Prolactine effect on CD69 and CD154 expression by CD4+ cells from systemic lupus erythematosus patients

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Abstract

Objective

The aim was to explore the role of prolactine (PRL) in the lymphocyte activation process in active and inactive systemic lupus erytematosous (SLE) patients in an in vitro *model.*

Methods

Peripheral blood mononuclear cells (PBMNC) were isolated from SLE patients and healthy individuals. The mRNA for prolactine and its receptor, obtained by standard techniques with an appropriate primer, were subjected to PCR and visualised. The PBMC were cultured with: a) medium alone as a negative control, b) unspecific mitogen as a positive control (PMA-ionomycin for CD154 or concanavalin A for CD69), c) PRL alone, d) mitogen plus PRL, e) mitogen plus antibody anti-PRL (1:50) and f) mitogen plus an unrelated antibody. Then CD69 and CD154 were determined by flow cytometry analysis.

Results

Twelve inactive and 15 active SLE patients were studied. 25% of the active patients displayed hyperprolactinemia. Under basal conditions, CD69 expression was associated with disease activity. In contrast, CD154 did not show this association. The PBMNC activated in vitro *were capable of producing and secreting prolactine as measured by mRNA and Nb2 assay. In the same way the mRNA for prolactine receptor was visualized. Cells from SLE patients cultivated with PRL alone did not display increased CD69 or CD154 expression. The addition of PRL to the unspecific stimulated culture did not have an additive effect. In contrast, the addition of antibodies against PRL, in order to block the autocrine prolactine, resulted in a striking reduction in CD69 and CD154 expression.*

Conclusions

PRL is produced and secreted by the immune cell and acts just after the first trigger signal of activation in an autocrine way. The expression of CD69 and CD154 molecules depend partially on the prolactine.

Key words

Prolactine, SLE, prolactine receptor, CD69, CD154.

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Introduction

Systemic lupus erythematosus is an autoimmune rheumatic disease characterized by widespread inflammation and most commonly affects women. Virtually every organ and/or system may be involved. The course of the disease is characterized by remissions and exacerbation. The exacerbation of the disease has been linked to the activity of the immune system (1-4). The activation process of the immune system cells must be initiated by antigenic peptides bound to class I or II MHC molecules by antigen-presenting cells (APC) which are recognized by T cell receptor (TCR) (5). Its signal determines antigen specificity and plays a central role in initiating T cell activation. However, this interaction, by itself, is not sufficient to fully activate naïve T cells. Thus, subsequent non-antigen-specific co-stimulatory signals are necessary to trigger the cytokine gene expression. The best known co-stimulatory signal for T cells is provided by the interaction of CD28 on the T cell with members of the B7 family (CD80 and CD86) on the APC. On the other hand, B cells are also targets of co-stimulatory signals, mainly received through the CD40 receptor after engagement by its ligand CD154 (CD40-L) on activated T cells. This signal promotes growth, differentiation, survival and isotype switching on B cells. A third type of signal with a crucial role in T and B cell activation is mediated through the binding of soluble cytokines to their respective receptors (6, 7). The expression of these molecules is a marker of activated cells (8); this, plus the increased expression of CD69, is a common association in active SLE patients (9, 11).

Another association described in active SLE patients is the high serum level of prolactine (PRL) (12). Although the clinical trials have yielded conflicting information because some studies have found an association between the serum levels of PRL and the disease activity (12-15) but others could not confirm this relationship (16-19). Regarding the frequency of hyper-prolactinemia in SLE patients, there is a consensus that 15 to 30% of SLE patients display hyper-prolactinemia (12). Other evidence supports the participation of the prolactine in the SLE disease activity through the immune system activity, such as the hyper-prolactinemia patients without autoimmune disease showed an increased frequency of autoantibodies compared with healthy people (20); physiological hyper-prolactinemia states such as pregnancy and lactancy trigger lupus flares (21); elevated frequencies of hyperprolactinemia in male SLE patients (14, 22); the finding in the mouse model for SLE where implants of syngeneic pituitary glands induce a hyper-prolactinemia state, resulting in accelerated autoimmunity and early mortality (23, 24) and the treatment with bromocriptine which improves survival (24); or the experiment where PRL infusion induced a dramatic improvement in the survival of bromocriptine treated mice after intra-peritoneal injection of *Listeria monocytogenes* (25) and those studies *in vitro* where the aim was to show that the PRL acts as a co-mitogen for T, B and NK cells from both humans (26-28) and mice (29, 30). Moreover, we recently described that SLE patients with auto-antibodies against prolactine display less activity compared with those patients without these antibodies (31, 32).

