Journal of Cellular Biochemistry 57:290–298 (1995)

# Prolactin Induced Expression of Interleukin-1 Alpha, Tumor Necrosis Factor-Alpha, and Transforming Growth Factor-Alpha in Cultured Astrocytes

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**Abstract** Prolactin (PRL) is a potent mitogen in cultured astrocytes. Because one of the major effects of astrocyte proliferation is the expression of inflammatory cytokines, we examined the effect of PRL-induced mitogenesis on the expression of interleukin-1 (IL-1α), tumor necrosis factor-α (TNF-α), and transforming growth factor-α (TGF-α) in cultured astrocytes. Astrocytes were stimulated with PRL or growth hormone (GH), and the expression of cytokines was determined by immunohistochemistry and Western blot analysis. Following incubation of astrocytes with 1 nM PRL for 6 h, strong positive staining of IL-1α and TNF-α, but not TGF-α, was found. No detectable staining for the above cytokines was found in vehicle, or GH treated astrocytes. When astrocytes were incubated in the presence of 1 nM PRL for 18 h, strong positive staining for IL-1α and TGF-α was found. Immunocytochemical analysis of the expression of TNF-α and IL-1α in PRL stimulated astrocytes suggested that the expression of IL-1α preceded the expression of TNF-α. To confirm this observation, Western blot analyses were performed on extracts from astrocytes incubated with 1 nM PRL. In unstimulated astrocytes, IL-1α levels were not detectable. In astrocytes stimulated with 1 nM PRL, expression of IL-1α was clearly detected after 1 h of incubation, and IL-1α levels continued to increase during the course of the experiment (6 h). In contrast, in astrocytes stimulated with 1 nM PRL, an increase in the expression of TNF-α was first apparent after 2 h of incubation. TNF-α levels peaked 3 to 4 h after the addition of PRL, and returned to near control levels after 6 h. Finally, injection of PRL into a wound site in female rats increased the expression of glial fibrillary acid protein (GFAP), an astrocyte specific protein. These data suggest that PRL can stimulate astrogliosis at the wound site in vivo. These data clearly indicate that PRL can stimulate the expression of TNF-α and IL-1α in cultured astrocytes and suggest that PRL may play a role in the regulation of the neuroimmune response in vivo. © 1995 Wiley-Liss, Inc.

**Key words:** prolactin, astrocyte, cytokines, astrogliosis, interleukin-1, tumor necrosis factor

Prolactin (PRL) is a multifunctional hormone with effects on humoral and cellular immune responses. In vivo, hypoprolactinemia induced by hypophysectomy or by administration of bromocriptine impairs humoral, cell-mediated, and auto-immune responses. For example, hypoprolactinemia results in decreases in: (1) antibody production in rats [Lunkin, 1960]; (2) lymphocyte proliferation [Prentice et al., 1976]; (3) the number and activity of spleen natural killer (NK) cells [Cross et al., 1984]; (4) the development of adjuvant-induced arthritis [Nagy and Berczi, 1978]; (5) contact sensitivity induced by dinitrochlorobenzene [Nagy and Berczi, 1978]; (6) an increase in skin allograft survival; and (7) thymus weight [Gala, 1991]. Although the role of PRL in the regulation of the peripheral immune response has received considerable attention, there are few studies related to the role of PRL in the regulation of the neuroimmune response. In the adult, astrocytes are the most numerous cells in the brain and in response to infection, injury, or trauma, become "activated," resulting in astrocyte proliferation, hypertrophy, and increased synthesis of glial fibrillary acid protein (GFAP), an astrocyte specific protein [Benveniste, 1992]. The observations that activated astrocytes secrete cytokines, express MHC class I and II antigens, and present antigen to T cell clones in an MHC-restricted response [Chung et al., 1991], indicate that astrocytes

Received May 4, 1994; accepted June 30, 1994.

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can function as immunocompetent cells in the CNS. Although numerous studies demonstrate that activated astrocytes are found in many neurological disorders, including acquired immune deficiency syndrome dementia complex (ADC), multiple sclerosis (MS), and the animal model for MS experimental allergic encephalomyelitis (EAE) [Hickey et al., 1985; Benveniste, 1992; Martin et al., 1992], the mechanism by which astrogliosis is induced is poorly understood. Recent studies on astrogliosis have focused on the effects of cytokines, such as, IL-1α, IL-6, and TNF-α as stimulators of astrocyte proliferation [Chung et al., 1991]. For example, IL-1α stimulates astrocyte growth in vivo and in vitro, and induces the expression of TNF-α [Giulian et al., 1988; Giulian and Lachman, 1985]. We have recently shown that PRL is a potent mitogen in astrocyte cultures [DeVito et al., 1992b]. Because one of the major effects of stimulating astrocyte proliferation in the adult brain is the expression of inflammatory cytokines, we examined the effect of PRL on cytokine expression in cultured astrocytes to test the hypothesis that PRL functions as a neuroimmunoregulatory hormone. In the present study, we show that PRL-induces the expression of IL-1α, TNF-α, and TGF-α in cultured astrocytes and stimulates astrogliosis in vivo. We propose that PRL-induced mitogenesis of astrocytes and expression of IL-1α, TNF-α, and TGF by these cells play a role in the regulation of the neuroimmune response.

