Prolactin gene expression in mouse spleen helper T cells

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Abstract

Prolactin (PRL) is a single-chain polypeptide hormone that is generally secreted from prolactin cells of the anterior pituitary gland into the blood circulation. However, recent studies indicate that the gene expression of prolactin is ectopic in several tissues across several species. These studies found that lymphocytes also produce PRL, which is involved in the immunoregulatory system. Here, we searched for PRL messenger ribonucleic acid (mRNA), using the reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blotting in the spleens of mice at various growth stages. We also localized mouse prolactin (mPRL) and its mRNA in the spleens of 30- and 60-day-old mice by immunohistochemistry and *in situ* hybridization respectively. The mPRL gene was expressed in all spleen samples at 0–60 days postpartum. We localized mPRL mRNA in the sheathed artery, periarterial lymphatic sheath and the marginal zone of the spleen. Moreover, we detected mPRL in essentially the same area as its mRNA. Furthermore, we performed double-fluorescence immunohistochemical staining for mPRL and mouse CD4 that is specifically produced in helper T cells, or for mPRL and mouse CD19 or CD40 specified B cells. We colocalized mPRL immunoreactivity only in some CD4-immunopositive cells. These results clearly suggest that T cells synthesize mPRL in the mouse spleen.
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Introduction

The various functions of prolactin (PRL) in vertebrates include the growth and differentiation of mammary epithelium, lactation in mammals, osmoregulation and parental behavior in teleosts, amphibian development, broodiness in hens and crop sac production in pigeons (Banerjee & Menon 1987). PRL is also an important immunoregulatory hormone, since inhibiting endogenous PRL secretion by hypophysectomy and/or exogenous bromocriptine suppresses the immune response to *Salmonella typhimurium* (Edwards *et al.* 1991), T-cell proliferation (Nagy *et al.* 1983), the secretion of interferon-γ (Cesario *et al.* 1994) and the tumoricidal activation of macrophages (Bernton *et al.* 1988). The restoration of PRL can reverse such immunosuppression (Berczi *et al.* 1981, Bernton *et al.* 1988). The administration of PRL stimulates thymic function and hormone secretion, as well as antibody responses and interleukin-2 receptor expression, regulates B- and T-cell differentiation and proliferation, and augments macrophage cytotoxicity (Bernton *et al.* 1988, O'Neal *et al.* 1992).

Recent studies suggest that PRL or an immunoreactive PRL-like protein can be synthesized and secreted from nonpituitary tissues (Montgomery *et al.* 1992, O'Neal *et al.* 1992, Harigaya *et al.* 1997, 2002, Imaoka *et al.* 1998, Sakai *et al.* 1999). Evidence generated by these studies suggests that lymphocytes are a secretory source of PRL. Indeed, immune-competent cells from the thymus and spleen, as well as peripheral lymphocytes, contain PRL mRNA and release a bioactive molecule that is similar to pituitary PRL (Montgomery *et al.* 1992). Murine splenocytes produce proteins that stimulate the proliferation of the PRL-dependent Nb2 node lymphoma cell line (Montgomery *et al.* 1987, Shah *et al.* 1991). Furthermore, anti-PRL antiserum neutralized this activity, confirming the PRL-like nature of these proteins (Montgomery *et al.* 1990). A subsequent study identified a 22 kDa protein with PRL immunoreactivity in culture supernatants from mouse thymocytes and splenocytes, and demonstrated that these cells synthesize this protein. Lymphoid cells ectopically produce PRL-like proteins (Gellersen *et al.* 1991). Murine mononuclear cells express the PRL gene, and it appears to be mainly associated with the T-cell population (Clevenger *et al.* 1990, Murphy *et al.* 1993, Buckley 2001, Dugan *et al.* 2002). A murine T-helper cell line sequesters PRL from the culture medium and releases PRL-like proteins during interleukin-2 (IL-2)-driven growth (Clevenger *et al.* 1990). In this situation, PRL synergizes with IL-2 to augment cell mitogenesis. Thus, lymphoid cells can synthesize PRL-like proteins *in vitro*. Although the PRL gene is expressed in isolated lymphocytes *in vitro*,

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whether PRL is located in the spleen *in vivo* remains unknown.

