

Original Article

Flow Cytometric Analysis of *Leishmania* Reactive CD4⁺/CD8⁺ Lymphocyte Proliferation in Cutaneous Leishmaniasis

M Nateghi Rostami¹, A Khamesipour², SE Eskandari², A Miramin Mohammadi², A Sarraf Nejad³, *H Keshavarz¹

¹ Dept. Medical Parasitology and Mycology, School of Public Health; Tehran University of Medical Sciences, Iran

² Center for Research and Training in Skin Diseases and Leprosy; Tehran University of Medical Sciences, Iran

³ Dept. Immunology, School of Public Health; Tehran University of Medical Sciences, Iran

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Abstract

Background: Determination of the division history of T cells *in vitro* is helpful in the study of effector mechanisms against infections. Technique described here uses the intracellular fluorescent label carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor the proliferation.

Methods: In a cross sectional study, blood samples were collected from 7 volunteers with history of cutaneous leishmaniasis (CL) and one healthy control from endemic areas in Isfahan province who referred to the Center for Research and Training in Skin Diseases and Leprosy (CRTSDL), then CD4⁺/CD8⁺ lymphocytes and CD14⁺ monocytes were isolated from peripheral blood mononuclear cells (PBMC) using mAbs and magnetic nanoparticles. CFSE labeled CD4⁺ or CD8⁺ lymphocytes cultured with autologous monocytes in the presence of PHA, SLA, live *Leishmania major* or as control without stimulation. Cells were harvested after 7 days and were analyzed using flow cytometry.

Results: Five consecutive divisions were monitored separately. Stimulation of CD4⁺ or CD8⁺ lymphocytes from CL subjects with SLA showed a significant difference in proliferation comparing with unstimulated cells ($P < 0.05$). The significant difference in the percentages of CD4⁺ cells stimulated with SLA was revealed at different divisions for each subject. In CD8⁺ lymphocyte, significant stronger stimulation of SLA was evident later in the proliferation process. The mean number of divisions in both CD4⁺/CD8⁺ lymphocytes stimulated with SLA was significantly greater than when stimulated with live *L. major* ($P=0.007$ / $P=0.012$, respectively)

Conclusion: The percentage of divided cells might be calculated separately in each division. The cells remained active following CFSE staining and there is possibility of functional analysis simultaneously.

Keywords: CFSE, CD4⁺/CD8⁺ T cells, Proliferation, Cutaneous leishmaniasis

Introduction

Immune cells when encounter foreign antigens experience remarkable expansion and differentiation. The proliferation by itself is not a specific effector function of immune cells, the ability to determine the division history of cell population undergoing proliferation is particularly useful in the study of effector mechanisms

of immune cells against infections (1). There are a number of techniques available for determining cell division both *in vivo* and *in vitro*; however most of the techniques are only able to identify the proliferation of the cells through a limited number of divisions. By using incorporation of titrated thymidine technique, the overall division is quantified but it is not applicable to assess the division history of single cell. The

*Corresponding author: Tel: +98-21-816 33 799,
E-mail: hkeshavarz@tums.ac.ir

new technique described here uses the intracellular fluorescent label carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor proliferating sorted lymphocytes. Covalently bound CFSE is partitioned equally between daughter cells, allowing discrimination of up to 8 rounds of cell divisions, before the fluorescence is reach to background fluorescence of unstained cells (2). The method is applicable to *in vitro* cell division as well as to *in vivo* division of adoptively transferred lymphocytes (3). In the recent years, this technique is used in some studies preferentially on basic immunology in animal models (4). In the current study, CD4⁺ and CD8⁺ lymphocytes were purified from human PBMC of volunteers recently recovered from cutaneous leishmaniasis (CL) and the possibility of using flow cytometry method for monitoring the proliferation of CD4⁺ and/or CD8⁺ T cells in response to *Leishmania* antigens was explored *in vitro*.

