

Identification of prolactin as a novel immunomodulator on the expression of co-stimulatory molecules and cytokine secretions on T and B human lymphocytes

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Received 8 October 2004; accepted with revision 21 March 2005

Available online 28 April 2005

Abstract

We investigate the immunomodulator role of prolactin (PRL) on CD4⁺ and B cell activation from healthy subjects in comparison with hyperprolactinemic patients. Peripheral blood mononuclear cells, CD4⁺ or B cells, purified, were cultured under different conditions, as follows: with mitogen, without stimulus, with different concentrations of human PRL, with unspecific mitogen plus PRL, or with antibodies against PRL. The results revealed that PRL is produced by lymphocytes, the expression of CD69 and CD154 molecules, and interleukin secretions depend partially on the autocrine PRL, this is supported by the findings that secretions of IL-2, IFN γ , and co-stimulatory molecule expression were markedly reduced when autocrine PRL was blocked with anti-PRL antibodies. Furthermore, PRL activity was only observed during the first 2 h after activation. In contrast, B cell culture did not show any alteration by adding or blocking PRL in the expression of CD40 and CD86 in both groups: healthy subject and hyperprolactinemic patients.

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Keywords: Prolactin; CD69; CD154; CD40; CD86; Hyperprolactinemia; Immunomodulatory; Cell activation; IL-2; IFN γ

Introduction

Communication between cells is mediated by endocrine, nervous, and immune systems, which constitute an interlocking network. Several evidences have shown that the neuro-endocrine peptide hormone, prolactin (PRL), participates in the immune response. PRL is mainly synthesized by the anterior pituitary. However, the decidua, breast, and the lymphocytes [1–4] are also capable of synthesizing this hormone. Structural analysis of the PRL receptor has demonstrated that it is related to the cytokine/hematopoietin family, like the growth hormone (GH), erythropoietin,

granulocyte-macrophage colony stimulating factor (GM-CSF), and some interleukins (IL), such as IL-2 to IL-7, IL-9, IL-13, and IL-15 [5]. The receptor for PRL is also present in T- and B-lymphocytes and macrophages [6–9]. Excess prolactin (hyperprolactinemia) can be caused by diverse conditions, which can be divided into three groups: physiological (pregnancy), drugs (metoclopramide), and disease (prolactinomas and chronic renal failure). Hyperprolactinemia in women can cause irregular menses, galactorrhea, hypogonadism, and infertility. In contrast, clinical manifestations in men are widely variable and include decreased libido, impotence, and infertility [10]. Recently, it has been described that some hyperprolactinemic patients develop autoimmune rheumatic diseases [11] and some women develop natural autoantibodies during

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pregnancy probably due to the hyperprolactinemic state [12], suggesting a strong PRL participation in immune system activation.

Other evidences about PRL's role in the immune system are: in vitro, PRL acts as a co-mitogen for T, B, and NK cells in both human [13–16] and mice [17,18], independently from stimulation with antigen and/or mitogen. In NZB/W mice, which display a disease similar to systemic lupus erythematosus (SLE), implants of syngenic pituitary glands induce a hyperprolactinemic state, resulting in accelerated autoimmunity and early mortality [19,20] that can be improved with bromocriptine treatment [20]. In humans, clinical trials have shown that a subset of SLE patients course with hyperprolactinemia during disease activity [21,22]. In contrast, hypophysectomized rats decreased responses to both red blood cells and *Escherichia coli* lipopolysaccharides, which can be restored by the administration of exogenous PRL, but not with other pituitary hormones [23], suggesting that PRL's low levels are associated with immunodeficiency.

The adaptive immune response is a complex process, in which activation of the immune cells is fundamental. The process is initiated by antigen presentation of antigenic peptides bound to class I or II MHC molecules by professional antigen-presenting cells (APC) [24]. Then the antigen is recognized by the T cell receptor (TCR) who dictates antigen specificity and plays a central role in initiating T cell activation [24]. However, this interaction, by itself, is not sufficient to fully activate naive T cells. Thus, for virgin T cell activation, subsequent non-antigen-specific co-stimulatory signals are necessary to trigger cytokine gene expression. The best-known co-stimulatory signal for T cells is provided by the interaction of CD28 on the T cell with the members of the B7 family (CD80 and CD86) on the APC. On the other hand, B cells and other antigen-presenting cells are also targets of co-stimulatory signals, mainly received through the CD40 receptor after engagement by its ligand CD154 (CD40L) 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 binding of soluble cytokines to their respective receptors [25,26]. To further define the participation of prolactin in the mechanism of immune cell response activation, cells from two different sources were studied: (a) from hyperprolactinemic patients with high serum levels of prolactin at the moment the samples were taken and (b) from healthy humans with normal serum levels of PRL. Cells were in vitro cultivated under different stimuli to recreate different levels of PRL at the moment that cell activation is achieved. The markers for CD4⁺ T cell activation were the expression of co-stimulatory molecules, such as CD69, CD154, and cytokine secretions; CD86, CD40, and immunoglobulin production were used as markers of B cell activation.

Materials and methods

Patients

The criteria of five idiopathic hyperprolactinemia patients were high PRL serum level (>20 ng/ml) plus menstrual disorder and galactorrhea without any evidence of conditions otherwise associated with elevated PRL, such as pregnancy, PRL-secreting pituitary adenoma (prolactinoma), intracranial tumors compressing the pituitary stalk or hypothalamus, drugs, hypothyroidism, chest wall diseases, or hepatorenal disorders. The criteria of ten healthy subjects were to be disease-free, women without menstrual disorders, and normal PRL serum levels (<20 ng/ml). Venous blood samples were drawn between 8:00 AM and 10:00 AM.

