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A CFSE-based assay of proliferative response of lymphocytes to stimulation with dengue viruses

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Summary. – Dengue viruses (DENVs) are human pathogens that constitute a significant threat worldwide. Since they up-regulate MHC class I molecules; the cell-mediated immunity may play an important role in the defense against viruses. In this work, we tested a CFSE-based assay in determining proliferative response of lymphocytes isolated from mice or monkeys previously immunized with various DENV antigens to *in vitro* stimulation with DENVs. A positive proliferative response was obtained with lymphocytes of animals immunized with either live DENV-2 or its recombinant proteins. A similar result was also obtained with CD8+ T cells from mice immunized with live DENV-1 or DENV-2 following stimulation with homologous viruses. A comparison of the carboxyfluorescein diacetate succinimidyl ester (CFSE)-based and a ^{3}H-thymidine incorporation-based assays of proliferative response of total lymphocytes showed a fair correlation of results of both assays. However, the CSFE-based assay offers in addition the determination of contribution of the CD8+ or other subsets of T cells to total proliferative response. These results represent the first and successful application of a CFSE-based assay to the evaluation of cell-mediated immunity to DENVs. This assay might be also exploited in testing candidate DENV vaccines.

Keywords: cell-mediated immunity; CFSE, dengue viruses; lymphocytes; CD8+ T cells

Introduction

DENVs including DENV-1 to DENV-4 (the genus *Flavivirus*, the family *Flaviviridae*) are transmitted to humans following a bite from an infected mosquito, usually *Aedes aegypti*. The infection may result in a disease of various severity, from asymptomatic to life-threatening syndrome, known as dengue hemorrhagic fever (DHF) (Gubler and Kuno, 1997). Epidemiological data suggest a greater risk of DHF during secondary infection and immunopathological mechanisms involved in this phenomenon (Vauhgn *et al*., 2000).

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Abbreviations: CDI = cell division index; CFSE = carboxyfluorescein diacetate succinimidyl ester; CDI = cell division index; ConA = concanavalin A; DENV(s) = dengue virus(es); DHF = dengue hemorrhagic fever; PBMCs = peripheral blood mononuclear cells; SI = stimulation index

Antibody-dependent enhancement of secondary viral infections is a phenomenon that can explain the association between severe disease and preexisting immunity to DENV (Halstead, 1979). Other parameters such as viral load (Libraty *et al*., 2002) and features of the virus and the host may also be important (Leitmeyer *et al*., 1999; Gamble *et al*., 2000; Loke *et al*., 2001). Moreover, recent reports indicate that serotype cross-reactive memory T cells play an important role in the pathogenesis of the disease (Zivny *et al*., 1999; Mongkolsapaya *et al*., 2003, 2006; Beaumier *et al*., 2008). However, dengue is a non-cytopathic virus that, similarly to other flaviviruses, up-regulates the expression of MHC class I molecules on the surface of infected cells (Cheng *et al*., 2004; Lobigs *et al*., 2004). Therefore, the cell-mediated immunity has been proposed as an important protection mechanism against DENV infections. Nevertheless, only a few studies have been reported on this topic (Gagnon *et al*., 1996; Van der Most *et al*., 2000).

Measurement of antigen-specific proliferative response is essential for assessment of cell-mediated immunity. Over

the last 30 years, this response has been measured by the incorporation of ^{3}H-thymidine into the DNA of proliferating cells (Frome *et al*., 1996). However, although the level of ^{3}H-thymidine incorporation directly correlates with the number of cells that synthesize DNA during the pulse period, this method does not allow to detect proliferative responses of inndividual subsets of T cells. This problem can be circumvented by labeling cell population with CFSE prior to culture setup. CFSE reacts with secondary amino groups of intracellular proteins providing a uniform fluorescent label to cells (Lyons and Parish, 1994). Upon cell division a CFSE^{high} cell loses half of its CFSE label and yields CFSE^{low} daughter cells and these two cell populations can be differentiated by flow cytometry. Coupled to parallel analysis of other properties such as expression of surface molecules using specific fluorophore-conjugated antibodies, the CFSE-based assay provides additional information on proliferation profiles and differentiation.

In the present work, we evaluated the potential of a CFSE-based assay as an indirect measure of the cell-mediated immunity to DENVs. Using this assay, we (i) determined the proliferative response of lymphocytes or their CD8+ subset isolated from different animals previously immunized with various DENV antigens to *in vitro* stimulation with live homologous virus, and (ii) compared the CFSE-based and ^{3}H-thymidine incorporation assays in relation to proliferative response of total lymphocytes. This work constitutes the first report on use of a CFSE-based assay for determination of the proliferative response to *in vitro* stimulation with DENVs.

