**Transgenic prolactin**−/−** mice: effect of trauma-hemorrhage on splenocyte functions**


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**Matsutani, Takeshi, T. S. Anantha Samy, Loring W. Rue III, Kirby I. Bland, and Irshad H. Chaudry. Transgenic prolactin**−/−** mice: effect of trauma-hemorrhage on splenocyte functions. Am J Physiol Cell Physiol 288: C1109–C1116, 2005. First published December 15, 2004; doi:10.1152/ajpcell.00478.2004.—Prolactin (PRL) is involved in the regulation of immune functions under normal and pathological conditions. Trauma-hemorrhage (T-H) produces profound immunosuppression in male mice but not in proestrus female mice. Administration of PRL in males after T-H, however, restores immune functions. In this study, PRL+/+ and transgenic (PRL−/−) male and female mice were used to assess immune suppression after T-H and to determine the reasons for the hormone’s beneficial effect. In vitro lymphoproliferation assay with Nb2 cells showed complete absence of PRL in the circulation of the transgenic PRL−/− mice of both sexes, whereas very high levels of the hormone were detected in the wild-type PRL+/+ mice of both sexes. Moreover, T-H resulted in the appearance of significant levels of the hormone in circulation, but only in PRL+/+ mice. Splenocyte proliferation in male PRL−/− mice was significantly lower than in PRL+/+ mice after T-H. Marginal differences between PRL+/+ and PRL−/− mice were observed in the release of IL-2 and IFN-γ by splenocytes, while the release of IL-10 was significantly higher in PRL−/− than in PRL+/+ mice. A significant observation of our study is the release of a ~25-kDa protein in the concanavalin A-stimulated splenocytes of male PRL+/+ and PRL−/− mice that was active in the in vitro lymphoproliferation assay with Nb2 cells. It is unlikely that this protein is PRL because it is also present in the splenocyte extracts of PRL+/+ transgenic mice. Nonetheless, because control of lymphoid cell proliferation is considered one of the characteristics of the immune system, the local release of this protein may be significant in the differences observed in splenocyte cytokine release after T-H in wild-type as well as transgenic mice.

**Nb2 cells; cytokines; immune functions**

**PROLACTIN (PRL), a protein hormone produced in the anterior pituitary gland, possesses diverse physiological functions that include reproduction, osmoregulation, and cell proliferation and survival (4, 8, 12). Although PRL is produced primarily in the pituitary, studies have shown its synthesis in other tissues also, including those associated with the immune system (3, 4, 38). Furthermore, extrapituitary cells express receptors for PRL, implying the ability of the hormone to modulate immune functions. This observation is substantiated by studies showing that PRL 1) acts as a growth factor for lymphocyte proliferation and function, 2) helps in the promotion of Th helper (Th1) response, 3) is involved in the regulation of mononuclear and polymorphonuclear leukocyte functions, and 4) plays a role in the suppression of thymic and T lymphocyte apoptosis (1, 6, 7, 10, 15, 20–22). Because of its participation in the regulation of immune functions, PRL has emerged as a potential target for intensive research in diseases in which immune dysfunction is apparent (3, 13, 34). Trauma-hemorrhage (T-H) causes severe and prolonged immunosuppression in young males (36, 39) but not in proestrus females, indicating gender dimorphism in the response to injury. Previous studies have also shown that administration of PRL or metoclopramide (a dopamine antagonist that increases PRL release) in male mice after T-H restores immune functions and decreases mortality from subsequent sepsis (17, 40–42). Besides PRL, administration of 17β-estradiol (18, 19) and the androgen receptor antagonist, such as flutamide (35), in males after T-H also has been shown to restore immune functions. Thus a close association between the endocrine and immune systems is apparent after T-H.