Cells

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

The used human PRL (hPRL) and polyclonal antibodies against prolactin were kindly donated by Dr. A.F. Parlow from the National Hormone and Pituitary Program Harbor-UCLA Medical Center (batch # AFP3855A). The non-related antibody was obtained through serum precipitation from normal rabbits.

T and B cell isolation

Isolation of human CD4⁺ T cells from PBMC was achieved by depletion of non-CD4⁺ cells. PBMCs were reacted with a cocktail of hapten-conjugated monoclonal antibodies (mAb) against CD8, CD11b, CD16, CD19, CD36, and CD56. B cells (95% CD19⁺) were negatively selected after reacting PBMC with hapten-labeled mAb to non-B cells, including anti-CD2, CD4, CD11b, CD16, CD36, and anti-IgE. These cells were exposed to magnetic beads coupled to an anti-hapten monoclonal antibody (Miltenyi Biotec, Auburn, CA). The magnetically labeled cells were depleted on a MACS column (Miltenyi Biotec, Auburn, CA) with the magnetic MidiMACS field (Miltenyi Biotec, Auburn, CA).

Cell proliferation assay

PBMNCs were plated at 2×10^6 cells/ml, 0.1 ml/well, in 96-well plates and cultured in synthetic serum-free medium with 0, 0.5, 1, and 2 μ g/ml of concanavalin A (conA, Sigma, St Louis, MO). For the rest of the experiment, we decided to

use 2 $\mu\text{g/ml}$ of conA because it optimally enhanced cell proliferation. For the conA plus hPRL cultures (10, 50, 100, 250, 500, 1000 ng/ml), we chose 50 ng/ml of hPRL because it yielded the best additive effect. $^3\text{H-TdR}$ (1.0 μCi) was added during the last 18 h of culture. The $^3\text{H-TdR}$ incorporation was determined in a liquid scintillation analyzer (Packard 1900 TR).

Activation assay

Cells from hyperprolactinemic patients and from normal subjects were cultured in 24-well plates (5×10^5 PBMC/well) and 96-well plates (1×10^5 cells/well) at 37°C . Cells were stimulated under different experimental conditions, as follows: (a) medium alone as a negative control, (b) hPRL alone, (c) unspecific mitogen as a positive control (PMA-ionomycin for CD154, CD40, CD86, or conA for CD69), (d) mitogen plus hPRL, (e) mitogen plus antibody anti-hPRL (1:50), and (f) mitogen plus non-related antibody (1:50). IRMA and Nb2 bioassay were performed as quality control for AIMV medium.

Flow cytometry analysis

After the aforementioned different experimental conditions, cells were re-suspended in PBS-BSA-AZ (PBS, pH 7.4, 0.2% bovine serum albumin, 0.2% sodium azide) and stained with varying FITC-labeled mAb, including anti-CD4, anti-CD69, anti-CD154, anti-CD19, anti-CD40, and anti-CD86 (PharMingen, San Diego, CA) for 20 min at 4°C , after which 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 analyzed with Cell Quest Software.

Intracellular cytokine analysis

Purified T cells were cultured in 96-well plates (1×10^5 cells/well) for 4 h at 37°C under one of the following conditions: (a) hPRL (50 ng/ml), (b) PMA (5 ng/ml) and ionomycin (0.4 $\mu\text{g/ml}$), (c) PMA-ionomycin and hPRL (50 ng/ml), and (d) PMA-ionomycin and antibody anti-hPRL (1:50). Brefeldin A (1 $\mu\text{g/ml}$) was added 4 h before the end of culture time. Cultured cells were washed in PBS-BSA-AZ, fixed, and permeabilized with cytofix/cytoperm solution according to manufacturer's instructions (PharMingen, San Diego, CA). Permeabilizations of cells were FITC-labeled with mAb specific for IL-2 or IFN- γ (PharMingen, San Diego, CA) and examined in a flow cytometer as described above.

Competitive test

In separate assay tubes, a constant concentration of anti-hPRL was pre-incubated (at 37°C for 1 h) with increasing

concentrations of hPRL in order to obstruct the binding site of the antibodies. The cells were then incubated with PMA-ionomycin and the complex (anti-PRL antibody with PRL in the binding site) was added to the culture. Then, the cells were washed, incubated, and fixed. Fluorescence analysis was performed using a FACScalibur cytometer to show that the inhibition observed in the experiment was due to PRL uptake by the antibodies, since the antibodies with the binding site engaged with prolactin did not display inhibition.

PRL assays using Nb2 lymphoma cells

PRL bioactivity was measured using Nb2 lymphoma cell assay as described by Tanaka et al. [27]. Briefly, 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. After that, cells were arrested in the early G_1 phase of the cell cycle by 24 h of pre-incubation in lactogen-free medium. In order to obtain the standard curve, we add increasing concentrations of purified human pituitary PRL (NIDDK hPRL) (from 0 to 8 ng/ml). PRL-like bioactivity was assayed in (a) aliquots of culture medium from PBMC stimulated with conA at different times (0, 1, 2, 3, and 4 h) and (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 [^3H] thymidine (1 μCi) into Nb2 cells. The sensitivity of this assay for PRL was 3 pg/ml.

Results

The population in study consisted of five hyperprolactinemic female patients (mean \pm SD of age, 35 ± 7 years). All patients showed clinical manifestations, such as irregular menses and galactorrhea, only one with an infertility status. The mean of PRL serum levels was 44.4 ± 8.4 ng/ml. All hyperprolactinemia cases were classified as idiopathic because no one showed any of the associated conditions for elevated PRL. The ten healthy women studied had a mean age of 34 ± 10 years and displayed less than 20 ng/ml of serum PRL levels.

