

Proliferative Response of Hamster CD4+ T Cells after Different Mitogenic Stimulation

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Summary

The measurement of effector cells involved in immune responses is an important parameter in various fields of lymphocyte research using the Syrian Golden hamster as the experimental model. The aim of the present study was to identify the percentage of peripheral hamster CD4+ cells that undergo proliferation and the number of cell divisions induced by four different mitogenic compounds: phorbol-myristate acetate plus ionomycin (PMA/ionomycin), concanavalin A (Con A), phytohaemagglutinin (PHA) and the superantigen Staphylococcal enterotoxin B (SEB). The proliferative response was investigated by the use of the cross-reactive mouse anti-CD4 antibody (clone H129.9) and analyzed by flow cytometry using the dye carboxyfluorescein diacetate succinimidyl ester (CFSE). PMA/ionomycin induced the highest proliferative response of CD4+ cells. Interestingly, the down-regulation in the CD4 expression described after PMA/ionomycin activation both in human and in mouse lymphocytes was not seen in hamster cells. Stimulation with SEB and with Con A also induced up to 6 to 7 proliferation cycles in cells, whereas PHA induced the lowest proliferation. These results provide useful tools for the study of CD4+ cells in the hamster model.

Introduction

The Syrian golden hamster (*Mesocricetus auratus*) is used as an experimental model to evaluate diseases caused by viruses, bacteria, fungi and parasites (Li *et al.*, 2008; Yan *et al.*, 2009; Parise-Fortes *et al.*, 2000; Maravilla *et al.*, 1998) as well as for the evaluation of vaccine candidates (Mendez *et al.*, 2005; Azizi *et al.*, 2006; León-Cabrera *et al.*, 2009). The measurement of cell proliferation after mitogenic or antigen specific stimulation is an important parameter to evaluate immune responses. However, studies in the hamster model can not be achieved at

phenotype, activation, and cytokine level due to the lack of available specific markers. To circumvent this problem, the use of cross-reactive antibodies developed for other species is an option to evaluate the immune system function of hamsters.

Cell proliferation is still most commonly measured by incorporation of tritiated thymidine (3H) into lymphocyte DNA despite the disadvantage of dealing with radioactive material and that the phenotype of proliferating cells can not be identified. One method to overcome the conventional (3H) radioactive assay is the use of the fluorochrome carboxyfluorescein diacetate succinimidyl ester (CFSE). CFSE spontaneously and irreversibly couples to cellular proteins by reaction with lysine side chains and other available amines. This highly amine-reactive product then forms dye-protein adducts that are retained by the cells throughout their development. CFSE is inherited equally by daughter cells after division, resulting in the sequential halving of mean

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fluorescence with each generation (*Weston and Parish, 1990*). When analyzed by flow cytometry, this sequential halving of fluorescence is visualized as distinct peaks and can be used to track division progression (*Lyons and Parish, 1994*). The advantage of assessing proliferation by CFSE is that, combined with flow cytometry, the phenotypes of cells in proliferation can be identified and characterized by cell surface or intracellular markers and, simultaneously it is possible to calculate the number of induced cell cycles and the percentage of proliferative cells in each cycle. Here, we report the use of a cross-reactive anti-CD4 antibody for mouse cells (*Liu H, 1991*) to evaluate the proliferative response of hamster CD4⁺ CFSE stained cells to different polyclonal activators with mitogenic activity.

Materials and Methods

Out-bred female golden hamsters (8 to 12 week-old) were supplied by the animal house of the Faculty of Medicine. Animals were maintained in a conventional breeding facility and were fed with commercial pellets and water *ad libitum*. All procedures were endorsed by the Ethics Committee of the Faculty of Medicine, UNAM and followed the Guidelines for Care and Use of Laboratory Animals translated into Spanish by the Office for Protection from Research Risks, Division of Animal Welfare, National Institutes of Health, NIH publication No. 90-23Sm 1985, USA.

