

Effects of Prolactin Deficiency on Myelopoiesis and Splenic T Lymphocyte Proliferation in Thermally Injured Mice

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The importance of prolactin (PRL) in mammopoiesis and milk production is undisputed. However, previous studies investigating the role of PRL in immune function have yielded inconsistencies. These inconsistencies have led to our hypothesis that the immunomodulatory effects of PRL are only manifest under conditions in which the organism is subjected to stress. Thermal injury is a well-known stressor. The goal of this study was to determine whether the lack of PRL enhanced the negative effects of thermal injury-induced immune alterations utilizing a mouse model in which the PRL gene had been disrupted. Mice received either sham or burn treatment, and were sacrificed 4 days later. The immune parameters studied were the capacity of bone marrow cells to form granulocyte-macrophage colony forming units (GM-CFU) in the presence of granulocyte-macrophage colony stimulating

factor, and the ability of the splenic T lymphocytes to proliferate in response to phytohemagglutinin (PHA). As shown by others, our results reveal that burn increased the number of GM-CFU compared to sham controls; however, this elevation was only significant in the PRL^{-/-} mice. Thermal injury increased PHA-stimulated proliferation of splenic T lymphocytes, however this increase was only significant in the PRL^{+/-} group. We conclude that under conditions of a controlled stress event (thermal injury) [a] the increase in the GM-CFU is exaggerated in the absence of PRL, and [b] the enhancement of PHA-induced proliferation of splenic lymphocytes required PRL. This study supports the hypothesis that the immunomodulatory effects of PRL are manifest when the organism is subjected to stress.

The hormone prolactin (PRL) has been implicated as an immunomodulatory molecule; however, the effects of PRL on the immune system have produced conflicting results. Part of the reason for the inconsistencies is due to differences in study design (1-9). It has been proposed that PRL mediates its immune modulating effects only under conditions in which the organism is subjected to stress (10). Utilizing a PRL^{-/-} mouse, we tested this hypothesis with thermal injury serving as the stressor.

In spite of modern therapeutic advances, sepsis remains a significant cause of death following thermal injury. Severe thermal injury negatively affects immune system function (11-12). These immunosuppressive effects are multiple, affecting both humoral and cell-mediated immunity (13). Most notably, thermal injury alters myelopoiesis, and the proliferative ability of T Helper-1 lymphocytes (Th1) in response to antigenic or mitogenic stimulation (12, 14-18).

Given the propensity for the thermal injury-induced immunosuppression to result in sepsis, many potential therapeutic interventions have been investigated (19). The effects of PRL
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in thermal injury-induced immune sequelae has not been documented. The goal of this study was to elucidate the consequences, if any, that the lack of PRL has on the immune system of thermally injured mice. This study focused on two immunological parameters, bone marrow myelopoiesis and splenic lymphocyte response, in which previous studies have documented important thermal injury effects (12, 14-18, 20). The results presented show that PRL produced significant immunomodulatory effects on both myelopoiesis and T cell proliferation in thermally injured mice.

Materials and Methods

Mice

Experiments were conducted using 6-week old male C57Bl/6 mice at Shriners Hospital for Children, Cincinnati Burn Institute, in barrier housing within AAALAC-accredited animal quarters under protocols that were reviewed and approved by the Institutional Animal Care and Use Committee. The use of thermally injured animal models has been studied and supported by the Research Committee of the American Burn Association (21). The mice had free access to food and water. They were either +/- or -/- genotype with respect to the PRL gene. The mice were randomized into 4 groups: sham^{+/-}, sham^{-/-}, burn^{+/-}, or burn^{-/-}.

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There were 5 mice per group, with the exception of the sham-/- group, which had only 4 mice. Generation of the PRL-/- mouse is discussed elsewhere (4). On the day of treatment, mice were shaved on their backs while awake. All mice received 3% Isoflurane at 1L/min for 10 minutes. Their backs were covered with a fireproof template, which allowed for a controlled full skin thickness burn on an area equivalent to 30% of the total body surface area. The burn group was doused in the treatment area with 1ml of 100% ethanol, followed by a flame-induced burn for 10 seconds. All mice then received 0.5 ml of saline intraperitoneally for fluid resuscitation. To avoid additional experimental variables such as analgesics or secondary non-burn stresses, mice that showed any signs of distress after the burn treatment (lethargy, anorexia, scratching at wound, etc.) were eliminated from the study and humanely euthanized by CO₂ asphyxiation. The sham treated group received the same treatment as the burn group, excluding the alcohol dousing and flame burn. The sham and burn treated mice were kept in separate cages. Four days after treatment, all mice were sacrificed by cervical dislocation; their abdomens and one leg were shaved. The following samples were harvested from each mouse: approximately 100 μ l of blood from the heart and inferior vena cava, the spleen, and one femoral bone with muscle and tissues removed.