Materials and Methods

Patients and sampling

In a cross sectional study, seven volunteers (M=5, F=2) with history of CL caused by *L. major* with a positive Leishmanin skin test (LST) who had recovery from CL between 2-24 months before sampling and one healthy male volunteer with no response to LST as control from an endemic area in Isfahan province, who referred to CRTSDL were included in this study

SLA preparation

Soluble *Leishmania* antigen (SLA) was prepared from *L. major* (MRHO/IR/75/ER) using Scott method (5). Briefly, *L. major* was cultured on RPMI 1640 supplemented with 10% FCS, and promastigote were harvested at stationary phase and adjusted to 1x10⁹ promastigotes/ml. Parasites were washed with cold PBS (pH 7.2), and then protease inhibitor cocktail enzyme (Sigma, USA) was added and repeatedly freeze-thawed followed by sonication at 4 °C with ten 20-s blasts. Parasite suspension was

centrifuged at 30,000 g for 20 min, the supernatant was collected and re-centrifuged at 100,000 g for 4 h. Finally the supernatant was sterilized using 0.22 µm membrane filter.

Isolation of CD4⁺/CD8⁺/CD14⁺ cells

Twenty ml of heparinized blood sample was collected from each volunteer; PBMCs were isolated using Ficoll–Hypaque density gradient centrifugation. CD4⁺/CD8⁺ lymphocytes isolation from PBMC were performed using magnetic nanoparticles system (StemCell Technologies Inc., Canada) by positive selection using anti-CD4 and anti-CD8 coated magnetic beads. Monocytes (CD14⁺) isolated from autologous PBMC using magnetic beads by negative selection using monocyte enrichment cocktail antibodies according to manufacturer's instruction. Monocytes freshly used as antigen presenting cells (APCs) and cultured with sorted CD4⁺ or CD8⁺ lymphocytes (1:10 ratio). The purity of yielded lymphocytes and monocytes were found to be between 85-96% by flow cytometry using conjugated mAbs.

CFSE staining

For proliferation assay, purified CD4⁺ or CD8⁺ T cell subpopulations were adjusted to 1x10⁵ cells/ml in pre-warmed PBS + 0.1% BSA. 5 mM stock CFSE (5 and 6-carboxyfluorescein diacetate succinimidyl ester) (Molecular Probes, Eugene, USA) solution in DMSO was added at 2 µl per milliliter of cells for a final working concentration of 10 µM followed by incubation at 37 °C for 10 min. The staining was quenched by adding (5 fold to cell volume) of ice-cold RPMI 1640 + 10% FBS to the tube. After 3 times washing with RPMI 1640, the cells were resuspended in fresh medium and cultured.

Purified lymphocyte culture

The culture medium used was RPMI 1640 supplemented with 10% heat-inactivated human AB Rh+ serum, 10 mM/L Hepes, 2 mM L-glutamine, 100 U/ml penicillin G and 100 µg/ml streptomycin. CFSE labeled CD4⁺ or CD8⁺ lymphocytes were adjusted to 1-2 x10⁵ cells/well in the medium and dispensed in a 96-

well plate with 1:10 of autologous monocytes in the presence of 2 µg/well PHA, or 20 µg/well of SLA or 5×10^4 /well live *L. major* or as control without stimulation in a final volume of 200 µL. The medium was replaced every other day with fresh medium. The plates were incubated at 37 °C with 5% CO₂ in a humidified atmosphere up to 7 days.

Flow cytometric analysis

Cells were harvested after 7 days of culture, washed with PBS and fixed with 1% PFA (Paraformaldehyde), and were analyzed using Partec flow cytometer with 488 nm excitation. Green fluorescence was collected with a 525-nm band-pass filter. A minimum of 20,000 events were acquired for each sample. Two sets of controls were used, one control was the cells activated under the same conditions but not labeled with CFSE which served as autofluorescence (background) control. Events with green fluorescence above this background were collected. The second control was unstimulated but stained cells. Using an unstimulated CFSE stained control sample, the parent generation was set (see Fig. 1). FACS data analysis was performed using FloMax (DAKO cytometry, Denmark) and ModFit (Verity Software House, USA) software. The numerical values for proportions of proliferated cells at each cell division were obtained by ModFit analysis and used for statistical analysis (Fig. 1).

Statistical analysis

Non-parametric tests of Mann-Whitney and Kruskal-Wallis were used for comparison of different proliferation values induced in CFSE cell culture. Data were analyzed using SPSS (version 11.5) software (SPSS Inc., USA). *P* value of <0.05 is regarded as significant.