In spite of this information, even if there is very little knowledge about the participation of the PRL in the phenomenon of activation of the cells from the immune system, the aim of this research is to explore the participation of prolactine in the lymphocytes activation process from active or inactive SLE patients using the *in vitro* approach; the lymphocytes from patients and their controls were stimulated under different conditions in order to determine if the prolactine that participates in the immune response is of pituitary or lymphoid origin. As a marker of activated cells CD69 and CD254 expression were measured. In the same way, we explored the prolactine production at RNA and protein level.

Materials and methods

The human Ethical Committee and Medical Research of the Instituto Mexicano del Seguro Social (IMSS) ap-

proved the study protocol, and written informed consent was obtained from all the subjects who participated voluntarily in this study.

Patients

Twenty-seven SLE patients attended the lupus clinic at the Hospital de Especialidades en el Centro Medico Nacional "Siglo XXI" del IMSS for a long-term follow-up were included. All of them fulfilled four or more of the American College of Rheumatology (ACR) revised criteria for the classification of SLE (1). Recent medications, as well as those taken on the day of the evaluation were recorded. Any conditions associated with elevated PRL were noted including pregnancy, PRL-secreting pituitary adenoma (prolactinoma), intracranial tumours compressing the pituitary stalk or hypothalamus, drugs, hypothyroidism, chest wall diseases, hepatorenal disorders. All the hyperprolactinemic patients were classified as idiopathic because none showed any of the associated conditions for elevated PRL.

Disease activity was graded according to a previous published index (SLEDAI) (2). For the purpose of this study, any value above 0 was considered as disease activity. Twelve of the SLE patients were inactive and 15 were active. At the time the samples were taken, treatment was with cloroquine (2.5 mg/Kg) and prednisone (10-20 mg) and none of the SLE had taken an immunosuppressor drug. In the case of the active patients, the samples were taken at the moment of hospitalisation before standard treatments for active disease were used. As a control, cells and serum from 12 healthy subjects were used. The criteria for the healthy subjects was the absence of disease and menstrual disorder, and normal serum levels of prolactin (normal levels of PRL are 5 to 20 ng/ml). Venous blood samples were drawn between 8:00 am and 10:00 am after an overnight fast.

Cells

Human peripheral blood mononuclear cells (PBMNC) were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma AS, and Oslo Norway). PBMNC were recovered from the interface, washed in PBS, and resuspended in serum-free culture medium (AIM-V medium, Life Technologies, Grand Island, NY), a synthetic medium without prolactine. Cell viability, determined by trypan blue exclusion, was always above 95%.

PRL

The human PRL (hPRL) and polyclonal antibodies from rabbits against prolactin we used were kindly donated by Dr. A.F. Parlow from the National Hormone & pituitary program Harbor-UCLA Medical Center (lot# AFP 3855 A). The non-related antibody was obtained from the serum precipitation from normal rabbits.

RNA for prolactine and its receptor

Four microliters (4μg) of cellular RNA, obtained from PBMNC (6 x 10^6) stimulated with PMA 5 ng/ml-Ionomicina 200 ng/ml, for 0, 1, 2, 3, 4, 5, 6 and 24 h, was used for cDNA strand–Synthesis reaction. Twenty microliters of the cDNA were added to the PCR mixture (final volume, 100 μl) containing Taq polymerase (2.5 unit; GIBCO-BRL) for prolactine we used 5'CAAGAAGAATCGGAACATACAGGCTTT and 3'GCAGTTGTTGTTGTGGATGATTCGGCA primers (25 pM) and 5'GTCTGGGCAGTGGCTTTGAAGGGC 3'CACTTGCTTGATGTTGCAGTGAAGTTG for the prolactine receptor in a PCR Buffer (1.5 mM $MgCL_2$, 50mM KCl, 20mM Tris HCl, pH 8.4, GIBCOBRL) and dNTP (0.2 mM, GIBCOBRL). The reaction mixture was subjected to 30 cycles of PCR (each consisting of denaturation at 94° C for 1 min, annealing at 65°C for 2 min and extension at 72°C for 2 min for prolactin. For the prolactin receptor we used 94°C for 30 sec, 65°C for 1 min and 72°C for 1.5 min. In-gel amplification was then performed in 1.5% remelted low-melting-point agarose (GIBCO/BRL). Double-distilled water served as a negative control for amplification.