## MATERIALS AND METHODS

### Cell Culture

Astrocytes were prepared from cerebral hemispheres of 1-day-old rat pups as previously described [DeVito et al., 1992b]. Cells were originally seeded at $2 \times 10^5$ cells/cm$^2$ in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, and cultured for 7 days at 37°C, under an atmosphere of 5% $CO_2$, 95% air. Cells were subcultured every 7 days, and were used between the 2nd and 6th passage. Prior to use, cells were dispersed by trypsin, counted in a hemocytometer, and cell viability determined by trypan blue exclusion. For immunocytochemical analysis, cells were plated at a density of 20,000 cells/ml in DMEM containing 10% calf serum into slide chambers (Nunc, Inc., Naperville, IL) and allowed to attach for 24 h. For Western blot analysis, cells were plated at a density of $2 \times 10^5$ cells/cm$^2$ and grown to subconfluence. The media was replaced with fresh media containing 1% serum and cultured for 24 h. After the 24 h incubation period, cells were incubated with vehicle (DMEM), recombinant human PRL (generously provided by Dr. S. Richards, Genzyme Corp., Boston, MA) or rat growth hormone (NIDDK) for 6 or 18 h.

### Immunocytochemistry

Indirect immunofluorescent detection of IL-1α, TNF-α, and TGF was performed using antisera against mouse IL-1α, TNF-α (Genzyme), and TGF-α (Oncogene Science, Manhasset, NY) as previously described [DeVito et al., 1992b]. Briefly, cells were fixed in 1% paraformaldehyde, permeabilized in absolute methanol, washed in 0.1 M phosphate buffered saline (PBS), and incubated with antibodies (1:500) overnight. Cells were then washed in PBS, and incubated with biotinylated anti-mouse or anti-rabbit IgG (Vector Labs, Burlingame, CA) for 1 h (1:100). The cells were then washed and incubated with Streptavidin Texas Red (Amersham, Arlington Heights, IL) for 45 min. Specificity for immunostaining was verified by incubation of cells in the absence of primary and/or secondary antibodies, and with preimmune mouse or rat sera. Antibody specificity was verified by Western blot analysis.

### Induction of Astrogliosis

Female rats were anesthetized with Ketamine (80 mg/kg) and Xylazine (15 mg/kg) and an intracerebral wound was made with a 22 g blunted needle. All procedures were approved by the IAUCC at our medical center. The needle was lowered using a stereotaxic device into the CNS through a burr hole placed 4.0 mm caudal to bregma, 2.0 mm lateral from the midline suture, at a depth of 1.2 mm from the surface of the brain. The needle was moved 0.3 mm caudal and rostral from the insertion point. On one side of the brain, 5 μl of 100 nM PRL was injected into the center of the wound. The same procedure was preformed on the contralateral side of the brain, except that 5 μl of saline was injected, and served as the control. Five days after the wound, each animal received an injection of dye into the center of the wound, and the brain was excised and a block of tissue surrounding the wound, approximately 0.5 and 1.2 mm wide and 2.0 mm deep, was removed. The expression of GFAP, a 49,000 Mr protein whose expression is restricted to astrocytes, was determined by Western blot analysis, using a monoclonal antibody from Signet Laboratories (Dedham, MA).

### Western Blot Analysis

For Western blot analysis of TNF-α, IL-1α, and GFAP in cultured astrocytes, tissues were homogenized in 25 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 1 mM phenylmethylsulfonylfluoride (PMSF) and aprotinin (100 kIU), and centrifuged for 15 min at 1,000*g* at 4°C. The resulting supernatant was centrifuged for 30 min at 100,000*g* at 4°C. The resulting microsomal fractions were dissolved in sample buffer (0.65 M Tris, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% b-mercaptoethanol, and 0.01% bromophenol blue) and 250 μg of protein was separated by SDS polyacrylamide gel electrophoresis on 0.75 mm thick slab gels, using a 4% polyacrylamide stacking gel and a 15% resolving gel. Electrophoresis was carried out at 20 mA for approximately 2 h using a Hoefer sturdier electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA), as previously described [DeVito, 1989a]. Proteins were transferred electrophoretically onto nitrocellulose at 100 V for 2 h at 4°C. The blots were incubated with 3% dry fat-free milk, 5% BSA in 1% ethanolamine, pH 7.0, for 6 h and then incubated with anti-IL-1α or anti-TNF-α for 18 h at room temperature. Blots were then extensively washed with PBS, developed using enhanced chemiluminescence detection reagents from Amersham Life Sciences according to the manufacturers' instructions, and exposed to Kodak X-Omat film.