The spleen generates immunologic responses to blood-borne antigens, and removes particulate matter and aged or defective blood cells, particularly erythrocytes, from the circulation. In the human fetus, the spleen is an important site of hemopoiesis and this function may be resumed in adulthood during some pathologic states. The structure of the spleen allows intimate contact between blood and lymphocytes, which constitute 5–20% of the total mass of the spleen. Although the spleen is involved in the removal of aged or defective blood cells from the circulation, whether this function is mechanical or acts through an immunologic recognition system remains obscure.

In this study, we investigated the presence of PRL mRNA in the mouse spleen by means of RT-PCR and Southern blot analysis (Southern 1975). We also localized mPRL protein and mRNA by immunohistochemistry and *in situ* hybridization respectively. We investigated by double-labeled immunohistochemistry whether mPRL colocalizes with lymphoid cells. We also determined the size of PRL protein in the mouse spleen by immunoblotting from birth to adulthood.

Materials and Methods

Animals

The experimental design of this study proceeded according to the guidelines for animal experiments of this institution. We housed ICR mice under a controlled temperature (22 ± 2 °C) in an artificially illuminated room (12-h light/12-h dark). Food and tap water were freely available. At 70–90 days of age, females were mated with males, and the day a vaginal plug was present was designated as day 0 of pregnancy. Parturition regularly occurred on day 19 of pregnancy. The day of birth was designated as infant day 0. The mice were killed by ether anesthesia on day 18 of pregnancy and 0, 2, 4, 6, 10, 30 and 60 days after birth. The pituitary and spleen were removed and used immediately or stored at −80 °C.

Detection of PRL mRNA

We estimated the concentration of total RNA isolated from 100 mg of spleen or pooled pituitary gland with an RNA extraction Kit (ISOGEN; Nippon Gene, Tokyo, Japan) by monitoring absorbance at 260 nm. We assessed PRL gene expression in the tissues by RT-PCR. We synthesized mouse PRL cDNA in 20 μl buffer containing 50 mM Tris–HCl (pH 8·3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 200 U/μl RNase inhibitor, 200 U/μl Superscript II Reverse transcriptase (Invitrogen), 0·1 mM oligo $(dT)_{18}$ primer and 5 μg total RNA. The reaction proceeded at 42 °C for 60 min and was terminated by heating for 5 min at 90 °C. Samples were then stored at 4 °C. Target cDNA obtained with reverse transcriptase was amplified by PCR for 30 cycles (one cycle: 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min) in a Thermal Cycler (Funakoshi, Tokyo, Japan). The buffer contained synthesized cDNA, 2 mM $MgCl_2$, 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 2·5 U *Taq* DNA polymerase (Takara, Kyoto, Japan) and 0·75 μM sense primer for mPRL in a total volume of 10 μl. The mPRL primers corresponded to the mPRL cDNA sequences reported by Harigaya *et al.* (1986). For mPRL, the nucleotide sequences of the primer are as follows: sense, 5′-CAAGCCCTGAAAGTCCCT CCGGAAG-3′; antisense, 5′-CTCAGAAAGAGATGG ACTGAATGT-3′. The predicted size of the PCR product was 418 bp. The RT-PCR control included glyceraldehyde 3-phosphate dehydrogenase (G3 PDH) primers (sense, 5′-CACCCAGAAGACTGTGGA-3′; antisense, 5′-TGTTGAAGTCGCAGGAGA-3′). The predicted size of the G3 PDH PCR product was 313 bp. For the specificity of RT-PCR assay, the negative control experiments were performed without reverse transcriptase in each reaction buffer.

DNA blotting

We performed Southern blotting as follows (Southern 1975). The PCR products resolved by electrophoresis on 2% agarose gels were blotted onto Hybond-N (Amersham) membranes. We detected mPRL cDNA with the NEBlot Phototope Kit (New England BioLabs, Beverly, MA, USA) according to the supplier's recommendations, and the RT-PCR product of mPRL cDNA with the same primers isolated from the pituitary gland as probes. The primers were labeled with biotin with a kit (New England BioLabs).