Activation assay

Cells were cultured in 24 (5 x 10^5 PBMNC/well) and 96-well plates (1 x 10^5 cells/well) at 37°C under the following conditions: a) medium alone as a negative control, b) hPRL alone, c) unspecific mitogen as a positive control (PMA-ionomycin for CD154, or concanavalin A for CD69), d) mitogen plus hPRL, e) mitogen plus anti-hPRL antibody (1:50) and f) mitogen plus non-related antibody (1:50). As a quality control for the AIMV medium, IRMA and Nb2 bioassays were performed.

Flow cytometry analysis

After the different experimental conditions mentioned above, the cells were re-suspended in PBS-BSA-AZ (PBS pH 7.4, 0.2% bovine serum albumin, 0.2% sodium azide) and were stained with varying FITC-labelled mAb, including anti-CD4, anti-CD69, and anti-CD154 (PharMingen, San Diego, CA) for 20 min at 4°C, after which, the cells were fixed in 1% paraformaldehyde for further analysis. Fluorescence analysis was performed in a FACScalibur flow cytometer (Becton Dickinson). A minimum of 10,000 lymphocyte-gated events were acquired in list mode and analysed with Cell Quest Software.

Nb2 assay

PRL bioactivity was measured using Nb2 lymphoma cell assay as described by Tanaka *et al*. (33). Briefly, the cells were kept at 37°C in Fisher's medium containing 10% FBS as a source of lactogen, 10% horse serum, 10^{-4} M 2-mercaptoethanol, 50 IU/ml penicillin. Those cells were arrested in the early G_1 phase of the cell cycle by 24 h of preincubation in lactogen-free medium. Resumption of the cell cycle was achieved by the addition of increasing concentrations of purified human pituitary PRL (NIDDK hPRL). PRL-like bioactivity was assayed in: a) aliquots of culture medium from PBMNC stimulated with concanavalin A at different times (0, 1, 2, 3 h), b) supernatant plus antibody anti-hPRL, at different dilutions to ascertain parallelism with the standard curve. To inhibit the lactogenic effect of human growth hormone (hGH) rabbit antiserum to hGH (NIDDK-anti-hGH-IC-3 A.S., CYTO

[AFPC11981A]) was added to a final dilution of 1:4000. Cultures per triplicate were further maintained in an atmosphere of 95% air-5% CO_2 at 37°C for 72 h. The effects of hPRL on cell proliferation were analyzed by the incorporation of [^{3}H] thymidine (1μCi) into Nb2 cells. The sensitivity of this assay for PRL was 3 pg/ml.

The competitive test

In separate tubes a constant concentration of anti-PRL was pre-incubated (at 37°C for 1 h) with increasing concentrations of PRL in order to obstruct the binding site of the antibodies. The cells were then incubated with PMA-ionomycin and the complex (anti-PRL antibody-hPRL) was added to the culture. Then, the cells were washed, incubated and fixed. Fluorescence analysis was performed using a FACScalibur cytometer. It was done in order to show that the inhibitions observed in the experiment were due to the capture of PRL by the antibodies.

Statistical analysis

The significance of the differences between the variables in two independent groups was determined by the nonparametric statistic Mann Whitney U and for the three groups Kruskal Wallis was used.

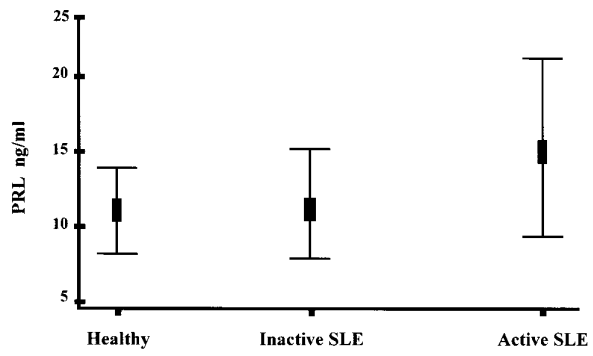

Fig. 1. Serum prolactine levels measured by Nb2 assay in inactive and active SLE patients compared with a group of healthy individuals. The concentration is expressed in ng/ml.