Blood serum

The blood serum was isolated and then stored at -80°C until the PRL Nb2 bioassay and corticosterone radioimmunobinding assays could be performed to measure serum PRL and corticosterone levels respectively. The PRL Nb2 bioassay procedure is detailed elsewhere (22). The corticosterone concentrations were assayed by a commercial solid-phase radioimmunoassay (RIA) which was performed by the Endocrinology Laboratory in the Diagnostic Lab, at the College of Veterinary Medicine, Cornell University, Ithaca NY.

Femoral bone marrow cells

The femoral bone was cut at both ends and the marrow cavity was flushed with 3ml of ice cold PBS. The cell suspension was filtered through a 100 μm nylon mesh (Falcon, Becton Dickinson), washed with an additional 2ml of cold PBS, and centrifuged 10 minutes at 400 \times g and 4 $^{\circ}\text{C}$. Following centrifugation, the pellet was resuspended with 1ml of IMDM 2% FBS (2.5ml penicillin/streptomycin 125U/ml and 125 $\mu\text{g}/\text{ml}$ final, 1.25ml gentamycin 125 $\mu\text{g}/\text{ml}$). Cells were counted via trypan blue exclusion using a hemocytometer. The desired cell number was 3×10^4 cells/300 μl total volume (using IMDM 2% FBS as the media). To each cell sample was added 3ml of Methocult (2.4ml M3234, 2.4 μl GM-CSF + 597.6 μl IMDM 2% FBS; each 3ml tube contained GM-CSF at 20 ng/ml). The mixture was subsequently plated in a 35 X 10mm culture treated well (Falcon, Becton Dickinson). The culture was then incubated 5-7 days at 37 $^{\circ}\text{C}$ and 5% CO₂. Following incubation, the number of GM-CFU were counted using a 20X dissecting microscope. Aggregates containing 50 or more cells were counted as colonies.

Splenocyte proliferation

The spleen was crushed between 2 sterile frosted glass slides in 2ml of ice cold PBS. The cell suspension was filtered through a 100 μm nylon mesh (Falcon, Becton Dickinson) followed by centrifugation at 400 \times g and 4 $^{\circ}\text{C}$ for 10 min. After centrifugation, the pellet was resuspended in 1ml of DMEM/F-12 (10% FBS + penicillin/streptomycin 2.5ml/100ml, gentamycin 1.25ml/100ml). Cells were counted via trypan blue exclusion using a hemocytometer. The desired cell number was 500,000 cells/200 μl total volume using DMEM/F-12 as media. The cell suspension was treated with one of 4 conditions: 1) no-mitogen, 2) PHA 2 $\mu\text{g}/\text{ml}$, 3) lipopolysaccharide (LPS) 10 $\mu\text{g}/\text{ml}$, 4) *Staphylococcus aureus* Cowan (SAC). Each cell suspension was then placed in a 96 well non-culture treated plate (Falcon, Becton Dickinson). Each condition was cultured in replicates of 5. The plate was then incubated for 48 h at 37 $^{\circ}\text{C}$ and 5% CO₂. After 48 hours, 1 μCi of 3[H]thymidine at 1 $\mu\text{Ci}/50 \mu\text{l}$ of DMEM/F-12 was added to each well. Subsequently, the plate was incubated for 16 h at 37 $^{\circ}\text{C}$ and 5% CO₂. Following incubation, the cells were harvested onto fiberglass filters that were dried and placed in vials filled with scintillation fluid. Beta- decay was detected by liquid scintillation counting.

Statistics

Comparisons among multiple treatment groups were made by Analysis of Variance (Two-Way, or One-Way with Tukey's test between groups). Comparisons between two means were performed by Student's t-test. For the standard curves, linear regression analysis was used.

Results

Serum prolactin and corticosterone concentrations 4 days after sham or burn treatment

Serum PRL was undetectable in the PRL-/- mice, and averaged 25 ng/ml in the PRL+/- mice. Serum PRL levels in burn injured PRL+/- mice were not significantly different from sham treated controls. Corticosterone levels were similar in all groups, averaging 154 ng/ml, indicating that neither the PRL genotype nor burn injury altered basal corticosterone levels on day 4.