Ethical Considerations

The proposal was approved by Institutional Ethical Committee as well as Ethical Committee of Tehran University of Medical Sciences. Potential candidates were informed about the study and those who were willing to participate and sign an informed consent were recruited.

Results

Fig. 2 (a-h) and Fig. 3 (a-h) show the percentage of sorted CD4⁺ and CD8⁺ in serial divisions after 7 days of stimulation in culture in volunteers recently healed from cutaneous leishmaniasis and healthy control volunteer. The proportion of proliferating cells at each generation determined using ModFit analysis. Figures summarize the percentage of cells at a total of 5 cell generations for each stimulation. Under the stimulation of SLA, purified CD4⁺ and CD8⁺ lymphocytes from all volunteers with history of CL showed a significantly (*P*<0.05) higher cell divisions in comparison with unstimulated cells.

There was a significantly (*P*<0.05) higher percentages of CD4⁺ cells stimulated with SLA compared to unstimulated cells in culture from generation 3 to generation 5 for patients 1 and 2 and from generation 2 to generation 4 for patients 3, 4, 6 and 7. Patient 5 (Fig. 3-e) showed a significantly (*P*<0.05) higher CD4⁺ cells in generation 3 and 4 compared to unstimulated cells.

In CD8⁺ lymphocyte culture stimulated with SLA, the significantly higher number of cells (*P*<0.05) compared to unstimulated cells are evident later in the culture at final cell divisions (generation 4 or later).

The results of all CL volunteers were pooled and the mean division number of stimulated and unstimulated CD4⁺/CD8⁺ lymphocytes was calculated (Table 1). When CD4⁺ or CD8⁺ lymphocytes stimulated with SLA, a significantly greater number of divisions was induced compared with unstimulated control cells (*P*=0.002 and *P*=0.003 for CD4⁺ and CD8⁺, respectively); no significant difference was seen when the cells were stimulated with live *L. major* and the mean division number was not significantly different from that of unstimulated cells for both CD4⁺ and CD8⁺ lymphocyte in culture.

The purified CD4⁺ and CD8⁺ lymphocytes from healthy control volunteer were undergone up to 3 divisions (g3) upon SLA or live *L. ma-*

major stimulation (Fig. 3-h and Fig. 4-h) that was higher than unstimulated cells which undergone just one division (g1).

In both CD4⁺ and CD8⁺ lymphocytes culture, stimulated with live *L. major* no significant difference was seen compared to unstimulated cells in proliferation analysis.

Comparing between the two antigens, the mean number of divisions of CD4⁺ lymphocytes stimulated with SLA (4.1±0.64087) was signifi-

cantly ($P=0.007$) greater than that of stimulated with live *L. major* (2.125±1.3562).

In regard to CD8⁺ lymphocytes, the mean number of divisions induced by SLA stimulation (3.75±0.88641) was significantly ($P=0.012$) greater than that of live *L. major* stimulation (1.875±0.83452).

No significant difference was seen in overall mean number of divisions between CD4⁺ and CD8⁺ lymphocytes in culture.

Table 1: Mean number of divisions of purified CFSE stained lymphocytes after culture. Purified lymphocytes were labeled with CFSE and cultured under the stimulation of PHA, SLA, live *L. major* or without antigen. Data obtained from CL volunteers by analysis of ModFit software were pooled and summarized.

Type of anti- gen	CD4+ T cells			P value	CD8+ T cells			P value
	Mean	95% Confidence Interval	In-		Mean	95% Confidence Interval	Inter-	
PHA	4.0	3.5531-4.4469		0.002	3.625	3.1923-4.0577		0.002
SLA	4.1	3.5892-4.6608		0.002	3.75	3.0089-4.4911		0.003
Live <i>L. m</i>	2.125	0.9912-3.2588		0.777	2.25	1.3846-3.1154		0.473
Unstimulated	1.875	0.9336-2.8164			1.875	1.1773-2.5727		