In situ *hybridization*

Frozen spleens that had been immediately fixed in 4% paraformaldehyde were cut into 8 μm sections with a cryostat (HM 505 E; Zeiss, Overkochen, German), immersed in 0·2 mol/l HCl for 20 min at room temperature (RT), and then digested with 10 ng/ml proteinase K in 0·01 mol/l PBS for 20 min at 37 °C. The tissue sections were acetylated in 0·1 M triethanolamine/acetic anhydride buffer for 10 min at RT. Hybridization proceeded in 1 × Denhardt's solution containing 2 × SSC (150 mmol/ml NaCl, 15 mmol/l sodium citrate, pH 7·0), 50% deionized formamide, 1·25 mg/ml transfer RNA (tRNA), 1·0 mg/ml salmon sperm DNA and biotin-labeled PRL cDNA probe (5′-CTTCCGGAGGGAC TTTCAGGGCTTG-3′) for 16 h at 42 °C. Negative control sections were incubated with only hybridization buffer. Thereafter, the sections were washed twice with 2 × SSC, 1 × SSC, 0·5 × SSC and 100 mM Tris–HCl for 20 min. Hybridized biotinylated cDNA probes were

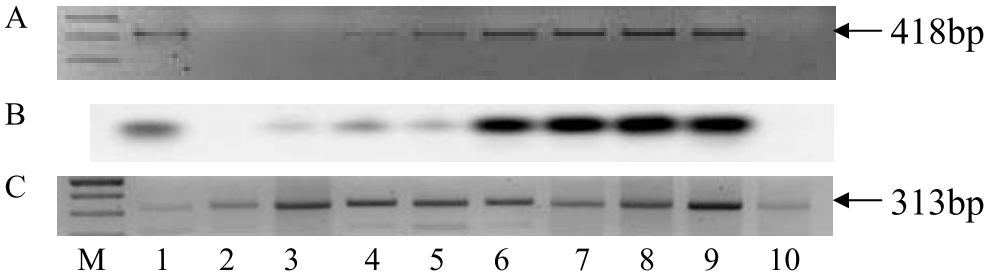

Figure 1 Products amplified by RT-PCR analyzed by agarose gel electrophoresis (A) and Southern blot hybridization (B) with mPRL cDNA probe. Predicted size of the amplified products was 418 bp. Lanes contain PCR products obtained from pituitary glands of adult female mice (lane 1); from spleen at 18 days of viviparity (lane 2); and from 0-, 2-, 4-, 6-, 10-, 30- and 60-day-old mice (lanes 3, 4, 5, 6, 7, 8 and 9 respectively), adult mouse liver as control (lane 10), and standard DNA size marker (lane M). Stained PCR products are faint in lanes 3, 4 and 5, but obviously hybridized with mPRL cDNA probe. (C) G3 PDH amplification as RT-PCR control.

detected with the horseradish peroxidase-conjugated antibiotin antibody supplied with the ABC kit (Vector, Burlingame, CA, USA) and then visualized with 3,3′-diaminobenzidine (DAB).

Immunohistochemistry

The sections were prepared as described for *in situ* hybridization. Endogenous peroxidase was removed by exposure to 0·3% H_2O_2 in 100% methanol, and then the sections were rehydrated, washed in PBS (pH 7·6) and incubated overnight at 4 °C with rabbit anti-mPRL antibody. Control sections were incubated with normal rabbit serum in instead of primary antibody. All sections incubated with biotinylated goat antirabbit IgG and avidin–biotin–peroxidase complex were finally visualized by a 4 min incubation at 40 °C with 0·03% DAB in 0·05 M Tris–HCl buffer (pH 7·4) containing 0·02% H_2O_2.

Sections were prepared in the same manner for double-staining immunohistochemistry. To increase antibody penetrability, the sections were exposed to 100% methanol for 30 min at –20 °C and then washed twice in PBS for 5 min. Nonspecific binding was blocked with 5% normal bovine serum in PBS for 15 min at RT and rinsed with PBS. The sections were incubated overnight at 4 °C with rabbit anti-mPRL antibody and rat antimouse CD4 antibody, or with rabbit anti-mPRL antibody and rat antimouse CD19 antibody, or with rabbit anti-mPRL antibody and antimouse CD40 antibody. Control sections were incubated with normal rabbit serum instead of primary antibody. After rinsing with PBS, the sections were incubated for 1 h with rhodamine-conjugated goat antirabbit IgG diluted 1:400 and fluorescein isothiocyanate-conjugated goat antirat IgG diluted 1:400, rinsed with PBS, and then mounted with FluoroGuard Antifade (BioRad).