Bone marrow GM-CFU following thermal injury (Figure 1)

To determine the effect of PRL on myelopoiesis following thermal injury, we cultured bone marrow cells (3×10^4 cells/300 μl) for 5-7 days in the presence of GM-CSF (20 ng/ml). Cells from thermally injured mice exhibited a highly significant ($p < 0.01$) increase in the numbers of GM-CFU compared to the sham-treated group, which is consistent with previous findings (14, 17-18, 23). Notably, the burn-induced increase in GM-CFU numbers was elevated more than 2-fold in the PRL-/- mice, compared to the PRL+/- mice ($p < 0.05$). These results indicate that the effects of thermal injury on bone marrow GM-CFU formation is exaggerated in the absence of PRL.

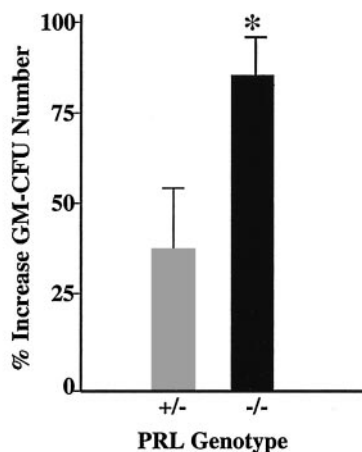


FIG. 1. Clonogenic assay for the quantitation of GM-CFU of bone marrow cells from thermally injured mice. Bone marrow cells (3×10^4 cells/300 μ l) were incubated in the presence of GM-CSF (20 ng/ml). Colonies were counted after 5–7 days. Thermal injury elicited an increase in GM-CFU compared to sham-treated mice (not shown). The increase was > 2-fold higher in the PRL $^{-/-}$ mice compared to PRL $^{+/-}$ mice, * $p < 0.05$.

Splenocyte proliferation responses (Figure 2)

Non-adherent cells from the spleen were studied to investigate the relationship between thermal injury and the effects of PRL deficiency on lymphocyte proliferation. Splenic cultures were exposed to one of three mitogens: LPS, SAC, and PHA, which act primarily on macrophages, B cells, and T cells respectively. PHA-induced proliferation was dramatically affected by both burn injury and PRL. In the PRL $^{+/-}$ mice, burn resulted in a 5-fold higher PHA-induced proliferative response compared to splenocytes obtained from sham-treated controls ($p < 0.004$), however, the proliferative response to PHA was not significantly elevated in the burned PRL $^{-/-}$ mice compared to sham-treated controls. There was not a significant difference in the PHA-induced proliferative response among the sham controls (PRL $^{+/-}$ vs. PRL $^{-/-}$) (Fig. 2). These data imply that under conditions of thermal injury-induced stress, PRL enhances PHA-induced proliferation of splenic T lymphocytes. Neither LPS nor SAC stimulated proliferation above that seen in the no-mitogen controls, and there were no significant differences among the various treatment groups (data not shown).

Discussion

It has been shown that stressors, in addition to activating the neuroendocrine system, also alter immune system function (24). PRL has been implicated as an immunomodulatory hormone, but previous studies have focused on unstressed ani-

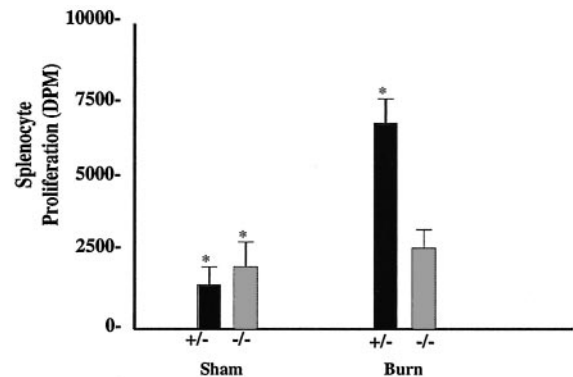


FIG. 2. Proliferation rates of splenocytes (500,000 cells/200 μ l) cultured 48 h in the presence of PHA (2 μ g/ml), followed by 16 h incubation in 1 μ Ci 3[H]thymidine. Assay was performed in replicates of 5. The results were analyzed via Two-Way ANOVA. Burn injury resulted in a 5-fold higher proliferation rate in the PRL $^{+/-}$ mice compared to the sham controls, * $p < 0.004$; no significant differences were seen among the sham controls, or between burn PRL $^{-/-}$ mice and sham controls.

mal models, poorly controlled stress conditions, or in vitro paradigms (10). Utilizing PRL $^{-/-}$ mice, we sought to evaluate the role of PRL in immune system function in animals subjected to a well-understood stressor. Thermal injury is known to act as an acute stressor in rodents (25) and as an acute and chronic form of stress in humans (26–27).

