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14. As in the case of c-*src* overexpression, the expression of Tyr^{527} mutants or c-*src*/v-*src* chimeras did not affect nonjunctional membrane permeability; the rates of loss of Lucifer Yellow were negligible and not different from controls (six photometric measurements as in Fig. 2, C and D, were performed for each condition).
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Suppression of Macrophage Activation and T-Lymphocyte Function in Hypoprolactinemic Mice

EDWARD W. BERNTON,* MONTE S. MELTZER, JOHN W. HOLADAY

The effects of prolactin on lactation and reproductive organs are well known. However, the other possible target organs and physiological consequences of altered levels of circulating prolactin remain poorly understood. In this study, mice were treated with bromocryptine, a dopamine receptor agonist that inhibits pituitary prolactin secretion. Bromocryptine treatment prevented T-cell–dependent induction of macrophage tumoricidal activity after the intraperitoneal injection of *Listeria monocytogenes* or *Mycobacterium bovis*. Coincident treatment with ovine prolactin reversed this effect. Of the multiple events leading to macrophage activation in vivo, the production by T-lymphocytes of γ-interferon was the most impaired in bromocryptine-treated mice. Lymphocyte proliferation after stimulation with mitogens in vitro was also depressed in spleens of bromocryptine-treated mice, and coadministration of prolactin also reversed this effect. Bromocryptine treatment also reduced the number of deaths resulting from inoculation of mice with *Listeria*; exogenous prolactin significantly reversed this effect. The critical influence of pituitary prolactin release on maintenance of lymphocyte function and on lymphokine-dependent macrophage activation suggests that, in mice, lymphocytes are an important target tissue for circulating prolactin.

A NUMBER OF REGULATORY INTERactions among the central nervous system (CNS), the neuroendocrine axis, and the immune system have recently been described. Endocrine and autocrine mediators and receptors shared in common by the immune and endocrine systems may explain changes in immune function in response to environmental stimuli perceived by the CNS (*1*). Although high levels of endogenous (adrenal) or exogenous corticosteroids can suppress many immune responses, immunosuppression induced in rats by repeated periods of tail-shock persists after adrenalectomy (*2*). Such data suggest that other neural and endocrine mechanisms under CNS control may have immunomodulatory roles beyond the activation of the pituitary-adrenocortical axis as a result of stress.

Pituitary prolactin secretion is regulated not only by reproductive hormones, but also by CNS pathways affected by both acute and chronic stressors as well as by many CNS-active drugs, such as opiates and aminergic agonists and antagonists (*3*). Several lines of evidence indicate that prolactin (PRL) may be an important immunoregulatory hormone. In rats, both hypophysectomy and treatment with the dopamine agonist bromocryptine inhibit the development of delayed cutaneous hypersensitivity, experimental allergic encephalitis, or adjuvant-induced arthritis; treatment with exogenous PRL reverses these immunosuppressive effects (*4*). Cyclosporine, an immunosuppressive fungal peptide that inhibits T-cell function, inhibits PRL binding to lymphocytes (*5, 6*). Two hypopituitary strains of mice, the Ames and Snell dwarfs, develop impaired cellular immunity and hypotrophic thymuses after weaning; this immunodeficiency is prevented by injections of milk, a PRL source (*7*). However, the mechanisms mediating immunosuppression in hypoprolactinemic animals have not been elucidated.

We therefore examined the T cell–dependent induction of activated, tumoricidal macrophages in mice infected with either *Mycobacterium bovis* (strain BCG) or *Listeria monocytogenes* (LM), or inoculated with killed *Proprionibacterium acnes*. Bromocryptine, a dopamine type 2 (DA-2) agonist, was used to continuously suppress serum PRL to less than 2.0 ng/ml (*8*). Injections of bromocryptine on days −1 to 3 before and after inoculation with either BCG, LM, or *P. acnes* on day 0 prevented induction of tumoricidal macrophages (Fig. 1). However, the timing of DA-2 agonist administration was critical. Daily bromocryptine treatment either on days 0 and 1 or on days 4 through 7 did not alter macrophage activation. This time course indicated that one or more early steps in the pathway of macrophage activation in vivo were blocked by DA-2 agonist administration.