Immunoblotting

Ultrasonically disrupted pituitaries and spleens were separated by centrifugation at 15 000 ***g*** for 10 min, and the supernatant was heated at 95 °C for 5 min in sample buffer (62·5 mM Tris–HCl (pH 6·8) containing 5 mM EDTA, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) 2-mercaptoethanol (2-ME) and 0·01% (w/v) bromophenol blue). The samples were resolved by electrophoresis on 12·5% SDS–polyacrylamide gels. Separated proteins were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) and immersed in primary rabbit antiserum raised against recombinant mPRL. Antigen–antibody complexes were detected by a biotinylated secondary antibody and streptavidin–biotin–horseradish peroxidase complex using the Vectastain ABC kit (Vector, CA, USA). Specific proteins detected by the mPRL antibody were stained with DAB as a chromogen.

Results

PRL gene expression in mouse spleen

The length of the amplified fragment of RT-PCR corresponded to the size predicted from the mPRL primers in the pituitary glands and spleens from 0–60-day-old mice (Fig. 1A). To confirm the accuracy of these findings, we performed Southern blotting after agarose gel electrophoresis. These PCR products were hybridized with a probe containing the mPRL cDNA sequence, and signals were identified in all pituitary glands and spleens after birth (Fig. 1B). Negative controls of RT-PCR without reverse transcriptase showed no signal in all samples (data not shown).

Localization of mPRL mRNA and mPRL protein in mouse spleen

We performed *in situ* hybridization for mPRL mRNA, using a biotin-labeled mPRL cDNA probe to determine the site of PRL gene expression. We detected signals in the spleens of 10-, 30- (data not shown) and 60-day-old

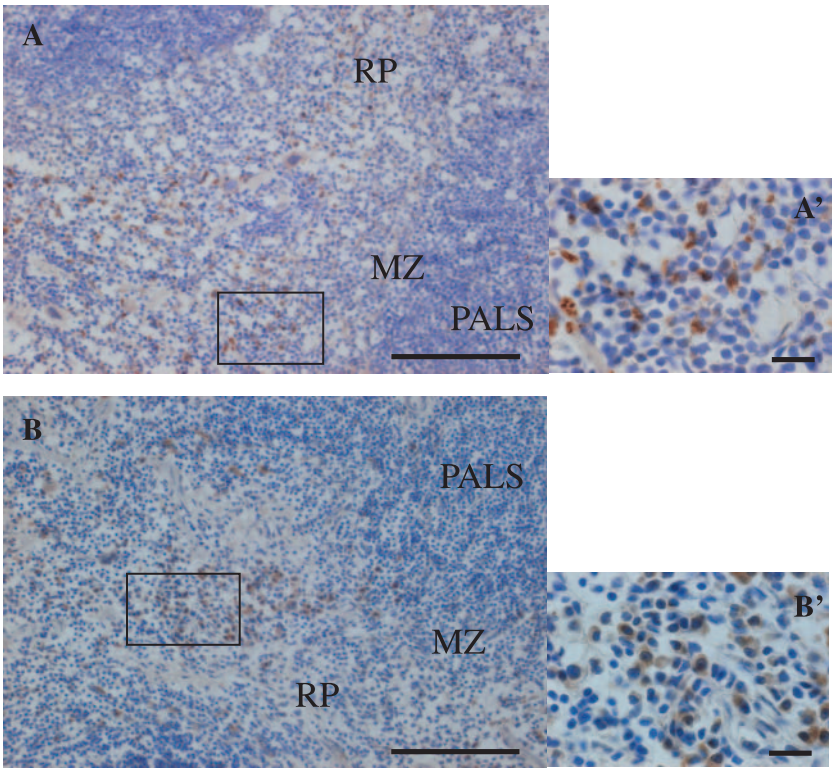

Figure 2 *In situ* hybridization for mPRL mRNA and immunohistochemical findings of splenic mPRL. Signals were positive in spleens from adult (A; boxed region in A corresponds to A′ (inset)) mice. Cells positive for PRL mRNA are located in splenic sheathed artery, periarterial lymphatic sheath and marginal zone. Immunostained cells are located in red pulp, marginal zone and periarterial lymphatic sheath of 10-, 30- (data not shown) and 60- (B; boxed region in B corresponds to B′ (inset)) day-old mice. The scale bars are 100 μm (A and B) and 20 μm (A′ and B′). RP, red pulp; PALS, periarterial lymphatic sheath; MZ, marginal zone.

mice (Fig. 2A). Cells were positive for mPRL mRNA in the red pulp, periarterial lymphatic sheath and marginal zone of the spleen. Although the cells containing PRL mRNA appeared to be lymphocytes, they could not be identified in the spleen.