In proliferation assay of the PBMC from hyperprolactinemic patients and healthy subjects, the hPRL by itself was unable to induce proliferation at concentrations ranging from 10 to 1000 ng/ml. However, the hPRL displayed an additive effect on PBMC proliferation produced by unspecific mitogen (conA) stimulation, showing a statistically significant difference ($P < 0.005$) compared with those cultures without hPRL. In contrast, the addition of anti-

bodies against hPRL to PBMC cultures showed a 30% decrease in ^3H -TdR incorporation in response to conA with statistically significant difference ($P < 0.005$) (Fig. 1). The addition of not related antibodies does not induce inhibition, suggesting that PRL is produced locally during cell proliferation (data not shown).

In the activation assay, the PBMCs from five hyperprolactinemic patients and ten healthy subjects were tested for CD69 expression under different conditions. We found a similar pattern of cell activation in both groups studied as can be seen in Fig. 2a. The addition of hPRL alone to the cell culture did not induce changes in CD69 expression on CD4^+ cells from either patients or healthy subjects. ConA stimulation revealed that 34.8% of CD4^+ cells expressed CD69 in healthy subjects and 28.5% in hyperprolactinemic patients; these expressions were used as a positive control for each group. In cells from both groups, the addition of hPRL to conA-stimulated cells did not affect CD69 expression (33% and 29%, respectively). However, addition of antibodies against hPRL to the unspecific-stimulated culture resulted in a striking reduction (about 52%) of CD69 expression in cells from healthy subjects. Meanwhile, the cells from hyperprolactinemic patients display an inhibition with anti-hPRL antibodies around 38%. However, in both cases, statistically significant differences were found ($P < 0.05$). In contrast, the addition of not related antibodies to the culture did not induce changes in CD69 expression in both groups (Fig. 2b).

In order to determine whether the inhibition was due to anti-hPRL antibody activity, the binding site of the anti-hPRL antibodies was engaged with hPRL. In different assay tubes, a constant concentration of antibodies against hPRL

was pre-incubated with increased concentrations of hPRL, varying from 12 ng/ml to 100 ng/ml. Fig. 3a shows that the blocking of antibody binding site with hPRL reduces the inhibition of CD69 expression. As we engaged the antibody binding site with increased amount of hPRL, the CD69 expression returned to mitogen response levels, indicating that the reduction in CD69 expression was due to blockage or deletion of prolactin and not due to the Fc antibody portion, also indicating that addition of the antibody-hPRL complex to the culture did not play any role in the cell activation process.

In order to determine at what time hPRL exerted its activity during in vitro PBMC activation, the anti-hPRL antibodies were added at different moments after ConA activation. Fig. 3b shows that prolactin exerts its effect during the first hour after the immune response was triggered. In contrast, addition of antibodies against hPRL 2 h after stimulation did not exert an inhibitory effect on CD69 expression.

The autocrine PRL production was measured by Nb2 assay in the supernatant from stimulated cell cultures collected at 0, 1, 2, 3, and 4 h, the concentrations were 0, 121, 157, 110.7, and 96 pg/ml of PRL, respectively. In contrast, no PRL was detected in cultures from non-stimulated cells or in those cultures to which anti-hPRL antibodies had been added to the supernatant to block the lymphoid prolactin (data not shown), demonstrating that PBMCs are capable to produce and secrete PRL.

To examine whether hPRL had an effect on the expression of CD154, PBMCs from five hyperprolactinemic patients and ten healthy individuals were cultured in the presence of PMA-ionomycin (5 ng/ml and 0.2 $\mu\text{g}/\text{ml}$), which in preliminary experiments had been found optimal response, with 54% of PBMCs expressing CD154. Addition of hPRL alone or the addition to PMA-ionomycin-stimulated cells had no effect on CD154 expression. However, addition of anti-hPRL antibodies resulted in a reduction of CD154 expression in PBMC of around 47% in cells of either patients or healthy individuals (Fig. 2a). The inhibitory activity of anti-hPRL antibodies was also ablated by absorption with hPRL. Fig. 4a shows the results of adding anti-hPRL antibodies at different times during in vitro PBMC activation with PMA-ionomycin; their addition at time 0 and 1 h later reduced CD154 expression in 29%. In contrast, the addition of antibodies after 2 and 3 h had no effect on CD154 expression.

Another possibility to explore was whether hPRL could perpetuate CD154 expression after the initial stimulus, which could result in an enhanced B–T cell interaction. The cells were cultured with PMA-ionomycin and with PMA-ionomycin plus hPRL, harvested at 2, 4, 6, and 18 h, finding a similar pattern of CD154 expression in both conditions (Fig. 4b), suggesting that prolactin does not perpetuate CD54 expression. Likewise, addition of anti-hPRL antibody decreased CD154 expression throughout the 18 h of culture.