### TNF-α Bioassay

TNF-α activity was determined in a biologic assay using WEHI 164 mouse fibrosarcoma cells as described by Chung and Benveniste [1990]. The absolute concentration of TNF-α activity was determined by extrapolation from the standard curve, which was generated using known amounts of recombinant mouse TNF-α.

### Statistical Analysis

The data were analyzed using analyses of variance. The size of the region of rejection of the null hypothesis was set by an alpha error of 5%.

## RESULTS

As illustrated in Figure 1, in astrocytes grown in 1% serum, there was no detectable staining of TNF-α, IL-1α, and TGF-α (top panels). Incubation of astrocytes with 1 nM human recombinant PRL for 6 h, however, resulted in the expression of TNF-α and IL-1α, but not TGFα (bottom panels). Similar results were obtained using purified rat PRL (NIDDK; data not shown). Preincubation of PRL with PRL antiserum prevented the PRL-induced cytokine expression (data not shown). As illustrated in Figure 2, when incubated for 18 h with 1 nM PRL, astrocytes showed only faint staining of TNF-α. In contrast, IL-1α and TGF-α staining were clearly detectable.

To further examine the relative expression and physicochemical properties of these cytokines, Western blot analyses were also performed. Figure 3 illustrates a Western blot analysis of the effect of different concentrations of PRL on the expression of TNF-α. In less than 5% of the studies performed, as illustrated in Figure 3A, low but detectable levels of TNFα were found in unstimulated controls. However, in 95% of the studies, the signal in unstimulated cultures was similar to background (presented in the figure legends). Incubation of astrocytes with PRL for 6 h resulted in a significant dose dependent increase in immunoreactive TNF-α, with an apparent molecular weight of 26 kDa (Fig. 3). When compared to the signal in control cultures, there was a marked increase (20-fold) in the relative level of TNF-α in astrocytes incubated with 100 nM PRL for 6 h. Incubation of astrocytes with 100 nM GH had no effect on TNF-α levels. Western blot analysis of the same cell extracts with anti-IL-1α did not reveal detectable levels of IL-1α in control or GH treated astrocytes. However, incubation of astrocytes with PRL resulted in a marked dose dependent increase in immunoreactive IL-1α, with an apparent molecular weight of 17 kD (Fig. 4).

We next determined the time course for the expression of IL-1α and TNF-α in cultured astrocytes. Western blot analyses were performed on cell extracts from astrocytes incubated with 1 nM PRL for 0 to 6 h. As illustrated in Figure 5, incubation of astrocytes with 1 nM PRL for 1 h increased the expression of IL-1α to easily detectable levels, and IL-1α levels continued to increase during the time course of the experiment (6 h). In contrast, in astrocytes stimulated with 1 nM PRL, the expression of TNF-α was first apparent after 2 h of incubation, reached a peak at 4 h, and subsequently decreased.