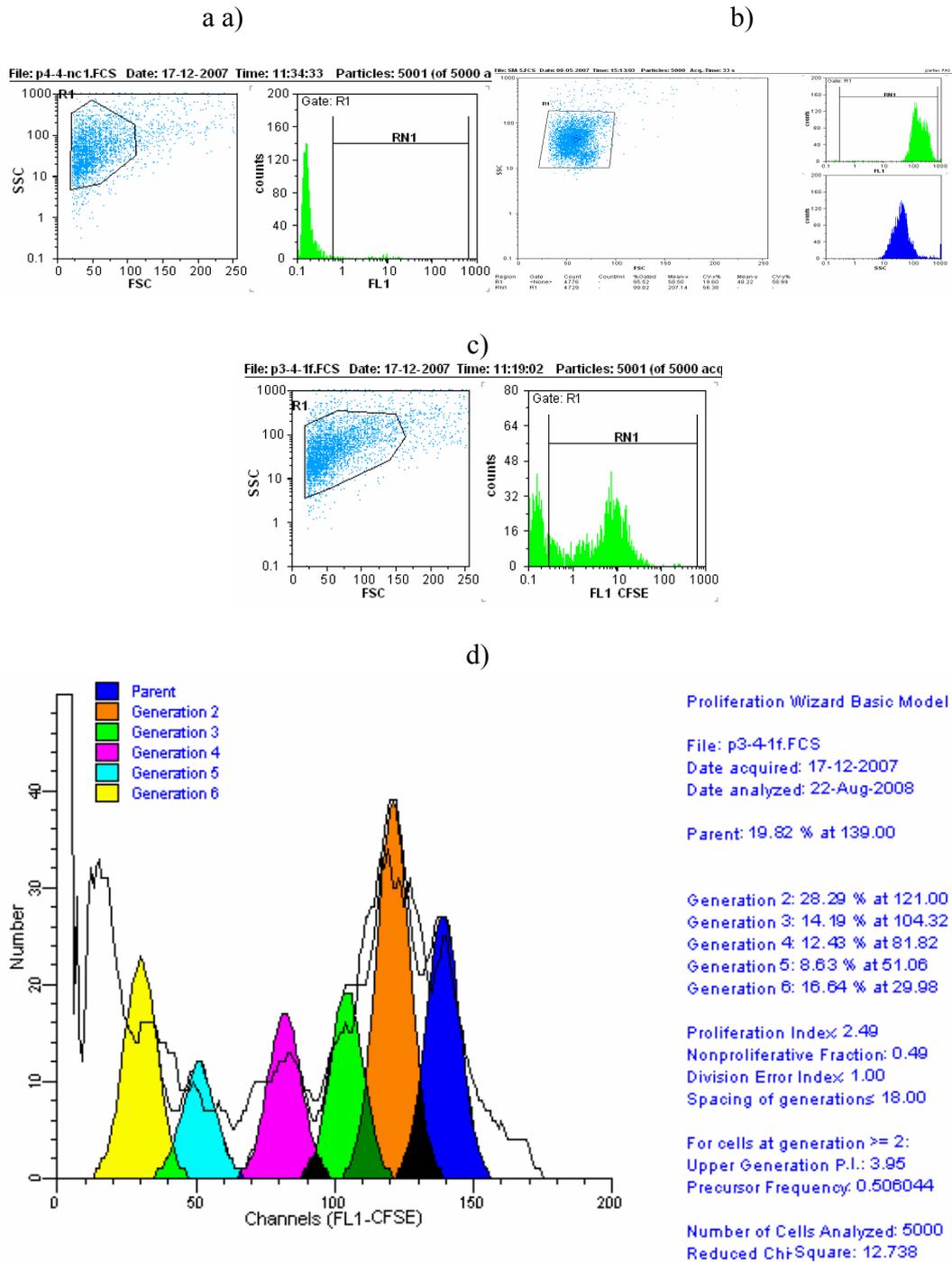


Fig. 1: CFSE labeled proliferation profile of lymphocytes using ModFit software analysis.

- a) Setting unstimulated CFSE-labeled control. b) Setting unstained PHA stimulated control.
 c) Histogram shows CFSE stained cell as seen in FloMax analysis software.
 d) Analysis result using ModFit software based on data obtained from FloMax histogram of CFSE stained cells. The peak on the right corresponds to the unstimulated CFSE-labeled control cells. The unfilled peak on the left corresponds to the autofluorescence of unstained stimulated cells. The percentage of cells in each division is seen on the right.