The restoration of immune functions in males by PRL or metoclopramide administration after T-H suggests an important role of this hormone in immune function after injury. However, the mechanism by which PRL restores immune function remains unclear. Thus evaluation of immunosuppression effects of T-H in male PRL−/− mice in which pituitary hormone release is expected to be absent may provide useful information concerning the role of this hormone in the response to injury. Studies were therefore performed to assess changes in the splenic T lymphocyte functions after T-H in the transgenic PRL−/− and wild-type PRL+/+ male and female mice. Significant changes in the release of IL-2, IL-10, and IFN-γ by splenocytes after T-H were observed, especially in the males of both wild-type and transgenic mice. The results demonstrate a release of ~25-kDa protein that was active in the proliferation of Nb2 cells in vitro by the concanavalin A (ConA)-stimulated splenocytes of both wild-type and transgenic mice. Because of the splenocyte release and the inherent lymphocyte stimulatory activity, the local release of the ~25-kDa protein may play a significant role in the changes in splenocyte function after T-H.

**MATERIALS AND METHODS**

**Animals.** Transgenic male (PRL−/−) and heterozygous female (PRL+/−) mice, provided by Dr. Nelson D. Horseman (University of Cincinnati, Cincinnati, OH), were housed and bred in a temperature-controlled room, kept on a 12:12-h light-dark cycle, had free access to standard laboratory chow and water, and were maintained as breeders in a pathogen-free animal facility. The severed tailpiece of the 4-wk-old litters was assessed by performing RT-PCR analysis for the expressivity of PRL. Animals were then assigned to experimental groups, which included a sham group (n = 20) and T-H group (n = 20). Animals were anesthetized with ketamine and xylazine, and the tail was raised and held between the fingers of a blunt forceps to bleed the posterior end of the tail. Blood was collected into heparinized syringes and allowed to clot for 2 h to obtain well-formed clots. The clots were centrifuged at 1600g for 15 min, and the plasma samples were separated and stored at -80°C until use. Plasma samples were serially diluted two-fold with PBS and assayed using a PRL enzyme-linked immunosorbent assay (ELISA) kit (Alpco), as per the manufacturer’s instructions. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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presence of the pituitary PRL (420 bp) or mutated (PGK-neo; 930 bp) genes in the breeder mice according to a previously described procedure (11, 14). The three oligonucleotide primers used in each PCR assay were neo-6, 5'-CCATACATGGCAGTCATGCAATGGC-3' and PRL4-2, 5'-CTCAACTCCCCCTGAAGCTTCG-3' and PRL3-2, 5'-CAAGAGACGATGATATACAC-3'

The results obtained from one such analysis of animals are shown in Fig. 1. The data show that the homozygous PRL+/+ mice exhibited only the 420-bp band, the homozygous PRL−/− mice exhibited only the 930-bp band, and the heterozygous PRL+/− mice exhibited both the 420- and 930-bp bands. PRL−/− and age-matched PRL+/− mice (C57BL/6 mice; The Jackson Laboratory, Bar Harbor, ME), 10-15 wk old and weighing 20–25 g, were used in the experiments. PRL−/− and PRL+/− mice, male or female, did not manifest any abnormal behavior. The major phenotypic changes resulting from target disruption of the PRL gene were observed only in the transgenic female mice. The estrus cycles of PRL−/− females were irregular, with longer or shorter duration, and these mice were infertile. Although the mammary glands of the mutant females developed a normal ductal tree, the ducts failed to develop lobular decorations. To the contrary, PRL-deficient mice were fertile. Primary lymphopoiesis and myeloopoiesis, however, were normal in both mutant mice (14). The females that were found to be in the proestrus stage on the basis of vaginal smear analysis were used in these studies. Animal experiments were conducted in accordance with guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH) and approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

**Experimental groups.** Animals were assigned to the following groups (6–8 mice/group): male PRL+/+ sham, male PRL+/+ subjected to T-H, male PRL−/− sham, male PRL−/− subjected to T-H, proestrus female PRL+/+ sham, proestrus female PRL+/+ subjected to T-H, proestrus female PRL−/− sham, and proestrus female PRL−/− subjected to T-H.