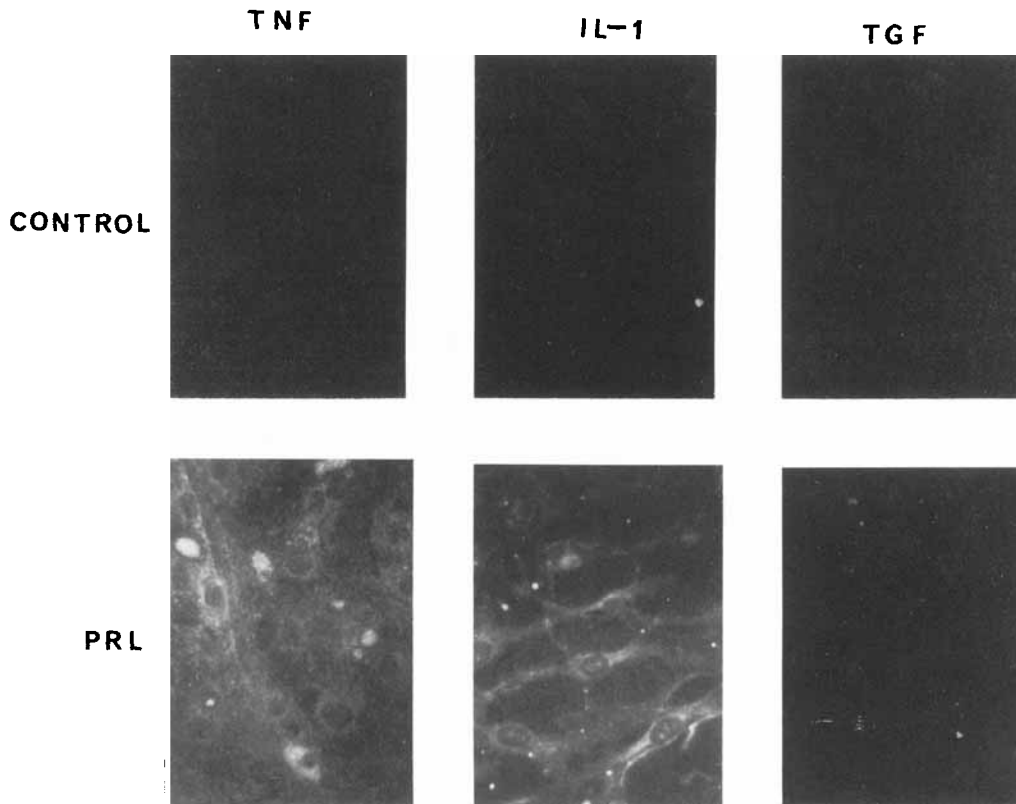


**Fig. 1.** The expression of TNF-α, IL-1α, and TGF-α in astrocytes grown in 1% serum in the absence of PRL (top panels), and in the presence of 1 nM hrPRL for 6 h (bottom panels). Magnification, ×400.

Immunocytochemical and Western blot analysis clearly indicate that PRL-induces the expression of TNF-α and IL-1α in cultured astrocytes. To determine if the expression of these cytokines results in their secretion, we examined the effect of PRL on the release of TNF-α from cultured astrocytes using a TNF-α bioassay. No detectable TNF-α activity (less than the minimal detectable dose of the assay, 5 pg/ml) was found in media from astrocytes incubated in 1% serum for 4 h. However, incubation of astrocytes with 1 or 100 nM PRL for 4 h resulted in an increase in biologically active TNF-α from undetectable levels to 105 ± 21 and 275 ± 41 pg/ml in the media, respectively (n = 3). Further, the concentration of TNF-α released into the media in response to 100 nM PRL was significantly ($P < 0.05$) greater then that released in response to 1 nM PRL.

The cellular events at the site of a wound in the CNS include the rapid appearance of inflammatory cells and the subsequent growth of astroglia. To determine if PRL stimulates astroglia proliferation in vivo, we examined the effect of PRL injection on astrocyte proliferation in response to a CNS wound. As shown in Figure 6, infusion of PRL into a CNS wound site resulted in a 4.5 ± 0.7 fold increase in GFAP levels compared to the contralateral wound site infused with saline.

## DISCUSSION

In response to infection or trauma astrocytes become "activated," resulting in cell proliferation and hypertrophy [Benveniste, 1992]. We have shown that PRL is a potent mitogen in cultured astrocytes, with low concentrations (1 nM) stimulating mitogenesis, and higher concentrations (100 nM) inhibiting mitogenesis [DeVito et al., 1992b]. In the present study, we show that incubation of astrocytes with PRL stimulates the expression of IL-1α, TNF-α, and TGFα, and that their expression is dependent on the concentration and duration of PRL stimulation. That is, when simulated with either a high or low concentration of PRL, the expression of IL-1α and TNF-α precedes the expression of TGFα. At high PRL concentrations the expression of IL-1α and TNF-α were markedly increased. In contrast, preliminary studies (data