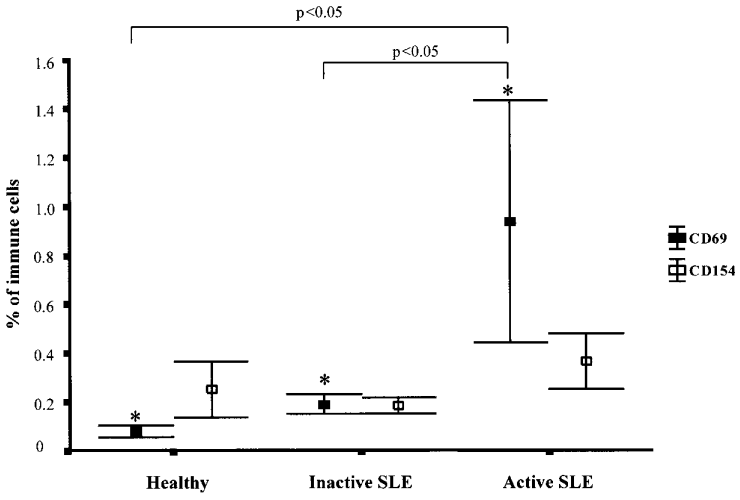

Fig. 2. The percentage of CD69 and expression of CD154 in cells from SLE patients and healthy controls at the moment in which the samples were taken. The expression of CD69 was in CD4+ whereas CD154 were stained on PBMNC. Statistically significant differences ($p < 0.05$) were found between the patients with active SLE compared with inactive patients and healthy controls.

Results

The study population consisted of 27 SLE patients (mean ± SD age 36 ± 7 years) similar to the twelve healthy control subjects with a mean age of 31 ± 10 years old. The patients were divided using SLEDAI in twelve inactive (42 ± 8 years) and fifteen active (34 ± 9 years). The clinical features of the history of the disease in both groups of patients (active and inactive) are in Table I. The mean of the serum levels of prolactine in non-active SLE patients was 12 ± 1.9 ng/ml, similar to the mean of the healthy control group 11.1 ± 1.2 ng/ml. In contrast, the mean of the sera levels of PRL was higher 16.8 ± 2.9 ng/ml. Moreover, 25% of the active SLE patients had hyperprolactinemia (Fig. 1). The mean of SLEDAI in the group of active SLE patients was 14.5 compared with 0 from the non-active patients.

At the moment the sample was taken and before the realization of the culture, the expressions of CD69 and CD 154 by CD4$^+$ cells from SLE patients and controls were analyzed. We did not find statistically significant differences in the expressions of CD154 among the active, inactive, and healthy groups (Fig. 2). In contrast, the CD69 expression was higher in the CD4$^+$ from patients with the disease activity (1 ± 0.45%), it was statistically significantly different ($p < 0.05$) compared with the percentage of CD4$^+$ from the inactive SLE patients (0.29 ± 0.09%) and healthy group (0.07 ± 0.02%). CD69 is an early cell activation marker that could show the quantity of active immune cells in the disease activity.

To confirm that the cells of the immune system are capable of producing and secreting PRL, PBMNC from healthy people were activated *in vitro* using unspecific mitogen, then the supernatant was collected at intervals of 0, 1, 2, and 3 h., finding concentrations of 0, 45.8, 26.2 and 63.4 pg/ml respectively ,measured by Nb2 assay. In contrast, we did not find Nb2 activation in those cultures with supernatant without stimulus and in those where anti-PRL antibody was added to the supernatant (data not shown). In the same way, the mRNA for PRL was obtained at: 0, 1, 2, 3, 4 and 5 h. after the PBMNC was stimulated with unspecific mitogen. Then RT-PCR and Dot Blotting was performed, as can be seen in Figure 3a. We found mRNA for PRL at time zero. In the period of time after the cells were activated an increased amount of mRNA for PRL was observed. In the same way, the mRNA for PRL receptor was found in activated PBMNC. The amount of mRNA for receptor appears un-