In preliminary experiments, we tested several commercial monoclonal antibodies against mouse T and B lymphocyte molecules: two anti-CD3 (clones 17A2 and 145-2C12), two anti-CD4 (clones GK1.5 and H129.9), two anti-CD8 (clones 53-6.7 and KT15), one anti-CD19 (clone ID3), one anti-CD45R/B220 (clone RA3-6B2) but only the anti-CD4 antibody clone H129.9 exhibited cross-reactivity against the hamster cells, therefore, this was the antibody used in the experiments.

Fifteen hamsters, in 3 separate experiments of 5 hamsters, were studied. Hamsters were sacrificed by an over-exposure to sevoflourane (Sevoran, Abbott Laboratories, Queenborough Kent, UK), spleens

were harvested and individually filtered through a 70-mm cell strainer (Falcon, Carlsbad, Ca). Cell suspensions were placed on lymphoprep (Axis-Shield PoC AS, Oslo, Norway) gradient to separate mononuclear cells. The purified cells were washed twice with complete RPMI medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA) and stained with 10µM CFSE (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Briefly, 10⁷ cells were suspended in 1 mL of PBS with 0.1% BSA and 4 µl of 5 mM stock CFSE solution were added, cells were incubated for 10 minutes at 37°C and 5 mL of cold complete medium was added to sequester any free CFSE. Cells were then washed, placed in 48-well plates at 1x10⁶ cells/mL and activated with the following stimuli: a) none, b) 2.5 µg/mL phytohaemagglutinin (PHA), c) 2 µg/ml concanavalin A (con A), d) 25 ng/mL phorbol-myristate acetate (PMA)+ 0.5 µg /mL ionomycin and, e) 10 µg /ml Staphylococcal enterotoxin B (SEB) (all mitogens purchased from Sigma Chemical, St. Louis, MO, USA). Cells were incubated at 37°C in a 5% CO₂ atmosphere.

Five days after *in vitro* CFSE labelling and stimulation, cells were washed twice as above, stained with 40 ng/10⁶ cells of phycoerythrin-conjugated anti-mouse CD4 antibody, clone H129.9 (BD-PharMingen San Jose, CA) and incubated for 20 min in the dark, washed twice, fixed with 1% paraformaldehyde in PBS and analyzed in a FACSort flow cytometer (Becton-Dickinson, San Jose, Ca) with graphics generated using the Cell Quest Pro software version 5.2.1. Statistical analysis was done using the GraphPad Prism software version 3.03. Two group comparisons of data were conducted using the Mann Whitney *U*-test. *P* values of < 0.05 were considered significant.

Results

Proliferation studies were evaluated by flow cytometry five days after *in vitro* CFSE labelling and stimulation, which is considered the standard time to evaluate antigen-specific responses (*Schneider*

et al., 2002; Ghosh et al., 2006). Both total splenocytes and CD4⁺ cells were analyzed in their proliferative capacity. Figures 1-A and 1-B show representative dot-plot graphics of non-stimulated and PMA/ionomycin stimulated cells, respectively. Similar graphics were generated with cells stimulated with PHA, Con A and SEB. From this kind of graphic a gate was established to define the CD4⁺ cells, and histograms (Figure 1 C to G) were generated to determine the number of cell divisions (each cycle marked from M1 to M7) and the average percentage of divided cells in each cycle. Cells were able to undergo proliferative cycles when stimulated with each of the mitogens tested. Table 1 shows the cell division capacities of total splenocytes (1A), and CD4⁺ splenocytes (1B) in each of the CFSE peaks. About 80% of total and 70% of CD4⁺ splenocytes from the non-stimulated group were included in peaks M2 and M3, after 5 days of stimulation. In contrast, the highest percentage of proliferating splenocytes in response to PHA at this time was found in peak M5 (19.9%), while for CD4⁺ cells there was similar distribution of cells

between peaks M3 and M6. An equal percentage (24%) of total splenocytes were found in peaks M5 and M6 when stimulated with Con A, although only 9% were found in peak M6 for CD4⁺ cells.