The use of the PRL $^{-/-}$ mouse model has several advantages over other models of PRL deficiency. Disruption of the PRL gene by homologous recombination resulted in the expression of a truncated transcript that encodes the synthesis of an N-terminal 11Kda fragment that has no PRL bioactivity, but which could conceivably have unknown actions (4). In most previous studies, PRL deficiency has been induced by surgical or pharmacological methods, which alter or eliminate pituitary PRL (28). However, it is possible that PRL also acts via an autocrine and/or paracrine mechanism, since PRL is synthesized by cells within the immune system and has been reported to regulate immune cell function (29). The dopamine 2-receptor agonists and antagonists may not affect these extrapituitary PRL sources. The PRL receptor (PRLR) $^{-/-}$ mouse model is another useful genetic model of defective PRL signaling (2). The effects of stress, acute or chronic, on these extrapituitary sources of PRL and/or PRLR expression levels is currently unknown.

Both PRL and glucocorticoids are known to be elevated following thermal injury (25–26, 30–32). We found in this study that neither corticosterone nor PRL levels were significantly elevated in thermally injured mice compared to sham controls 4 days after burn. These results are in agreement with other burn studies performed on rodents (25, 31), but contrast with those

shown in thermally injured human patients, which exhibit sustained elevations (26, 30). This indicates that thermal injury, while acting as an acute stressor in rodents, elicits a more chronic form of stress in humans. The role of PRL in immune system function under chronic stress warrants further research.

It is well known that thermal injury leads to an increased ability of bone marrow cells to form GM-CFU when cultured in the presence of GM-CSF (14, 17, 18, 23). The ability to form CFU when cultured in the presence of a specific growth factor such as GM-CSF is dependent on environmental factors such as other cytokines present in the culture (11, 13, 18). With regard to the role of PRL in myelopoiesis, it has been shown that about 80% of bone marrow cells, which includes all hematopoietic lineage precursors, express high levels of PRL receptors (33). The significance of these receptors has not been elucidated. A prior study utilizing the PRL^{-/-} mouse model concluded that PRL does not play a role in the formation of GM-CFU (4), which is in agreement with the data obtained utilizing PRLR^{-/-} mice (2). However, neither study was performed in association with a controlled exogenous stress. The current study is the first to address the effects of PRL on myelopoiesis under a controlled stress condition. The role of PRL in myelopoiesis is still unclear; however, our studies indicate that PRL absence significantly increased the stimulatory effect of burn injury on GM-CFU (Fig. 1). This observation does not address any direct effects that PRL might have on hematopoiesis, but it implies that the normal secretion of PRL, either steady-state levels or post-stress elevation, significantly attenuates the response of bone marrow cells to cytokines released in response to a burn injury.

About 70% of T cells express PRL receptors (34). Also, murine splenocytes induced by a T cell mitogen have been shown to produce PRL (29, 35). Many studies have been performed to determine the effect of PRL on T lymphocyte function. While some studies (6, 36) indicate that PRL stimulates splenic T-cell proliferation, most studies have shown that PRL does not directly influence proliferation of lymphocytes obtained from the spleen (2, 3, 8, 9). The sham-treated controls in our study are consistent with the latter findings. The proliferation rates measured in the burn groups showed an increase in the PHA-induced proliferation compared to the sham groups. However, the burn effect was only significant in the burn PRL^{+/-} mice, $p < 0.004$ (Fig. 2). The results from our study design suggest that thermal injury activates as yet unidentified stimulatory factor(s), which enhance the PHA-induced proliferation. This was only found to be significant in the PRL^{+/-} mice, which implies that PRL may be required for the activation of this unknown stimulatory factor(s).

Our study is the first to evaluate the effect of PRL on the immune system under a controlled stress condition. It is noteworthy that PRL deficiency did not manifest any significant effect on the immune parameters studied when comparing sham +/- to sham -/- mice. This is consistent with other studies involving non-stressed organisms (2, 4). The immunomodulatory effects of PRL were only observed when stressed animals were com-

pared to sham controls. This supports the hypothesis that PRL has a positive influence on the immune system, which is manifest when the organism is subjected to stress. Our results in this current study apply to acute stress circumstances because corticosterone and PRL levels were normal on day 4 after burn when the blood was harvested. Additional studies are needed to address the effect of PRL deficiency during chronic stress.

In conclusion, our results implicate a role for PRL in modulating myelopoietic aberrations seen following thermal injury. The precise mechanism by which PRL attenuates the increase in GM-CFU numbers normally seen following thermal injury remains to be elucidated. In addition, the apparent stimulatory factor(s) of T cell proliferation induced by burn in our study design needs further investigation. Because of the necessity for caution when extrapolating the results of animal studies to those expected in human patients, the use of PRL as a therapeutic agent for human burn patients warrants further study.

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