**Trauma-hemorrhage.** Animals were anesthetized with isoflurane (IsoSol; Vedco, St. Joseph, MO) and restrained in a supine position. A 2.5-cm midline laparotomy (i.e., induction of soft tissue trauma) was performed and then closed aseptically in two layers using 6-0 sutures (Ethilon; Ethicon, Somerville, NJ). Subsequently, both femoral arteries were aseptically catheterized with polyethylene 10 tubing (Clay-Adams, Parsippany, NJ), and the animals were allowed to awaken. Blood pressure was monitored continuously through one of the femoral catheters using a blood pressure analyzer (Digi-Med BPA-190; Micro-Med, Louisville, KY). Upon awakening, the animals were bled through the other catheter to a mean arterial pressure (MAP) of 35 ± 5 mm Hg and 95% ± 3.1 on June 12, 2017 http://ajpcell.physiology.org/Downloaded from
Briefly, a PCR assay was performed using 5 µl of the cDNA mixture. RT products were used in a total volume of 50 µl containing 5 µl of 10× PCR buffer, 10 µl of Q-Solution, 0.2 mM of each dNTP, 1.5 units of HotStar Taq polymerase (Qiagen), and 1 µM of each of the primers. PCR reactions were performed in a gradient Mastercycler (Eppendorf, Westbury, NY). For each PCR reaction, preheating was performed at 95°C for 15 min, then at 94°C for 40 s, at 55°C for 45 s, and at 75°C for 1 min, with a final extension phase at 72°C for 10 min. Each reaction was analyzed for amplification between 25 and 40 cycles. The amplification of β-actin (Clontech) was used as the housekeeping gene. The PCR products were analyzed using electrophoresis on 1.5% agarose gels in 1× TBE buffer. The intensity of the bands was measured in the 500 Fluorescence Chemilumines (Alpha Diagnostics). The relative absorbance of the PCR products was corrected against the absorbance obtained for β-actin.

**Splenocytes IL-2, IL-10, and IFN-γ release.** Splenocytes were cultured at 1 × 10⁶ cells/well in 24-well plates using serum-free RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 2.5 µg/ml ConA for 48 h at 37°C with 5% CO₂ and 90% humidity. IFN-γ and IL-2 were chosen as representative Th1 cytokines, whereas IL-10 was chosen as a representative Th2 cytokine. The levels of INF-γ, IL-2, and IL-10 in culture supernatants of stimulated splenocytes were determined using sandwiched enzyme-linked immunosorbent assays (ELISA; BD Pharmingen, San Diego, CA). After the incubation period, cell-free supernatants were harvested and kept frozen at −80°C until assayed for the cytokines.

**Analyses of splenocytes for PRL-like protein.** Splenocytes were cultured at 1 × 10⁶ cells/well in 24-well plates using serum-free RPMI 1640 medium constituted with the serum replacement medium (BD Nu-Serum serum replacements, which provide low-protein alternatives to newborn calf, fetal bovine, and other sera routinely used for cell culture; BD Biosciences, San Jose, CA) and 2.5 µg/ml ConA for 48 h at 37°C in an incubator in 5% CO₂ and 90% humidity. The protein content of the supernatant was determined by performing a colorimetric assay (Bio-Rad, Hercules, CA). For the immunoprecipitation reaction, the assay mixture consisted of an aliquot of the supernatant equivalent to 200 µg, 1 µg/ml rabbit polyclonal anti-mouse PRL antibody (provided by Dr. Albert F. Parlow, National Hormone & Peptide Program, NIH), and PBS to a final volume of 200 µl. After overnight incubation in a shaker at 4°C, 50 µl of 10% protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Uppsala, Sweden) were added. The reaction mixture was then shaken at room temperature for 2 h, centrifuged at 12,000 g for 1 min, and washed three times with PBS. The resulting pellet was suspended in 40 µl of 3× SDS sample buffer, boiled for 5 min at 95°C, and centrifuged at 12,000 g for 10 min, and then the supernatant was collected for electrophoresis. Splenocyte extracts (20 µg) and recombinant mouse PRL that acted as a positive control were loaded on a Nu-PAGE 12% Bis-Tris gel and electrophoresed at 200 V. Western blot analysis of the electrophoresed proteins was performed essentially using the procedure described above.