changed throughout the time (Fig. 3b). The cells culture conditions were synthetic medium, non-supplemented with foetal bovine serum (FBS), in order to avoid interference due to growth factors in the foetal serum. Before each experiment, the synthetic medium culture was tested for PRL in an Nb2 assay as a quality control. In the same way, the antibodies against PRL were capable of blockine the biological activity of PRL on the Nb2 cells (data not shown). Figure 4 shows the behaviour of PBMC from SLE patients in culture with human pituitary PRL or without prolactine (depleting with anti-PRL antibodies): The CD69 expression in the negative control (cells with medium) was similar to the CD69 expression in culture with only hPRL, meaning that hPRL alone is not capable of activating cells from active or inactive SLE patients. In the positive control, PBMNC stimulated with unspecific mitogen showed high levels of CD69 expression: In the healthy group, 30% of $CD4^+$ display CD69 closly similar to the 40% finding in the inactive SLE patients. Inexplicably, only 15% of cells from active patients display the expression of CD69. To find the effect additive described in the assays of proliferation with PRL, the experiments were carried out adding hPRL to the cultures stimulated with mitogen unspecific, but we did not find this effect, the expression of CD69 did not change. In contrast, the addition of antibodies against PRL resulted in a striking reduction of CD69 expression in about 50% in patients and controls with statistically significant differences ($p < 0.05$); the addition of no related antibody did not affect the CD69 expression.

In spite of the control with non-related antibodies which did not show changes in the CD69 expression, we decide to prove that the inhibition of CD69 expression was due to anti-PRL antibody activity: to do that, the binding site of the anti-PRL antibody was engaged with hPRL. Different concentrations of hPRL were pre-incubated with a constant concentration of antibody against PRL and then were added to the culture.

In the measurement in which the binding site of the antibody anti PRL is blocked, the inhibition in the expression of CD69 returns to the levels of the response to the mitogen. We found statistically significant differences ($p < 0.005$) between the inhibition of CD69 expression with the antibody alone against the complex of antibodies with 12 ng/ml of PRL. In contrast, no statistically significant differences were found in the CD69 expression with the addition of the complex formed by anti-prolactine antibodies and 25, 50 and 100 ng/ml. It demonstrated that the reduction of the CD69 expression was due to the blocking of prolactine produced by lymphocyte and not for another reason, like the activity of the Fc antibody portion or by the complex formed between antibody and the PRL (Fig. 5).

In PBMNC from SLE patients and healthy subjects the hPRL alone was not capable of inducing the CD154 expression. By contrast, PMA induced the CD154 expression in about 50% of PBMNC in SLE patients and controls. The addition of hPRL to PMA-stimulated cells had no effect on the CD154 expression. However, the supplement

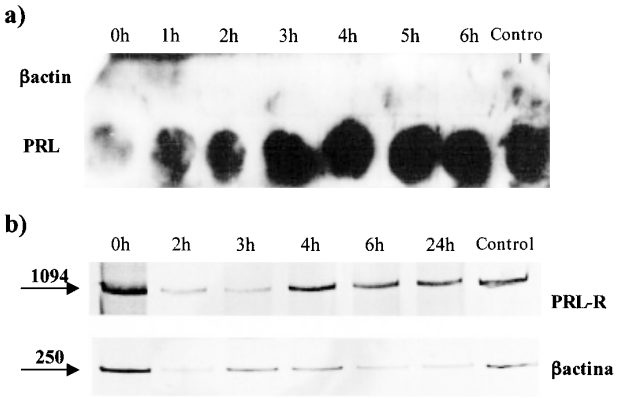

Fig. 3. (**a**) mRNA for prolactine visualized by dot blotting; the expression of RNA was found in cells at baseline time (without stimulus). After the stimulus the amount of mRNA for prolactine appeared to increase. (**b**) mRNA for the PRL receptor; here the amount of RNA did not increase after the stimulus.