In the spleen of 10-, 30- (data not shown) and 60-day-old mice (Fig. 2A), immunohistochemistry with the mPRL antibody detected an intensely reactive product in the cells of the red pulp, marginal zone and periarterial lymphatic sheath. These specific signals were located in the same area as PRL mRNA.

Double-labeled immunohistochemistry

Double-staining immunohistochemistry determined whether all lymphocytes synthesize mPRL. Mouse CD4 is a 55 kDa cell-surface receptor expressed by most helper T cell subpopulations of mature T cells. We therefore used anti-CD4 antibody as a marker. Mouse CD19 is a 95 kDa transmembrane protein bearing two extracellular immunoglobulin (Ig) domains and an extensive cytoplasmic tail. Onset of CD19 expression occurs at the earliest stages of B-cell development before the expression of the pre-B-cell receptor. On mature B cells, CD19 is found in a complex with the complement receptor. Mouse CD40 is a 45–50 kDa type I transmembrane glycoprotein that is a member of the TNFR family expressed by mouse B cells. CD40 regulates B-cell development and maturation. We therefore used anti-CD4, anti-CD19 and anti-CD40 antibodies as helper T- and B-cell markers respectively. Figure 3 shows several mPRL-immunoreactive cells (rhodamine label) that are also immunoreactive for mouse CD4 (fluorescein isothiocyanate labeled) (Fig. 3A, B and C). However, none of the mPRL-immunoreactive cells reacted to mouse CD19 antibody (Fig. 3D, E and F) and CD40 antibody (Fig. 3G, H and I).

Immunoblotting

Immunoblotting also detected mPRL in the spleen from days 0–60 after birth (Fig. 4). However, the molecular size of the positive band from the spleen was approximately