Materials and Methods

Viruses and recombinant viral proteins. DENV-1 Jamaica strain and DENV-2 SB8553 strain (kindly provided by Dr. M.J. Cardosa, University of Sarawak, Kuching, Malaysia) were employed for *in vitro* stimulation of lymphocytes. Stock viruses were prepared from Vero cell cultures infected at a MOI of 10 PFU/cell in standard way. DENV-1 Hawaii strain and DENV-2 NGC strain passaged in suckling mice brain were used for immunization of mice. Viral titers were determined by plaque assay in BHK-21 cells. Recombinant DENV-2 capsid protein and DENV-2 PD5 protein (DENV-2 envelope fragment fused to the C-terminus of meningococcal P64k protein) were prepared by the Development Group of CIGB as described (López *et al*., 2004, 2009).

Preparation of lymphocytes. Groups of five 6–8 week-old BALB/c mice (CENPALAB, Cuba) were inoculated intraperitoneally with DENV-1 Hawaii strain or DENV-2 NGC strain (one dose of 10^{4} PFU/0.5 ml) or with recombinant DENV-2 capsid protein (3 doses of 10 μg at days 0, 15, and 30) as previously reported (Lazo *et al*., 2007). A mock-inoculated group of mice served as negative control. Thirty days after the last dose, the animals were splenectomized under aseptic conditions. Lymphocytes were prepared from spleens in standard way.

A group of three American green monkeys (*Cercopithecus aethiops sabaeus*) (CENPALAB, Cuba) were inoculated subcutaneously with recombinant DENV-2 proteins (four doses of 50 μg of capsid protein and 100 μg of PD5 protein at days 0, 30, 90, and 150). Another group inoculated with 100 μg doses of meningococcal P64k protein served as negative control. Blood was collected by venepuncture 15 days after the last dose and lymphocytes were isolated with Ficoll-Paque™ Plus (Amersham Biosciences AB, Sweden) by density gradient centrifugation.

CFSE-based proliferation assay. Lymphocytes were stained with CFSE (Molecular Probe, USA) as previously reported (Venken *et al*., 2007). The stained cells were seeded in 96-well round-bottom plates (2×10^{5} cells/well) in RPMI-1640 medium (Sigma) supplemented with 2 mmol/l glutamine, 5×10^{-5} mol/l 2-mercaptoethanol, 5% FBS and antibiotics. The cells were stimulated by adding DENV (0.05 PFU/cell) for 96 hrs. Mock-infected and concanavalin A (ConA, Sigma) -treated cells served as negative and positive controls, respectively. In all experimental series, triplicate wells were employed.

Flow cytometry. To assay proliferative response of total lymphocytes they were collected following stimulation, washed with PBS and subjected to flow cytometry in standard way. Dead cells were excluded by propidium iodide incorporation. Duplicate samples were analyzed in PasIII flow cytometer (Partec GmbH, Germany) using the WinMDI software version 2.8 (Purdue University, USA). Results were expressed as cell division index (CDI) defined as a ratio of the percentage of antigen-stimulated CFSE^{low} cells to the percentage of antigen-unstimulated CFSE^{low} cells. A response with CDI ≥3 and [antigen-stimulated CFSE^{low} cells (%)] – [antigen-unstimulated CFSE^{low} cells (%)] ≥1 was considered positive.

To assay proliferative response of CD8+ T cells, lymphocytes were collected following stimulation, washed with PBS, stained with a PE-conjugated monoclonal antibody toCD8+ T cells (BD Pharmingen, USA) and subjected to flow cytometry in standard way.

^{3}H-thymidine incorporation assay was conducted as previously described (Jordan and Merigan, 1974; Lechmann and Liang, 2000). Briefly, lymphocytes (2×10^{5}/well) were cultured in 96-well round-bottom plates with DENV-2 (0.05 PFU/cell) for 96 hrs. Mock-infected and ConA-exposed cells served as negative and positive controls, respectively. Results were expressed as SI defined as a ratio of the count of antigen-stimulated cells to the count of antigen-unstimulated cells. SI ≥3 was considered a positive result.

Statistical analysis. CDI and SI values were compared by the Pearson correlation test using the GraphPad Prism version 5.00 for Windows (GraphPad Software, USA) (www.graphpad.com).