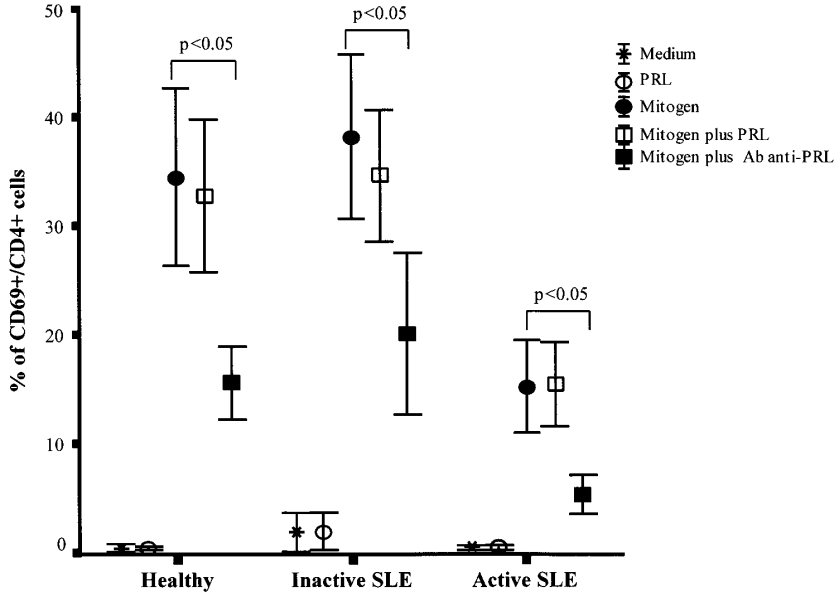

Fig. 4. PBMNC from active and inactive SLE patients and controls cultured under different conditions. Statistically significant differences were found in the response to mitogen between active SLE patients compared with inactive patients and healthy individuals ($p < 0.005$). The performance was closely similar in the 3 groups studied; statistically significant differences were found between the responses to mitogen compared with those cultures where prolactine was blocked ($p < 0.005$).

with anti-PRL antibodies resulted in a reduction of the CD154 expression on PBMNC in about 59% as can be seen in Figure 6. The responses to unspecific stimulus display a similar pattern in both SLE patients and healthy subjects. However, the cells from SLE patients display less CD154 expression than the cells from healthy controls. In fact, cells from active SLE patients display a weak response to unspecific mitogen, but display a higher reduction of the CD154 expression with anti-PRL antibodies compared with the inactive and healthy control group. In the same way, in the three groups studied, the inhibitory activity of anti-PRL antibody was ablated by absorption with PRL.

Table I. Demographic data and clinic characteristic of 27 patients with SLE.

Clinical feature	Active (n = 15)	Inactive (n = 12)
Age. in yrs, mean ± SD	34 ± 9	42 ± 8
Women (%)	100	100
Disease duration mo. media	70	75
Mean of SLEDAI	14.5	0
Neurologic disorder (%)	53	36
Renal disorder (%)	60	58
Serositis (%)	26	16
Joint (%)	80	83
Oral ulcers (%)	33	25
Alopecia (%)	40	41
Photosensitivity (%)	46	50
Skin rash (%)	66	41
Hematology disorder (%)	46	25
ANA (+) (%)	80	90
Prolactine, ng/ml mean ± SD	12 ± 1.9	6.8 ± 2.9
Hyperprolactinemia (%)	25	0

The clinical data are those that the patients display in some moment along the history of the disease. At the time that the samples were taken all the patients were being treated with chloroquine (2.5 mg/Kg) and prednisone (10-20 mg); none of the SLE patients were on immunosuppressive drugs.

Discussion

The information about the relationship between PRL and lupus activity in SLE patients has been inconsistent (12). The SLE patients in our research showed a similar pattern to previous trials: 25% of active SLE patients showed hyperprolactinemia, which is similar to the ranges in the previous reports (12-14, 31). The group of active SLE patients showed higher serum levels of PRL compared with the non-active patients and healthy group similar to the previous results from our laboratory (31). In fact, we did not find hyperprolactinemia in any inactive SLE patients, which supports the idea that PRL plays a role in the disease activity. We also found an association between CD69 expressions with disease activity in the SLE patients, probably as reflection of the immune system activity; similar to the previous report (9, 11). Surprising, the amount of active immune cells measured through the CD69 and CD 154 expression in SLE patients at the moment the samples were taken (basal) was less than 2% of the cells, it probably denotes the amount of auto-reactive immune cells in the disease.

Previous research suggests that the hyperprolactinemic state could activate the immune system (23, 24). In contrast, a low level of PRL has been associated with deficient immune response (34). The current study was carried out using cells from active and inactive SLE patients emulating a micro-environment with or without PRL in order to discriminate the activity of pituitary and lymphoid prolactine in the immune cells activation process. The original expectation was that the human PRL (pituitary) could trigger the lymphocyte activation especially in inactive SLE patients or perpetuate the immune cells activation in cells from active SLE patients. In contrast, we found that the hPRL by itself is not capable of inducing activation in cells from SLE pa-