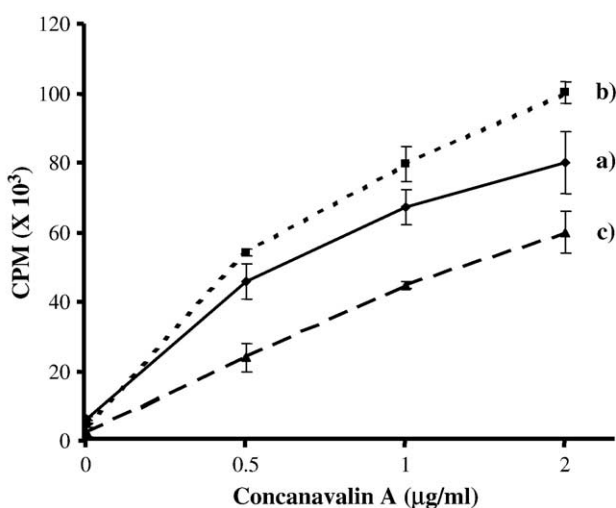


Fig. 1. PBMC proliferation measured by ^3H -TdR incorporation. Line a, cells stimulated with different concentrations of ConA (0, 0.5, 1, and 2 $\mu\text{g}/\text{ml}$); line b, cells stimulated with ConA plus PRL; and line c, cells with ConA plus anti-PRL antibodies. The values represent the mean plus one standard deviation of healthy human subjects. Statistically significant differences were found ($P < 0.005$) among the three groups.

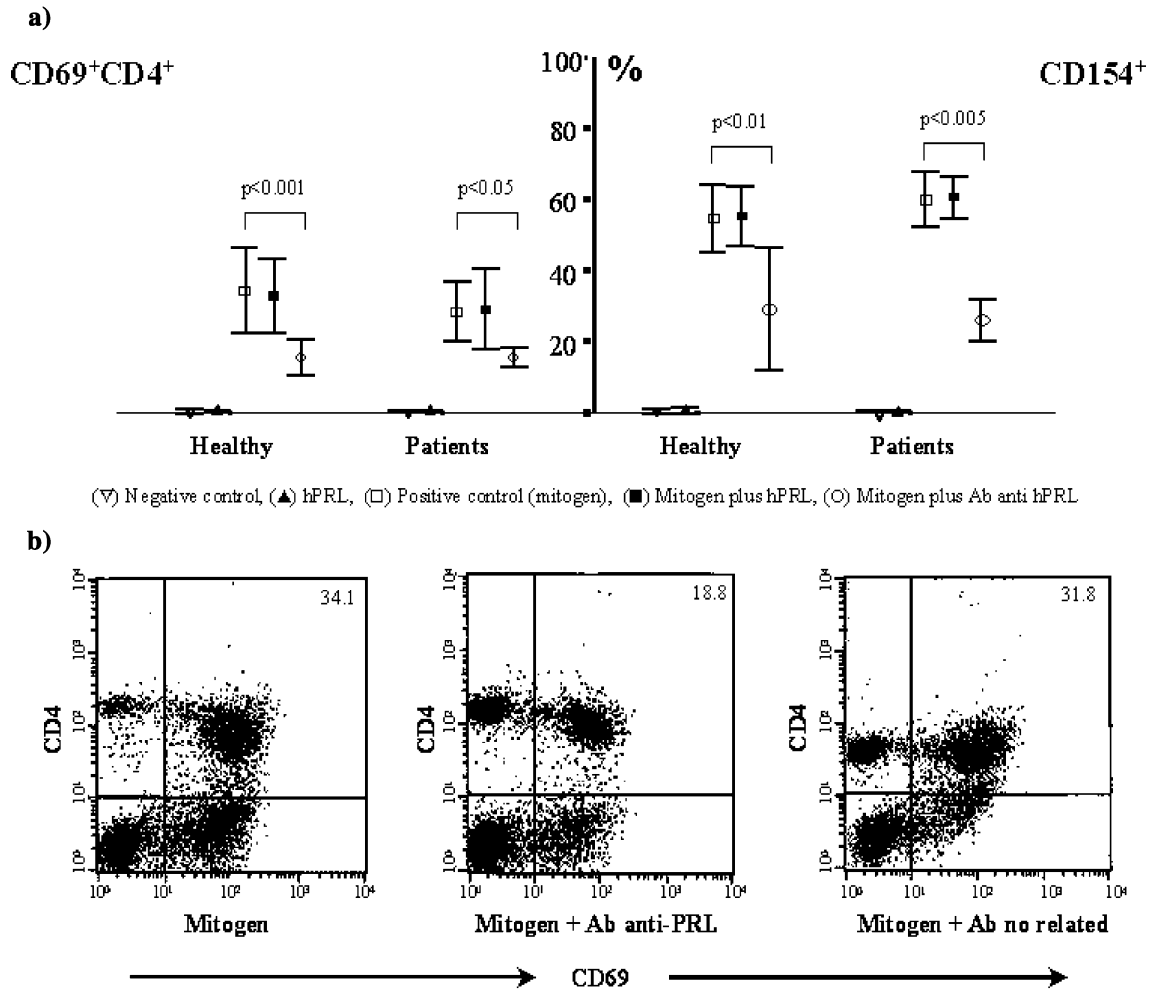


Fig. 2. Percentage of CD69 expression on lymphocytes CD4⁺ and CD154 on PBMNC from ten healthy subject and five hyperprolactinemic patients. Panel a compares cell behaviors from healthy subject with patients. Panel b displays a sample of the distribution of CD69 on CD4 under ConA stimulus, mitogen plus antibody against PRL, and mitogen plus one antibody not related.

Using the same model of activation, we explored CD40 and CD86 expression on purified B cells from five hyperprolactinemic patients and ten healthy individuals. Figs. 5a and b show that CD40 expression was not affected by any of the culture conditions (hPRL alone, unspecific mitogen [PMA-ionomycin], mitogen plus hPRL, mitogen plus anti-hPRL antibodies, and non-related antibodies). In contrast, PMA-ionomycin was able to induce CD86 expression in 38.6% of the B cells, but its expression was not affected by the addition of hPRL or anti-hPRL antibodies, suggesting that PRL does not participate in the expression pathway of these two molecules.