Since DA-2 agonists exert physiological effects other than the inhibition of PRL release, we examined whether exogenous PRL could reverse the effects of bromocryptine treatment. Simultaneous daily intraperitoneal injection of ovine PRL (100 μg/day) (*9*) restored to normal the induction of tumoricidal macrophages during BCG or LM infection in bromocryptine-treated ani-

E. W. Bernton and J. W. Holaday, Neuropharmacology Branch, Department of Medical Neurosciences, Division of Neuropsychiatry, Walter Reed Army Institute of Research, Washington, DC 20307.
M. S. Meltzer, Department of Immunology, Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Washington, DC 20307.

*To whom correspondence should be addressed.

mals (Fig. 1A). These data indicate that hypoprolactinemia may be the direct cause of suppressed immune function in mice after treatment with DA-2 agonist.

The induction of tumoricidal peritoneal macrophages by BCG, LM, or *P. acnes* depends on a precise sequence of immunologic events. A peritoneal inflammatory response is elicited, and phagocyte cells, for example, macrophages, process and present antigen to antigen-specific T-helper cells. These then secrete their own growth factor, interleukin-2 (IL-2). Clonal expansion and IL-2 production by T-helper cells leads to increased secretion of γ-interferon (γ-IFN) and other less well characterized macrophage activating factors (MAFs). MAFs act on macrophages to induce changes generically known as "activation." These include enhanced expression of membrane Ia antigen, enhanced ability to kill intracellular parasites and bacteria, and increased cytotoxicity for tumor cells.

Inhibition of PRL release by bromocryptine could affect any of the events in this cascade. Therefore, we examined each of the steps involved in the activation of macrophages. We noted that the total number of peritoneal exudate cells that accumulate during BCG or LM infection (including relative percentages of macrophages, lymphocytes, and polymorphonuclear leukocytes) were the same in mice in all treatment groups. Thus, the effects of bromocryptine or PRL were not directly mediated by changes in the early inflammatory response to infectious challenge.

Since T-cell proliferation often precedes and amplifies secretion of MAFs, we investigated the effects of bromocryptine and PRL treatment on splenocyte proliferative responses to the T-cell mitogen concanavalin A (con A) as well as the B-cell mitogen *Salmonella* lipopolysaccharide (LPS). Four daily subcutaneous injections of bromocryptine dissolved in sesame oil caused a dose-related suppression of both serum PRL and lymphocyte proliferation in response to both mitogens (Fig. 2). Lymphocyte proliferation was directly correlated to serum PRL. Daily injections of 20 μg of mouse PRL on days 3 and 4 caused a partial but significant reversal of the effects of the highest dose of bromocryptine. This dose of PRL resulted in a serum PRL of 26.2 ± 4.7 ng/ml at 4 hours after injection. Mice treated with bromocryptine alone had PRL levels of 2.2 ± 0.3 ng/ml at the same time point. By 24 hours after the last injection of PRL, this group had serum PRL levels similar to those of the group treated with bromocryptine alone (Fig. 2). Significant suppression of lymphocyte proliferation was noted after 48 but not 24 hours of bromocryptine treat-

