Age-Related Effects of Ectopic Pituitary Transplants on the Activation of Ames Dwarf Mouse Lymphocytes *In Vitro* (43956)

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Abstract. **Prolactin (PRL), one of the anterior pituitary hormones, has been implicated in the development and maintenance of immune system function. The following experiments were conducted to evaluate age-related effects of PRL on immune system activity in a PRL-deficient mouse model, the Ames dwarf. Two- and ten-month-old dwarf and phenotypically normal male mice were used. Six dwarf mice from each age group received a surgically implanted pituitary (under the kidney capsule) from a normal donor female mouse. Six different dwarfs were similarly operated but had no pituitary graft inserted and will be referred to as sham-operated dwarfs. Three weeks following surgery, spleens were removed from the dwarfs and age-matched normal mice and splenocytes isolated. The two age groups will subsequently be referred to as "3-month-old" and "11-month-old", respectively. The splenocytes were used in mitogen-induced (concanavalin A [Con A]; phytohemagglutinin [PHA]; lipopolysaccharide [LPS]) proliferation assays, and histopaque isolated lymphocytes were used for T cell surface marker determination. Pituitary grafting increased body weights of dwarf mice although the weights were less than control mice independent of age. A similar pattern was observed for total number of splenocytes per spleen. In young animals, the relative number of splenocytes per gram of body weight increased in pituitary grafted dwarfs reaching values of control mice, whereas much smaller differences were observed in older animals. The relative percentage of CD_4+ cells was reduced ($P < 0.01$) in 3-month-old pituitary-grafted mice, compared with sham-operated dwarf mice, while no differences were observed between sham-operated dwarf and normal mice. Pituitary grafting did not affect the numbers of CD_8+ cells. In 11-month-old animals, the relative percentages of CD_4+ and CD_8+ cells were greater ($P < 0.05$) in sham-operated dwarf than in normal mice but not affected by pituitary grafting. At 3 months of age, proliferation of splenocytes in response to Con A was increased ($P < 0.05$) in sham-operated dwarfs and reduced ($P < 0.05$) in pituitary-grafted dwarfs compared with normal mice. In contrast, PHA stimulation of splenocytes was decreased ($P < 0.05$) in sham-operated compared with normal, and was still lower in mice receiving an ectopic pituitary. Substantially different responses were obtained in 11-month-old animals. In response to Con A, splenocytes from sham-operated dwarfs exhibited a reduced ($P < 0.05$) proliferative capacity as compared with normals, and proliferation was increased in pituitary-grafted as compared with sham-operated dwarfs. In the presence of PHA, the proliferative capacity of splenocytes was greater ($P < 0.05$) in sham-operated dwarfs as compared to normals and pituitary grafting normalized this parameter. These results demonstrate differential effects of PRL deficiency (sham-operated dwarfs versus normal) and of PRL replacement (pituitary-grafted versus sham-operated) in young as compared with old dwarf mice on immune system activation.**

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There is increasing evidence implicating prolactin (PRL) and growth hormone (GH) in the control of development and maintenance of the immune response (1, 2). However the mechanisms involved are not fully understood. Both cellular and humoral responses are deficient in hypophysectomized rats and administration of either PRL or GH restores immunocompetence (3). Hereditary defi-

ciency of GH, PRL, and thyroid-stimulating hormone in dwarf mice (4–6) is associated with impaired immune responses (7). This was reported to lead to drastically shortened life span (8), although subsequent studies have shown that dwarf mice can survive at least as long as their normal siblings if they are weaned later than 21 days of age (6). Recent studies confirmed immunodeficiency of both Snell (Snell-Bagg) and Ames dwarf mice regardless of the age of weaning (9, 10). We have previously reported that ectopic transplants of pituitaries from normal mice increased thymus and spleen cellularity in Ames dwarf mice (10) and that PRL and GH interact to regulate immunocompetence in these animals (11). Data obtained in different experimental models indicate that PRL can exert inhibitory effects on immune activity as well (12, 13; Brown-Borg *et al.*, submitted). Recent findings from our laboratory suggest that the effects of PRL on the activity of the immune system are age dependent (13). The present study was undertaken to analyze age-related effects of PRL on immune system activity in the Ames dwarf mouse.