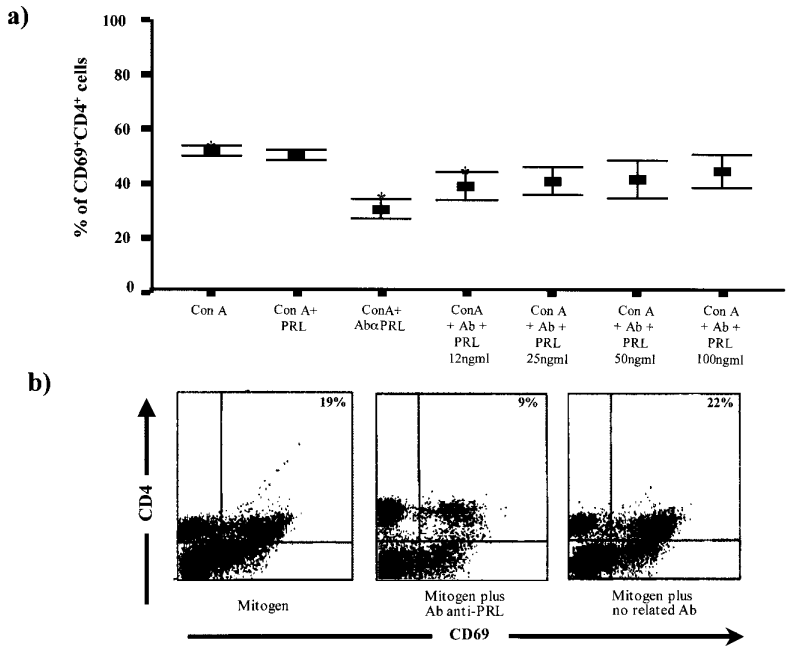

Fig. 5. (**a**) Inhibition test where the binding site of anti-PRL antibody was engaged with 12, 25, 50 and 100 ng/ml of hPRL. Statistically significant differences were found in the inhibition of CD69 expression with the antibody alone and with the complex of antibodies plus 12 ng/ml hPRL ($p < 0.005$). In contrast, we did not find statistically significant differences with the complex formed by anti-prolactine antibodies and 25, 50 and 100 ng/ml hPRL. (**b**) An example of flow cytometry analysis with double staining CD4/CD69 stimulating with mitogen, blocking the prolactine with anti-PRL antibody and with non-related antibodies.

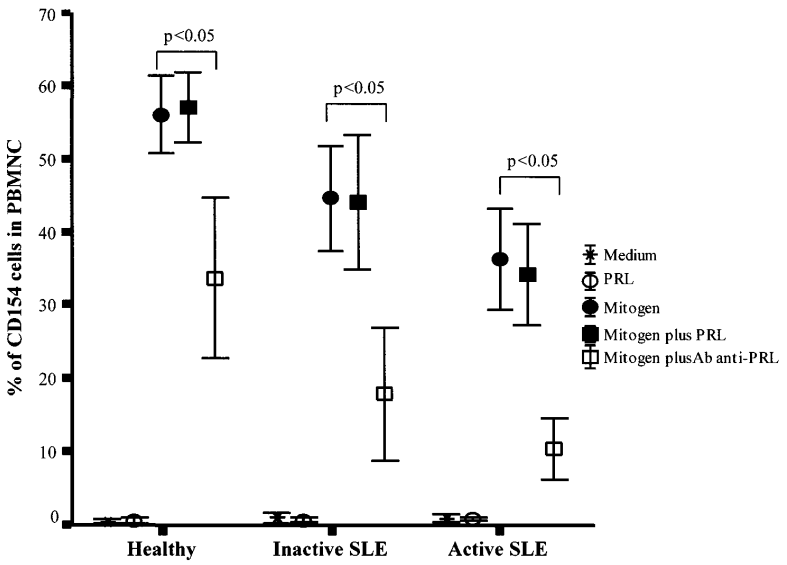

Fig. 6. PBMNC from active and inactive SLE patients and healthy subjects cultured under different conditions. Statistically significant differences were found in the response to mitogen between cells from inactive SLE patients and healthy controls compared with the response in cells from active SLE patients ($p < 0.005$). In the 3 groups statistically significant differences were found ($p < 0.005$) between the responses to mitogen compared with those cultures where the prolactine was blocked.

tients and healthy subjects. The hPRL activity was found just after the trigger the immune response (in this case with unspecific mitogen). In the performed experiments the PRL has autocrine and paracrine comportment similar to those previously reported in the literature (28, 29). Moreover, the cells from the immune system were capable of producing and secreting PRL similar to previous research (28, 29).