**PRL bioassay.** Plasma PRL as well as the protein from splenocyte extracts for in vitro proliferation activity was assayed in the laboratories of Dr. Arthur R. Buckley (University of Cincinnati, Cincinnati, OH) using the lymphoma Nb2 cell line (30). In brief, Nb2 cells were grown in 96-well tissue culture plates in the presence of either PRL standard (NIH sheep PRL-20, 31 IU/mg; a gift from the National Hormone and Pituitary Program) or the splenocyte extracts for 48 h at 37°C in a humidified atmosphere. Proliferation of Nb2 cells in the plates was quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye conversion assay. The minimum detectable level of PRL in this assay was 1 pg/ml. The minimum detectable level of PRL in this assay was 1 pg.

**Statistical analyses.** Data are presented as means ± SE for each group. One-way ANOVA for multiple comparisons, followed by Tukey’s or Dunn’s test, was used to determine the significance of the differences between experimental means. P < 0.05 was considered significant for all statistical analyses.

**RESULTS**

**Assessment of transgenic PRL⁻/⁻ mice for PRL activity.** The amount of PRL in the pituitary gland as well as in the circulation was determined to assess the status of hormone activity in the wild-type and transgenic mice. Analysis of plasma in vitro using Nb2 lymphoma cell proliferation assay (Table 1) indicated the absence of PRL in the circulation of male and proestrus female transgenic PRL⁻/⁻, while the wild-type PRL⁺/+ strains from both sexes showed the presence of the hormone in significant amounts. The hormone levels in the plasma of PRL⁺/+ proestrus females were 20-fold those of the PRL⁺/+ males. T-H significantly increased the plasma hormone levels about fivefold in both PRL⁺/+ males and females, but the increase in hormone levels of proestrus females could not be established, because the amount exceeded the assay detection limit. There was no change in the hormone levels of PRL⁻/⁻ males and proestrus females after T-H. In contrast, no such increase was observed in the plasma PRL of CS/HelN male mice after T-H in our previous study, in which assays were run using the ELISA kit for rats (21).

Western blot analysis of pituitary extracts from PRL⁺/⁺ and PRL⁻/⁻ mice revealed the absence of this protein in both sexes in sham control animals and after T-H are shown in Fig. 2. Male and female PRL⁺/⁺ showed the presence of a 25-kDa protein, identical in mobility to mouse PRL, in the pituitaries, whereas the corresponding transgenic PRL⁻/⁻ mice of both sexes showed the absence of this protein. There was no change in the PRL content of sham control animals and after T-H. In contrast, no such increase was observed in the plasma PRL of CS/HelN male mice after T-H in our previous study, in which assays were run using the ELISA kit for rats (21).

**Splenocyte proliferation in PRL⁺/⁺ and PRL⁻/⁻ mice.** ConA-stimulated proliferation of splenocytes in PRL⁺/⁺ and PRL⁻/⁻ male and female mice after sham treatment and after T-H is shown in Fig. 3. Splenocyte proliferation in proestrus female PRL⁺/⁺ and PRL⁻/⁻ sham mice was significantly lower compared with the male counterparts. Moreover, while there was no difference in the splenocyte proliferation between PRL⁺/⁺ and PRL⁻/⁻ males (sham controls), a significant ~50% decrease in proliferation was observed in proestrus females in

| Table 1. PRL levels in plasma 24 h after T-H determined in vitro by Nb2 lymphoma cell proliferation assay |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | Male            | Proestrus Female |                  |                  |                  |
|                                | PRL⁺/⁺          | PRL⁻/⁻          | PRL⁺/⁺          | PRL⁻/⁻          |                  |
|                                | S   | T-H | S   | T-H | S   | T-H | S   | T-H |
| PRL levels, ng/ml              | 9.8±3.6*       | 52.2±10.0       | ND             | ND             | 197.2±45.3      | >200.00         | ND             | ND             |

Data are means ± SE; n = 6–8/group. PRL, prolactin; S, sham, T-H, trauma-hemorrhage; ND, not detected. *P < 0.05.

