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Flow Cytometry for the Analysis of T Cells Expressing CD69 after Stimulation with Glutamic Acid Decarboxylase

Watip Boonyasrisawat[1], Napatawn Banchuin[1], Kovit Pattanapanyasat[2], Chaicharn Deerochanawong[3], Pa-thai Yenchitsomanus[2], Sirirat Ploybutr[4] and Sathit Vannasaeng[4]

SUMMARY Type 1 diabetes mellitus is a T-cell mediated autoimmune disease in which the insulin-producing pancreatic beta cells are selectively destroyed. We recently found that the detection of cell-mediated immune response to glutamic acid decarboxylase (GAD) was more useful than the detection of specific autoantibodies for the diagnosis of type 1 diabetes mellitus. In this study, we established a flow cytometric analysis for the detection of activated T cells in whole venous blood, obtained from diabetic patients and normal controls after stimulation by GAD. Two millitiers of peripheral venous blood and 6 hours incubation time were used for performing the test. It was found that 33% (3/9) type 1 diabetic patients, 7.7% (1/13) type 2 diabetic patients and neither patients with fibrocalculous pancreatopathy nor normal controls had ≥ 20% CD8+ T cells expressing CD69. The results suggest that flow cytometry may be a useful tool for the detection of surrogate markers of type 1 diabetes mellitus.

Immune-mediated type 1 diabetes mellitus ensues from the selective aggression against insulin secreting beta cells of the islets of Langerhans by autoreactive T cells.[1,2] Both CD4 and CD8 T cells are involved in the pathogenesis of the spontaneous disease. In both animal models and human, the islet lesions are characterized by cellular infiltrates of mononuclear cells, predominantly T cells and macrophages. It is one subtype of type 1 diabetes mellitus, which is generally diagnosed in childhood and adolescence. Another subtype of type 1 diabetes is idiopathic diabetes in which the patients have permanent insulinopenia and are prone to develop ketoacidosis, just as those with immune-mediated type 1 diabetes, but in this case there is no evidence of autoimmunity nor any other etiology.[3]

In immune-mediated type 1 diabetes, circulating autoantibodies to beta cell autoantigens can be found several years before the appearance of clinical disease. The currently used immunological markers of immune-mediated type 1 diabetes are various autoantibodies, including islet cell antibodies (ICAs), insulin autoantibodies (IAAs) and antibodies to glutamic acid decarboxylase (anti-GAD).[4-11] However, the frequency of positive autoantibodies in Asians is generally lower than in Caucasians.[5,9-12] We recently found that the prevalence of antibodies to GAD was higher than the prevalence of all autoantibody markers for type 1 diabetes previously reported in Thailand.[13,14] However, the lymphoproliferation assay for determination of antibodies to GAD is time consuming and requires a large volume of blood, which may not be suitable for use with pediatric patients.

Using a flow cytometric method, Maino *et al.*[15] were able to

From the [1]Department of Immunology, [2]Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. [3]Department of Medicine, Rajavithi Hospital, Ministry of Public Health, Bangkok, Thailand, [4]Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University.
Correspondence: Napatawn Banchuin

show similar dose-response profiles between the percentage of CD69 (EA-1/Leu 23) expressing lymphocytes at 4 hour stimulation and the ^{3}H-TdR incorporation assay following 72 hours of activation using the T-cell specific stimulus, CD2/CD2R. The amount of specimen required for such a type of test can be as little as 2 ml of whole blood.[16] Thus, a flow cytometric method using whole blood appeared to be suitable for our purpose.

In this study, we report the establishment of flow cytometry for the analysis of CD69-expressing T cells after stimulation with GAD, using blood obtained from diabetic patients and normal controls.

MATERIALS AND METHODS

Subjects

A total of 9 type 1 diabetic patients, 13 type 2 diabetic patients, 2 patients with fibrocalculous pancreatopathy and 8 normal controls were recruited from two hospitals in Bangkok, Thailand. Five patients attended diabetic clinics or were hospitalized at Siriraj Hospital and 19 were patients at Rajavithi Hospital. Type 1 diabetes mellitus was diagnosed from a positive history for at least one episode of ketoacidosis on insulin withdrawal or from a deficient or absent C-peptide response following a glucagon stimulation test. All of the patients in this group are females between 15 and 46 years of age. The duration of diabetes ranged from 1 to 30 years.