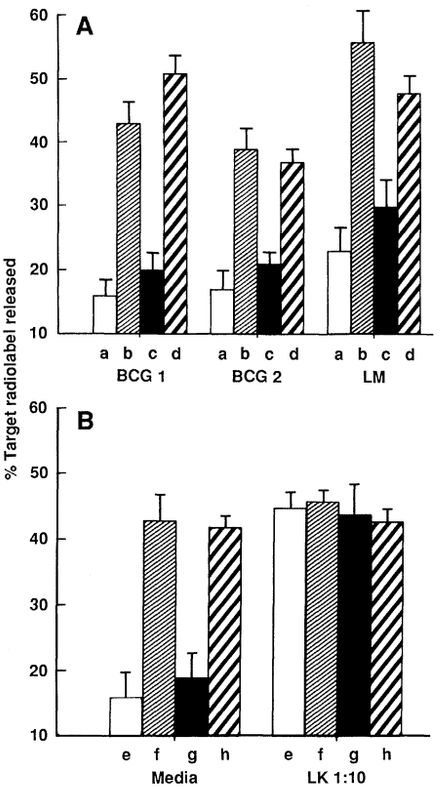

Fig. 1. (**A**) Effect of bromocryptine treatment on induction of tumoricidal macrophages during BCG or LM infection. In separate experiments, groups of five male C3H/HeN male mice were inoculated (day 0) intraperitoneally with either viable *M. bovis*, strain BCG, or with LM (4500 CFU, 0.2 LD_{50}). After 10 days, equal numbers of adherent peritoneal cells recovered by lavage were cultured with radiolabeled TU-5 tumor target cells (*10*). Mice were treated on days −1 to 3 with daily injections of (b) vehicle (0.1% bovine serum albumin in sterile 0.05*M* tartaric acid); (c) bromocryptine mesylate (0.2 mg in 0.2 ml of vehicle, Sandoz Research); or (d) bromocryptine 0.2 mg and ovine PRL 0.1 mg (in 0.1 ml of vehicle). Tumor cytotoxicity of macrophages was assessed by measurement of radiolabel release in triplicate cultures at 48 hours and expressed as mean percent of total counts of tumor-cell radiolabel ± the SEM. Bars labeled "a" represent the assay baseline, radiolabel released from target cells incubated with identical numbers of starch-elicited (unactivated) macrophages. Spontaneous label release (target cells alone) varied from 5 to 15% of total label in all assays. Radiolabel release greater than twice spontaneous release was always associated with microscopically visible target cell lysis. BCG1 and BCG2 represent separate experiments. (**B**) Inhibition of induction of tumoricidal macrophages by bromocryptine after *P. acnes* treatment and reversal in vitro by addition of lymphokines. Ten days after intraperitoneal injection of heat-killed *P. acnes* (50 μg per mouse, Burroughs-Wellcome), equal numbers of adherent peritoneal cells from treatment groups of five mice were cultured with radiolabeled tumor target cells with and without culture fluids from PPD-stimulated BCG-immune splenocytes (lymphokines) (*10*). Treatment groups were (f) vehicle, (g) bromocryptine, 200 μg, and (h) bromocryptine and ovine PRL, 100 μg; treatments were given on days −1 through 3. Bars labeled "e" represent tumor cells incubated with starch-elicited macrophages. Tumor cytotoxicity was estimated by measurement of radiolabel release in triplicate cultures at 48 hours and expressed as percentage of total counts. Two groups of four mice each received each treatment. Error bars represent SEM for triplicate determinations on two groups per treatment.

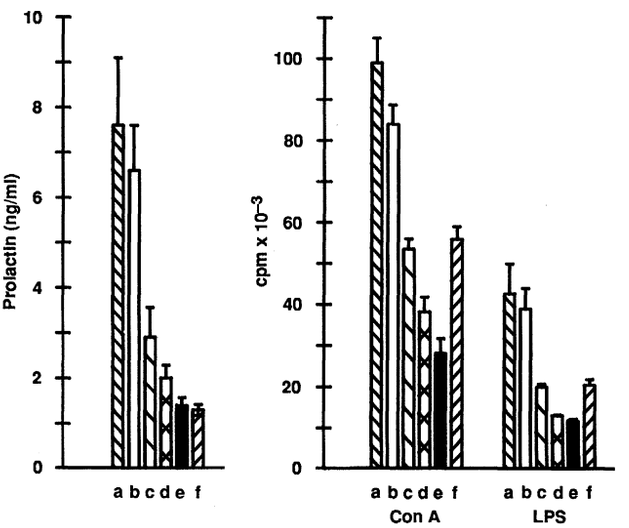

Fig. 2. Dose-related suppression of serum PRL levels and splenocyte proliferative responses in mice after 4-day treatment with graded doses of bromocryptine, and partial reversal with 20 μg/day of exogenous mouse PRL. Groups of five to six male C3H/HeN mice were injected subcutaneously each day with (a) 0.1 ml of sesame oil; (b) 25 μg of bromocryptine in sesame oil; (c) 50 μg of bromocryptine; (d) 100 μg of bromocryptine; or (e and f) 250 μg of bromocryptine. Mice in (f) also received 20 μg/day of mouse PRL on days 3 and 4 only. On day 5, 24 hours after the last treatment, immediately after mild stress (3-minute swim in 20°C water), mice were bled by decapitation for determination of serum PRL. Spleens were then dissociated, pooled, adjusted to equal cell concentrations, and cocultured with con A or LPS for determination of blastogenic responses (*15*). Responses to 2 μg/ml of con A and 5 μg/ml of LPS are illustrated. Background counts (no mitogen) were less than 800 cpm in all groups. A seventh group of mice was treated with 10 μg/day of bromocryptine. A paradoxical increase in serum PRL (11.0 ± 1.2 ng/ml) was seen in this group, presumably due to drug clearance in less than the 24-hour dosing interval. Splenocyte proliferation was also increased above vehicle controls in this group (133,000 ± 4,500 cpm with 2 μg/ml of con A). Error bars represent SEM for five to six mice for PRL, and quadruplicate cultures for blastogenic responses.

ment (250 μg/day). Forty-eight hours after ending 4 days of bromocryptine treatment, serum PRL rebounded to almost double that of vehicle-treated controls, and splenocyte-proliferative responses to con A were proportionately elevated. Bromocryptine mesylate added directly to con A– and LPS-stimulated splenocyte cultures at concentrations ranging from 0.08 to 3.0 μg/ml did not significantly alter proliferative responses.

