index showed an increase

from 1.04±0.03 to 1.33±0.42 with stimulation in these patients. Six of eighteen CID patients had low values for CD3%. Neither M% nor PI after stimulation had relation to CD3 low or normal percentages (P=1.000). Sixteen of CID patients (n=18) had impaired proliferative response and seven of them had low M% values below 5%.

4 | DISCUSSION

As highlighted in our previous studies,^{17,18} high prevalence of PID is seen due to high percentage of consanguineous marriages in Turkey. Twenty patients (43.5%) were born to consanguineous parents, and family history of PID was positive for twelve patients (26.1%) in this study.

Primary immunodeficiencies are a group of chronic disorders that present in childhood and T-cell disorders present as combined T- and B-cell immunodeficiency with susceptibility to bacterial, viral, and fungal pathogens.¹ A total of 70.5% of our patients had infections on admission. The sinopulmonary infections were the most frequent type similar to literature^{17,19,20}, and a total of 54.3% of patients had minimum of six infections per year.

In many laboratories, routine laboratory techniques (quantitative determination of immunoglobulins, immunophenotyping, etc.) for investigating PID are feasible, but functional studies are not prevalent in general use. CFSE-based assay for the assessment of mitogenic and antigenic T-cell proliferation responses provides us to diagnose and classify T-cell defects according to IUIS.¹⁰ This assay also gives an idea for both cell function and viability more effectively as opposed to radioactive assays such as thymidine uptake assays. These cumbersome radioisotope-based assays are replaced with CFSE method as later one is less labor-intensive, cheaper to perform, is able to focus analysis on particular lymphocyte subsets and is able to measure expression of activation markers directly and in terms of cell cycle.²¹ Evrard et al.⁴ suggested that thymidine test was not sufficiently accurate and CFSE method using an apoptosis marker together would yield more information about targeted lymphocytic proliferation in quality control of extracorporeal photopheresis. Similarly, Faivre et al.²² emphasized the CFSE method's benefits besides reference thymidine method in this practice.

Decreased mean values for immunoglobulins (45.7%, 53.3%, and 41.3% of patients for IgG, IgA, and IgM) were expected findings in our patients and changes in T- and B-cell percentages, naive and memory T-cell pool or T lymphocyte cell receptor profile also helped us for definitive diagnosis. On the other hand, in vitro T-cell proliferation responses especially in patients with normal T-cell counts gave us an idea for detecting prior exposure of T cells to antigens.

Lymphocyte proliferation response to mitogens may be affected by the use of steroids, immunosuppressive or immunomodulatory agents, alcohol, physiological-social stress, and the time elapsed since blood collection. These were taken into consideration during evaluation of our patients' data for these factors and collected all samples on the day of study. Samples were diluted in the presence of excess neutrophils or nonviable cells to avoid falsely diminished results.

TABLE 3 The laboratory data and flow cytometric T lymphocyte proliferation findings in different PID diagnosis groups

	IgG (mg/dL)	IgA (mg/dL)	IgM (mg/dL)	CD3 ⁺ T cells%	CD3 ⁺ CD4 ⁺ %	CD3 ⁺ CD8 ⁺ %	CD19 ⁺ B cells%	NK cells ⁺ %	Active T cells ⁺ %	Proliferation index	
										Before	After
Combined immunodeficiency (n=19)	670±363	59.0±105	118.7±153.3	64.3±21.5	32.3±19.9	24.2±18.3	15.5±15.8	18.2±14.6	19.5±18.5	21.2±22.7	1.04±1.32
CID with associated or syndromic features (n=9)	958±664	65.5±81.6	123.4±98.9	58.9±19.5	36.6±15.5	20.3±7.65	23.2±19.4	12.8±12.8	12.5±8.25	42.2±24.7	1.02±0.01
Predominantly antibody deficiency (n=4)	394±164	32.9±16.6	30.4±5.08	66.9±5.84	37.5±7.66	28.1±7.76	14.3±4.64	13.3±3.32	3.06±1.40	30.8±34.9	1.04±0.04
Disease of immune dysregulation (n=2)	1010±0	103.5±37.4	161±101.8	51.5±6.36	25.9±18.5	23.5±13.4	32±8.48	12.7±14.5	13.8±13	40.8±57.5	1.02±0.00
Intrinsic and innate immunity defects (n=11)	1001±310	76.2±29.3	112.8±63.1	67.9±14.4	35±9.33	29.5±10.2	18±1.9	9.29±4.74	14.4±14.8	40.0±33.4	1.03±0.03
Autoinflammatory syndromes (n=1)	988	208	158	71±0	33±0	23±0	13±0	9±0	36±0	0.10	1.04
P	.008*	.043*	.181	.622	.830	.506	.374	.599	.454	.213	.796
											.387

*P<.05.