Materials and Methods

Animals and Treatment. Ames dwarf (df/df) and phenotypically normal (?/+) male mice from a stock maintained in our vivarium were used in all studies. The animals were adults, approximately 2 or 10 months of age. The mice were maintained in the same room at a temperature of 23° ± 2°C, were exposed to a regulated 12:12-hr light:dark lighting regimen, and were provided with food (Purina Lab Chow, Purina Mills, St. Louis, MO) and water *ad libitum*. Six dwarf mice from each age group were anesthetized with ether and had one pituitary gland from a normal adult female of the same strain surgically placed under the capsule of the right kidney. The remaining dwarf mice were similarly anesthetized and operated, but no pituitary graft was inserted. These latter animals will be subsequently referred to as sham-operated dwarf mice. Twenty-one days later, both groups of dwarfs along with untreated age-matched normal male mice (n = 6/group) were sacrificed via cervical dislocation and the spleen was quickly removed under sterile conditions (using institutionally reviewed and approved protocols). The retention of the pituitary graft was visually confirmed. In subsequent sections of this paper, the two age groups will be referred to as "3-month-old" and "11-month-old," respectively.

Cell Preparation. The spleens were removed aseptically and dispersed into a single cell suspension by passage through a stainless steel mesh. Cells were suspended in phosphate-buffered saline (PBS). The red blood cells were lysed with 0.83% Tris-buffered ammonium chloride solution for 2 min at room temperature and the viable cells spun through fetal bovine serum (FBS; viability determined via trypan blue exclusion: >95%). Erythrocyte depleted cells were washed three times with cold PBS and counted.

Proliferation Responses to Mitogens. For studies of proliferation, erythrocyte depleted spleen cells were resuspended in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat inactivated FBS (Hyclone, Logan, UT), 50 μg/ml of gentamycin and 5 mM of 2-mercaptoethanol. Lymphocytes from each spleen were adjusted to a concentration of 5×10^6 cells/ml. One hundred microliters of these suspensions were added to wells of 96-well flat-bottomed microtiter plates containing predetermined optimal concentrations of mitogens for T cells (2.5 μg/ml of concanavalin A [Con A; Sigma] and 5 μg/ml of phytohemagglutinin [PHA; Sigma]) and B cells (10 μg/ml of lipopolysaccharide [LPS; Sigma]). The cells were harvested at 48 hr following a 5-hr pulse with 1 μCi of [^{3}H]thymidine. The average incorporation of [^{3}H]thymidine was determined using a scintillation counter. The proliferative indices were calculated by dividing the cpm of mitogen stimulated wells by the corresponding cpm values from wells with basal proliferation.

Determination of T Cell Surface Markers. For these studies, monoclonal antibodies (mAb) GK 1.5, which is reactive with L3T4, a mouse T helper cell differentiation antigen, and 53–6.7, which is reactive with Lyt-2 antigen expressed on mouse T cytotoxic/suppressor cells were purchased from Becton Dickinson (Mountain View, CA). Purified rat IgG2a and IgG2b (Pharmingen, San Diego, CA) were used for the assessment of nonspecific binding of rat monoclonal antibodies to mouse surface antigens. The unlabeled mAb were used in an indirect immunofluorescence assay with goat anti-rat IgG labeled with fluorescein isothiocyanate (FITC; Accurate Chemicals, Westbury, NY). Lymphocytes isolated on histopaque (Sigma) gradients were washed in cold PBS with 0.02% sodium azide, and incubated with appropriate primary antibodies for 30 min at 4°C, using 1×10^6 cells/tube. Following two washes, the cells were incubated with fluorescein-conjugated goat anti-rat IgG for 30 min at 4°C, washed three times, resuspended in 1% paraformaldehyde in PBS and analyzed using FACS Vantage (Becton Dickinson).

Statistical Analysis. The results are reported as means ± SEM. Significance of the differences between groups was calculated by a two-way analysis of variance, followed by a Fisher's protected least significant difference test; using $P < 0.05$ as criteria for statistical significance.

Results

In the 3- or 11-month-old age groups, body weights significantly increased after pituitary grafting ($P <$

0.05) but did not reach values of normal mice at either age (Table I). Spleen cellularity increased after pituitary grafting in 3-month-old Ames dwarf ($P < 0.05$) as compared with sham-operated control dwarf mice (Table II). Relative splenocyte number per g body weight significantly increased in 3-month-old pituitary grafted dwarfs ($P < 0.05$) as compared with sham-operated dwarfs reaching values observed in normal mice (Table III). Pituitary grafting increased the relative number of splenocytes per gram body weight also in 11-month-old dwarfs but this effect was numerically very modest (Table III).

In the 3-month-old age group, the relative percentage of CD_4+ lymphocytes was significantly reduced ($P < 0.01$) in pituitary-grafted dwarf mice when compared with either sham-operated dwarfs or normal mice (Fig. 1). No differences in the relative percentage of CD_4+ lymphocytes were observed between sham-operated dwarf and normal animals (Fig. 1). The relative percentage of CD_8+ cytotoxic T cells in dwarf mice was not affected by pituitary transplants (Fig. 1). However, the relative percentage of CD_8+ cells was significantly ($P < 0.05$) lower in sham-operated dwarf than in normal mice (Fig. 1).

