Expression of Cyclin B1 and Cyclin Dependent Kinase Inhibitor p21 in Lymphocytes in Patients with Systemic Lupus Erythematosus

CHEONG-YIP HO, CHUN-KWOK WONG, EDMUND KWOK-MING LI, LAI-SHAN TAM, and CHRISTOPHER WAI-KEI LAM

ABSTRACT. ***Objective.*** The roles of cyclins and cyclin dependent kinase (CDK) inhibitors of lymphocytes in the pathogenesis of systemic lupus erythematosus (SLE) are unclear. We measured the expression of cyclin B1 and CDK inhibitor p21 in peripheral blood lymphocytes (PBL) from patients with SLE and controls.

Methods. PBL from 40 SLE patients with renal disease (RSLE), 40 SLE patients without renal disease (SLE), and 28 healthy control subjects were cultured with phytohemagglutinin (PHA). Bivariate distribution of cyclin B1 or p21 expression versus cellular DNA content was assessed by flow cytometry.

Results. Expression of p21 in lymphocytes was significantly lower in patients with RSLE and with SLE than controls (RSLE vs controls and SLE vs controls, both $p < 0.001$). Expression of cyclin B1 was similar in all groups. The percentages of RSLE lymphocytes in G0/G1 and S phase were significantly reduced and elevated, respectively, compared with controls.

Conclusion. Downregulated p21 in PHA stimulated PBL from patients with SLE may be closely related to aberration of cell division in SLE lymphocytes. (J Rheumatol 2002;29:2537–44)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS CYCLIN B1
CYCLIN DEPENDENT KINASE INHIBITOR p21 LYMPHOCYTES

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by various immunological abnormalities, including polyclonal activation of circulating B lymphocytes that produce a large quantity of autoreactive antibodies[1,2]. It is suggested that B cell proliferation in SLE is T cell dependent, and the persistence of autoreactive B and T lymphocytes is thought to be responsible for the production of autoantibodies in SLE[3]. Following antigen exposure, mature lymphocytes require intense and repeated proliferation to generate a rapid immune response and immunological memory. It has been established that dysregulation of apoptosis in lymphocytes might lead to autoimmunity, as illustrated by studies on lupus prone mice and patients with SLE[4,5]. *In vivo* overproliferation of autoreactive T cells of MRL-lpr/lpr mice suggested that defective apoptosis might lead to increased cell cycling and hence disrupted tolerance toward self-antigens[6,7]. Another group has reported that activated T cells from patients with SLE differ in their cell cycle distribution[8]. Loss of tolerance and autoimmunity might thus stem from defects in cell division and dysregulated expression of cell cycle regulators.

Following mitogenic stimulation, quiescent cells (G0 state) progress through 4 cell cycle phases: G1, the first gap phase; S, DNA synthesis; G2, the second gap phase; and M, mitosis. Control of this progress involves positive regulators such as cyclins and cyclin dependent kinases (CDK), and negative regulators like CDK inhibitors[9,10]. During G1/S phase progression, D cyclins (D1–D3) act in mid-G1, followed by cyclin E and cyclin A involved at the G1/S boundary; cyclins A and B (B1 and B2) act during the S and G2/M phases. CDK require complexing with cyclins as well as phosphorylation for activity. CDK inhibitors function by repressing the activity of cyclin-CDK complexes[11]. Based on their structural characteristics and CDK targets, 2 classes of CDK inhibitors have been defined, Ink4 (inhibitors of CDK4) and Cip/Kip (CDK interacting protein). Ink4 proteins including p15, p16, p18, and p19 associate solely with CDK4 and CDK6, whereas the Cip/Kip proteins (p21, p27, and p57) interfere with cycling by binding to cyclin/CDK complexes and inhibiting all CDK involved in G1/S transition[12,13].

From the Department of Chemical Pathology and Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, NT, Hong Kong.

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C.Y. Ho, PhD, Postdoctoral Fellow; C.K. Wong, PhD, Associate Professor, Department of Chemical Pathology; E.K. Li, MD, Associate Professor; L.S. Tam, MD, Medical Officer, Department of Medicine and Therapeutics; C.W.K. Lam, PhD, Professor, Department of Chemical Pathology.

Address reprint requests to Prof. C.W.K. Lam, Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, NT, Hong Kong. E-mail: waikeilam@cuhk.edu.hk

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It is thought that p21 mediates cell cycle arrest by forming a quaternary complex with cyclins and their complementary CDK, and thereby inhibiting the kinase activity[14]. It also binds to proliferating-cell nuclear antigen (PCNA) for inhibiting DNA replication[10]. Malfunction of the p21 protein may contribute to the pathogenesis of systemic autoimmunity, e.g., SLE, through a variety of mechanisms. Inactivating mutations of p21 may allow overactivation and proliferation of otherwise quiescent, low avidity, self-reactive T cells in the peripheral lymphoid organs of healthy individuals[15]. Studies on a p21–/– mouse model showed that enhanced p21 deficient T cell proliferation was evident only after prolonged mitogenic T cell stimulation, a circumstance akin to repeated presentation of self-antigens to T cells[11]. In the absence of p21, prolonged exposure to antigen might thus lead to a disruption of tolerance towards self-antigens, and the resulting accumulation of CD4+ memory T cells might represent the conversion of autoreactive cells to the memory phenotype[16,17].