Results

Proliferative response of lymphocytes from mice immunized with DENV-2 to in vitro stimulation with homologous virus as determined by the CSFE-based assay

To assess the antigen-specific cell-mediated immunity in mice immunized with DENV-2 we determined their proliferative response to the virus *in vitro* by the CFSE-based assay (Table 1). As expected, DENV-2-immunized animals showed after 96 hrs of stimulation a positive proliferative

Table 1. Proliferative response of lymphocytes from animals immunized with various DENV antigens to *in vitro* stimulation with homologous virus

Immunization		Stimulation *in vitro*[a]	CDI			
			Lymphocytes		CD8+ T cells	
Animals	Antigen		Immunized animals	Non-immunized animals	Immunized animals	Non-immunized animals
Mice	DENV-2	DENV-2	5.05±1.06	1.6±0.28	–	–
	DENV-2 recomb. capsid protein		6.85±1.75	2.45±0.21	–	–
Monkeys	DENV-2 recomb. capsid and PD5 proteins		9.8±2.0	1.7±0.9	–	–
Mice	DENV-1	DENV-1	–	–	5.2±2.8	1.3±0.55
	DENV-2	DENV-2	–	–	5.6±1.5	1.8±0.94

[a]Lymphocytes were stimulated for 96 hrs.

response in contrast to negative response of non-immunized animals. A positive control with 5 μg/m ConA showed strong proliferative response. Similar results were obtained after 72 hrs but not 144 hrs, when an increased number of dead cells was observed (data not shown). Therefore, in further experiments a 96-hrs stimulation was used.

Proliferative response of lymphocytes from mice immunized with recombinant DENV-2 capsid protein to in vitro stimulation with homologous virus as determined by the CSFE-based assay

In the next experiment, a more defined system, in particular mice immunized with viral antigen instead of live virus were employed for testing the usefulness of the CFSE-based proliferation assay. As viral antigen a particulate recombinant DENV-2 capsid protein was chosen (López *et al*., 2009). In general, recombinant proteins are expected to have a lower capacity to induce antigen-specific cell-mediated immunity as compared to that of live virus (Wang *et al*., 1999). However, the chosen particulate antigen may be cross-presented by dendritic cells more efficiently than soluble antigen and consequently induce a fair antigen-specific cell-mediated immunity (Ackerman *et al*., 2006). In the experiment, the mice immunized with the recombinant DENV-2 capsid protein generated lymphocytes proliferating strongly after virus stimulation *in vitro* (Table 1). Negative and positive controls gave expected results (data not shown).

Proliferative response of lymphocytes from monkeys immunized with recombinant DENV-2 proteins to in vitro stimulation with homologous virus as determined by the CSFE-based assay

To test the suitability of the CFSE-based proliferation assay for lymphocytes of different sources, monkeys were immunized with recombinant DENV-2 capsid and PD5 proteins and their PBMCs were stimulated *in vitro* with live DENV-2. The assay yielded a positive result for the lymphocytes from animals immunized with recombinant viral proteins and a negative result for the lymphocytes from non-immunized animals (Table 1). Positive control gave expected result (data not shown).

Proliferative response of CD8+ T cells from mice immunized with DENV-1 or DENV-2 to in vitro stimulation with homologous viruses as determined by the CSFE-based assay

Regarding the potential of the CFSE-based assay to determine individual subsets of proliferating cells, we selected CD8+ T cells. In this case, proliferative response of lymphocytes from mice immunized with DENV-1 or DENV-2 to *in vitro* stimulation with homologous viruses was assayed (Table 1). The results showed that CD8+ T cells from mice immunized with DENV-1 or DENV-2 gave a similarly strong proliferative response (Table 1). Negative and positive controls gave expected results (data not shown).

Comparison of the CFSE-based and ^{3}H-thymidine incorporation assays of proliferative response of lymphocytes from mice immunized with DENV-2 and stimulated with homologous virus

The previous experiments demonstrated the applicability of the CFSE-based assay to determination of the proliferative response of lymphocytes from two animal models immunized with different DENV antigens to *in vitro* stimulation with homologous live DENVs. Regarding the wide use of ^{3}H-thymidine incorporation assays in the field of DENVs (Kurane *et al*., 1991; Rothman *et al*., 1996) we compared the CFSE-based assay with this method. A number of samples of lymphocytes from mice immunized with DENV-2 stimulated with homologous virus were employed. As a result, both the

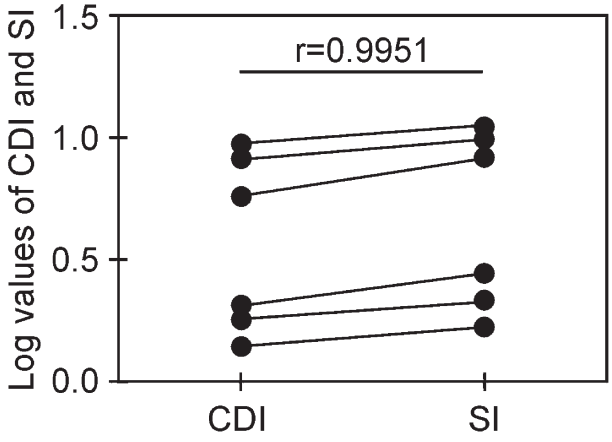

Fig. 1

Comparison of the CFSE-based (CDI) and the ^{3}H-thymidine incorporation (SI) assays of proliferative response of lymphocytes from mice immunized with DENV-2 to stimulation with homologous virus

^{3}H-thymidine incorporation and the CFSE-based assays gave the same positivity of proliferative response of lymphocyte samples, although SI values (the ^{3}H-thymidine incorporation) were higher than CDI ones (the CFSE-based assay). However, a significant correlation between these values was detected ($r = 0.9951$, $p < 0.001$) (Fig. 1).