The results obtained in 11-month-old animals were substantially different. The relative percentage of CD_4+ lymphocytes was greater ($P < 0.05$) in sham-operated dwarfs than in normal mice and was not significantly affected by pituitary grafting (Fig. 2). The relative percentages of CD_8+ cells were similarly increased in both groups of dwarf mice ($P < 0.01$) as compared with normals and not altered by treatment with pituitary transplants (Fig. 2).

In 3-month-old animals, the basal proliferative capacity of splenocytes was significantly greater ($P < 0.05$) in sham-operated dwarfs than in normal mice (Table IV). Pituitary transplants reduced this capability to values lower ($P < 0.05$) than those found in age-matched normal mice (Table IV). The blastogenic proliferation in the presence of Con A was increased ($P < 0.05$) in sham-operated dwarfs as compared with normal mice, and reduced in pituitary grafted dwarfs as compared with either sham-operated dwarf ($P < 0.05$) or normal ($P < 0.05$) mice. In contrast, the PHA-stimulated proliferative capacity was lower in sham-operated dwarfs ($P < 0.05$) as compared with normals (Table IV) and was further reduced by treatment of dwarf mice with an ectopic pituitary transplant. Blastogenic proliferation of B lymphocytes did not differ between sham-operated dwarfs and normal mice but was ($P < 0.05$) decreased in pituitary-grafted as compared with sham-operated or normal animals (Table IV).

Similarly to the results obtained in young animals, the basal proliferative capacity of splenocytes was increased ($P < 0.05$) in 11-month-old sham-operated dwarfs as compared with normal mice (Table V). However, treatment with pituitary transplants further increased, rather than decreased this parameter ($P < 0.05$ vs sham-operated dwarf and $P < 0.01$ vs normals; Table V). The effects of hereditary dwarfism and the effects of pituitary transplants on proliferative responses to mitogens were also drastically different in 11- as compared to 3-month-old animals. The blastogenic proliferation in 11-month-old animals stimulated by Con A was lower ($P < 0.05$) in sham-operated dwarfs than in normals. Proliferation was increased in pituitary-grafted as compared with sham-operated dwarfs but without reaching the values measured in normal mice (Table V). In the presence of PHA, the proliferative capacity was significantly greater in sham-operated dwarfs as compared with normal mice ($P < 0.05$, Table V) and pituitary grafting normalized this parameter. Similarly, the blastogenic proliferation of B lymphocytes was increased in sham-operated dwarfs as compared with normal mice ($P < 0.01$) and normalized by the presence of an ectopic pituitary (Table V).

In 3-month-old sham-operated dwarfs, the proliferative index in response to Con A was reduced as compared with normal mice ($P < 0.05$) and markedly increased by treatment of dwarf mice with pituitary transplants ($P < 0.05$ vs normals and vs sham-operated dwarfs; Fig. 3). In the presence of PHA, the proliferation index was lower ($P < 0.05$) in sham-operated dwarf than in normal mice while dwarf mice bearing an ectopic pituitary did not differ from normal mice with respect to this parameter (Fig. 3). The proliferative indices of B lymphocytes (LPS stimulation) were comparable to those observed for Con A. A reduction was observed in sham-operated dwarfs as compared with the values measured in normal mice ($P < 0.05$) and a marked increase was found following treatment of dwarf mice with ectopic pituitary transplants (Fig. 3).

In 11-month-old animals, the proliferation index in the presence of Con A was lower in sham-operated

Table I. Body Weights (g) of 3- and 11-Month-Old Normal, Sham-Operated Dwarf and Pituitary-Grafted Dwarf Mice

Age	Normal	Sham-operated dwarf	Pituitary-grafted dwarf
3 months	32.0 ± 1.2^a	12.0 ± 1.2^b	16.6 ± 1.1^c
11 months	45.0 ± 1.5^d	25.0 ± 3.0^e	33.1 ± 1.0^f

Note. Values represent mean ± SEM. Rows and columns lacking a common letter differ ($P < 0.05$).

Table II. The Number of Splenocytes ($\times 10^6$) in 3- and 11-Month-Old-Normal, Sham-Operated Dwarf and Pituitary-Grafted Dwarf Mice

Age	Normal	Sham-operated dwarf	Pituitary-grafted dwarf
3 months	8.0 ± 0.7^a	1.8 ± 0.4^b	4.0 ± 0.3^c
11 months	4.5 ± 2.0^b	2.0 ± 0.8^b	$3.1 \pm 0.2^{b,c}$

Note. Values represent means ± SEM. Rows and columns lacking a common letter differ ($P < 0.05$).