Cyclin B1 is a mitotic cyclin that complexes with CDK1 ($p34^{cdc2}$) to form the mitosis-promoting factor required for cells to enter mitosis. The onset of cyclin B1 synthesis starts in G2 phase, with its expression peaking during the G2/M transition, and its degradation being completed at the transition to anaphase of mitosis[18]. Several studies have confirmed that cyclin B1 is a key cell cycle regulator of the G2/M checkpoint[19-21]. Cyclin B2 is a membrane associated B-type cyclin that can also bind and activate CDK1. A recent study has suggested that cyclin B1 may compensate for the loss of cyclin B2 in mutant cyclin B2-null mice and implies that cyclin B1 is capable of targeting the CDK1 to the essential substrates of cyclin B2[22]. An abundant level of cyclin B1 has been found in the thymus of lupus prone MRL-lpr/lpr mice compared to other strains, and southern blot analysis of cyclin B1 gene showed restriction fragment length polymorphism and hence multiple forms of cyclin B1 related sequences in various murine genomes[23]. However, to our knowledge no study on the expression of cyclin B1 in SLE lymphocytes has been conducted.

We examined the expression of CDK inhibitor p21 and cyclin B1 with respect to the cell cycle positions, i.e., the G1/S and G2/M checkpoints, in lymphocytes from patients with SLE and controls stimulated with a T cell mitogen phytohemagglutinin (PHA) *in vitro*. Using multiparameter flow cytometry as described[24-26], we evaluated the direct relationship between the status of individual cells in the cell cycle (DNA content) and their cyclin content as a function of cell cycle phase. We are the first group using this technique to investigate the bivariate distribution of CDK inhibitor (p21) expression versus DNA content in lymphocytes from patients with SLE.

MATERIALS AND METHODS

Patients, controls, and blood samples. Eighty Chinese patients with SLE were recruited at the Rheumatology Outpatient Clinic of the Prince of Wales Hospital, Hong Kong. Diagnosis of SLE was established according to the 1982 revised American Rheumatism Association criteria[27], and disease activity was evaluated by the SLE Disease Activity Index (SLEDAI)[28]. The patients were divided in 2 groups: 40 SLE patients with (or having the history of) renal disease (RSLE group) and 40 SLE patients without renal disease (SLE group). Twenty-eight sex and age matched healthy Chinese volunteers were recruited as controls. Twenty milliliters of heparinized venous peripheral blood were collected from each patient and control. The protocol of this study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong and informed consent was obtained from all participants.

Cell preparation. Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood by Ficoll-Paque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). The cells were washed twice with phosphate buffered saline (PBS; Sigma Chemical Co., St. Louis, MO, USA), cultured in RPMI 1640 (Gibco Laboratories, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Gibco), 20 mM HEPES (Gibco), and 10 µg/ml PHA (Calbiochem, San Diego, CA, USA) in a density of 10^6 cells/ml at 37°C, 5% CO_2. After 48 h, the cells were harvested for flow cytometric analyses of cyclin B1 and p21.

Immunocytochemistry. After 48 h incubation with PHA, the cells were rinsed with ice cold PBS and fixed in 80% ethanol at –20°C. After several washings with PBS, the cells were permeabilized with 0.25% Triton X-100 (Sigma) in PBS for 5 min on ice. They were then washed and incubated overnight at 4°C with murine monoclonal antibody to human cyclin B1 (FITC conjugated IgG1, clone GNS-1; Becton Dickinson Pharmingen, San Diego, CA, USA) and p21 (IgG1, clone SX118; BD Pharmingen). The anti-p21 Mab was diluted in PBS containing 1% bovine serum albumin (BSA) to obtain 0.25 µg/10^6 cells and both types of Mab were added in 100 µl. For p21 staining, the cells were washed and incubated with a FITC conjugated goat anti-mouse IgG antibody (Zymed Laboratories, South San Francisco, CA, USA) diluted 1:40 in PBS containing 1% BSA for 30 min at room temperature. The isotypic control was prepared identically as described above, except that an isotype-specific antibody was used instead of cyclin B1 or p21 Mab (mouse IgG1 for p21 Mab, Sigma; FITC conjugated mouse IgG1 of clone MOPC-21 for cyclin B1 Mab, BD Pharmingen). After immunofluorescence staining, the cells were washed with PBS and resuspended in 10 µg/ml propidium iodide (Sigma) and 0.1% RNase A (Calbiochem) in PBS, and incubated at room temperature for 20 min prior to flow cytometric measurement.

Flow cytometry. The immunofluorescence stained cells were analyzed with a Becton Dickinson FACSCalibur™ flow cytometer. The red (propidium iodide) and green (FITC) emissions from each cell were separated and quantitated using the built-in FACSCalibur optics. A forward scatter/side scatter gate was set for lymphocytes (with relative abundance > 90%) to exclude monocytes. Among the lymphocyte population, most were T cells. Bivariate scatter plot of cyclin B1 (or p21)-FITC versus DNA content-propidium iodide was acquired in list modes. Each acquisition and analysis was performed on at least 10,000 events using CellQuest™ software (Becton Dickinson). Each measurement of cyclin expression as a function of the DNA content was obtained from the bivariate cyclin B1 (or p21)-FITC/DNA-propidium iodide scatter plot gated to exclude debris and doublets on the scatter plot of FL-2 width/FL-2 area DNA content. After subtracting the background fluorescence of isotypic controls, results of cellular fluorescence were expressed as median, interquartile range (IQR), minimum and maximum values of mean fluorescence intensity (MFI). Data analysis of cell cycle phase distribution was with ModFit LT™ software (Verity Software House Inc., Topsham, ME, USA).