The effect of hPRL on IFN γ secretion by CD4⁺ lymphocytes was also examined in both groups. The hPRL alone did not induce IFN γ release. Also, hPRL plus PMA-ionomycin did not show additive effects (data not shown). However, when anti-hPRL antibodies were added to cultures, a 60% decrease in IFN γ ⁺-stained cells was found. This decrease was due to hPRL, since blocking the anti-hPRL antibody binding site with hPRL displayed a dose-

dependent inhibition (Fig. 6a). Similar results were obtained in those experiments designed for IL2 expression on lymphocyte CD4⁺ (data not shown). Kinetic studies showed that the inhibitory activity of anti-hPRL antibodies on the IL-2 expression decreased progressively with time, supporting our previous results. The inhibitory effect found with the addition of anti-PRL antibodies after 1 h of activation was 55%, and 40% after 2 h; it was no longer present after 4 h (Fig. 6b). The same results were found in the experiment designed for IFN γ , as can be seen in Fig. 6b, the addition of anti-hPRL antibodies at time zero showed almost 60% of inhibition in IFN γ expression. In contrast, addition of the antibodies after 1 or 2 h of activation induced less inhibition.

Finally, we examined in vitro antibody production by B-lymphocytes stimulated in the presence or absence of hPRL. As seen in Fig. 7, B cells from healthy subjects were stimulated with mitogen and with mitogen plus the addition of hPRL; the antibody productions were measured in the supernatant using ELISA method. We found that the

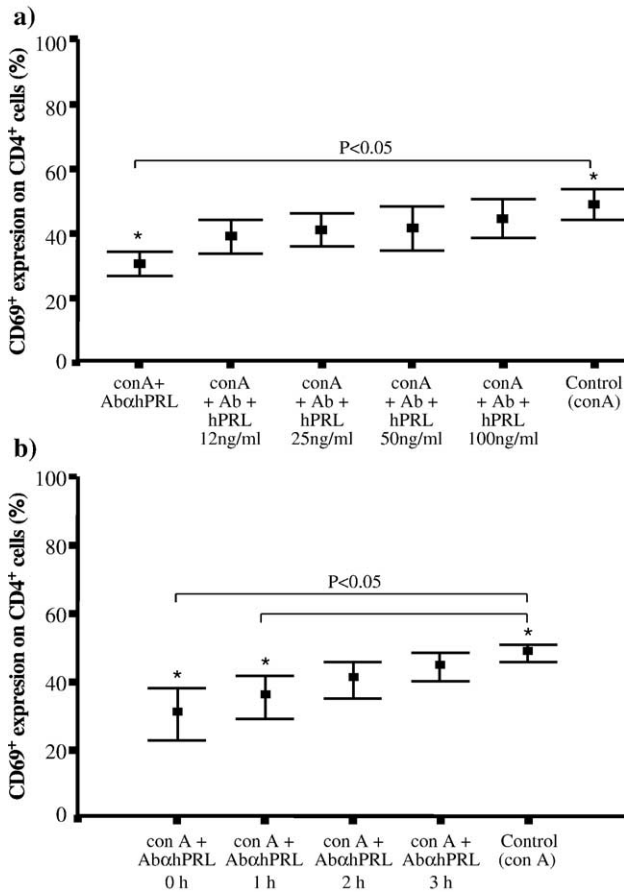


Fig. 3. CD69 inhibition on stimulated lymphocytes CD4⁺ with anti-PRL antibody; increased concentrations of PRL were added to a solution containing predetermined quantities of anti-PRL antibody, then added to the cells stimulated with conA (a). Panel b illustrates a kinetic of time: the anti-PRL antibodies added at different times after the cells were activated with conA.

cultures with PRL had an additive effect on antibody secretion that was not statistically significant.

Discussion

The clinical features (galactorrhea and hypogonadism) of patients with hyperprolactinemia are due to the effects of the hormone in the target organ. However, the immune system alterations reported in hyperprolactinemic patients, such as autoimmune rheumatic diseases [11], and the fact that some women have developed natural autoantibodies during pregnancy, probably due to the hyperprolactinemic state [12], are difficult to associate to PRL. We explored the immune response in cells from patients with hyperprolactinemia, because a large number of evidence suggest PRL contributes in immune responses activity like: (a) additive effect in lymphocyte proliferation [17,18], (b) immunodeficiency observed in animal models with hypoprolactinemia [14,23]; (c) association of hyperprolactinemia with autoimmune diseases both in animal models and humans [19–

21]; (d) regulatory feedback exerted by some cytokines on serum PRL levels [28]; and (e) the fact that lymphocytes and other immunocompetent cells express PRL receptor besides being able to release PRL [7–9]. All this evidence strongly suggests that prolactin could participate in triggering the immune response; this action could be exerted through the secondary signal, such as the expression of co-stimulatory molecules and/or interleukin secretion.

