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Adel N. Hanna
25 Wadi El-Nil St. 12411,
Giza, Egypt

Tel: +20123440310

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The Study of CD 69 as an Early Marker of SLE Activity in Pediatrics

[1]Kh Saber, [2]Z. El-Khayat, [2]T. Ramzy and [1]A.N. Hanna

A major problem in the management of Systemic Lupus Erythematosus (SLE) patients is to assess disease activity. Widely used serological markers are sometimes normal while the patient exhibits obvious signs and symptoms. The CD69 antigen is an integral membrane protein rapidly induced on the surface of activated lymphocytes and is thought to be implicated in the pathogenesis of SLE. We aimed to investigate the dynamic changes in peripheral blood $CD8^+$ T cells expressing CD69 antigen in pediatric SLE in relation to disease activity. $CD3^+$ $CD8^+$ T cells were collected from 25 children and adolescents with SLE and from 20 healthy controls. The percentage of CD69 expressing in freshly isolated cells was quantified by three color immunofluorescent staining. Patients were also subjected to clinical evaluation for disease activity by Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and system involvement. The ESR, peripheral blood cell count, Anti DNA antibody, complement 3, creatinine clearance and 24 h urinary proteins were assessed as well as renal biopsy in selected cases. CD69 was over-expressed in $CD3^+$ $CD8^+$ T cell subsets of SLE patients (median = 41.5%, mean±SD = 44.10±14.41%) when compared with control values (median = 13.9%, mean±SD = 13.73±3.78%; $p<0.01$). SLE patients also had significantly higher CD69/CD3 ratio (median = 0.5, mean±SD = 0.54±0.2) as compared to controls (median = 0.2, mean±SD = 0.2±0.1). A significant positive correlation could link CD69 expression to ESR and SLEDAI score. Patients with clinical neuropsychiatric involvement had higher CD69 levels than others. CD69 expression was neither affected by the presence of lupus nephritis nor by intake of cytotoxic drugs. In conclusion the CD69 expression in peripheral blood lymphocytes of SLE patients could be a reliable marker for monitoring disease activity and is probably related to the development of neuropsychiatric disease.

Key words: SLE, CD69, T cells, activity marker, flow cytometry

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[1]Department of Child Health, [2]Department of Medical Biochemistry,
Medical Division, National Research Center, Cairo, Egypt

INTRODUCTION

Abnormal expression of key signaling molecules and defective function of T lymphocytes play a significant role in the pathogenesis of Systemic Lupus Erythematosus (SLE) (Nambiar *et al*., 2004). T cells from patients with SLE exhibit aberrant responses to stimuli that contribute to the persistence of autoreactive clones and the lack of productive immune responses. It seems likely that a defect or defects in the mechanisms that closely regulate the activation and subsequent proliferation of T lymphocytes (peripheral tolerance) could be responsible for the myriad of alterations observed in these patients (Kyttaris and Tsokos, 2004).

Lymphoid activation follows a complex mechanism which is only partially understood. It is the process by which a lymphocyte undergoes the differentiation that will allow it to become an effector cell and the proliferation that will augment the number of lymphocytes specific for the antigen that elicits the response. One of the earliest changes that appears in an activated cell population is the shift in the pattern of expression of some surface molecules that are absent in resting cells. The α subunit of the receptor for interleukin (IL)-2 (CD25), transferrin receptors (CD71) and CD69 are examples. These molecules are called cell activation markers (Crispin *et al*., 1998).

CD69, one of the earliest specific antigens acquired during lymphoid activation, is a widely expressed type II transmembrane glycoprotein related to the C-type animal lectins that exhibits regulated expression on a variety of cells of the hematopoietic lineage, including neutrophils, monocytes, T cells, B cells, natural killer (NK) cells and platelets (Natarajan *et al*., 2000). CD69 belongs to a family of receptors that modulate the immune response and whose genes are clustered in the natural killer gene complex, present in humans on chromosome 12 (Renedo *et al*., 2000). Activation of T lymphocytes results the induced expression of CD69 at the cell surface. In addition, cross-linking of CD69 by specific antibodies leads to the activation of cells bearing this receptor and to the induction of effector functions. However, the physiological ligand of CD69 is unknown (Natarajan *et al*., 2000).