Statistical analyses. Since cyclin B1 and p21 expressions (MFI) and cell cycle phase distribution in terms of percentage were not in a Gaussian distribution, the Mann-Whitney rank sum test was used to assess differences in cyclin B1 and p21 expressions and cell cycle phase distribution between patients and controls. Results were expressed as median (IQR) or mean ± standard deviation. All analyses were performed using the

Statistical Package for the Social Sciences (SPSS) for Windows, Version 9.0 (SPSS Inc., Chicago, IL, USA). A probability (p) less than 0.05 was considered significantly different.

RESULTS

Patients and controls. The age, sex, SLEDAI score, duration of diagnosis, anti-DNA antibody titer, and drug treatment of the study populations are summarized in Table 1. Forty SLE patients with renal disease (39 women, one man, mean ± SD age 38.5 ± 10.3 yrs, range 20–59) and 40 SLE patients (40 women, age 38.4 ± 11.3 yrs, range 20–67) were studied. The mean duration of the diagnosis of SLE at the time when patients were evaluated for this study was 12.4 ± 6.4 years (range 1.7–26.6) and 9.0 ± 6.8 years (range 0.3–25.6) for RSLE and SLE patients, respectively. The mean SLEDAI scores of RSLE and SLE patients were 7.3 ± 5.6 (range 1–20) and 3.2 ± 5.3 (range 1–32), respectively. Patients were undergoing treatment with prednisolone (RSLE 6.7 ± 5.1 mg daily, 92.5%; SLE 3.2 ± 6.6 mg daily, 50.0%), hydroxychloroquine (RSLE 87.5 ± 99.0 mg daily, 50.0%; SLE 112.0 ± 108.0 mg daily, 60.0%), azathioprine (RSLE 20.0 ± 30.0 mg daily, 40.0%; SLE 9.0 ± 30.0 mg daily, 12.5%), cyclosporin A (RSLE 20.9 ± 43.5 mg daily, 22.5%; SLE 0 mg daily, 0%), or in combination. Twenty-eight sex and age matched healthy controls (27 women, one man, age 38.5 ± 7.9 yrs, range 22–51) were studied. The mean concentrations of anti-DNA autoantibodies in RSLE and SLE patients were 401.5 ± 302.9 IU/ml (range 71–1000) and 348.9 ± 325.1 IU/ml (range 77–1000), respectively.

Distribution of lymphocytes in 3 cell cycle phases. Distribution into 3 cell cycle phases (G0/G1, S, and G2/M) of 48 h PHA stimulated PBL from patients and controls was evaluated according to the frequency of DNA content distribution. Table 2 clearly shows that the percentage of RSLE lymphocytes in S phase was significantly elevated compared with the controls [median (IQR) percentages: RSLE 21.6% (15.7–25.0) vs controls 14.8% (9.7–20.9); p = 0.022]. On the other hand, the percentage of RSLE lymphocytes in G0/G1 phase was significantly lower than in controls [RSLE 71.0% (68.8–80.3) vs controls 79.8% (73.0–87.4); p = 0.028]. However, no significant difference could be found in the percentage of cells in G2/M phase between RSLE and control lymphocytes (p > 0.05). Although the percentages of SLE lymphocytes in the 3 phases were different from those of controls, no significant difference was observed (p > 0.05).

Expression of cyclin B1 and p21 in relation to cell progression through S phase. Figure 1 shows the representative scatter plots of bivariate distribution for expression of cyclin B1 or p21 versus DNA content in PBL from RSLE patients, SLE patients, and controls after 48 h PHA stimulation. Expression of cyclin B1 was found to be higher in the G2/M cell population of PBL, while expression of p21 was similar in the G0/G1, S, and G2/M phases. Figure 2A indicates that the expression of cyclin B1 in the G2/M phase was similar in lymphocytes from RSLE and SLE patients and controls (p > 0.05). Figure 2C illustrates that the p21 expression of

Table 1. Characteristics of RSLE and SLE patients and controls.