The current study was performed in vitro using cells from hyperprolactinemic patients and healthy individuals in order to determine differences in cell activation. Our model emulates different concentrations of PRL (low or high) in the medium, where the activation takes place, as it has been suggested that the hyperprolactinemic state elicits the immune response [19,20] and the hypoprolactinemia has been associated with a deficient immune response [23]. We found, however, that lymphocytes obtained from healthy subjects and from hyperprolactinemic patients had a similar pattern of response, suggesting that the amount of prolactin in the microenvironment does not affect the immune response. But when immune cells start the activation process (by mitogen or antigen), the levels of PRL affect the quality of lymphocyte activation through three probably mechanisms: the first by an additive effect on PBMC proliferation, as revealed our results and previous works

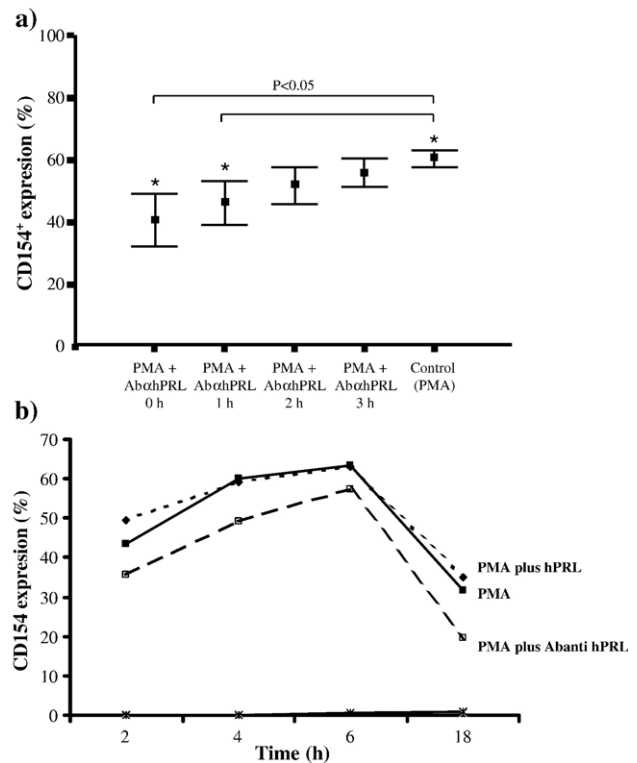


Fig. 4. Kinetic of time of CD154 on PBMC. Anti-PRL antibodies were added at different times after the cells were activated (a). Statistically significant differences was found using ANOVA ($P < 0.05$) among the positive control compared with those culture with antibody at time zero and 1 h after the stimulus. Panel b displays the expression of CD154 on PBMC along 18 h after the different stimulus.

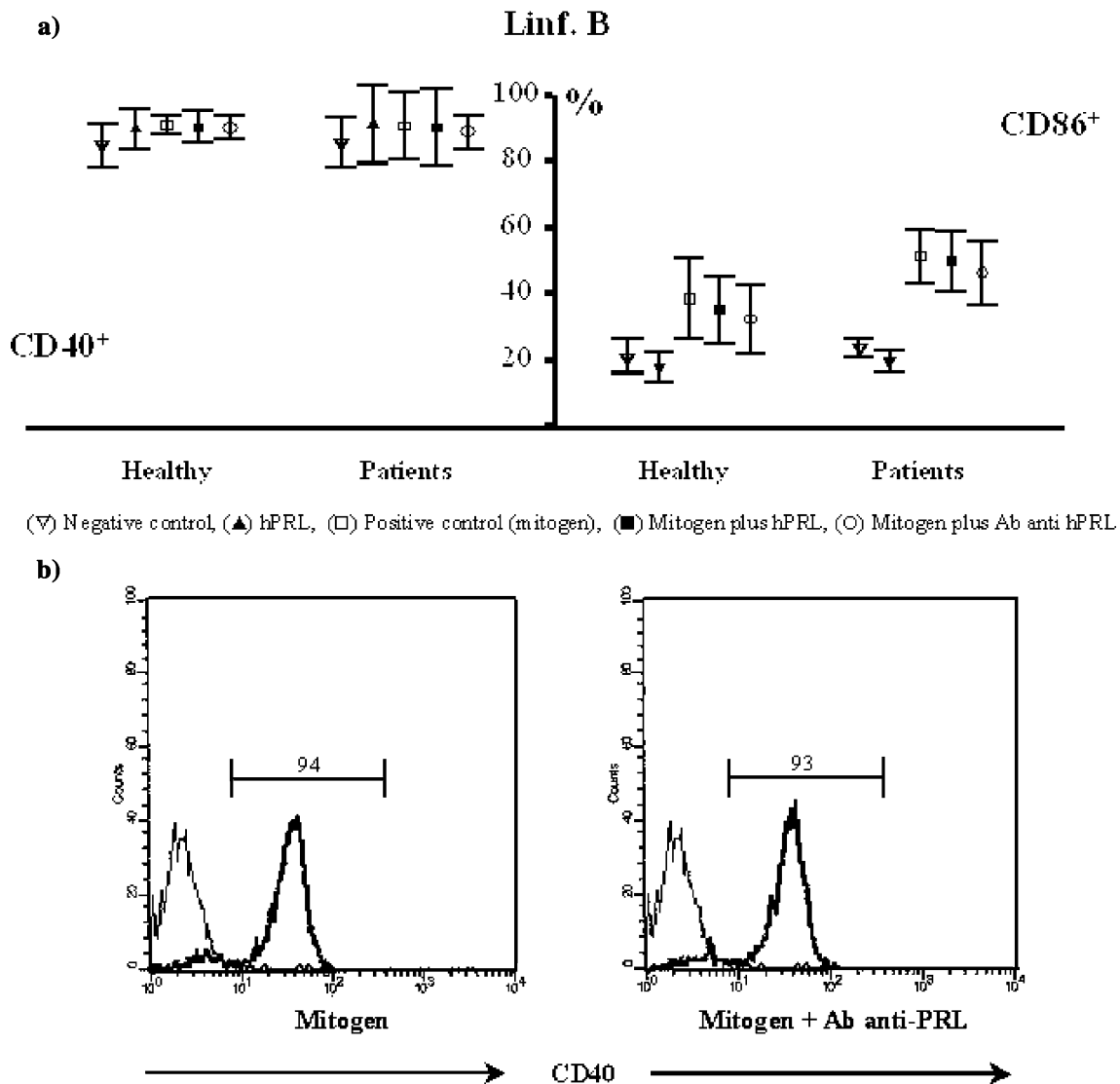


Fig. 5. Panel a displays CD40 and CD86 expression: a similar expression for CD40 was found in healthy patients under different conditions (cells without stimuli, with PRL alone, with mitogen, with mitogen plus PRL, and with mitogen plus anti-PRL antibodies). In contrast, the expression of CD86 on B cell does not suffer any changes with the addition of PRL or with antibodies against to PRL. Panel b is a sample of CD40 distribution on stimulated B cells with mitogen and the blocking with antibody against to PRL.