In the present study, we used flow cytometry to study the expression of CD69 in peripheral blood lymphocytes obtained from patients with SLE and from normal donors. The relation of CD69 expression to disease activity and system involvement was evaluated.

MATERIALS AND METHODS

The study comprised 25 children and adolescents with SLE and 20 controls. Subject selection followed the stratified non-random method. An informed consent was obtained from their parents or care-givers before enrollment. The patients were recruited form the pediatric Allergy and Immunology Unit of the Children's Hospital of Al-Azhar University, Cairo, during the period from April 2005 to November 2005. Their demographic data were as follows:

Patients: The patients were 25 children and adolescents with SLE (18 females and 7 males). SLE was considered to be present if a patient had four of the 11 criteria of the American College of Rheumatology (ACR) (Hochberg, 1997; Petri, 2005). Their ages ranged from 7.0 to 18.0 years with a mean value of 13.8±3.1 years. The disease duration ranged from 1.5 to 14.0 years with a mean value of 5.6±3.4 years. All patients were receiving corticosteroids in the form of prednisone (1-2 mg kg^{-1} day^{-}), either alone (n = 15), or in combination with other immunosuppressives in the form of intravenous cyclophosphamide (600 mg m^{-2} $month^{-1}$) in 9 patients, or azathioprine (2 mg kg^{-1} day^{-1}) in a single patient.

Controls: These were 16 females and 4 males with an age range of 9.0-16.0 years (mean±SD = 12.1±2.4) years. Exclusion criteria for controls were the presence of personal or family history of rheumatological diseases, chronic illness, or weights or heights below the 5th or above the 95th percentiles for age (Needlman, 2004).

Clinical methods: Evaluation was performed by detailed history through personal interview with patients and their parents besides the clinical examination and laboratory results obtained at the time of the study, aided by the medical records of the Pediatric Allergy and Immunology Outpatient Clinic. Activity of SLE was assessed by the SLE Disease Activity Index (SLE DAI) (Bombardier *et al*., 1992). The disease was considered active in all patients (SLE DAI>8) (Ibanez *et al*., 2005). Lupus nephritis was diagnosed in 15 patients by the presence of one or more of the following : protein in urine ≥0.2 g/24 h, hematuria, pyuria, urinary casts and/or abnormal creatinine clearance. Renal biopsy was done for 13 of them and revealed world Health Organization (WHO) class II b (pure mesangiopathy) in 6 patients, class III (segmental and focal proliferative) in a single patient and class IV (diffuse proliferative) in 3 patients, while 3 patients suffered class V (diffuse membranous) lupus nephritis. Neuropsychiatric SLE was diagnosed (n = 11) when a significant change in the base line neurological and/or psychiatric function was identified by history and physical examination. Evidence of cutaneous vasculitis was recorded in 5 patients by the presence of any of the following: Purpura, ulcers, subcutaneous nodules or livedo reticularis. System involvement was recorded

according to the definitions of the American College of Rheumatology (American College of Rheumatology Ad Hoc Committee on Systemic Lupus Erythematosus Response Criteria, 2004; Renal Disease Subcommittee of the American College of Rheumatology Ad Hoc Committee on Systemic Lupus Erythematosus Response Criteria, 2006; Kasitanon *et al*., 2006).

Laboratory methods

Sample collection: Five milliliter of venous blood were collected under complete aseptic conditions and were used as follows:

- Two milliliter were added to EDTA anticoagulant and were used for ESR, CBC and flow cytometric immunophenotype analysis.
- Three mL were left to clot and the serum was obtained by flicking off after centrifugation for 15 min at 3000 rpm. This was used for assessment of serum creatinine, ANA, anti-DNA and serum complement 3(C3).