PMA/ionomycin stimulation allowed 7 cycles of cell division both in total and CD4⁺ cells resulting the most potent mitogen for total and CD4⁺ spleen cells as demonstrated by the high percentage of proliferating cells at the seventh cell cycle (14.5 and 12.7%, respectively). PMA/ionomycin stimulation did not cause alterations in cell size and cell granularity of hamster cells when analyzed by flow cytometry (data not shown), neither down-regulation of the surface marker CD4 (Figure 1B), as occurred in the control human PBMC (peripheral blood mononuclear cells) analyzed, where both alterations and down-regulation of CD4 were observed (data not shown). SEB was able to elicit a strong proliferation with a high percentage (13.5%) of total splenocytes proliferating at the seventh peak, while 19% of CD4⁺ cells were found proliferating at peaks five and six and 5.7% of CD4⁺ cells at peak seven.

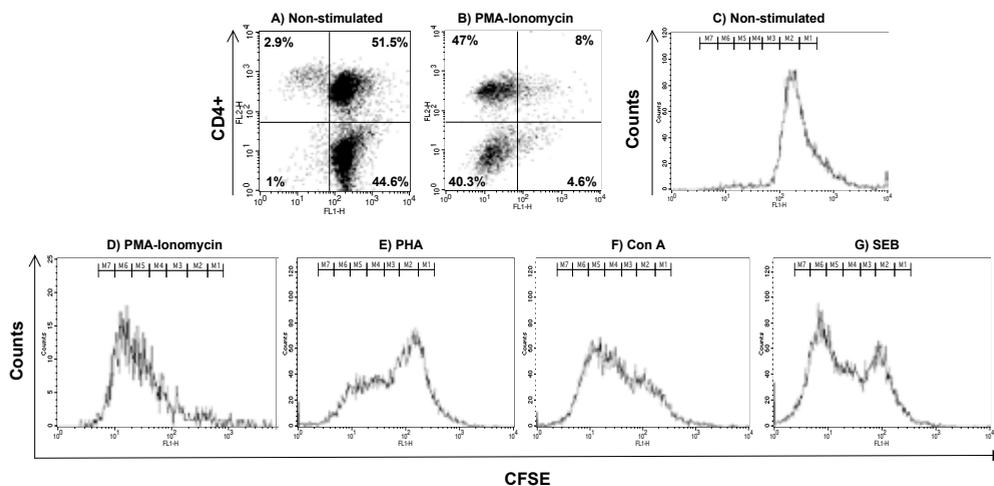


Figure 1. Quantitative analysis of lymphocyte cell division of CD4⁺ splenocytes obtained from Golden Syrian hamsters non-stimulated (A and C) and stimulated with PHA (E); Con A (F); SEB (G) and PMA-Ionomycin (B and D). Cells were labeled with CFSE and incubated for 5 days. Figures show the representative histograms of lymphocytes from one animal in each group. Similar data were obtained from the others hamsters in three separate experiments. Cell division cycles are represented by markers M1 to M7.

Table 1. Percentage of proliferating cells in each cell cycle (M1-M7) of total and CD4⁺-non-stimulated and mitogen stimulated hamster cells.