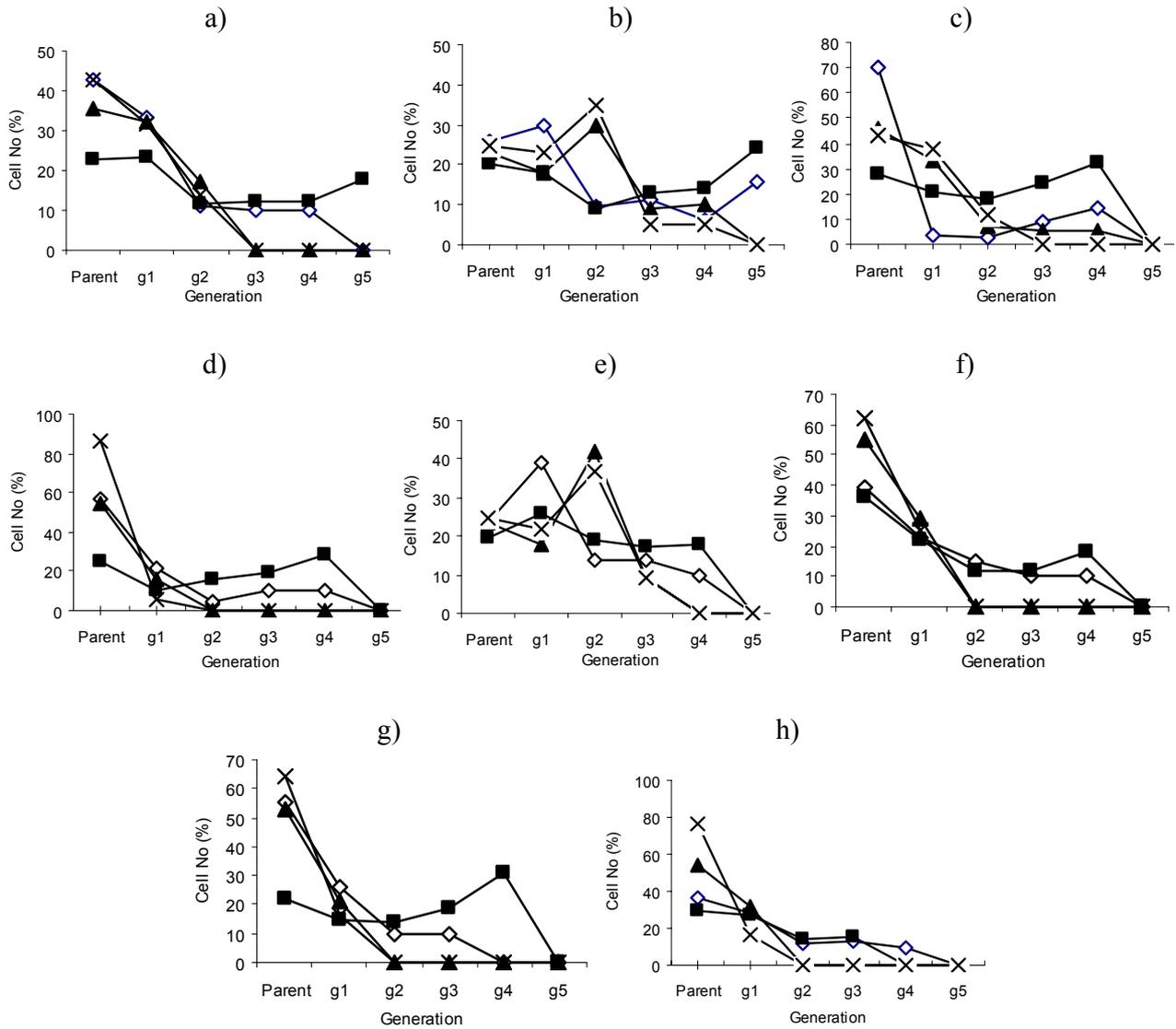


Fig. 2: Proliferation of CFSE stained CD4+ lymphocytes after culture. Purified lymphocytes were labeled with CFSE and cultured with PHA, SLA, live *L. major* or without antigen. Cells were harvested after 7 days of incubation and analyzed using Partec flow cytometer. A minimum of 20,000 events were acquired for each sample. The numerical values for proportions of proliferated cells at each cell division were obtained by ModFit software analysis. “a” to “g” show Patients 1 to 7, respectively. “h” shows Healthy control.