AJP-Cell Physiol • VOL 288 • MAY 2005 • www.ajpcell.org
sham control PRL$^{-/^{-}}$ (vs. sham control PRL$^{+/+}$). T-H caused a significant decrease in splenocyte proliferation in males of both PRL$^{+/+}$ and PRL$^{-/^{-}}$ strains; the decrease was profound and significant ($P < 0.01$) in PRL$^{-/^{-}}$ mice compared with wild-type mice. In proestrus females, however, T-H produced an increase in proliferation only in the transgenic PRL$^{-/^{-}}$ mice, while there was no change in the wild-type PRL$^{+/+}$ strain.

Cytokine expression and release by splenocytes. The expression and release of IL-2 and IL-10 by splenocytes from PRL$^{+/+}$ and PRL$^{-/^{-}}$ male and female sham controls, and in those after T-H, are shown in Figs. 4 and 5. No significant changes in the expression of IL-2 and IL-10 by splenocytes were observed between the PRL$^{+/+}$ and PRL$^{-/^{-}}$ males, and T-H did not alter their expression. T-H led to decreased expression of IL-2, but not IL-10, in the female transgenic mice.

The release of IL-2 was unaltered between the PRL$^{+/+}$ and PRL$^{-/^{-}}$ male and female sham controls (Fig. 4B). Regarding IL-10, it increased approximately twofold in PRL$^{-/^{-}}$ male sham-treated controls (Fig. 5B). However, the effect of T-H on the release was different with respect to IL-2 (Fig. 4B). There was a significant decrease in IL-2 release in both PRL$^{+/+}$ and PRL$^{-/^{-}}$ males after T-H. IL-2 release also decreased in PRL$^{+/+}$ females after T-H; however, such a decrease was not observed in PRL$^{-/^{-}}$ females. The release of IL-10 after T-H in PRL$^{-/^{-}}$ males was similar to that observed in sham controls, in which an approximately twofold increase was observed (compared with PRL$^{+/+}$ male mice after T-H). There was no significant effect of T-H on the release of IL-10 in proestrus wild-type and transgenic females, although an apparent decrease in release was observed (Fig. 5B). However, in all of these investigations, the release of cytokines did not correspond with their expression. Although the decrease in the release of IL-2 was detectable in both PRL$^{+/+}$ and PRL$^{-/^{-}}$ females after T-H, the decrease was not statistically significant in T-H PRL$^{-/^{-}}$ proestrus females (Fig. 4B). It is of interest to note, however, that between male and female PRL$^{-/^{-}}$ mice, an increased release of IL-10 occurred in the male transgenic strain of animals, suggesting an opposite effect between the sexes (Fig. 5B).

Expression and release of IFN-$\gamma$ by splenocytes. There was no significant difference in IFN-$\gamma$ expression between PRL$^{+/+}$ and PRL$^{-/^{-}}$ in either male or female shams (Fig. 6A). T-H, however, caused increased IFN-$\gamma$ expression only in male wild-type and transgenic strains, but not in corresponding proestrus female strains (Fig. 6A). Similarly, there was no significant difference in the release of IFN-$\gamma$ between the strains in males and proestrus females (Fig. 6B). However, in contrast to what we observed with IFN-$\gamma$ expression, T-H led to decreased IFN-$\gamma$ release in PRL$^{+/+}$ and PRL$^{-/^{-}}$ male strains. The release of IFN-$\gamma$ by splenocytes in wild-type and
knockout males followed the opposite pattern regarding gene expression. The release of IFN-\(\gamma\)/H9253 by splenocytes of PRL\(+/+\)/H11001/H11001/H11001 and PRL\(-/-\)/H11002/H11002/H11002 females was not altered after T-H. Presence of an active lymphoproliferative protein in the splenocytes.