A) Total splenocytes					
CFSE peak	Non stimulation	Mitogen stimulation			
		PHA	Con A	PMA/ionomycin	SEB
M1	14.8±1.2	6.8±0.9 ^a	4.4±1.8 ^a	1.5±0.9 ^a	3.5±1.6 ^a
M2	68.2±6.2	29.5±2.5 ^a	16.6±4.3 ^a	2.4±0.9 ^a	19.3±2.6 ^a
M3	9.3±3.8	12.2±2.4	11.5±3.5	4.2±1.4 ^a	9.3±2.1
M4	3.0±3.0	13.1±2.1 ^a	13.8±3.2 ^a	12.2±4.4 ^a	7.8±3.6
M5	1.3±0.6	19.9±0.8 ^a	24.4±3.2 ^a	29.2±9.0 ^a	15.9±3.9 ^a
M6	0.7±0.4	13.8±3.5 ^a	23.2±9.3 ^a	35.2±7.3 ^a	28.7±5.8 ^a
M7	0.3±0.2	4.0±0.9 ^a	4.2±1.6 ^a	14.5±8.5 ^a	13.5±3.7 ^a
B) CD4 ⁺ splenocytes					
CFSE peak	Non stimulation	Mitogen stimulation			
		PHA	Con A	PMA/ionomycin	SEB
M1	18.6±6.6	13.8±2.3	12.6±3.5	1.3±1.1 ^a	7.5±3.1 ^a
M2	60.1±9.6	27.7±3.7 ^a	21.0±4.0 ^a	3.0±1.6 ^a	19.0±2.6 ^a
M3	9.8±3.5	14.4±1.2 ^a	13.2±2.3	5.8±1.9	11.9±3.2
M4	2.6±2.7	12.6±1.3 ^a	17.3±2.7 ^a	13.9±4.9 ^a	13.1±1.8 ^a
M5	1.1±1.1	11.2±2.1 ^a	18.8±5.2 ^a	29.4±5.1 ^a	19.0±1.4 ^a
M6	0.8±0.8	8.4±2.0 ^a	9.0±3.7 ^a	32.8±7.4 ^a	19.1±5.0 ^a
M7	0.4±0.3	1.8±0.7 ^a	1.9±1.0 ^a	12.7±7.3 ^a	5.7±2.5 ^a

^a Statistical significance ($P < 0.032$ Mann-Whitney test) in the same peak comparing the different stimuli against the non-stimulated cells.

Discussion

CD4⁺ cells are of crucial importance in humoral and cellular immunity, they may differentiate into Th1, Th2, TH17 phenotypes (the effector Th cell triade), with distinct cytokine products and biological functions, or evolve into the inducible regulatory T (Treg) lineage, with immunomodulatory functions (*Fietta*

and *Delsante, 2009*). The capacity of CD4⁺ hamster and total spleen cells to proliferate in response to polyclonal stimuli was established through CFSE staining. Con A and PHA are the mitogens most commonly used as polyclonal activators. Our results demonstrated that Con A induced a higher proliferative response compared to PHA in CD4⁺ cells, but

less proliferation as compared with SEB and PMA/ionomycin, where cells underwent proliferation up to 6 to 7 cycles. Indeed, PMA/ionomycin stimulation elicited the strongest proliferative response. PMA in combination with ionomycin is known to induce a loss in CD4 expression at the surface of both murine and human peripheral T cells (*Hoxie et al., 1986; Wang et al., 1987*). In mouse peripheral T cells the stimulation with PMA alone induces only a partial (approximately 60%) and transient (10 to 12 h) loss of CD4 from the cell surface, and the combination of PMA-ionomycin virtually induces the total loss of surface CD4 for a period of at least 24 h. Human PBMC respond to PMA alone with a complete and sustained surface CD4 disappearance, and the addition of ionomycin has no synergistic effect on CD4 expression (*Anderson and Coleclough, 1993*). In hamster cells, the loss of surface CD4 expression after 5 days of incubation with PMA/ionomycin was not detected. Future studies are needed to clarify this subject.

We also describe that SEB induces proliferation of hamster CD4 lymphocytes and as far as we know, this is the first report of proliferative responses to a superantigen in the hamster model. SEB belongs to the family of superantigens which comprise a group of microbial products with powerful immunomodulatory properties possibly playing a key role in the pathogenesis of several human diseases (*Krakauer, 2005*). Our results provide a useful tool that could be used to determine the effects of this superantigen in the study of hamster models of infectious and non-infectious diseases.

In conclusion, the use of the murine cross-reactive antibody to target the hamster CD4 molecule with the use of the dye CFSE established the number of cell divisions and the percentage of proliferative CD4+ cells in each cycle after PHA, PMA/iono, Con-A and the superantigen SEB.

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