—◇— PHA —■— SLA
 —▲— Live L.m —×— Unstimulated

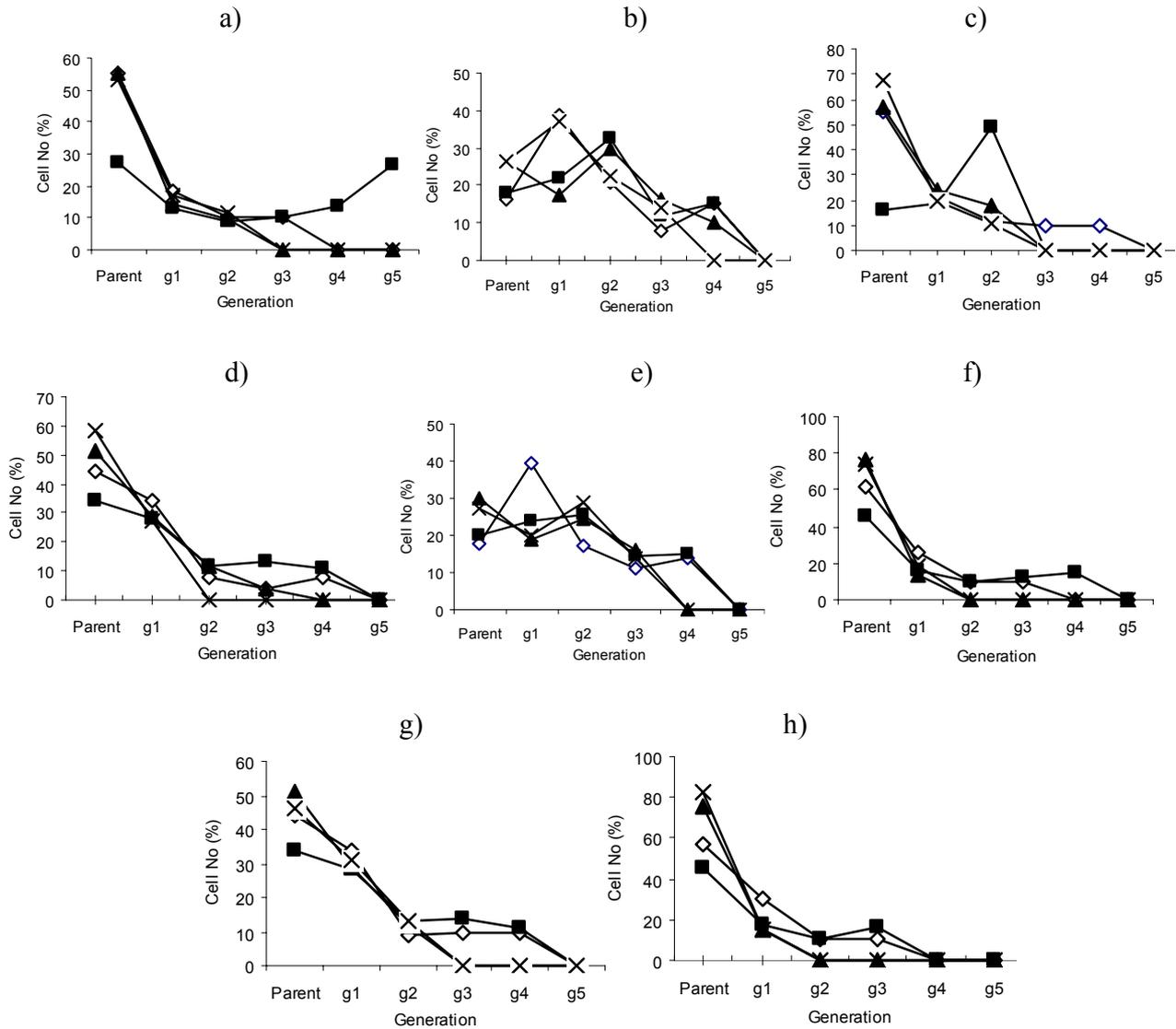


Fig. 3: Proliferation of CFSE stained CD8+ lymphocytes after culture. Purified lymphocytes were labeled with CFSE and cultured with PHA, SLA, live *L. major* or without antigen. Cells were harvested after 7 days of incubation and analyzed using Partec flow cytometer. A minimum of 20,000 events were acquired for each sample. The numerical values for proportions of proliferated cells at each cell division were obtained by ModFit software analysis. “a” to “g” show Patients 1 to 7, respectively. “h” shows Healthy control.