The observations that suggest that the PRL through its receptor play a role as grow factor are the hybridomas proliferation and the additive effect in the proliferation assay using mitogen or antigen in immune cells from both human and mice (35, 36). Our results confirm that the immune cells are capable of producing and secreting prolactine and this production is related to the amount of activation since we found doses response between PBMNC proliferation with the amount of PRL in the supernatant and at the mRNA levels. In the supernatant we found a correlation between the time of collection after the cell activation (0, 1, 2, and 3 h.) with PRL concentrations (0, 45.8, 26.2 and 63.4 pg/ml respectively), supported by the result obtained with RT-PCR and Dot Blotting where the amount of mRNA from PBMNC stimulated with unspecific mitogen increased over time. Moreover, the mRNA for prolactine and its receptor were found in cells without stimulus, suggesting that the PRL is constitutive of the lymphocyte and in the measure that the cell acquire activation his production and secretion is increased

The central axle in the immune response is the trigger, which is initiated normally antigen presentation of antigenic peptides bound to class I or II MHC molecules to the T cell receptor, by professional antigen-presenting cells (6, 7). This interaction is not enough to fully activate naïve T cells. A subsequent non-antigen-specific co-stimulatory signal is necessary to produce an adequate activation. Both moments are relevant because this is when the specificity and the magnitude of the immune response is determined. Here the objective was to explore the role of PRL in the immune cells activation, using the *in vitro* model described above. The status of activation was measured through the expression of CD69 and CD154. These molecules provided the second signal in the cell activation process by the interaction of CD28 on the T cell. This signal promotes growth, differentiation, survival and isotype switching on B cells. Our results shown that human prolactine added to cells from SLE patients (active or inactive) do not increase the expression of CD69 and CD 154. Likewise, the lymphocytes obtained from SLE patients and healthy subjects had a similar pattern of immune response. In contrast, the depletion of prolactine produced by the lymphoid with anti-PRL antibodies shows a decreased expression in the activation mark molecules in about ~50%, indicating that PRL has a role in between the trigger (antigen presentation) and co-stimulatory signal in the immune cells activation and it could explain why SLE patients with autoantibodies against prolactine have less disease activity (31). Moreover, it clearly shows that PRL used in the activation process is secreted by the lymphocyte itself. This result is comparable to those experiments carried out with tirocytos where PRL stimulus alone does not change the HLA-DR and CD40 expression (37). These findings are difficult to understand in the context of the literature because in the knockout mouse for PRL there are no evident abnormalities in the anatomy, in the development in B and T cell and the innate immunity (38). However, the adaptive immune response, where most of the effects of PRL have been demonstrated including our results, was not studied in the knockout mouse (38). Moreover, in our laboratory we have found that the deletion of PRL with anti-PRL antibody also produces a striking reduction in the secretion of IL-2 and IFNγ by $CD4^+$ which supports these results (submitted).

T cell activation is a fundamental step for the onset of the adaptive immune response. This is characterized by profound alterations in cell surface expression of activation and adhesion molecules: increased CD69, CD25, CD40L, FAS-L and CD44 and decreased CD 62L and CD45R expression. Some of these changes are necessary for T cells to enter and progress in the cell-cycle (5); these alterations in the outer membrane are observed in lymphocytes from SLE patients, probably as a mirror image of the immune system activation. Moreover, some of these changes are related to the disease activity as

the CD69 expression (9, 11). In fact we found an increased CD69 expression in active SLE patients compared with in active patients and healthy subjects before the culture. In the stimulated culture it was observed that the CD69 expression decreased with the addition of anti-PRL antibody. This reduction was due to PRL depletion and to no ther cause because, when the binding site on the antibody against PRL was engaged with PRL, a restored expression of CD69 was observed. In the same way, the addition of a non-related antibody did not affect CD69 expression, demonstrating at least that in our model the reduction of the CD69 expression was due to the blocking of prolactine produced by the lymphocyte and not to the possible activity of the Fc antibody portion or immune complex of antibody-PRL.

Further studies are necessary in order to determine the steps and signal where the PRL is active in T cell activation. This model clearly displays that hPRL do not trigger the immune response in resting human cells. However, after the initial stimulus, the PRL appear to play a role in order to continue the event of activation like the co-stimulatory molecule expression and interleukin secretion necessary for a full response. In addition, the PRL that takes effect in the immune response is produced and secreted by the lymphocytes. It could suggest that the hyperprolactinemic states in lupus patients could be secondary in part by the immune cell activation as has been suggested for other hormones (39). In contrast, in agreement with our model, the hyperprolactinemic state is not capable of initiating the immune cells activation. Moreover, the SLE patients with autoantibodies against PRL that display a less clinical manifestation than those without antibodies, could be due to the it antibodies blocking the prolactine in a similar manner to our *in vitro* model (31, 32).

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