	RSLE	SLE	Control
Number	40	40	28
Female/male	39/1	40/0	27/1
Age, yrs, mean ± SD (range)	38.5 ± 10.3 (20–59)	38.4 ± 11.3 (20–67)	38.5 ± 7.9 (22–51)
SLEDAI score, mean ± SD (range)	7.3 ± 5.6 (1–20)	3.2 ± 5.3 (1–32)	NA
Duration of diagnosis, yrs, mean ± SD (range)	12.4 ± 6.3 (1.7–26.6)	9.0 ± 6.8 (0.3–25.6)	NA
Anti-DNA antibody titer, IU/ml, mean ± SD (range)	401.5 ± 302.9 (71–1000)	348.9 ± 325.1 (77–1000)	NA
Treatment with prednisolone			
Patients, n (%)	37 (92.5)	20 (50.0)	NA
Daily dose, mg, mean ± SD	6.7 ± 5.1	3.2 ± 6.6	
Treatment with hydroxychloroquine			
Patients, n (%)	20 (50.0)	24 (60.0)	NA
Daily dose, mg, mean ± SD	87.5 ± 99.0	112.0 ± 108.0	
Treatment with azathioprine			
Patients, n (%)	16 (40.0)	5 (12.5)	NA
Daily dose, mg, mean ± SD	20.0 ± 30.0	9.0 ± 30.0	
Treatment with cyclosporin A			
Patients, n (%)	9 (22.5)	0 (0.0)	NA
Daily dose, mg, mean ± SD	20.9 ± 43.5	0	

NA: not applicable.

Table 2. Cell distribution in the 3 phases of the cell cycle. Results are expressed as median (interquartile range) of the percentage distributed in 3 cell cycle phases.

Cell Cycle Phase	RSLE, n = 20	SLE, n = 20	Controls, n = 18
G0/G1	71.0 (68.7–80.3)*	71.8 (67.3–86.4)	79.8 (73.0–87.4)
S	21.2 (15.7–25.0)**	20.7 (10.7–25.3)	14.8 (9.7–20.9)
G2/M	6.0 (4.8–7.7)	6.3 (3.0–7.4)	4.9 (2.9–6.9)

Statistical difference between RSLE or SLE patients and controls in 2 independent experiments for each donor was determined by Mann-Whitney rank sum test. * p = 0.028, ** p = 0.022.