Patients who did not meet the criteria for type 1 diabetes were diagnosed as type 2 diabetes. This group included 3 males and 10 females with ages ranging from 30 to 79 years and 37 to 75 years, respectively. The duration of diabetes ranged from 1 to 15 years among males and 2 to 23 years among females.

Fibrocalculous pancreatopathy was diagnosed by the presence of pancreatic calcifications on a plain abdominal roentgenogram, requiring insulin treatment since the onset of diabetes and having neither ketonuria nor ketosis despite insulin withdrawal by themselves for several weeks. This group of subjects comprised two females aged 44 and 48 years. The duration of diabetes was 14 and 8 years.

Normal controls were recruited from 1 blood donor and 7 laboratory personnel at Siriraj Hospital. All of them were females between 23 and 40 years of age.

Ethical approval

Approval for the study was granted by the Faculty of Medicine Siriraj Hospital Research Ethics Committee. Written consent was obtained from all the subjects studied.

Blood samples

Two milliliters of peripheral venous blood were collected from each subject, into a sterile test tube containing heparin with a concentration of 10 units/ml blood.

T-lymphocyte activation assay

Fifty microliters of heparinized whole blood in a 12 x 75 mm polystyrene tube was cultured in the absence or presence of either 100 μl of a mitogen, phytohemagglutinin (PHA), or GAD antigen (Sigma Chemical Co., St. Louis, USA). PHA was used at a concentration of 2 μg/ml and GAD was used at two concentrations, 80 and 160 μg/ml. The incubation was performed at 37°C in humidified air with 5% CO_2 for 6 hours. After incubation, the cells were stained with 7 μl of fluorochrome-conjugated monoclonal antibodies, either CD3FITC/CD4PerCP/CD69PE or CD3FITC/CD8PerCP/CD69PE (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA [BDIS]) (Table 1), for 15 minutes at room temperature in the dark. Red blood cells in the blood samples were then lysed by adding 2 ml of FACS lysing solution (BDIS) and stored for 15 minutes at room temperature in the dark, then centrifuged at 300 x *g* for 5 minutes. The supernatant was discarded and the white blood cell pellet was washed once with 1 ml PBS. After washing, the cells were fixed by adding 300 μl of 1% paraformaldehyde in PBS prior to analysis by a fluorescence activated cell sorter (FACS).

Flow cytometric analysis

Stained samples were analyzed by three-color analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, USA). Linear amplification of the forward and side scatter (FSC/SSC) signals, as well as logarithmic amplification of the fluorescence 1 (FITC; band pass filter wavelength 530 nm), fluorescence 2 (PE, 585 nm), and fluorescence 3 (PerCP, 650 nm) emissions in green, orange, and red fluorescence, respectively, were obtained. Data was processed with CellQuest software (Becton Dickinson), using fluorescence triggering in green channel (FITC) on CD3 to gate on the lymphocyte populations. Data were displayed as two-color plots (PerCP versus PE) to enumerate the lymphocyte subsets and quantify the proportion of

Table 1 Monoclonal antibody combinations and their identifications

Cluster designation of monoclonal antibodies FITC/PE/PerCP*	Reactive target cells
CD3/CD69/CD4	Early activation T-helper cells
CD3/CD69/CD8	Early activation T-cytotoxic cells

*FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein

Table 2 Percentage of CD4+ and CD8+ T cells with CD69 expression, in patients with type 1 DM, type 2 DM, fibrocalculous pancreatopathy, and normal control

Normal controls		Type 1 DM		Type 2 DM		Fibrocalculous pancreatopathy	
CD4/CD69	CD8/CD69	CD4/CD69	CD8/CD69	CD4/CD69	CD8/CD69	CD4/CD69	CD8/CD69
25.9	11.3	10.86	20.76	16.46	9.33	1.46	2.05
9.35	11.01	9.6	9.38	17.87	8.69	5.18	11.46
7.75	6.43	0.03	22.76	0.19	8.03		
10.06	13.46	2.93	2.92	0.03	10.81		
3.74	2.5	11.92	20.04	9.07	10.13		
2.85	4.5	10.87	8.20	5.32	9.41		
2.06	1.71	15.18	4.26	13.11	12.96		
0.44	0.9	12.77	16.27	9.59	28.73		
		6.14	11.53	7.12	7.86		
				4.52	2.98		
				9.05	1.25		
				0.25	1.3		
				10.19	13.42		

activated $CD4^+$ and $CD8^+$ T-lymphocytes that expressed CD69. Instrument settings and three-color compensation were performed prior to running the samples, using CaliBRITE 3 beads (Becton Dickinson). Data of 3,000-5,000 events per sample in the trigger gate were acquired and stored in CellQuest software with an Apple Mcintosh computer.