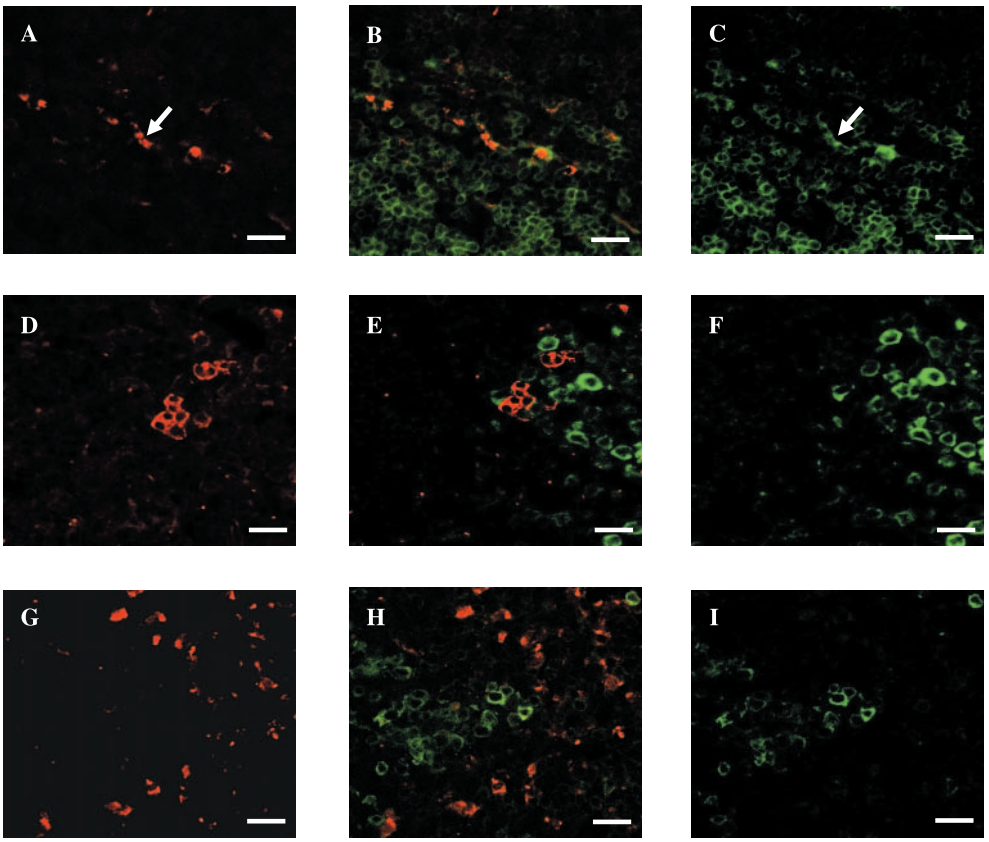

Figure 3 Immunohistochemical double staining for mPRL and CD4, CD19 or CD40. Sections were incubated with rabbit polyclonal anti-mPRL and rat antimouse CD4 antibodies (A and C respectively) or with rabbit anti-mPRL and rat antimouse CD19 antibodies (D and F respectively) or with rabbit anti-mPRL and rat antimouse CD40 antibodies (G and I respectively) followed by goat rhodamine-conjugated antirabbit IgG and goat fluorescein-conjugated antirat IgG. Most rhodamine-labeled cells containing mPRL (A) are also fluorescence labeled for mouse CD4 (C), indicated by arrows. Cells containing mPRL (D) do not contain detectable fluorescence-labeled mouse CD19 and CD40 (F and I respectively). The scale bars are 20 μm.

16 kDa, which was smaller than that from the pituitary gland. In addition, very small amounts of PRL were detectable in the spleens of mice at any age.

Discussion

The PRL gene is expressed in the human thymus, lymph nodes, tonsils and spleen (Wu *et al.* 1996). Lymphocytes can also be a source of PRL (Montgomery *et al.* 1990). The present study uncovered evidence that the mouse spleen produces PRL. Specific RT-PCR, Southern blotting and *in situ* hybridization located mPRL mRNA.

To define the cell types expressing the PRL gene and their localization in the spleen, we performed *in situ* hybridization with a biotin-labeled cDNA probe. Our data showed that cells containing PRL mRNA localized in the sheathed artery, periarterial lymphatic sheath and marginal zone of the spleen. We also localized PRL by immunohistochemistry and found specific signals in essentially the same locations as mPRL mRNA. Mouse PRL mRNA and mPRL might be localized in lymphoid cells. We performed double-staining immunohistochemistry for mPRL and mouse CD4 that is specifically produced in helper T cells, and for mPRL and mouse CD40 in B cells to determine where mPRL localizes. Immunoreactivity for mPRL was colocalized in cells that were immunopositive for CD4, but not for CD19 and CD40. The white pulp of the spleen consists mainly of B cells. On the other hand, the marginal zone comprises small lymphatics arising in the T-cell area, and the periarterial lymphatic sheath is also rich in T cells. The red pulp of the spleen consists of disseminated T and B cells. The PRL gene was expressed in both of these areas. However, mPRL was localized only in T cells, the proliferation of which is regulated by various peptide growth factors, including

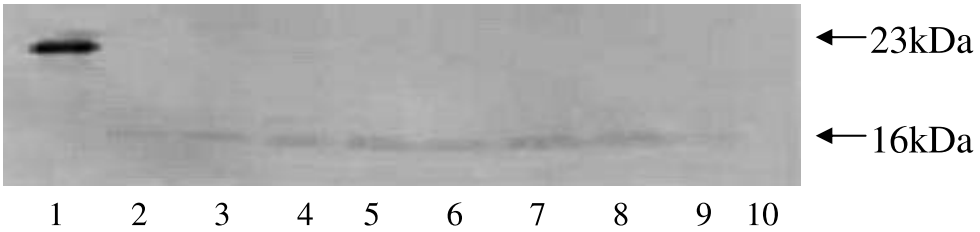

Figure 4 Immunoblots of spleen PRL. Samples resolved by 12·5% SDS–PAGE were immunoblotted and reacted with rabbit mPRL antiserum. Extracts are from pituitary gland (lane 1) and spleen of 18-day fetus (lane 2) and from 0-, 2-, 4-, 6-, 10-, 30- and 60-day-old mice (lanes 3–9 respectively); lane 10 is liver as control. The molecular mass of the positive band from the spleen was approximately 16 kDa.