To assess whether the mouse splenocytes make a protein similar to PRL in activity, splenocytes were extracted with RPMI medium after ConA stimulation, and the extracts were reacted with NIH antiserum to mouse PRL. The immunoprecipitates were recovered by centrifugation of the reaction mixture, extracted with the disassociation buffer, assessed using Western blot analysis, and examined for activity by performing Nb2 lymphoma cell proliferation assay. The results of these experiments are shown in Fig. 7. Western blot analysis showed that the splenocyte extracts from both PRL\(+/+\)/H11001/H11001 and PRL\(-/-\)/H11002/H11002 male mice, with or without T-H, displayed a \(-25\)-kDa protein band with mobility similar to that of recombinant mouse PRL, which was used as a positive control. This \(-25\)-kDa protein was active in vitro in the proliferation of Nb2 cells assayed at \(-2.7\) ng/ml. Because this protein was also detected in the splenocyte extracts of the PRL\(-/-\) mice, it is not chemically or structurally similar to pituitary PRL. A chemical and biological characterization of the protein in relation to PRL is definitely necessary.

**DISCUSSION**

PRL is a mediator of the neuroendocrine-immune network whereby the nervous, endocrine, and immune systems interact with each other (23). It plays an important role in the regulation of cellular immune responses in physiological as well as pathological states, such as in autoimmune diseases (3, 13, 34). PRL may also be considered a cytokine because it reacts with its own cell surface receptor (PRLR), which is functionally similar to the cytokine or hematopoietic receptors (9, 16). Like cytokine receptors, PRLR transduces signals inside the cell with a wide variety of protein phosphokinases, which in turn activate the downstream effects. Generally, the cascade of events involve the Jak/Stat pathway, the Ras/Raf/MAPK pathway, and the Src tyrosine kinases (9, 16). Our previous studies using C3H/HeN mice showed severe depression of immune function in young males after T-H (39). The depression in immune function was associated with alterations in the lymphocyte and Kupffer cell functions, which were reflected in the inordinate release of pro- and anti-inflammatory cytokines. The beneficial effects of PRL on the restoration of immune function in males after T-H point out the hormone’s capability of modulating the immune system after injury. Because PRL can regulate lymphocyte function by a paracrine or autocrine process, the action of this hormone appears to be quite com-
plex. In addition, studies show PRL involvement in apoptosis of thymocytes, splenocytes, and lymphocytes in T-H as well as in sepsis (1, 15).

The PRL−/− mouse used in this study is a C57BL/6 transgenic mouse whose pituitary PRL gene is disrupted by homologous recombination with the neomycin cassette insert (14). Earlier studies to assess the effects of T-H on immune responses were performed with the C3H/HeN strain. Because PRL−/− transgenic mice of the C3H/HeN strain were not available, C57BL/6 transgenic mice were used in this study. Our recent studies have shown that C57BL6 mice were also immunodepressed, as were the C3H/HeN mice, after T-H, with the exception that the former were slightly hyperresponsive to T-H (25). The behavior of PRL−/− male and females was normal, except that the estrus cycle of transgenic females was irregular and they were infertile. PRL expression was absent in the pituitaries of transgenic male and female mice. Furthermore, circulatory levels of PRL in transgenic mice of both sexes were so low that they were not quantifiable using the in vitro Nb2 cell proliferation assay.

Previous studies of PRL−/− mice have indicated that PRL is not required for somatic growth, male reproduction, and the maintenance of homeostasis but that it is essential for reproductive and mammary gland development in adult females (11). Moreover, the absence of significant changes in the lymphocytes or in vitro proliferation activity using Nb2 lymphoma cells. Recombinant mouse PRL was the positive control. Data are means ± SE; n = 4/group. *P < 0.01 vs. sham control.

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The critical role of cytokines and growth factors in the regulation of immune functions after T-H is well known. However, IL-2 and IFN-γ deserve special attention because the former acts in vivo to constrain lymphoid growth by promoting apoptosis and maintain peripheral tolerance by promoting cell-mediated immunity (32). A decrease in these cytokines released by splenocytes is evident after T-H, implying a proinflammatory Th1 response. This observation is corroborated in the present study and shows that both IL-2 and IFN-γ release are significantly lowered in both the wild-type and transgenic strains. This is evident in males, but not in proestrus females, after T-H. Studies demonstrate PRL synergism with IL-2 in eliciting IFN-γ production as well as the Th1 response (24, 26, 44). Because PRL release is lowered after T-H, it is not surprising that release of IL-2 and IFN-γ decreases after T-H. The absence of such effects in proestrus females, in the wild-type as well as transgenic animals, may be due to 1) maintenance of estrogen release in the proestrus females after T-H (33), and 2) the effect of estrogen on the lymphocyte functions, either alone or in synergy with PRL.