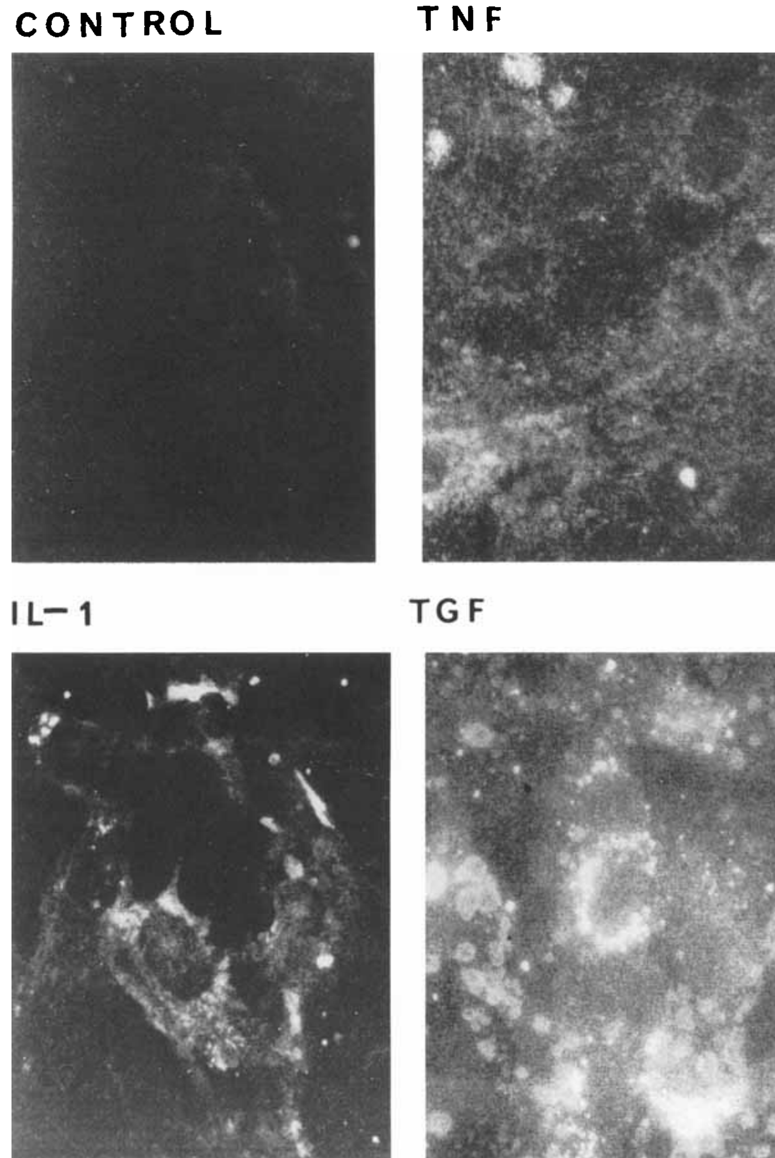


**Fig. 2.** The expression of TNF-α, IL-1α, and TGF-α in astrocytes grown in 1% serum and presence of 1 nM hrPRL for 18 h. The top left panel represents astrocytes stimulated with PRL and immunohistochemistry performed using preimmune serum as a control. Magnification, ×630.

not presented) suggest that when stimulated with high concentrations of PRL, TGF-α expression is suppressed. However, further analysis is required to verify this effect and to clarify TGF-α's role on astrocyte function.

Over the last decade, a large body of evidence has shown that there is a multidirectional flow of information among the central nervous system (CNS), neuroendocrine, and immune systems. The interrelationship between the immune and neuroendocrine systems was first demonstrated over 60 years ago when Smith reported that the thymus glands of rats ceased to grow immediately after hypophysectomy and regressed to less then one half of the weight of controls [Smith, 1930]. Subsequent studies revealed that the immunodeficiencies which result from hypophysectomy can be ameliorated by administration of GH or PRL [Nagy and Berczi, 1978; Nagy et al., 1983a; Berczi et al., 1981]. Similarly, in the dwarf Snell-Bagg mouse with defective pituitary regulation, normal ontogeny of the immune system is disrupted and these mice display immune deficiencies consisting of early thymic involution, diminished production of lymphocytes, atrophy of peripheral lymphoid organs, and severe impairment of antibody production [Baroni et al., 1967]. Administration of