Laboratory investigations included:

- The erythrocyte sedimentation rate (ESR) was measured by Westergren method.
- A complete blood count was performed using MaxM cell counter, coulter electronics, Florida, USA. Data included TlC (cells $\times 10^9$ L^{-1}), Hb (g dL^{-1}) and platelets count (cells $\times 10^{12}$ L^{-1}). Peripheral blood smears were stained using leishman stain of differential leucocyte counting with special emphasis on lymphocyte morphology.
- Serum ANA and anti-DNA were assessed by indirect immunofluorescent microscopy (IMMCO Diagnostics, USA).
- Serum C3 was estimated by turbidimetry (Turbiquant C3, Behringwerke Diagnostics, Marburg, Germany).
- Serum and urinary creatinine were measured by synchron Cx7 autoanalyzer (Beckman Instruments Brea, California, USA).
- Urinary proteins 24 h quantification was performed by the turbidimetric method (Stanbio Laboratory Inc., San Antonio, TX, USA).
- Complete microscopic urine analysis for WBCs, RBCs and casts.
- Three color flow cytometric immunophenotype analysis (Bijl *et al*., 2001).

In all cases sample preparation and flow cytometric (FCM) data acquisition were performed within the first 24 h from collection. The antibodies' clones were carefully selected on the basis of their reactivity patterns and absence of background staining as follows: CD3 (Leu4), CD8 (3B5) and CD69 (H12F3) and were obtained from BD pharmingen, BD biosciences, Heidelberg, Germany. A software protocol for the FCM analysis was constructed so that a triple fluorochrome conjugated monoclonal antibody combination was established as CD3 PC5/CD8 PE/CD69 FITC. Labeling of the cells was done by incubating 2×10^6 nucleated cells suspended in a 100 μL volume of Phosphate Buffer Saline (PBS) with saturating amounts of the fluorochrome conjugated mouse anti-human antibodies for 20 min at room temperature in the dark. This was followed by incubation for 2 min in 1 mL of 0.83% ammonium chloride for the purpose of erythrocyte lysis. After a 5 min centrifugation at 500×9 cells were re-suspended in a mixture of PBS pH 7.2 and 1% paraformaldehyde fixative. Murine isotype matched (Ig GI) antibodies obtained from the same source as the primary antibodies were used as isotype negative control. A total of 15,000 events were acquired by the instrument and analyzed by the operator in each sample using a coulter EPICS XL FCM (Beckman/Coulter Corporation, Coulter Electronics, Florida, USA) supplied with system II software, version 3, coulter electronics for data processing. Gating of cells was initially set according to light scatter on a forward scatter (Fs) and side scatter (SC) to exclude cellular debris and to identify the lymphocyte population. Lymphocytes were further gated according to their expression of CD3 to identify CD3 + sub-population of lymphocytes (T cells). Expression of CD8 and CD69 by CD3+ lymphocytes was measured. Data obtained was recorded in the form of percent of cells expressing the markers and the mean intensity of the expression termed Mean Fluorescence Intensity (MFI).

Statistical analysis: The results were statistically analyzed via a standard computer program (Stat View). The results are presented as median, interquartile range, mean and Standard Deviation (SD) values. For non-prarametric data, Mann-Whitney (u) test was used for inter-group analysis and the Spearman correlation coefficient (r) for intra-group analysis. For all tests p values <0.05 were considered significant.

RESULTS

Twenty five SLE patients were studied during active disease. Twenty healthy subjects served as control. Characteristics of the patients and markers of disease activity are given in Table 1.

Lymphocyte subsets in patients and controls: Although SLE patients had significantly lower absolute lymphocyte counts than controls, (mean±SD = 1601±989 cells versus

Table 1: Some clinical and laboratory data of the SLE patients

Age (years)	
Range	7-18
Median (mean±SD)	14.5 (13.8±3.0)
Gender : male/female	7/18
Disease duration years	
Median (mean±SD)	4.5 (5.6±3.4)
SLEDAI scores	
Range	14-54
Median (mean±SD)	27.0 (31.2±12.3)
Patients receiving cytotoxic drugs	
Number (%)	10 (40%)
Lupus nephritis	
Number (%)	15 (60%)
CNS disease	
Number (%)	11 (44%)
Markers of disease activity:	
ESR (mm h^{-1})	
Range	7.82
Median (mean±SD)	42.5 (43.8±21.4)
C3 level	
Range	3-91
Median (mean±SD)	61.0 (62.3±18.7)
Consumed (number (%) of patients	10 (40%)
Anti-DNA positivity	
Number (%) of patients	20%