Internal processing control

Data for quality control was obtained by verifying internal consistency of CD3+ lymphocytes present in each reaction tube containing the CD3 monoclonal antibody.

RESULTS

Establishment of flow cytometric analysis for the detection of GAD-activated T lymphocytes

The optimal antigen concentration for use in the test was determined by incubating GAD at various concentrations, being either 80, 160, or 320 μg/ml with 50 μl

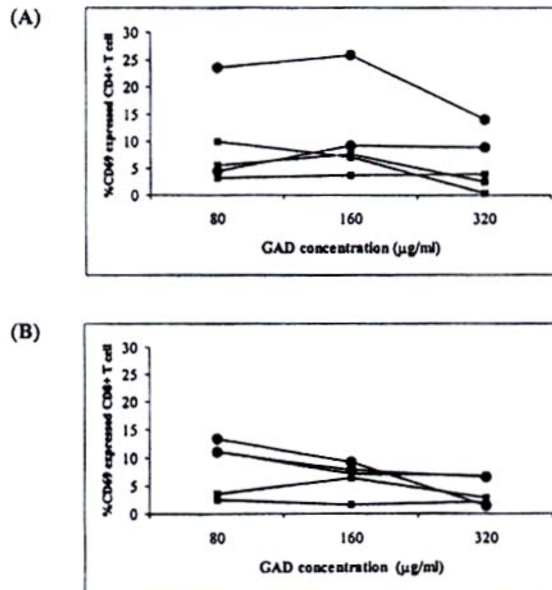

Fig. 1 Percentage of CD69 expressed CD4+ T cells (A) and CD8+ T cells (B) after stimulation with GAD at various concentrations. The test was performed with 50 μl heparinized blood, obtained from 2 normal controls (■) and 3 type 1 diabetic patients (●), and the incubation with GAD was carried out at 37 °C in humidified air with 5% CO_2 for 6 hours.