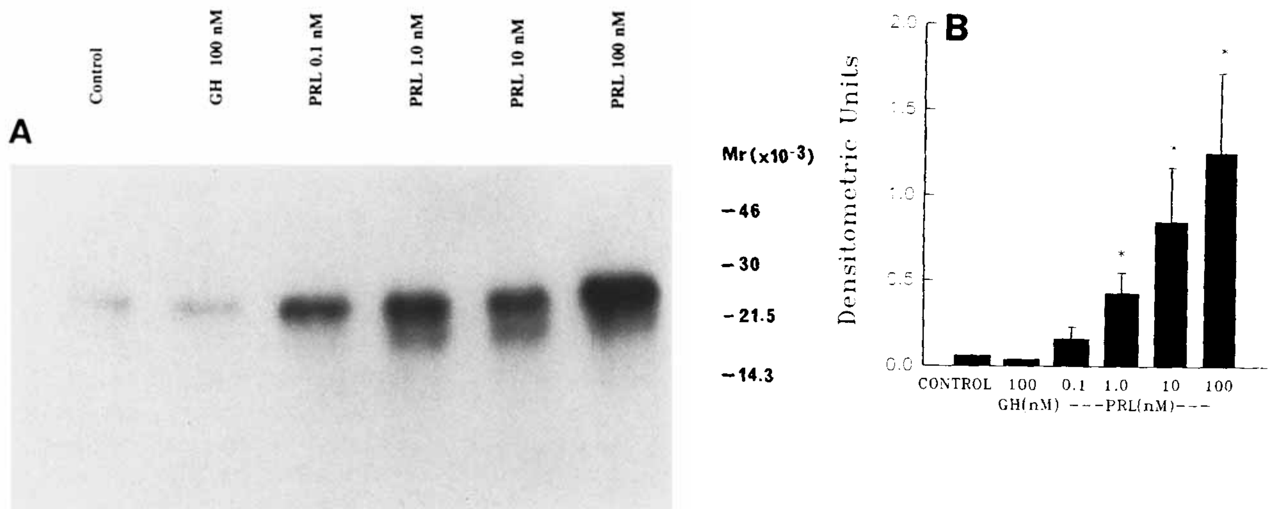


**Fig. 3.** **A:** Western blot analysis of TNF-α in extract preparations of astrocytes stimulated with increasing concentrations of PRL, or 100 nM GH, for 6 h. **B:** The results of three separate Western blot analyses. The data are expressed in densitometric units. The background of the autoradiograms ranged from 0.04 to 0.08 densitometric units. $^{*}P < 0.05$ vs. control. N = 3.

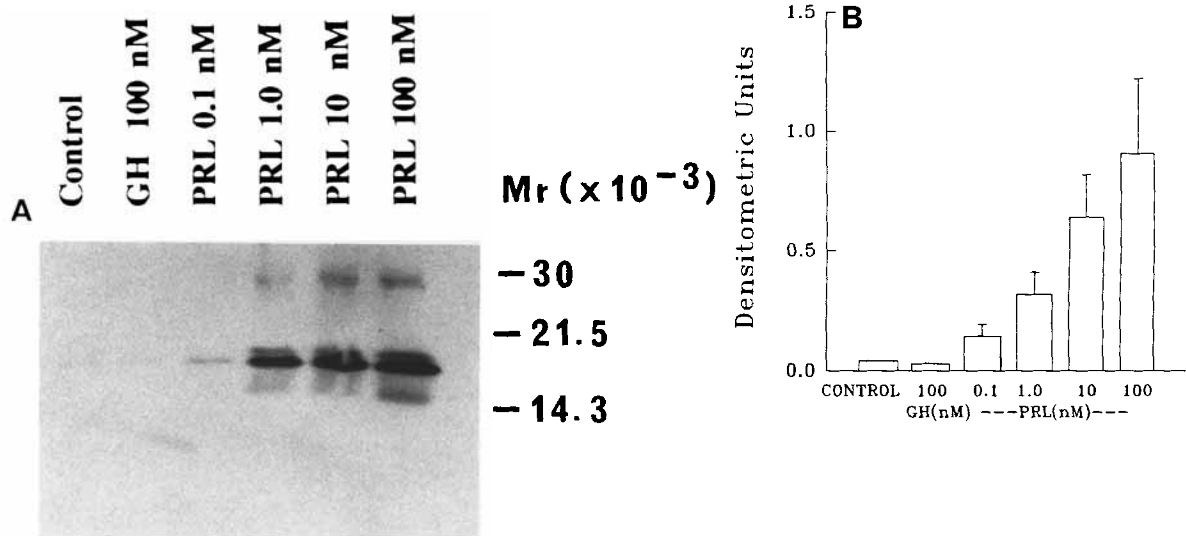


**Fig. 4.** **A:** Western blot analysis of IL-1α in extracts preparations of astrocytes stimulated with increasing concentrations of PRL, or 100 nM GH, for 6 h. **B:** The results of three separate Western blot analyses. The data are expressed in densitometric units. The background of the autoradiograms ranged from 0.03 to 0.06 densitometric units. $P < 0.05$ vs. control. N = 3.

PRL normalizes the lymphoid system [Pierpaoli et al., 1976]. Since the initial observations that suggested an effect of PRL on the ontogeny and function of the immune system, the immunoregulatory effects of PRL in mammals have been well documented and involve the regulation of the humoral and cellular immune systems. As discussed in the introduction, hypoprolactinemia induced by hypophysectomy or by administration of bromocriptine impairs humoral, cell-mediated, and auto-immune responses [Lunkin, 1960; Prentice et al., 1976; Cross et al., 1984; Nagy and Berczi, 1978; Gala, 1991]. Administration of PRL or GH, but not TSH, LH, FSH, or HCG, to hypophysectomized animals restores immune functions to normal levels [Gala, 1991; Nagy and Berczi, 1978; Nagy et al., 1983a; Bernton et al., 1988]. Administration of PRL to normal mice results in a dose-dependent stimulation of antibody production [Spangelo et al., 1987]. Treatment of mice with anti-PRL antiserum or bromocryptine results in a rapid and prolonged decrease in the percentage of splenic helper T cells, indicating that PRL is involved in