Anti-DNA = Anti-deoxyribonucleic acid; C3 = Complement 3; CNS = Central Nervous System; ESR = Erythrocyte Sedimentation Rate; SD = Standard Deviation; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index

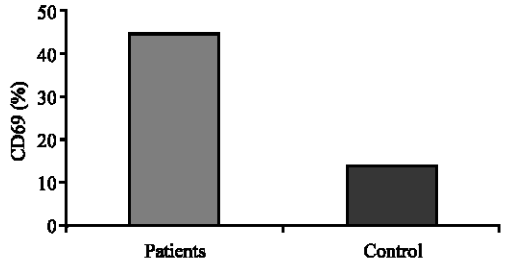

Fig. 1: Percentage of CD69 on activated lymphocytes in SLE patients and controls

2192±421 cells; $p<0.01$), patients had significantly higher percent of cells expressing CD3 and CD8 markers ($p<0.05$ and $p<0.01$, respectively) (Table 2).

Proportion of CD69 positive ($CD69^+$) peripheral blood T cells: Patients had significantly higher percentage of cells expressing CD69 antigen (Fig. 1) and higher CD69/CD3 ratio (Fig. 2) as compared to controls ($p<0.01$ in each comparison). CD8/CD69 co-expression on peripheral blood T cells was significantly increased as well in the patients as compared to the controls ($p<0.01$) (Table 2).

Table 3 shows the patient subgroups according to presence of renal or CNS involvement. In our current study, patients with CNS disease (44%) had a significantly higher SLEDAI score, CD 69% as well as higher CD69/CD3 ratio ($p<0.05$ in each comparison) and elevated ESR ($p<0.01$) than those free of CNS involvement. However, in all lupus nephritis patients (60%) all these parameters were not related ($p>0.05$).

Table 2: Comparisons between patients and controls as regards studied T-lymphocyte markers

	Patients	Control		
	Median (mean±SD)		Z-value	p-value
CD3 (%)	83.7 (82.5±7.8)	76.0 (76.5±5.7)	2.5	<0.05*
CD8(%)	51.4 (51.9±13.4)	33.9(36.7±8.0)	3.0	<0.01**
CD69(%)	41.5 (44.1±14.4)	13.9 (13.7±3.8)	4.4	<0.01**
CD8/CD69(%)	25.6 (29.1±13.3)	3.8 (3.5±1.4)	4.4	<0.01**
CD69/CD3 ratio	0.5 (0.54±0.2)	0.2 (0.2±0.1)	4.4	<0.01**
CD8 (MFI)	11.1 (14.22±7.5)	12.4 (15.4±9.3)	0.2	>0.05
CD69 (MFI)	2.0 (2.4±1.2)	2.4 (3.0±1.3)	1.2	>0.05
CD8/CD69 (MFI)	1.4 (1.4±0.6)	1.7 (1.7±0.2)	2.9	<0.01**

MFI = Mean Fluorescence Intensity; $p>0.05$ = not significant; *$p<0.05$ = significant; **$p<0.01$ = highly significant

Table 3: Comparison between patient subgroups according to presence of renal or CNS involvement