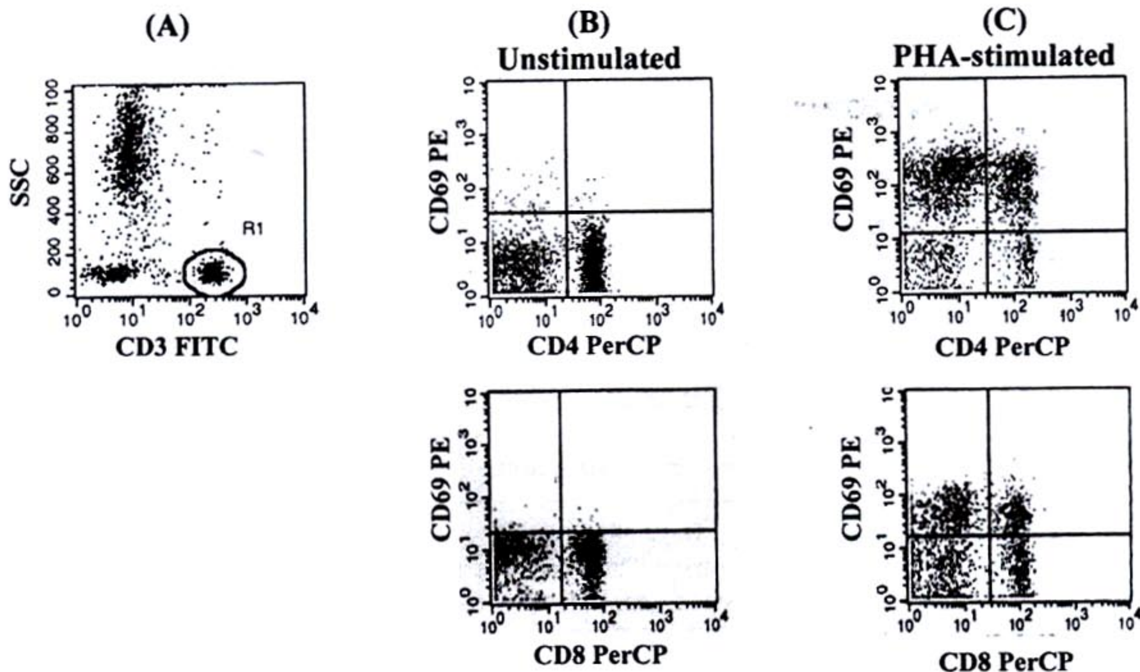

Fig. 2 Flow cytometric analysis of lymphocyte population, a trigger gate on CD3+ FITC lymphocytes (A) were analyzed for the CD4+ and CD8+ cells expressing CD69 in unstimulated (B) and PHA-stimulated (C) conditions.

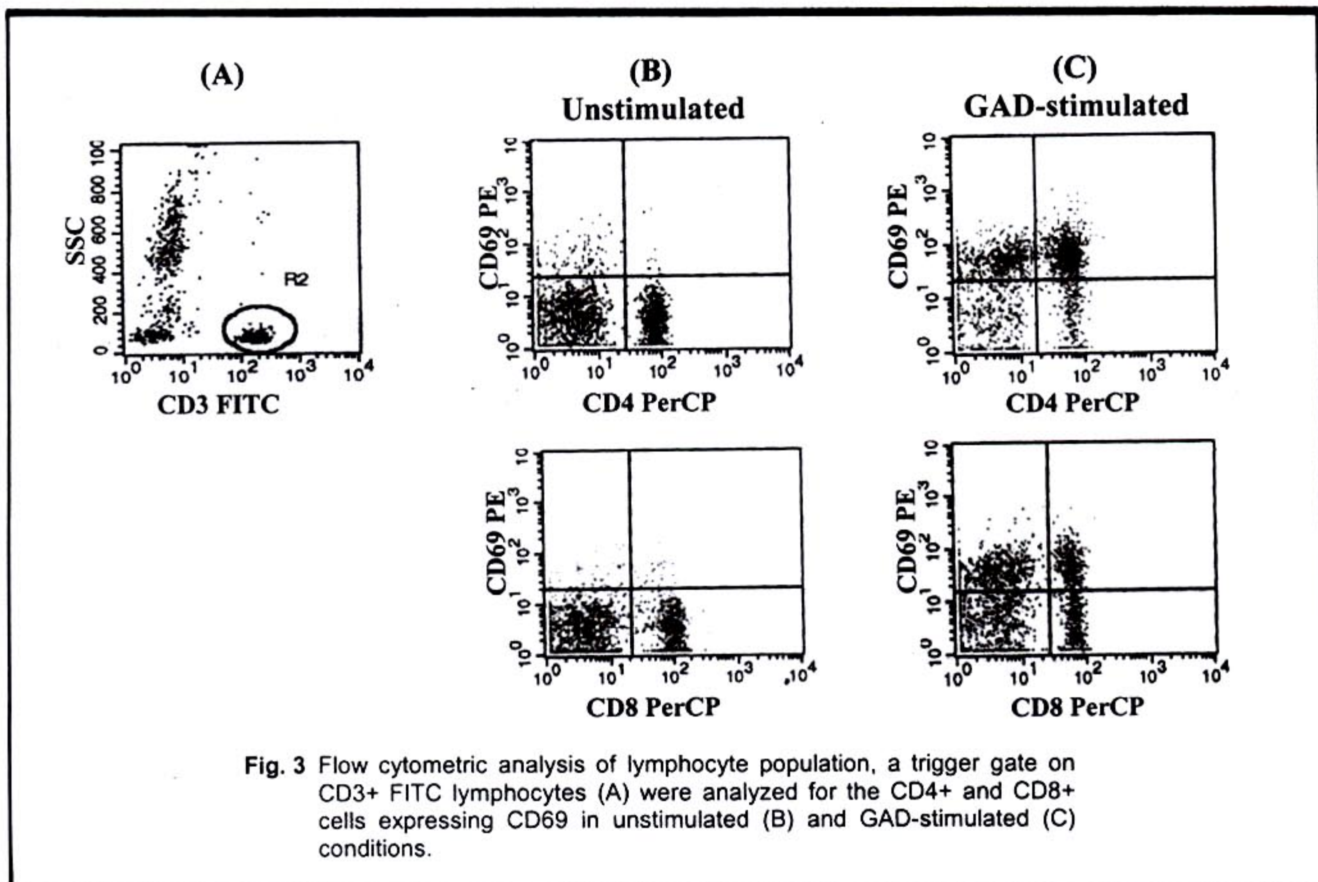

Fig. 3 Flow cytometric analysis of lymphocyte population, a trigger gate on CD3+ FITC lymphocytes (A) were analyzed for the CD4+ and CD8+ cells expressing CD69 in unstimulated (B) and GAD-stimulated (C) conditions.