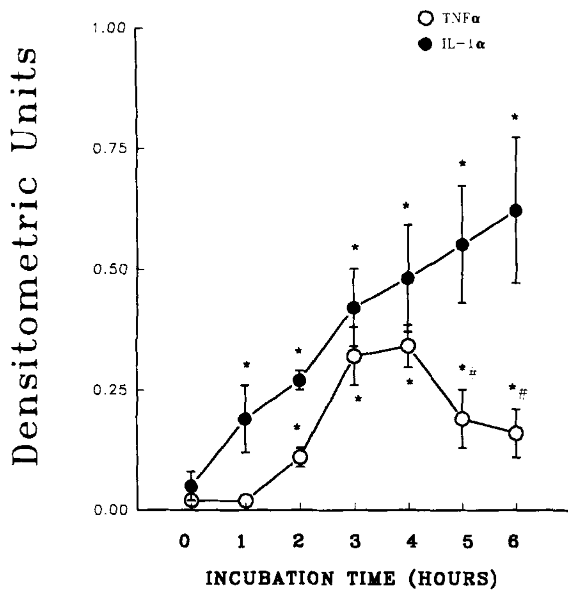


Fig. 5. Western blot analysis of the time course of PRL-induced expression of TNFα and IL-1α in cultured astrocytes. Astrocytes were incubated with 1 nM PRL for 0 to 6 h. The background of the autoradiograms ranged from 0.02 to 0.07 densitometric units. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. IL-1α at 3 and 4 h. N = 3.

the developmental expression of T and B lymphocyte populations in the thymus and spleen [Russell et al., 1988]. Implantation of GH3 cells, which produce GH and PRL, into aged rats results in the restoration of both thymic histology and interleukin 2 production [Kelley et al., 1993]. Further, PRL stimulates in vivo the production of the thymic hormone, thymulin, as well as in vitro in human and murine thymic epithelial cells [Dardenne et al., 1989].

One of the proposed mechanisms for the cause of the early lesions and demyelination in MS is an active immune response in the CNS. This is supported by studies which show macrophages, lymphocytes, and plasma cells surrounding demyelinated axons, and the expression of IL-2 receptors and MHC class II molecules on infiltrating immune cells and resident glial cells [Martin et al., 1992]. EAE is an autoimmune disease characterized by inflammation and demyelination, and is a well-established model for autoimmune demyelination. Interestingly, in EAE, serum PRL concentrations are elevated prior to the onset of signs of neurological disease [Riskind et al., 1991]. Inhibition of PRL secretion, by administration of bromocriptine, substantially reduces the incidence and severity of acute EAE and prevents the recurrent disease in female Lewis rats [Riskind et al., 1991]. The observation that elevations of serum PRL precede the clinical signs of neurological disease, suggests that elevations in PRL concentrations may be involved in the regulation of the immune response in the CNS. Consistent with a possible role of PRL as a regulator of the neuroimmune response, we found that PRL increased the expression of GFAP when injected into a CNS wound site. These data suggest that PRL plays a role in astrocyte proliferation in vivo. Although this hypothesis is supported by the demonstration of PRL-induced expression of IL-1α, TGFα, and TNF-α in cultured astrocytes, further in vivo studies will be required to substantiate the role of PRL in neuroimmune regulation.

Studies performed by a number of investigators indicate that the regulation of inflammatory cytokines within the CNS most likely entails activation of autocrine and/or paracrine loops involving several cytokines and their receptors [Benveniste, 1992]. For example, IL-1α can stimulate astrocyte growth in vitro [Giulian and Lachman, 1985] and when injected into the brain it can stimulate astrogliosis [Giulian et al., 1988]. In addition, IL-1α stimulates TNF-α secretion and gene expression in cultured astrocytes. Further, TNF-α also has a mitogenic effect on primary astrocytes and astroglioma cell lines, and may contribute to the reactive astrogliosis associated with various neurological diseases [Benveniste, 1992]. Taken together, these observations support the notion that the effect of PRL on astrocyte function may be mediated, in part, by the expression of TNF-α and IL-1α by PRL. Further studies are needed to test the hypothesis that the effect of PRL on astrocyte function may involve autocrine and/or paracrine effects on IL-1α and/or TNF-α.