	Lupus nephritis		CNS disease	
	Present (n = 15)	Absent (n = 10)	Present (n = 11)	Absent (n = 14)
	Median (mean±SD)		Median (mean±SD)	
SLEDAI scores	30 (31.7±13.3)	26 (30.3±11.1)	34(37.6±12.4)	26 (26.0±9.8)
	Z = 0.32	p>0.05	Z = 2.0	p<0.05*
ESR (mm/h)	45 (43.6±21.1)	39 (44.1±23.8)	60 (59.9±18)	36 (30.6±13.8)
	Z = 0.001	p>0.05	Z = 3.2	p<0.01**
C3	66 (63.8±20.3)	49 (56.6±16.1)	53 (60311±3.1)	69 (64.0±21.1)
	Z = 0.64	p>0.05	Z = 0.3	p>0.05
CD69%	40.4 (43.7±16.2)	43.5 (44.8±11.4)	51.6(52.3±16.9)	40.4 (37.4±7.6)
	Z = 0.67	p>0.05	Z = 2.2	p<0.05*
CD69/CD3 ratio	0.5 (0.54±0.2)	0.5(0.5±0.2)	0.7 (0.66±0.2)	0. (0.44±0.1)
	Z = 0.12	p>0.05	Z = 2.6	p<0.05*
CD8/CD69	24.2(29.6±14.6)	26.9(28.3±11.6)	31.7(35.7±17.1)	23.5(23.7±4.7)
Co-expression%	Z = 0.04	p>0.05	Z = 1.6	p>0.05

$p>0.05$ = Not significant; *$p<0.05$ = Significant; **$p<0.01$ = highly significant

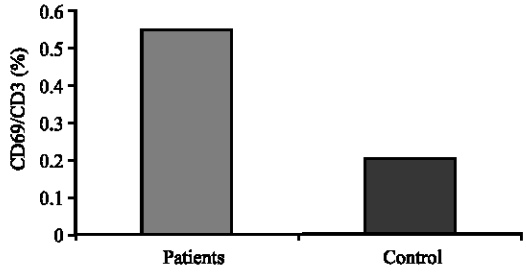

Fig. 2: CD69/CD3 ratio in SLE patients and controls

DISCUSSION

In this study, we studied the expression of CD69 antigen on $CD8^+$ T lymphocytes obtained from pediatric patients with SLE and compared it with the biological parameters of clinical exacerbations. The CD69 antigen was found to be up regulated on $CD8^+$ cells of the patients as compared to controls. This suggests that SLE cytotoxic T cells are a step ahead, already stimulated (activated *in vivo*), when studied in experimental conditions. This abnormal CD69 expression could result in abnormal proliferation of autoreactive lymphocytes that perpetuate the inflammatory response. In agreement with our results, previous studies on SLE adults showed that, the expression of CD69 was up-regulated in freshly isolated $CD4^+$ and $CD8^+$ peripheral blood lymphocytes but, the difference was statistically significant only for the $CD8^+$ subset (Crispin *et al*., 1998; Portales-Perez *et al*., 1997).

The evidence accumulated so far indicates that the immune system of SLE patients is in a hyperactive state. Several reports have revealed a higher expression of cell activation markers in lymphocytes obtained from patients with SLE than in normal controls. CD60 expression was reported on a higher percentage of $CD3^+$ $CD4^-$ $CD8^-$ (double negative) T cells. These cells help to enhance anti-DNA antibody production in SLE patients (Anand *et al*., 2002). Also, patients with SLE were reported to have statistically lower levels of peripheral blood $CD4^+$ $CD25^+$ regulatory T cells than did normal controls. The over whelming majority of $CD4^+$ $CD25^+$ T cells play a crucial role in the suppression of abnormal immune responses (Fathy *et al*., 2005). Viallard and associates (2001), observed a significantly higher percentage of $CD8^+$ cells that bore the DR $^+$molecule in patients with active SLE than in patients with quiescent disease.

Whether the increased CD69 expression on T cells is an intrinsic T cell defect or is due to abnormal humoral factors in patients' sera is a matter of discussion. *In vitro* studies had previously illustrated the contribution of IL-10 to CD69 expression. The addition of IL-10 blocking antibody to the peripheral blood mononuclear cell culture further increased the CD69 expression in cell cultures of SLE patients compared to controls. The increase was observed in the cells with higher basal CD69 expression. This suggests that the IL-10 plays an inhibitory role compensating for T-cell hyperactivity; thus when it is neutralized, the quantity of activated T cells rises even further (Wang *et al*., 2005). In a relevant study involving T cell stimulation assays, T cells from patients with active SLE were found to have an intrinsic defect that impairs their activation process, probably involving an alteration in the activation of protein kinase C^3. It seems that the abnormal T cell responses in SLE could be due to both intrinsic T cell defects and abnormal cytokine milieu.