In separate experiments, mice were treated for 8 days with placebo pellets or pellets continuously releasing bromocryptine (125 μg/day), implanted subcutaneously. Serum PRL levels at 4 hours, 2 days, and 4 days, respectively, were 6 ± 1.7, 14 ± 4.2, and 9 ± 3.2 ng/ml in the placebo-treated mice and 2.1 ± 0.2, 1.2 ± 0.2, and 1.3 ± 0.3 ng/ml in the bromocryptine-treated mice. Proliferative responses to con A, phytohemagglutinin (PHA), two T-cell mitogens, and the B-cell mitogen LPS were all depressed severalfold in bromocryptine-treated animals (Fig. 3A). Other mice treated with bromocryptine pellets were injected intraperitoneally on days 6 and 7 with 100 μg of either PRL or a control solution of bovine serum albumin, and were also killed on day 8. Daily PRL treatment beginning 24 hours (day 7) or 48 hours (day 6) before the mice were killed reversed the bromocryptine-induced suppression of mitogen-stimulated proliferation, with the longer treatment period having greater effect (Fig. 3A). Depressed spleen lymphocyte proliferation was not a consequence of altered distribution of cells between lymphocyte subsets, since analysis of spleen cells for expression of lymphocyte markers for total T and B cell, suppressor/cytotoxic T-cell, and helper T-cell phenotypes by flow cytometry showed no statistically significant quantitative change in these functional subsets after immunosuppressive treatment with bromocryptine (Fig. 3B).

These experiments show that suppression of PRL secretion can reduce both T- and B-cell proliferation in response to mitogens without altering the relative numbers or ratios of total helper and suppressor phenotype T cells. Therefore, we investigated the subsequent steps in the immunologic cascade leading to macrophage activation. Seven to 10 days after intraperitoneal inoculation of mice with BCG, LM, or killed *P. acnes*, peritoneal macrophages develop that are cytotoxic against tumor target cells (*10*). Peritoneal inflammatory macrophages elicited by intraperitoneal injection of sterile irritants, such as starch or protease peptone, are not tumoricidal but can become so when activated in vitro by exposure to lymphokines such as γ-IFN. We found that bromocryptine (0.5 to 25 μg/ml) did not directly inhibit the cytotoxicity of BCG-activated macrophages in vitro and that ovine PRL itself (20 ng to 5 μg/ml) did not serve as a lymphokine to directly activate starch-elicited macrophages for cytotoxicity in vitro. However, BCG-elicited macrophages from bromocryptine-treated mice (which had no tumoricidal activity de novo) became fully cytotoxic after exposure in vitro to the MAFs in lymphokine preparations (*11*) (Fig. 1B) or to recombinant murine γ-IFN. This activation by lymphokines was also not affected by simultaneous exposure to bromocryptine or to PRL.

Since PRL and bromocryptine do not act directly on macrophages exposed to activation factors in vitro, the results of the experiments in vivo (Fig. 1) suggest that hypoprolactinemia abrogates the in vivo production of MAFs by T lymphocytes. This hypothesis was directly tested by measuring the amounts of γ-IFN secreted by spleen cells from BCG-infected mice treated with vehicle, bromocryptine, or bromocryptine plus PRL for 5 days at the doses used in prior experiments. Spleens were then removed and splenocytes were cultured with 50 μg/ml of PPD (purified protein derivative from *M. tuberculosis*) for 48 hours (*10*). Supernatants were assayed for γ-IFN by means of a specific enzyme-linked immunosorbent assay (ELISA) (*11*). Splenocytes from bromocryptine-treated mice produced one-fifth as much γ-IFN as an equal number of cells obtained from animals treated with a control vehicle (Fig. 3). The simultaneous administration of ovine PRL along with bromocryptine treatment restored γ-IFN secretion toward control values. These data support the hypothesis that PRL is required for the normal production of MAFs by antigen-stimulated T cells and further indicate that bromocryptine's inhibitory effects on macrophage activation in vivo reflect impaired T-cell production of MAFs such as γ-IFN.