Interestingly, Riskind et al. [1991] reported that in EAE there is a five-fold increase in serum PRL concentrations 4 and 10 days after immunization. Administration of bromocriptine, which reduced serum PRL concentrations to control levels, substantially reduces the incidence and severity of the neurological disease [Riskind et al., 1991]. Whereas their data indicate that elevations of serum PRL precede the clinical signs of neurological disease, and suggest that elevations in PRL concentrations may be involved in the regulation of the immune response in the CNS, they only address one source of PRL. That is, although PRL has been considered a reproductive hormone secreted from the anterior pituitary, it is well documented that PRL and PRL-like proteins are present in a number of tissues,

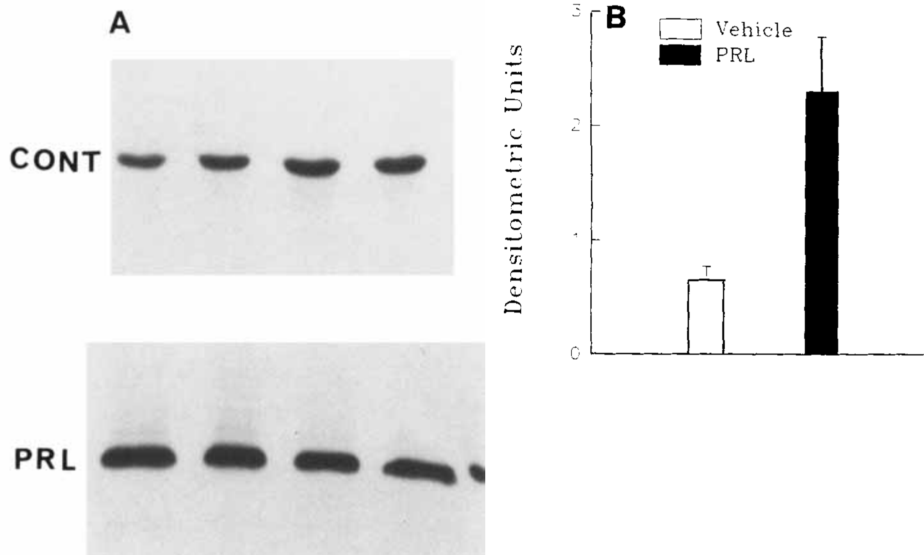


Fig. 6. Immunoblot (A) and autoradiographic analysis (B) of GAFP in cortical tissue surrounding a CNS wound site 5 days after injection with saline or 100 nM PRL. *$P < 0.05$ vs. control. N = 4.

including lymphocytes [Russell, 1989; Hiestand et al., 1986; Montgomery et al., 1992] and brain [DeVito et al., 1987; DeVito, 1988, 1989b; Emanuele et al., 1986, 1989]. We have shown that brain PRL is stored in large membrane bound granules [DeVito et al., 1987] at concentrations similar to that of norepinephrine, dopamine, AVP, and ACTH [DeVito et al., 1987; DeVito, 1988, 1989b]. The persistence of PRL in the brain after hypophysectomy, and the detection of PRL mRNA in the brain [Schacter et al., 1976; Emanuele et al., 1992; DeVito et al., 1992a], substantiate a brain PRL system independent of pituitary PRL. This raises the interesting possibility that PRL synthesized in the CNS, may play a role in the regulation of immunoregulatory proteins. Consistent with this hypothesis, we have found (unpublished observations) that PRL content increases at the site of a CNS wound. Studies are in progress to determine the role of endogenous brain PRL on the neuroimmune response to infection and/or trauma.

In summary, PRL is a multifunctional hormone with effects on humoral and cellular immune responses. Whereas our understanding of the effects of PRL on the immune system has recently attracted considerable attention, little is known about the possible role of PRL in the regulation of the neuroimmune system. It is clear that astrocyte proliferation and the expression of inflammatory cytokines play an important role in regulating the brain's response to injury or infection. In this study we show that: (1) in cultured astrocytes, physiological concentrations of PRL stimulate astrocyte proliferation and the expression of IL-1$\alpha$, TNF-$\alpha$, and TGF-$\alpha$; and (2) administration of PRL into a CNS wound site increases the expression of the astrocyte specific protein GFAP, a well-established marker for astrogliosis. To our knowledge, these are the first data that show PRL can activate or modulate the expression of immunoregulatory proteins in astrocytes, and are consistent with PRL's role as an immunoregulatory hormone. Further studies are required to clarify the cellular and molecular mechanisms involved in PRL-induced mitogenesis and cytokine expression stimulation in the CNS. These studies will provide new insights into the role of PRL in modulating brain function and the brain's response to injury or disease.

## ACKNOWLEDGMENTS

This work was supported by NIH Grants NS-26343 (to W.J.D.). A preliminary report was presented at the IV International Prolactin Congress, 1993.

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