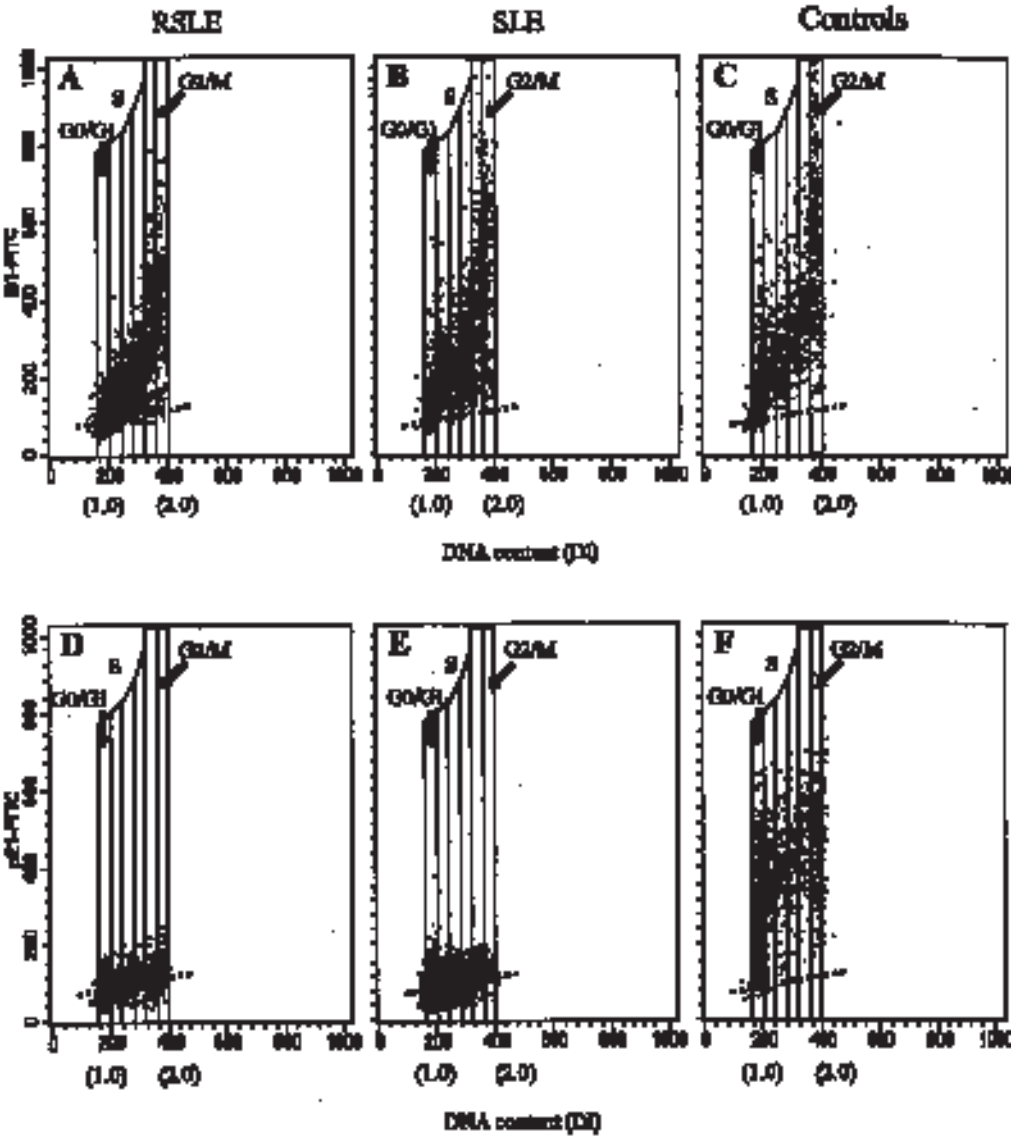

Figure 1. Expression of cyclin B1 (A–C) and p21 (D–F) in proliferating lymphocytes from a representative patient with RSLE (A, D) or SLE (B, E) and a control (C, F) in relation to cell cycle position (DNA content). Scatter plots represent bivariate distributions (cyclin B1 or p21 expression vs DNA content) of PBL after 48 h stimulation with PHA. Broken line in each plot represents fluorescence level of the respective isotypic control, i.e., the cells incubated with isotypic IgG. Three cell cycle phases are indicated as G0/G1, S, and G2/M in each plot. The vertical gating windows illustrate the principle of selection of cell subpopulations based on differences in cellular DNA content (the DNA index, DI) to obtain the mean values of cyclin B1 or p21 associated fluorescence per window.

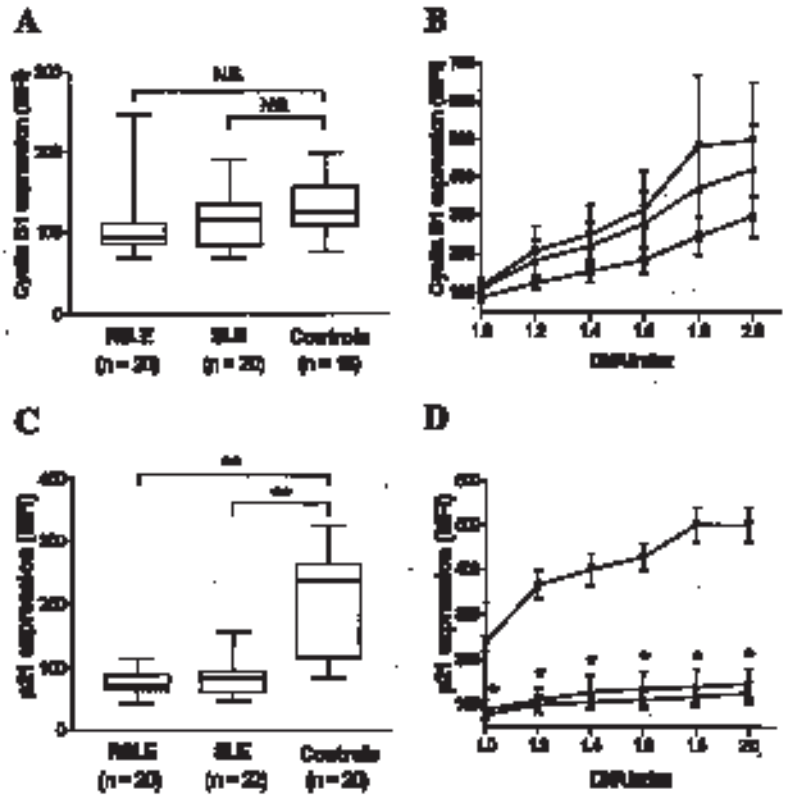

Figure 2. Expression of cyclin B1 (A, B) and p21 (C, D) in 48 h PHA stimulated lymphocytes from RSLE and SLE patients and controls in relation to cell progression through S phase. Results in (A) and (C) are expressed as mean fluorescence index (MFI) of cyclin B1 and p21 in the lymphocytes, respectively, using box-and-whisker plots. The extent of cell progression through S phase correlates with DNA content (DNA index). The MFI was calculated for G0/G1 cells (DNA index = 1) and for G2/M (DNA index = 2) phase cells, as well as for cells in discrete, narrow windows at various points in S phase. After subtracting the fluorescence intensity of isotypic controls in exactly equivalent windows, the remaining fluorescence intensity was plotted as a function of the mean DNA content for each window. Each point represents the mean MFI value of cyclin B1 (B) or p21 associated fluorescence (D) in each group (■: RSLE patients; ▲: SLE patients; ▼: controls) and the error bar represents the standard deviation. Statistical difference between RSLE or SLE patients and controls in a single experiment of each donor was determined by Mann-Whitney rank sum test. *p = 0.008; **p < 0.001 for both RSLE and SLE patients compared to controls. NS: not significant.

PHA stimulated PBL in RSLE and SLE patients was significantly lower compared with controls [median (IQR) of the MFI: RSLE 72.2 (64.1–88.9) vs controls 230.9 (96.0–283.9); $p < 0.001$; SLE 83.5 (59.6–84.6) vs controls 230.9 (96.0–283.9); $p < 0.001$].

Analysis of cyclin B1 or p21 during the S phase was done by electronic gating, i.e., selection of "slices" through the S phase compartment representing subpopulations differing in DNA content, in proportion to the fraction of replicated DNA. The mean value of cyclin or p21 associated fluorescence was estimated for each "slice" to evaluate the variation in the level of protein expression during progression through S phase. As shown in Figure 2B, the expression of cyclin B1 in all 3 groups was maximal in the cells with DNA index of 2.0, i.e., with DNA content equivalent to that of G2/M cells. The level of cyclin B1 rose sharply during the transition from S to G2 phase, especially in the second half of the S phase (DNA index > 1.5). The rate of increase in cyclin B1 level throughout the S phase was similar in patients and controls. In contrast, the expression of p21 in lymphocytes from RSLE and SLE patients was significantly lower compared with controls at various points from the G1 to G2/M phase through the S phase, as shown in Figure 2D ($p = 0.008$). The level of p21 in controls was upregulated at the G1/S phase transition (at DNA index 1.0–1.2) and rose modestly after the onset of S phase (DNA index > 1.2). The rate of increase in p21 expression during progression through the S phase in both RSLE and SLE patients was similar and was lower than for controls.