If bromocryptine treatment can suppress

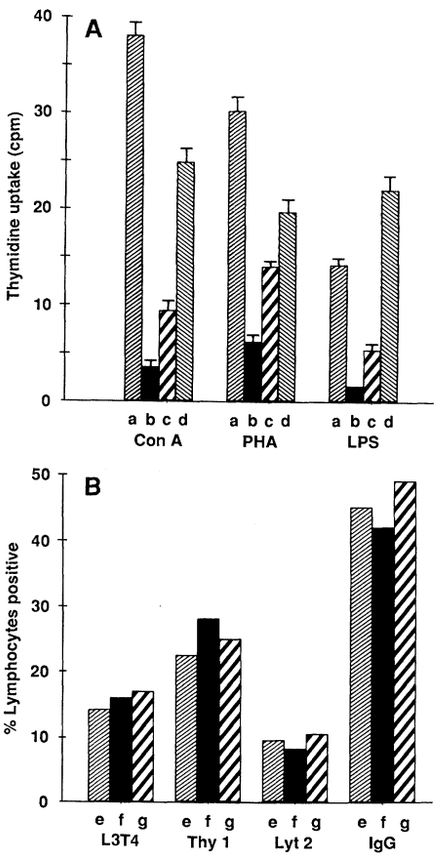

Fig. 3. (**A**) Effects of chronic bromocryptine treatment on lymphocyte proliferative responses to T- and B-cell mitogens and reversal by PRL treatment. On day 1 groups of five male C3H/HeN mice were implanted subcutaneously with (a) inert control pellets or (b) 2.5-mg pellets of bromocryptine (Innovative Research) that released approximately 125 μg of drug per 24 hours. Other groups of mice implanted with bromocryptine pellets were injected intraperitoneally with 100 μg of ovine PRL on day 7 (c) or on days 6 and 7 (d) or with 100 μg of bovine albumin as an inert control (a and d). On day 8, mice were killed and dissociated splenocytes from the mice in each group were pooled. These were adjusted to a standard cell concentration and cultured with or without various concentrations of mitogens for determination of blastogenesis (*15*). Results above (in cpm × 10^{-3}) were obtained with con A (1 μg/ml), PHA (10 μg/ml), and LPS (5 μg/ml). These values are representative of shifts in the entire dose-response curves to mitogens. This shift tended to be greatest with suboptimal mitogen stimulation, although still significant with supraoptimal mitogen concentrations. Error bars represent SEM of triplicate wells. These data are representative of several experiments. (**B**) Lack of effect of immunosuppressive bromocryptine treatment or PRL treatment on proportions of lymphocyte subsets. Groups of five mice received 4 daily intraperitoneal injections of (e) control vehicle, (f) bromocryptine 0.2 mg, or (g) bromocryptine plus ovine PRL 0.1 mg. Proliferative responses of splenocytes from the bromocryptine group were profoundly suppressed after culture with con A and LPS. Splenocytes from each group were washed three times in phosphate-buffered saline with 3% goat serum and 0.1% sodium azide (PBS/GS). Duplicates of 2 × 10^6 cells from each group were incubated with either PBS/GS or with monoclonal mouse antibodies to L3T4, Lyt 2.1, thy 1.2, or mouse immunoglobulin G (all from Becton Dickinson). The cells were washed twice, then incubated with fluorescein isothiocyanate–labeled second antibody, again washed twice, and fixed with 1% paraformaldehyde. Analyses were performed on a FACSTAR (Becton Dickinson) flow cytometer. Negative peak fluorescence for the PBS/GS control was used to delimit the flourescence channels containing true-positive cells. Coefficient of variation with replicate sample counts was always <5%.