—◇— PHA
 —▲— Live L.m
 —■— SLA
 —×— Unstimulated

Discussion

Techniques currently available to evaluate cell proliferation (Thymidine incorporation, BrdU assay) only show a limited number of cell divisions without giving any information about the

division history of individual cells (6). The combination of flow cytometry and CFSE (or CFDA-SE) labeling techniques is used to study cellular proliferation, including measurement of the percentage of proliferated lymphocytes and the number of cell divisions undergone by proliferated cells (7, 8).

T cell subtypes are heterogeneous in their ability to proliferate in response to antigens. In the case of CD4⁺ and CD8⁺ T cells, the two subpopulations represent diverse lineage of lymphocyte which is distinguished by the expression of different markers and functions (9, 10). The functions include class-specific MHC-restricted recognition of antigens, capacity to proliferate and produce a range of cytokines. Different features of proliferation might be seen in response to the same antigen by CD4⁺ vs CD8⁺ lymphocytes. In this study the proliferation history of both CD4⁺ and CD8⁺ lymphocytes in response to *Leishmania* antigens using a flow cytometry based method was successfully monitored. Both CD4⁺ and CD8⁺ T cells were analysed concerning the proportion of cells in each division. As CD8⁺ T cells might respond weaker to soluble antigens, either SLA or live *L. major* was used to stimulate lymphocytes. CD4⁺ lymphocytes of CL volunteers responded to SLA with significantly more number of divisions (compared to unstimulated cells) and more percentage of the cells undergone divisions early in the proliferation (from g2-so on, compared to CD8⁺ cells). Previously, the role of CD4⁺ cells in the proliferation to killed *Leishmania* antigens was showed when depletion of CD4⁺ cells before stimulation interfered with the *Leishmania* specific proliferative response (11). Most of CD8⁺ lymphocytes of CL volunteers reached g4 of division and significance of difference was divulged in this late step of division process. In leishmaniasis, antigen specific-CD8⁺ lymphocytes were reported to be present around the lesions (12) and in peripheral blood (10) during acute phase and during healing process of the lesion. Although it is believed that the main function of Ag-specific CD8⁺ T cells is to contribute in cytokine and especially IFN- γ production (13), it is not clear whether these cells proliferate upon exposure to *Leishmania* antigens (9).

In this experiment, either SLA or live *L. major* was used to stimulate cell proliferation. The

percentage of CD4⁺ and CD8⁺ lymphocytes in generation 3 and 4 of divisions showed that SLA induced expansion of the lymphocytes in culture condition. Unlike SLA, live *L. major* did not stimulate lymphocytes when compared with unstimulated cells. In agreement with this observation, results of another study showed that live and dead promastigotes differ in their ability to induce proliferation and cytokine production (14). Nysten *et al.* reported that there was apparent difference in the immune response evoked by dead and live *L. aethiops*, so that killed promastigotes preferentially induced proliferation rather than cytokine secretion. It was proposed that live *Leishmania* may inhibit presentation by MHC molecules, so that interfere with lymphocyte proliferation (15).

The cells from healthy control also showed a limited proliferation in response to *Leishmania* antigens. As expected, stimulation of purified lymphocytes with SLA provoked a weaker response and as such the cells were undergone up to three divisions (g3). It was demonstrated that cells from healthy individuals without prior exposure to *Leishmania* may proliferate following stimulation with *Leishmania* antigens *in vitro* (16). While using common well-known method of ³H-thymidine incorporation the overall proliferation of cultured lymphocytes is quantified, this technique showed the advantage that the percentage of the cells undergone proliferation could be calculated separately in each division; so that in some points of division's progression differential proliferation capacity of cells was seen in response to different stimulations. The cells remained alive and active following CFSE staining and the proliferation kinetics could be followed at different days of extended culture (17).