Although the majority of the circulating PRL is of pituitary origin, attention is now focused on the local production of the hormone in peripheral tissues or cells. Studies have shown the release of proteins exhibiting activity similar to that of PRL in normal lymphoid cells (28–30). We therefore examined whether PRL-like proteins were released by splenocytes from PRL+/+ and PRL−/− mice. Male mice were used in these experiments because they are immunosuppressed after T-H and respond to PRL administration to restore immune functions. Our results show the release of a protein (~25 kDa) in the ConA-stimulated splenocytes in both wild-type and transgenic male mice. This protein exhibited lymphoproliferative activity in the in vitro Nb2 assay similar to that of PRL. This ~25-kDa protein is unlikely to be related in any way to pituitary PRL, because it is found in the splenocyte extracts of PRL−/− transgenic mice. Moreover, it is uncertain whether this ~25-kDa protein possesses other PRL functions, namely, the lactogenic and osmoregulatory function. Purification and chemical characterization of the ~25-kDa protein and analysis of it for PRL-associated functions should be investigated. Because lymphocyte proliferation is generally associated with immune func-

**Fig. 7.** Analysis of extracts from splenocytes of PRL+/+ and PRL−/− males using Western blot analysis and Nb2 lymphoma cell proliferation assays. Splenocytes were stimulated with ConA for 48 h at 37°C before extraction with serum-free RPMI 1640 medium. The same extracts were analyzed for the release of protein and for in vitro proliferation activity using Nb2 lymphoma cells. Recombinant mouse PRL was the positive control. Data are means ± SE; n = 4/group. *P < 0.01 vs. sham control.

In the present study, PRL−/− transgenic mice were also immunodepressed, as were the C3H/HeN mice, after T-H, with the exception that the former were slightly hyperresponsive to T-H (25). The behavior of PRL−/− male and females was normal, except that the estrus cycle of transgenic females was irregular and they were infertile. PRL expression was absent in the pituitaries of transgenic male and female mice. Furthermore, circulatory levels of PRL in transgenic mice of both sexes were so low that they were not quantifiable using the in vitro Nb2 cell proliferation assay.

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IL-10 in male PRL−/− of sham control mice and after T-H (Fig. 5B). The differences observed in the ConA-stimulated splenocyte proliferation and in the cytokine release by splenocytes of transgenic mice suggest that stress or inflammation resulting from T-H may be the reason for alterations in splenocyte function observed in PRL−/− mice of both sexes.

Inflammatory response is assessed by Th cell cytokine release. The response is measured by the release of Th1 cytokines, TNF-α and IFN-γ and Th2 by the release cytokine such as IL-10. The immune response after T-H is thought to produce a shift from Th1 to Th2 cytokines with the release of IL-10 and TGF-β. Studies have also shown that PRL deficiency enhances inflammatory responses in vitro and exhibits Th1-type cytokine-like effects (24, 37). The results of this study do not appear to indicate a Th1-like effect, because splenocyte IL-2 and IFN-γ release was lowered in both PRL+/+ and PRL−/− male mice after T-H, with no significant changes in release observed in corresponding females (Figs. 4B and 6B). Furthermore, there was no effect of T-H on the release of IL-10 to indicate a Th2 cytokine response.