[17,18]. In contrast, depletion of PRL by the addition of anti-hPRL antibodies decreased the proliferation in about ~30%, indicating that PRL plays, at least, an accessory role in the proliferation. The second one, as shown by our results, could be through the expression of co-stimulatory molecules like CD69 and CD154 expression on CD4⁺ cells. Here we found that the depletion of PRL using anti-hPRL antibodies decreased the expression of activation marker molecules in about ~50% and the third one could be by affecting IL-2 and IFN γ secretion, our results showing a markedly reduced IL-2 and IFN γ when depleting PRL in the culture.

The presence of prolactin in the microenvironment could act as a growth factor in cell proliferation. However, the PRL that participates in the immune response activation process is secreted by the lymphocyte, as evidenced by the

finding that no additive effect was exerted when PRL was added to the culture, and a striking reduction in the key indicators of successful T cell activation was observed with the deletion of PRL. These results are in concordance with those obtained in a knockout mouse model for PRL, in which no evident abnormalities in the anatomy and development of B and T cells were found and the innate immunity was normal [29]; however, the adaptive immune response was not studied, where most of the effects of PRL have been demonstrated.

T cell activation is a fundamental step for the onset of adaptive immune response. This is characterized by alterations in cell surface expression molecules: increased CD69, CD25, CD154, FAS-L, and CD44 expression, and decreased CD62L and CD45R expression. Some of these changes are necessary for T cells to start and progress in the

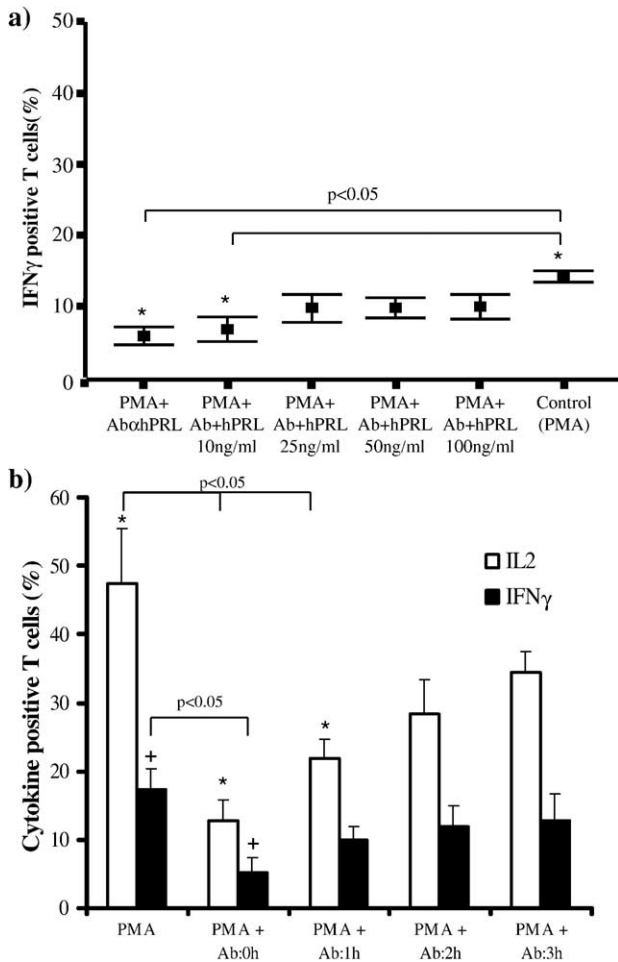


Fig. 6. IL2 and IFN γ secretion by CD4 $^+$ cell. Panel a shows the inhibition of IFN γ stained on lymphocyte CD4 $^+$ stimulated with PMA-ionomycin: increasing concentrations of PRL were added to a solution containing predetermined quantities of anti-PRL antibody, then added to stimulated culture. The IFN γ stain decreased in about 70%, it was statistically significant different using ANOVA ($P < 0.005$). Panel b illustrates a kinetic of IL2 and IFN γ production: here the anti-PRL antibodies were added at different times (0, 1, 2, and 3 h) after cell activation with PMA, statistically significant differences were found at time zero and 1 h after the stimulus using ANOVA ($P < 0.005$).

cell cycle [24]. Here, we explored the expression of CD69 on human CD4 $^+$ lymphocyte as an early activation indicator. Addition of hPRL to the culture did not affect CD69 expression. In contrast, the addition of anti-hPRL antibodies decreased CD69 expression, this was supported by the finding that engagement of the binding site of the antibody against hPRL by increasing concentrations of hPRL restored CD69 expression and the addition of non-related antibody did not affect its expression. Besides, PRL is necessary to initiate T cell activation, as revealed by the experiment in which anti-hPRL antibodies were added at different times after the stimulus.