DISCUSSION

These results illustrate that the percentage of RSLE lymphocytes in the G0/G1 phase was significantly lower compared to controls, while the percentage in S phase was

significantly higher (Table 2). However, no significant difference could be found in the patients with SLE. This might be explained by the difference in disease severity between RSLE and SLE patients (Table 1), although no significant correlation between SLEDAI score and the percentage distribution in either G0/G1 or S phase could be found (data not shown). Another study has shown a correlation between serum levels of soluble interleukin 2 (IL-2) receptor and number of proliferating-cell nuclear antigen positive lymphocytes in SLE patients having a higher proportion of T cells in the S phase[8]. In addition, we have described[29] aberrant apoptosis and Fas expression in activated T cells of SLE patients *in vitro*. These results suggest that the change in cell cycle phase distribution might be related to other proliferation and apoptosis markers in SLE T cells.

Recent studies have shown that in the p21–/– murine model, p21 deficiency results in high titer of anti-DNA autoantibodies, heavy IgG immune complex deposits, and development of severe lupus-like syndrome including lymphadenopathy and glomerulonephritis[11,16,17]. Balomenos, *et al*[16] illustrated that although p21 deficient T cells responded normally to polyclonal T stimulants (e.g., concanavalin A, phorbol-12-myristate-13-acetate), they failed to undergo p53-dependent cell cycle arrest following DNA damage. They also showed a proliferative advantage over wild-type cells following prolonged IL-2 stimulation, with an increased proportion of cells in the S phase of the cell cycle and accumulation of CD4+ memory cells and activated B cells, as well as loss of tolerance toward nuclear antigens.

Our findings indicate that CDK inhibitor p21 expression of PHA stimulated lymphocytes (predominantly T cells) from both RSLE and SLE patients was significantly downregulated compared with the controls (Figures 1 and 2). These findings suggest that the lack of p21 expression might contribute to dysregulation of the cell cycle and hence the sustained *in vitro* proliferation of T cells indicated by an increased proportion of cells in the S phase (Table 2). The bivariate distribution of p21 expression versus DNA content of normal lymphocytes showed that p21 was maintained at a high level during the G1/S transition (Figures 1 and 2), since p21 plays an important role in inhibiting the progress of a cell through the G1/S checkpoint in response to serum starvation, differentiation, and senescence[30-32]. p21 and other CDK inhibitors like p27 are known to interfere with cell cycling by complexing with cyclins (mainly D type) and CDK subunits and inhibit all CDK involved in G1/S transition. The defective expression of p21 in lymphocytes from RSLE and SLE patients might allow more cells to enter S phase due to the lack of inhibition of cyclins and CDK activities in the G1/S checkpoint, as evidenced by a decrease in the proportion of cells in the G0/G1 phase and an increase in S phase (Table 2).

Tolerance to self-antigens can be induced after activation of T cells under incomplete costimulatory conditions, e.g., absence of an inflammatory environment, leading to nonsustained T cell proliferation and deletion of activated cells[33]. Peripheral tolerance to self-antigens may occur in an analogous manner[34]. Due to the ubiquitous presence of self-antigens, impaired cell cycle regulation might lead to excessive proliferation and disruption of tolerance after repeated antigen presentation. Cell cycle inhibitors are thus essential in maintaining tolerance, and appear to act at 2 control points: (1) p21 negatively regulates T cell proliferation in response to self-antigens; and (2) p27 might maintain the anergic state in self-antigen-specific T cells[34]. We propose that following repeated stimulation by self-antigens, autoreactive p21 deficient T cells hyperproliferate and provoke a disruption of tolerance. This may result in an increase in helper and memory T cells and activated B cells, as well as production of autoantibodies (Table 1). Balomenos and Martinez[11] have also illustrated that p21 deletion results in a sex linked lupus-like syndrome in the murine model of SLE. Hence it is of interest to identify whether female hormones are directly associated to the p21 mediated disruption of tolerance or influence a specific type of immune cells or perhaps the generation of nuclear antigens. These data together suggest p21 plays a pivotal role in negatively regulating the proliferation of T cells and maintaining the tolerance toward self-antigens, and the downregulation of p21 possibly contributes to the pathogenesis of SLE. In addition, p21 deficiency may increase kidney T cell proliferation and deposition of immune complexes on the glomerular endothelium, contributing to inflammatory reactions and development of glomerulonephritis as seen in the RSLE group[16,17].