IL-2 (Glasebrook *et al.* 1981). The proliferation of IL-2-stimulated T cells *in vitro* depends upon the presence of other peptides, including transferrin and PRL. Removal of PRL *in vitro* with anti-PRL antiserum inhibits IL-2- or concanavalin A-driven T-cell proliferation. The helper T cell clone L2 is not transformed and remains dependent on repeated stimulation by allogeneic cells. In this cell line, exogenous PRL is required for IL-2-driven proliferation (Clevenger *et al.* 1990). The manipulation of PRL levels by hypophysectomy or bromocriptine therapy *in vivo* similarly inhibits T-cell proliferation and immune responses (Bernton *et al.* 1988). Furthermore, murine splenocytes induced by a T-cell mitogen produce PRL. About 70% of T cells express PRL receptors (Gagnerault *et al.* 1993, Touraine *et al.* 1994, Brown-Borg *et al.* 1996, Gunes *et al.* 1997, Feng *et al.* 1998). Many studies have examined the effect of PRL on T-cell function (Clevenger *et al.* 1992). While some studies indicate that PRL stimulates splenic T-cell proliferation, most have shown that PRL does not directly influence the proliferation of splenic lymphocytes (Bouchard *et al.* 1999). These data show that PRL plays a key role in regulating the immune response *in vivo* and in production of PRL by T-cell lines *in vitro*. On the other hand, PRL synergizes with IL-2 to enhance IL-2-receptor expression on stimulated T cells and increases IgG production by human B cells stimulated with anti-CD40 (Richards & Murphy 2000). However, mPRL did not colocalize in B cells under our experimental conditions.

Recently, the ontogeny and functional activity of the immune system was investigated in mouse models of targeted disruption of the PRL gene (Horseman *et al.* 1997) and its receptor (Bouchard *et al.* 1999). In each case, the females were profoundly defective in reproduction and mammary gland development, as would be expected from the classically described actions of PRL. However, thymic and splenic cellularity, and subset distributions of lymphocytes were not altered, nor was the capacity to produce antibodies, or proliferate in response to mitogenic stimuli *in vitro*. From these observations, some groups concluded that PRL most likely is not obligatory for the normal development and functioning of the immune response *in vivo* (Foster *et al.* 1999, Dorshkind & Horseman 2000). However, the possibility cannot be excluded that aberrations in immune function may have occurred that were not detected by the assays chosen, nor can we exclude functional redundancy by alternative cytokines, or other hormones in these animals. Thus, it is possible that these factors may support the function of PRL, or that PRL is affected only under some conditions of stress environment. For example, a study has indicated that PRL positively influences the immune system, as is manifest when the organism is stressed (Dugan *et al.* 2002).

We found the molecular mass of the immunoreactive PRL-like protein in the mouse spleen to be approximately 16 kDa. Post-translational modifications of the predominant form of PRL, which is a single, 23 kDa polypeptide chain with three disulfide loops, result in several molecular forms (Andries *et al.* 1992). Molecularly heterogeneous PRL arises not only in the anterior pituitary gland but also in the immune system and the brain (DeVito *et al.* 1988). The 16 kDa molecule can be derived from an enzymatically cleaved form of 23 kDa PRL, and it has unique functions not shared with the parent PRL. The specific effects of 16 kDa PRL include the inhibition of angiogenesis, which is stimulated by 23 kDa PRL both *in vivo* and *in vitro* (Ferrara *et al.* 1991, Clapp *et al.* 1993, Duenas *et al.* 1999, Struman *et al.* 1999). Furthermore, the anti-angiogenic 16 kDa PRL induces apoptosis as a means of blood vessel regression (Dimmeler & Zeiher 2000, Martini *et al.* 2000). Recombinant 16 kDa human PRL increases DNA fragmentation in cultured bovine brain capillary endothelial cells and in human umbilical vein endothelial cells time- and dose-dependently on the serum concentration. The 16 kDa PRL can inhibit tumor vascularization and growth (Bentzien *et al.* 2001). We observed an immunoreactive PRL-like protein in the spleen that was smaller than native mPRL. However, whether it is identical to the reported 16 kDa PRL remains unknown. The immunoreactive PRL-like protein appears to play an important role in some biologic functions of the mouse spleen. Thus, mPRL from the spleen might affect the cells that produce it or neighboring cells through an autocrine and/or paracrine pathway.

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