CD154 is a member of the tumor necrosis factor (TNF) family with a number of essential functions in the immune response after binding to its receptor, CD40. Activated

CD4 $^+$ cells, also some CD8 $^+$ T cells, dendritic cells, NK cells, human mast cells, platelets, and basophiles express mainly CD154. An important function of CD154–CD40 interaction is its relationship with the CD28 co-stimulatory pathway. Thus, CD154–CD40 interaction signals APC to up-regulate CD80 (B7-1) and, to a lesser extent, CD86 (B7-2), which in turn enhances the co-stimulatory activity of antigen presenting cells, including B cells, dendritic cells, and macrophages. CD28–CD80/86 and CD40–CD154 interactions synergize to initiate and amplify T cell-dependent immune responses [25,26]. Here, we explored both CD154 and CD40 expression in cells activated with PMA-ionomycin in culture with or without prolactin. Although expression of CD154 in response to PMA-ionomycin did not change with the addition of hPRL, we found that blocking lymphoid prolactin with antibody exerted an inhibitory effect on CD154 expression by PBMC, indicating that, most likely, T cell-derived PRL acts as an autocrine mediator on T cell activation leading to CD154 expression. Results are similar to those observed for CD69.

The PRL did not alter the expression of CD40 or CD86 in purified B cells. However, it induced a slight increase in antibody production by these B cells, suggesting that the mechanism of action of PRL in B cells could be different. Our model worked properly since the positive control displayed an increased expression of CD86 on stimulated cells, suggesting that PRL does not play any role in the co-stimulatory function through the induction or up-regulation of CD40 and B7 (including CD86) molecules on the surface of B cells (functioning as APC) for an appropriate activation [26,30].

The PRL role as a growth factor is supported by the additive effect in the proliferation assay and reduction of the proliferation by blocking the prolactin in synthetic medium.

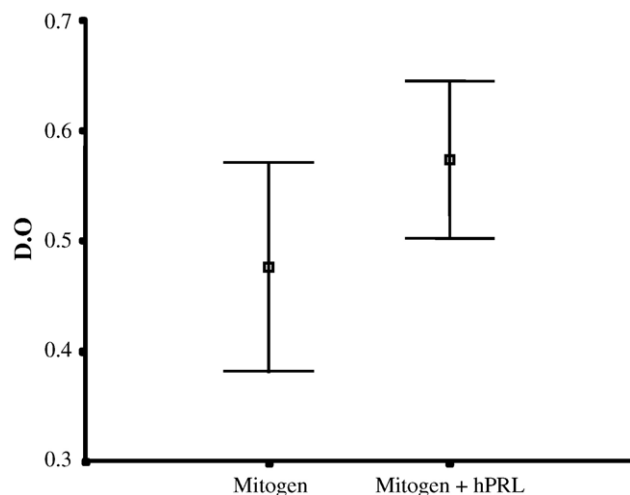


Fig. 7. B cell activation measured through antibody production by ELISA methods. B cells from healthy subject were stimulated with mitogen or mitogen plus PRL. Then the antibody production was measured in the supernatant. The PRL had an additive effect on antibody secretion, but that was not statistically significant.

In contrast, in the activation assay, addition of hPRL to the cell culture does not modify the activation process measured through CD69, CD154, CD40, and CD86 expression. However, the CD4⁺ activation measured through CD69 and CD154 expression depends mostly on the secretion of PRL by the lymphocyte, as supported by our results from the Nb2 assay in which PBMC from both hyperprolactinemic patients and healthy subjects produced and released PRL. Production of PRL correlated with both cell number and the degree of proliferation and the addition of anti-hPRL antibodies to the supernatants blocked the Nb2 proliferation, suggesting that the prolactin used by the PBMC is produced by themselves and that the Nb2 proliferation was not an effect of a nonspecific mitogen in the supernatants.

The secretion of IL-2 and IFN γ is strikingly reduced by blocking the prolactin. Besides, no additive effect was observed with the addition of hPRL to the culture. IL-2 is rapidly and potently induced after antigen presentation to resting T cells, the results of transcription and synthesis of IL-2 are often used as a key indicator of successful T cell activation [31]. In the same fashion, IFN γ could be an indicator of T cell activation [32,33]. Several questions remain to be answered, especially those related with the magnitude and type of immune response, because the antigen determines the specificity of the immune response, but the interaction of IL-2 with high-affinity IL-2 receptors regulates the magnitude and duration of the response. Cells from mouse and human in vitro models show that the presence of IFN γ in priming culture resulted in the outgrowth of Th1 but not Th2 clones [33]. We present evidence that in vitro blockage of PRL does not induce an appropriate T cell activation, as measured through the IL-2, IFN γ secretion, and CD69, CD154 expression.

In conclusion, it is evident that prolactin alone is unable to initiate an immune response in lymphocytes from patients and healthy subjects in spite of some clinical evidences [11,12]. In the same fashion, it is clear that PRL takes part in the trigger of T cell activation and that PRL is produced and secreted by the same cells acting predominantly in autocrine form collaborating in the expression of co-stimulatory molecules (CD69, CD154) and the IL-2, IFN γ secretion.

Further studies are needed in order to dissipate if the hyperprolactinemic condition in SLE patients is the result of cell activation of the immune system instead of the high levels of prolactin that trigger the immune response or the fact that some individuals develop autoimmune rheumatic disease after hyperprolactinemic state, it must address research about PRL participation just in auto-reactive clones.

Acknowledgments

This research was supported in part by Consejo Nacional de Ciencia y Tecnologia de Mexico (CONACYT): grant # 34454-N. We would like to thank Dr.

A.F. Parlow from the National Hormone and Pituitary Program, Harbor-UCLA Medical Center, for the donation of human PRL (hPRL) and polyclonal antibody against prolactin, and Prof. David A Isenberg for comments on the manuscript.

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