Cyclin B1, like cyclin A, is classified as a mitotic cyclin and functions exclusively during the G2/M phase. Both cyclins A and B interact with CDK1 to induce mitosis, and proteolysis of both proteins is required for completing mitosis[19,35]. Cyclin B1 accumulates at the time of cell exit from S phase, reaches maximal levels when the cell enters mitosis, and breaks down during the transition to anaphase[18]. Our results, in accord with others' findings, confirm that cyclin B1 expression in normal PHA stimulated PBL (Figure 1) is essentially limited to cells in the late S phase and those with DNA content of G2/M phase[20,24,26]. Our results indicate no significant difference in cyclin B1 expression between patients with SLE (RSLE or SLE) and the controls (Figure 2), suggesting that expression of the central G2/M regulator cyclin B1 is not defective in SLE lymphocytes. A similar pattern of increase in cyclin B1 expression throughout the S phase was found in lymphocytes from both patients and controls, suggesting that there is no defect in the kinetics of cyclin B1 expression in SLE lymphocytes (Figure 2). A recent study has illustrated a significantly higher level of cells in the G2/M phase and an

upregulation of cyclin B1 protein in thymocytes of lupus prone MRL-lpr/lpr mice compared with normal BALB/c mice, indicating an alteration of the cell cycle machinery in thymocytes and hence the aberrant development of T cells in these lupus prone mice[23]. Thus, the role of cyclin B1 in controlling the G2/M checkpoint may be related to the sustained proliferation of SLE T cells.

Although prednisolone, a synthetic glucocorticoid, has been shown to inhibit IL-2 synthesis during T cell proliferation[36], no previous studies have described any positive or negative effects of prednisolone on the cell cycle regulators of T cells in SLE. Hydroxychloroquine, an antimalarial that blocks antigen presentation and lowers cytokine production, has not yet been shown to affect the expression of cell cycle inhibitors and cyclins[37]. In addition, no recent findings have shown the influence of azathioprine on cell cycle regulation. However, the immunosuppressive drug cyclosporin A has been shown to indirectly induce the expression of p21 via transforming growth factor-ß[38]. In our cohort of RSLE and SLE patients, only a small number of patients received cyclosporin A treatment (RSLE 22.5%; SLE 0%), thus the influence of immunosuppressive drugs on p21 expression might be insignificant. A subgroup analysis may be carried out in the future to assess the possible effects of individual, rather than combined, drug treatment on patients with SLE.

A recent study has reported that an increase in the tumor suppressor protein p53 may influence the G2/M transition by repressing transcription of cyclin B and hence a cell arrest in G2 phase[39]. It also exerts its function in G1 checkpoint control through transcriptional activation of p21[40-42]. Thus the role of p53 in controlling G1/S and G2/M checkpoints through p21 and cyclin B in SLE is worth studying in the future. Recent genetic analyses of patients with SLE have identified several lupus susceptibility loci[43,44]. The p21 gene maps within a recently identified human MHC susceptibility locus 6p11-p21 for disease linkage, which may be related to genes other than human leukocyte antigen[44,45]. Thus, our future work will focus on the analysis of possible abnormalities in cyclin (e.g., D, E, A, and B) and CDK inhibitor genes (e.g., p21 and p27) of T lymphocytes in SLE. In addition, expression of cyclins and CDK inhibitors in subsets of activated T cells in SLE will be investigated.