The critical role of cytokines and growth factors in the regulation of immune functions after T-H is well known. However, IL-2 and IFN-γ deserve special attention because the former acts in vivo to constrain lymphoid growth by promoting apoptosis and maintain peripheral tolerance by promoting cell-mediated immunity (32). A decrease in these cytokines released by splenocytes is evident after T-H, implying a proinflammatory Th1 response. This observation is corroborated in the present study and shows that both IL-2 and IFN-γ release are significantly lowered in both the wild-type and transgenic strains. This is evident in males, but not in proestrus females, after T-H. Studies demonstrate PRL synergism with IL-2 in eliciting IFN-γ production as well as the Th1 response (24, 26, 44). Because PRL release is lowered after T-H, it is not surprising that release of IL-2 and IFN-γ decreases after T-H. The absence of such effects in proestrus females, in the wild-type as well as transgenic animals, may be due to 1) maintenance of estrogen release in the proestrus females after T-H (33), and 2) the effect of estrogen on the lymphocyte functions, either alone or in synergy with PRL.
tion, its release may be of significance in immune function after T-H. It should be pointed out that, similarly to PRL, IL-2 and IL-7 are also capable of stimulating Nb2 cells in vitro, and changes in the release of these cytokines are evident after T-H. The ~25-kDa protein appears not to be a cytokine, because it is prepared using an immunoprecipitation procedure with PRL antiserum. A detailed analysis of the ~25-kDa protein in relation to immune function is essential.

PRL is a paradoxical multifunctional hormone that is present in many discrete forms. It is produced in the pituitary as well as in other tissues and cells, including lymphocytes. PRL reacts with its own receptor, and its mechanism of action is similar to that of a cytokine or growth factor. Because the development and function of the immune system are normal in PRLR−/− and PRL−/− mice, PRL may not be an obligate lymphopoietic hormone. Previous studies conducted at our laboratory have demonstrated favorable effects of PRL in the restoration of immune function after T-H and decreased lethality as a result of subsequent sepsis (42). In contrast to T-H, Oberbeck et al. (31) showed that exogenous PRL increased mortality in a sepsis model. The reason why PRL decreases lethality as a result of sepsis induced after trauma but increases lethality resulting from sepsis per se, without prior T-H, is unclear. Because PRL receptor-knockout mice have normal immune function (5), it could be suggested that PRL may not play as important a role as previously suggested.

PRL secretion in the pituitary is under hypothalamic control. IL-1, IL-2, IL-6, and estrogen stimulate PRL production in the pituitary, while IFN-γ is inhibitory. Neurotransmitters released after T-H injury, such as catecholamines (epinephrine and norepinephrine) and dopamine, also are capable of regulating PRL release. Among these, dopamine is known to suppress the release of PRL. In fact, our earlier studies demonstrated salutary effects on the restoration of immune function in males after T-H by a dopamine antagonist, metoclopramide, suggesting the need for PRL in the maintenance of immune function after injury (14, 40). It is unclear whether local production of PRL in tissues, especially lymphocytes, is also influenced by cytokines, neurotransmitters, and sex steroids. The influence of mediators, especially the sex steroids, in the local synthesis and release of PRL has relevance to T-H because the administration of 17β-estradiol, similarly to PRL, restores the immune functions after T-H in male as well as ovariecotomized female mice (15–41). Unlike males, proestrus females are not immunosuppressed after T-H, owing to continued synthesis of 17β-estradiol in T lymphocytes even after injury (33). Because splenocytes, especially the T lymphocytes, have the intrinsic capability of producing cytokines, sex steroids (32, 33, 43), and PRL (2, 28, 29, 30) and, as observed in this study, also express their receptors, it is essential to know how they interact with each other in the maintenance of homeostasis under normal conditions and how the disruption of homeostasis occurs after stress or disease conditions. The interaction between estrogen and PRL in the regulation of immune function is critical because these hormones reciprocally stimulate each other (27). Both PRL and 17β-estradiol regulate immune function by interaction with their specific receptors present on the T lymphocyte surface. PRL influences the immune response, probably as a cytokine, because its receptor, PRLR, is a part of the cytokine hematopoietic receptor family. Thus PRL as an immunopotentiating agent may have a powerful therapeutic role in the treatment of patients after traumatic injury. Although much attention has been paid to the pituitary and circulatory PRL, none has been paid to the locally produced PRL or to proteins with PRL-like activity. Additional studies of the relationship between PRL-PRLR complexes, locally synthesized PRL, and PRL-like proteins and sex steroids in the regulation of the immune system after T-H may provide significant information concerning the potential of this hormone in the treatment of trauma patients.

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