Conclusively, by using flow cytometry method the proliferation history of both CD4⁺ and CD8⁺ T cells in response to *Leishmania* antigens was monitored in volunteers with history of CL. The percentage of divided cells might be calculated in each division. Using flow cytometry technique provides an opportunity to

analyze surface marker(s) or function of the cells simultaneously.

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References

1. Wyler DJ, Weinbaum FI, Herrod HR. Characterization of *in vitro* proliferative responses of human lymphocytes to *Leishmania* antigens. *J Infect Dis*. 1979;140: 215-221.
2. Hodgkin PD, Lyons BA. Tracking cell division using CFSE. *Today's Life Sci*. 2000;12(5):36-8.
3. Graziano M, Pierre Y, Beauchemin C, Desrosiers M, Potworowski EF. The fate of thymocytes labeled *in vivo* with CFSE. *Exp Cell Res*. 1998;240(1):75-85.
4. Lyons AB. Analyzing cell division *in vivo* and *in vitro* using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods* 2000;243(1-2):147-54.
5. Scott P, Pearce E, Natovitz P, Sher A. Vaccination against cutaneous leishmaniasis in a murine model I. Induction of protective immunity with a soluble extract of promastigotes. *J Immunol*. 1987;139:221-7.
6. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J Immunol Methods* 1994;171(1):131-7.
7. Yates A, Chan C, Strid J, Moon S, Callard R, George AJT, Stark J. Reconstruction of cell population dynamics using CFSE. *BMC Bioinform*. 2007;8:196.
8. Tsuge I, Okumura A, Kondo Y, Itomi S, Kakami M, Kawamura M, Nakajima Y, Komatsubara R, Urisu A. Allergen-specific T-cell response in patients with phenytoin hypersensitivity; simultaneous analysis of proliferation and cytokine production by Carboxyfluorescein Succinimidyl Ester (CFSE) dilution assay. *Allergol Int*. 2007; 56(2):149-55.
9. Ruiz JH, Becker I. CD8 cytotoxic T cells in cutaneous leishmaniasis. *Parasite Immunol*. 2007;29:671-8.
10. Da-Cruz AM, Conceicao-Silva F, Bertho AL, Coutinho SG. *Leishmania* reactive CD4+ and CD8+ T cells associated with cure of human cutaneous leishmaniasis. *Infect Immun*. 1994;62:2614-8.
11. Nylen S, Maasho K, Soderstorm K, Ilg T, Akuffo H. Live *Leishmania* promastigotes can directly activate primary human natural killer cells to produce interferon-gamma. *Clin Exp Immunol*. 2003;131: 457-67.
12. Da-Cruz AM, Bertho AL, Oliveira-Neto M, Coutinho S. Flow cytometric analysis of cellular infiltrate from American tegumentary leishmaniasis lesions. *British J Dermatol*. 2005;153:537-43.
13. Roger K, Titus R. Characterization of early cellular immune response to *L. major* using peripheral blood mononuclear cells from *Leishmania*-naive humans. *American J Trop Med Hyg*. 2004;71:568-76.
14. Nylen S, Mortberg U, Kovalenko D, Satti I, Engsterom K, Bakhiet M, Akuffo H. Differential induction of cellular responses by live and dead *Leishmania* promastigotes in healthy donors. *Clin Exp Immunol*. 2001(124):43-53.
15. Garcia MR, Graham S, Harris RA, Beverley SM, Kaye PM. Epitope cleavage by *Leishmania* endopeptidase limits the efficiency of the exogenous pathway of

- MHC-I associated antigen presentation.
Eur J Immunol. 1997;27:1005-13.
16. Kemp M, Hansen MB, Theander TG. Recognition of *Leishmania* antigens by T lymphocytes from nonexposed individuals. Infect Immun. 1992;60:2246-51.
 17. Asquith B, Debaq C, Florins A, Gillet N, Sanchez-Alcaraz T, Mosley A, Willems L. Quantifying lymphocyte kinetics in vivo using carboxyfluorescein diacetate succinimidyl ester (CFSE). Proc Biol Sci. 2006;273(1590):1165-71.