REFERENCES

1. Klinman DM, Steinberg AD. Systemic autoimmune disease arises from polyclonal B cell activation. J Exp Med 1987;165:1755-60.
2. Klinman DM, Steinman RA. Inquiry into murine and human lupus. Immunol Rev 1995;144:157-93.
3. Biji M, van Lopik T, Limburg PC, et al. Do elevated levels of serum-soluble Fas contribute to the persistence of activated lymphocytes in systemic lupus erythematosus? Autoimmunity 1998;11:457-63.
4. Cohen PL, Eisenberg RA. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Cell Death Differ 1991;9:243-69.
5. Salmon M, Gordon C. The role of apoptosis in systemic lupus erythematosus. Rheumatology 1999;38:1177-83.
6. Balomenos D, Rumold R, Theofilopoulos AN. The proliferative in vivo activities of lpr double-negative T cells and the primary role of $p59^{fyn}$ in their activation and expression. J Immunol 1997; 159:2265-73.
7. Balomenos D, Rumold R, Theofilopoulos AN. Interferon-γ is required for lupus-like disease and lymphoaccumulation. J Clin Invest 1998;101:364-71.
8. Tokano Y, Morimoto S, Hishikawa T, et al. Subsets of activated T cells in patients with systemic lupus erythematosus: the relation to cell cycle. Scand J Rheumatol 1997;26:37-42.
9. Sherr CJ. Cancer cell cycles. Science 1996;274:1671-6.
10. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G_1-phase progression. Genes Dev 1999;13:1501-12.
11. Balomenos D, Martinez-AC. Cell-cycle regulation in immunity, tolerance and autoimmunity. Immunol Today 2000;21:551-5.
12. Cheng M, Olivier P, Diehl JA, et al. The $p21^{Cip1}$ and $p27^{Kip1}$ CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J 1999;18:1571-83.
13. Hirai H, Roussel MF, Kato J-Y, Ashmun RA, Sherr CJ. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol Cell Biol 1995; 15:2672-81.
14. Harada K, Ogden GR. An overview of the cell cycle arrest protein, $p21^{WAF1}$. Oral Oncol 2000;36:3-7.
15. Bouneaud C, Kourilsky P, Bousso P. Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. Immunity 2000;13:829-40.
16. Balomenos D, Martin-Caballero J, Garcia MI, et al. The cell cycle inhibitor p21 controls T-cell proliferation and sex-linked lupus development. Nature Med 2000;6:171-6.
17. Santiago-Raber ML, Lawson BR, Dummer W, et al. Role of cyclin kinase inhibitor p21 in systemic autoimmunity. J Immunol 2001;167:4067-74.
18. Pines J, Hunter T. Human cyclins A and B1 are differentially located in the cell and undergo cell-cycle dependent nuclear transport. J Cell Biol 1991;115:1-17.
19. Sherwood SW, Rush DP, Kung AL, Schimke RT. Cyclin B1 expression in HeLa S3 cells studied by flow cytometry. Exp Cell Res 1994;211:275-81.
20. Viallard J-F, Lacombe F, Dupouy M, Ferry H, Belloc F, Reiffers J. Different expression profiles of human cyclin B1 in normal PHA-stimulated T lymphocytes and leukemic T cells. Cytometry 2000;39:117-25.
21. Yang J, Kornbluth S. All aboard the cyclin train: subcellular trafficking of cyclins and their CDK partners. Trends Cell Biol 1999;9:207-10.
22. Brandeis M, Rosewell I, Carrington M, et al. Cyclin B2-null mice develop normally and are fertile whereas cyclin B1-null mice die in utero. Proc Natl Acad Sci USA 1998;95:4344-9.
23. Hsu HC, Thomas TJ. Cyclins and autoimmunity: cyclin B1 gene expression and restriction fragment length polymorphism in lupus-prone mice. Autoimmunity 1995;22:17-26.
24. Darzynkiewicz Z, Gong J, Juan G, Ardelt B, Traganos F. Cytometry of cyclin proteins. Cytometry 1996;25:1-13.
25. Gong J, Traganos F, Darzynkiewicz Z. Expression of G_1 and G_2 cyclins measured in individual cells by multiparameter flow cytometry: a new tool in the analysis of the cell cycle. Cell Prolif 1994;27:357-71.
26. Gong J, Bhatia U, Traganos F, Darzynkiewicz Z. Expression of cyclins A, D2 and D3 in individual normal mitogen stimulated lymphocytes and in MOLT-4 leukemic cells analysed by multiparameter flow cytometry. Leukemia 1995;9:893-9.
27. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum

1982;25:1271-7.
28. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH, the Committee on Prognosis studies in SLE. Derivation of the SLEDAI: a disease activity index for lupus patients. Arthritis Rheum 1992;35:630-40.
29. Ho CY, Wong CK, Li EK, Lam CWK. Effects of dexamethasone on the expression of Fas molecules and apoptosis of lymphocytes in patients with systemic lupus erythematosus. Immunol Invest 2001;30:231-44.
30. Li Y, Jenkins CW, Nicolas MA, Xiong Y. Cell cycle expression and p53 regulation of cyclin-dependent kinase inhibitor p21. Oncogene 1994;9:2261-8.
31. Steinman RA, Hoffman B, Iro A, Guillouf C, Lieberman DA, El-Houseini ME. Induction of p21 (WAF-1/CIP1) during differentiation. Oncogene 1994;9:3389-96.
32. Zeng YX, El-Deiry WS. Regulation of $p21^{WAF1/Cip1}$ expression by p53-independent pathways. Oncogene 1996;12:1557-664.
33. Pape KA, Kearney ER, Khoruts A, et al. Use of adoptive transfer of T-cell-antigen-receptor-transgenic T cells for the study of T-cell activation in vivo. Immunol Rev 1997;156:67-78.
34. Ohashi PS, Sarvetnick N. Autoimmunity: a bias from tolerance to immunity. Curr Opin Immunol 1997;8:815-7.
35. Nurse P. Ordering S phase and M phase in the cell cycle. Cell 1994;79:547-50.
36. Kirou KA, Boumpas DT. Systemic glucocorticoid therapy in systemic lupus erythematosus. In: Wallace DJ, Hahn BH, editors. Dubois' lupus erythematosus. Philadelphia: Lippincott Williams and Wilkins; 2002:1173-94.
37. Wallace DJ. Antimalarial therapies. In: Wallace DJ, Hahn BH, editors. Dubois' lupus erythematosus. Philadelphia: Lippincott Williams and Wilkins; 2002:1149-72.
38. Khanna AK, Hosenpud JD. Cyclosporin induces the expression of the cyclin inhibitor p21. Transplantation 1999;67:1262-8.
39. Krause K, Wasner M, Reinhard W, et al. The tumour suppressor protein p53 can repress transcription of cyclin B. Nucl Acids Res 2000;28:4411-8.
40. Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature 1995;377:552-7.
41. Bunz F, Dutriaux A, Lengauer C, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 1998;282:1497-501.
42. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell 1995;82:675-84.
43. Gaffney PM, Kearns GM, Shark KB, et al. A genome-wide search for susceptibility genes in human systemic lupus erythematosus sib-pair families. Proc Natl Acad Sci USA 1998;95:14875-9.
44. Theofilopoulos AN, Kono DH. Mechanisms and genetics of autoimmunity. Ann NY Acad Sci 1998;13:225-35.
45. El-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-25.