The possibility that increased CD69% on T cells could be a mere reflection of the enlarged CD3 population in SLE led us to study also CD69/CD3 ratio which would be more informative of receptor expression. The parameters of disease activity (ESR and SLE DAI score) were positively correlated with each of the CD69% and CD69/CD3 ratios indicating that the more the activated phenotype was the severer got the disease. Similar findings were reported in two previous studies on SLE adults (Su *et al*., 1997; Crispin *et al*., 1998).

The expression of CD69 has been studied in other autoimmune diseases. In a recent study, the activation antigen CD69 was significantly expressed on peripheral blood and synovial fluid neutrophils from rheumatoid arthritis patients (Atzeni *et al*., 2004). In earlier reports, T cells obtained from synovial fluid of patients with rheumatoid arthritis and juvenile rheumatoid arthritis, over express $CD69^+$ antigen when compared to peripheral blood T cells from the same patients or to normal controls (Fernandez-Gutierrez *et al*., 1995; Minami *et al*., 2006). In this case, the up-regulation of the activation marker seems to be an indicator of the high proportion of activated lymphocytes that accumulate in the affected joints rather than the result of an intrinsic cell defect as in SLE.

Complement may have a deleterious role in the pathogenesis of SLE. Patients with SLE present with decreased circulating complement levels and complement deposition in inflamed tissues, suggestive of a harmful role of complement in the effector phase of disease (Manderson *et al*., 2004). In our series, although CD69%, CD69/CD3 ratio and C3 were all significantly correlated with SLE DAI scores, we could not establish a link between serum C3 and any of CD69%, CD69/CD3 ratio, or ESR. Hence, CD69%, CD69/CD3 ratio might share in the estimation of disease activity when levels of C3 fail to show good correlation with clinical manifestations of

disease activity. Also for a given patient, the $CD8^+$ CD69 percentage may be useful in the context of continuous monitoring of treatment, with the patient serving as this own control.

Central nervous system disease in SLE is driven by cross-talk between the peripheral immune system and the brain's innate immune system, which leads to overproduction of brain cytokines and ultimate tissue injury. Neuropsychiatric disturbances are present in up to one-half of SLE patients (Tomita *et al*., 2004). In the current study, patients with CNS disease (44%) had a significantly higher SLEDAI score, elevated ESR, intense CD8 expression as well as higher percent and intensity of CD69 expression than those free of CNS disease (Table 3). We could not cite published reports on the relationship between CD69 expression and CNS involvement in SLE which may place CD69 as a novel biomarker in favor of severe disease and CNS involvement. Further studies are needed to verify such observation.

A recent report showed that, SLE patients with lupus nephritis had significantly higher peripheral blood CD80 expression than patients without lupus nephritis (Hirashima *et al*., 2004). Others, provided evidence of expanded $CD4^+$ $CE69^+$ clones from the kidney of nephritic murine lupus model (Kikuchi *et al*., 2006). In our series, the level of CD69 expression was not related to the development of lupus nephritis which needs to be reassessed in wider scale studies.

Systemic lupus erythematosus is characterized by the development of multiple cytopenias secondary to cytolytic or cytotoxic antibodies directed toward red blood cells, platelets and white blood cells (Gehi *et al*., 2003). This could explain the lymphopenia observed in SLE patients when compared to the control group.

Taken together, the results of this study suggest that peripheral blood lymphocytes from pediatric patients with active SLE exhibit increased expression of the CD69 antigen. The positive correlation with ESR and SLEDAI score and its relation to neuro-psychiatric involvement, makes this molecule a good indicator of disease activity in pediatric systemic lupus. The findings are limited by the sample size. It might be rewarding to study the contribution of the disturbed immunological environment to the requirements of CD69 expression and T-cell activation in this complex disease. New findings in the pathophysiology of T cells in lupus and especially the application of novel techniques to correct immune cell aberrations on the transcriptional and transnational levels give hope for the development of non-conventional therapeutic options in systemic lupus erythematosus.

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