T-cell proliferation response is a functional marker of effective immune response and the degree of impairment may vary depending on T-cell capability. CD3⁺ T-cell proliferation responses, both M% and proliferation indexes that are recorded in patient group, were lower than controls after stimulation (Table 2), and the difference was significant between two groups (P<.001). M% values were decreased in 76.1% of patients in correlation with proliferation indexes.

Abnormal test results to mitogen stimulation are indicative of impaired T-cell function apart from T-cell counts, low or normal.²³ In patient group, M% values showed positive but weak correlation with CD3 and CD4 cell counts and also CD3 frequency. Thirteen patients (81.2%) with low CD3 values (n=13/16) showed decreased blastic transformation, and nine of them had low values below 5%. However, 73.3% (22/30) of patients with normal CD3 percentages showed decreased blastic transformation and four of them had values lower than 5%.

Neither M % (P=.213) nor PI (P=.387) showed statistically significant difference between the IUIS diagnosis groups (Table 3), indicating no correlation between clinically significant immunodeficiency and T lymphocyte proliferation response. Most prominent decrease was seen in CID group as expected. Sixteen of CID patients (n=18) had impaired proliferation responses and seven of them had low M% values lower than 5%.

Combined immunodeficiency is a heterogeneous group of immunodeficiencies that are characterized by defects of T-cell development and/or T-cell function, and can be associated with variable defects of B or natural killer cells. Bai et al.²⁴ showed that T-cell proliferation in response to PHA was undetectable in vitro in patients with RAG mutations consistent with the impaired V(D)J rearrangement and restricted TCR Vb repertoire diversity.

Chinese patients with LIG4 deficiency, who presented with T^{low}B^{low} combined immunodeficiency, also had low percentages of proliferating CD4⁺ and CD8⁺ T lymphocytes after in vitro PHA stimulation. CFSE fluorescence histograms generated by flow cytometry revealed no obvious peak of cell division suggesting impaired T-cell proliferation.²⁵

Roifman et al.²⁶ evaluated in vitro responses to PHA in patients with profound T-cell deficiency. They suggested that a total of 500 circulating T cells could be used as a cutoff level to distinguish severe combined immunodeficiency (SCID) from CID. They also studied the function of residual T cells in patients with SCID and CID and showed that all patients with SCID, regardless of genotype, had severely decreased (mostly <10%) in vitro responses to PHA. This function was also severely decreased in most patients with CIDs, including Omenn syndrome, ZAP70 deficiency, and IL2RA deficiency.

B-cell function may alter even in case of normal B-cell maturation due to impaired T-cell help. Izgi et al.²⁷ studied T- and B-cell proliferation in patients with common variable immunodeficiency and showed normal cell proliferation responses even after stimulation.

Our patients were evaluated individually with laboratory and clinical findings and were then directed to appropriate molecular genetical analysis.

So, there are still many cases of T-cell dysfunction in which the genetic basis of the condition remains unknown and there is not a single

laboratory test that can define impaired cellular immunity. Functional studies in primary immunodeficiencies contrary to routine laboratory methods are rarely performed in our country. CFSE-based assay for the assessment of mitogenic T-cell proliferation responses in specific primary immunodeficiency patients is reliable and practical for routine practice. There are some limitations of the method as difficulty in making standard operating conditions or laboratory staff, but this method assists clinicians in choosing the most appropriate genetical analyses required for the exact diagnosis and the most efficacious treatment.

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How to cite this article: Azarsiz E, Karaca N, Ergun B, Durmuscan M, Kutukculer N, Aksu G. In vitro T lymphocyte proliferation by carboxyfluorescein diacetate succinimidyl ester method is helpful in diagnosing and managing primary immunodeficiencies. *J Clin Lab Anal.* 2018;32:e22216. <https://doi.org/10.1002/jcla.22216>