Discussion

CFSE is a fluorescent dye that has been used to study cell division and other parameters such as surface expression of activation markers, cytokine and homing receptors, cytokine production, cytotoxic activity and indicators of apoptosis (Fazekas *et al*., 1999). Also, different researchers have employed this method to measure the proliferative response to *in vitro* stimulation with specific antigens, e.g. proliferation of human lymphocytes after stimulation with tetanus toxoid (Mannering *et al*., 2003; Venken *et al*., 2007).

In this work, we used a CFSE-based assay to determine the proliferation of lymphocytes isolated from different animals, previously immunized with diferent DENV antigens, after stimulation with live homologous DENVs. Measuring a proliferative response to stimulation with live virus is highly important, particularly after immunization with subunit vaccine candidates. When live virus is used, viral epitopes are presented in a natural form, thus resembling a hypothetical *in vivo* boosting caused by a viral infection. However, lymphoproliferation assays are generally performed by stimulation with immunodominant peptides, which may overestimate the stimulatory capacity of the immunogen (Simmons *et al*., 2005; Mongkolsapaya *et al*., 2006).

First we measured by a CFSE-based assay the proliferative response of virus stimulated lymphocytes from mice immunized with DENV-2. In all cases, a positive response was detected. A similar result was published by Rothman *et al.* (1996), who stimulated lymphocytes from DENV-2-immunized mice with inactivated DENV-2. However, these experiments were done by using the ^{3}H-thymidine incorporation assay.

When using a more rigorous system, mice immunized with recombinant DENV-2 capsid protein, we also found a proliferative response of lymphocytes after stimulation with homologous virus. This result is in accord with previous reports on CD4+ clones with cytotoxic activity specific for DENV-4 capsid protein (Gagnon *et al*., 1996). However, the present work is the first report on a recombinant protein induction of a strong proliferative response to stimulation with homologous virus. This result could explain previous experimental evidences of protection of mice immunized with capsid protein without induction of neutralizing antibodies (Lazo *et al*., 2007).

A proliferation response was also obtained with lymphocytes from monkeys co-immunized with DENV-2 capsid and PD5 proteins. This result confirmed the ability of the CFSE-based assay to measure proliferation of lymphocytes from different sources and represents the first report on determination of proliferative response to stimulation with a DENV in monkeys.

Additionally, we were able to detect a CD8+ T cell proliferative response after homologous virus stimulation in mice immunized with DENVs, demonstrating that the CFSE-based assay is essential for *in vitro* studies of the antigen-specific cell-mediated immunity in dengue. These results are consistent with those on other viruses reported previously (Svahn *et al*., 2003). For example, a protective role of CD8+ T cells has been observed for influenza virus encephalitis in mice (Hawke *et al*., 1998), lymphocytic choriomeningitis virus infection in mice (Williams *et al*., 2006), and primary infection of mice with a neurotropic strain of mouse hepatitis coronavirus (Marten *et al*., 2000). More recently, Yauch *et al.* (2009) have demonstrated a protective role of DENV-specific CD8+ T cells.

Summing up, our results demonstrate that the proliferative response measured by the CFSE-based assay correlates with that obtained by the^{3}H-thymidine incorporation assay. However, Mannering *et al*. (2003) reported that the CFSE-ased assay was more sensitive than the ^{3}H-thymidine incorporation assay in measuring the T cell proliferation from healthy donors in response to autoantigens. On the contrary, other researchers analyzing the correlation between activation markers on stimulated T cells and their proliferation concluded that both assays were complementary. Indeed, they recommended a combination of both methods to obtain a clear understanding of the events taking place in the

cell-mediated immune response (Caruso *et al.*, 1997). In the presented work, we did not observe any differences between the results of the two assays provided live DENV was used for stimulation purposes.

Analyzing the evidences described here, we can conclude that the CFSE-based assay is suitable for determining the proliferative response to live DENV-1 or DENV-2. Therefore, this method proves a powerful tool for further studies of the antigen-specific cell-mediated immunity to these flaviviruses. Moreover, it can be used to study T cell-mediated immunopathogenesis and develop new vaccine candidates.

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