heparinized blood obtained from 2 normal controls and 3 type 1 diabetic patients, for 6 hours, at 37°C in humidified air with 5% CO_2. Then the cells were stained with fluorochrome conjugated monoclonal antibodies and analyzed by FACScan. The results are shown in Figs. 1A and 1B, where it can be seen that the use of GAD concentrations of 80 and 160 µg/ml resulted in a higher expression of CD69 in most circumstances.

Flow cytometric analysis was also performed with unstimulated samples in order to assess the distribution of the different lymphocyte subsets in diabetic patients and controls. The total lymphocyte population was identified in the forward versus side scatter dot plot (FSC versus SSC). No difference was observed in the total lymphocyte population and lymphocyte subsets between the 2 groups of subjects.

Determination of GAD-activated lymphocytes in type 1 diabetic patients and controls

CD69 expression on CD4+ and CD8+ cells stimulated with either PHA or GAD at concentrations of 80 and 160 µg/ml was analyzed, using a trigger gate on positive CD3 FITC lymphocytes (Figs. 2 and 3). The maximum percentage of CD4+ and CD8+ cells expressing CD69 in patients with type 1 DM, type 2 DM, fibrocalculous pancreatopathy and normal controls was shown in Table 2. It can be seen that 3/9 (33%) type 1 diabetic patients had ≥ 20% CD8+ cells expressing CD69, while 1/13 (7.7%) type 2 diabetic patients and neither of 8 normal controls nor 2 fibrocalculous pancreatopathy patients gave such a result. CD4+ cells with CD69 expression were found to be less than 20% in all but one normal control, which was 25.9%.

DISCUSSION

The currently used immunological markers of immune-mediated type 1 diabetes mellitus are various autoantibodies, although they are thought to be secondary to the disease process.[4-11] These autoantibodies include islet cell antibodies (ICAs), insulin autoantibodies (IAAs) and antibodies to glutamic acid decarboxylase (anti-GAD). However, the prevalence of these autoantibodies, including anti-

GAD, are low in Asians, as compared to Caucasians.[7,11-13] We recently found that the prevalence of positive lymphoproliferation against GAD in Thai patients with type 1 DM was 76.3% which was higher than the prevalence of autoantibody markers of type 1 DM previously reported in Thailand.[13] It was previously found that the patients were positive for insulin autoantibodies in 45.4% while anti-GAD65 was found to be positive in 26.3-50%.[7,13,17] Thus, these results suggested that the detection of a lymphoproliferative response to GAD was useful for the diagnosis of type 1 DM in this population. However, the lymphoproliferation assay is time consuming, as it requires 5 days of incubation and at least 10 ml of peripheral venous blood, which may not be suitable for use with children who are the peak age group of type 1 DM patients. Therefore, we have established a flow cytometric analysis for the detection of activated T cells in whole venous blood after stimulation by GAD. Two milliliters of whole blood were required for the test, which is more suitable for pediatric patients. The incubation time required was shorter than for the ^{3}H-thymidine incorporation lymphoproliferation assay. The CD69 marker, which was previously reported to be the earliest activation antigen expressed on peripheral blood T cells following mitogen stimulation but undetectable or detectable at very low levels on non-stimulated lymphocytes, was detected on CD4+ and CD8+ cells. It was found that the percentages of CD8+ cells expressing CD69 in type 1 DM patients were higher than those in type 2 DM and normal controls. This was not so for CD4+ cells expressing CD69. The result suggested that flow cytometry is a useful tool for the detection of GAD-activated CD8+ T cells which may be a surrogate marker of type 1 diabetes mellitus. An incubation time longer than 6 hours may improve the sensitivity of the test. A study with a higher number of samples is required in order to show definite conclusion.

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