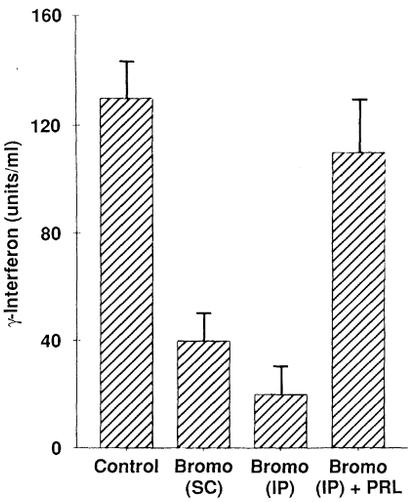

Fig. 4. Suppression of γ-IFN secretion by splenocytes from bromocryptine-treated BCG-immune mice. Spleens were removed from groups of C3H/HeN mice injected intraperitoneally with viable *M. bovis*, strain BCG, 8 days previously (day 0). Groups of BCG-infected mice were injected on days −1 to 3 with vehicle, bromocryptine [0.2 mg/day subcutaneously (SC)], bromocryptine [0.2 mg/day intraperitoneally (IP)] or bromocryptine (0.2 mg/day intraperitoneally) together with ovine PRL (0.1 mg/day intraperitoneally). Triplicate splenocyte suspensions (6×10^6 cells/ml) from pooled spleens of four mice per treatment group were cultured with or without (PPD) (50 μg/ml) (Connaught Laboratories) for 48 hours (*10*). Culture fluids were assayed for γ-IFN by an ELISA with recombinant murine γ-IFN as standard (*11*). Splenocytes of PPD-treated mice cultured without PPD released 30 ± 5 U/ml of γ-IFN. Error bars represent SEM of triplicate ELISA determinations each on duplicate cultures.

production of lymphokines required for macrophage activation, then such treatment should markedly increase susceptibility of mice to sublethal LM infection. Macrophage activation by lymphokines is required to contain and clear this bacterial infection (*11*). Groups of 23 male C3H/HeN mice, 4 to 6 weeks old, were inoculated with (i) bromocryptine alone (0.2 mg/day, daily, days 1 through 4); (ii) LM [0.2 LD_{50}, 4000 colony-forming units (CFU), intraperitoneally on day 1] plus vehicle days 1 through 4; (iii) LM plus bromocryptine; or (iv) LM plus bromocryptine plus ovine PRL (0.1 mg/day). As expected, no deaths occurred within 7 days in the group receiving bromocryptine alone. Survival on day 7 was 7 out of 23 in the LM plus bromocryptine group compared to 18 out of 23 in the LM plus vehicle group ($P < 0.01$, χ^2 test). Sixteen out of 23 survived in the group receiving bromocryptine plus ovine PRL ($P < 0.02$ compared to bromocryptine alone, χ^2 test). Thus, the suppression of PRL release by bromocryptine can significantly compromise host defenses and increase the lethality of an infectious challenge.

The results of these studies are consistent with recent reports that mixed-lymphocyte responses of splenocytes from bromocryptine-treated mice are suppressed (*6*), and that the thymic atrophy and suppressed splenocyte responses to T-cell mitogens in aged rats are reconstituted after implantation of a PRL-secreting pituitary tumor (*12*). We have recently found that treatment of mice with cysteamine, a drug that suppresses pituitary PRL secretion by a mechanism unrelated to dopamine agonists, also results in a suppression of splenocyte proliferative responses that can be reversed by exogenous PRL. This further suggests that in bromocryptine-treated mice, suppressed PRL secretion rather than some other action of the drug is responsible for immunosuppression. Thus our data indicate that, in mice, hypoprolactinemia impairs lymphocyte responses to antigenic stimuli (production of MAFs) central to host defense mechanisms in vivo.

These observations may be of clinical relevance. For example, it was recently reported that several patients with long-standing autoimmune uveitis underwent complete remission after incidental treatment with bromocryptine for unrelated conditions (*13*). Furthermore, in a rat model of autoimmune uveitis, bromocryptine greatly potentiates the immunosuppression resulting from treatment with low-dose cyclosporine (*14*). Drugs that stimulate PRL release can antagonize the immunosuppressive effects of cyclosporine in rodents (*6*). In primates, growth hormone (GH) also has PRL-like actions on classical PRL target tissues. The importance of PRL as an immunopermissive hormone in humans may therefore depend on whether or not human GH also has PRL actions on the immune system. We suggest that chronic stress, endogenous hormones such as estrogens and corticosteroids, and many pharmacotherapeutics (for example, opiates, neuroleptics, L-dopa, or bromocryptine), all of which affect PRL secretion, may alter immunologic function, perhaps with unrecognized adverse effects. Conversely, understanding the immunoregulatory effects of lactogenic hormones at the cellular level may lead to novel and useful methods for the modification of immune responses.

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9. Ovine PRL (NIADDK-oPRL-17) was supplied by A. F. Parlow. When 50-μg amounts were analyzed by SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue, 88% of the protein in this PRL formed a major band at M_r 23,000. Two minor bands, one at 52,000 and one at approximately 16,000, both stained with polyclonal antibody to human PRL on Western blots. Thus we could not detect non-PRL protein contaminating this preparation.
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