Table III. The Number of Splenocytes per Gram Body Weight ($\times 10^4$) in 3- and 11-Month-Old Normal, Sham-Operated Dwarf and Pituitary-Grafted Dwarf Mice

Age	Normal	Sham-operated dwarf	Pituitary-grafted dwarf
3 months	25.0 ± 0.6^a	15.0 ± 0.3^b	24.0 ± 0.3^a
11 months	10.0 ± 1.0^c	8.0 ± 0.3^d	9.0 ± 0.2^c

Note. Values represent means ± SEM. Rows and columns lacking a common letter differ ($P < 0.05$).

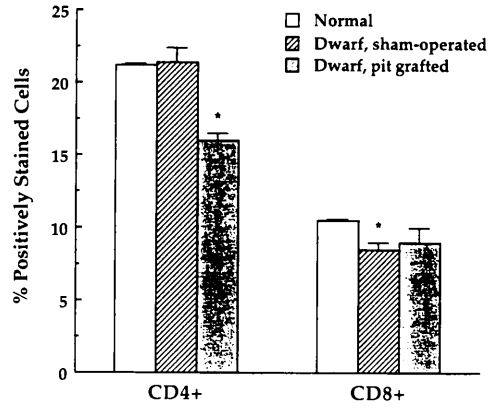

Figure 1. Percentage of lymphocytes expressing CD_4 and CD_8 antigens in 3-month-old normal, sham-operated dwarf and pituitary grafted dwarf mice. Bars represent means ± SEM. *Significant differences in comparison to normal mice ($P < 0.05$).

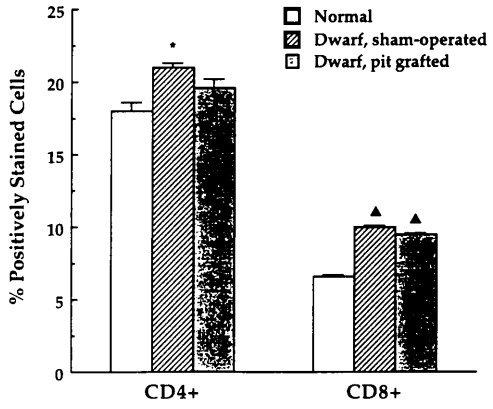

Figure 2. Percentage of lymphocytes expressing CD_4 and CD_8 antigens in 11-month-old normal, sham-operated dwarf and pituitary grafted dwarf mice. Bars represent means ± SEM. *,▲Significant differences in comparison with normal mice ($^*P < 0.05$; $^▲P < 0.01$).

dwarf animals than in normal controls ($P < 0.01$) and appeared to be further reduced by pituitary transplants (Fig. 4). The proliferative index of T lymphocytes stimulated by PHA did not differ between sham-operated dwarf and normal mice, while a decrease in this parameter was observed in dwarf mice given pituitary transplants ($P < 0.05$). Surprisingly, the proliferative index of B lymphocytes was increased in sham-operated dwarfs ($P < 0.05$) as compared with normal mice (Fig. 4), while pituitary transplants reduced this index to values significantly lower ($P < 0.05$) than those measured in normal mice (Fig. 4).

Discussion

The present results extend our studies of the effects of PRL on immunocompetence in Ames dwarf mice (10, 11). Prolactin has been shown to be the major hormone secreted by pituitary transplants (14). We have shown previously that ectopic transplants of normal pituitaries increase NK activity and cellularity of the thymus and the spleen in young adult dwarfs. In the present study, we analyzed the effects of pituitary transplants in young (approximately 2 months old at the time of grafting) and old (approximately 10 months old) dwarfs. The results demonstrate differential and, in several instances, opposite effects of pituitary hormone deficiency (sham-operated dwarfs versus normals) in these two age groups, along with differential effects of ectopic pituitary transplants in young versus old dwarfs. These age-related differences are consistent with previous reports concerning the effects of PRL on the neuroendocrine system (15) and with our studies (16) demonstrating that the age at which ectopic pituitary is placed in the recipient animal is a key determinant of the effects of this treatment. These data are supported by the results on cellularity in old versus young animals suggesting that ectopic pituitaries were less effective in old animals and agree with previous work in the Ames dwarf from this laboratory (10).

Young sham-operated dwarfs were immunodeficient, in agreement with previous results obtained in animals of the same age (10, 11), although the degree of immunodeficiency observed in the present study was of lesser magnitude than that described in Snell dwarf mice (7, 8). Because hormonal deficiencies in

Table IV. Blastogenic Proliferation of Splenocytes from 3-Month-Old-Sham-Operated and Pituitary-Grafted Ames Dwarf Mice Versus Normal Mice

	Medium	+Con A (2.5 μg/ml)	+PHA (5 μg/ml)	+LPS (10 μg/ml)
Normals	2,789 ± 409[a]	210,231 ± 1,721[a]	66,636 ± 1,202[a]	123,725 ± 3,881[a]
Sham-operated dwarf	3,688 ± 117[b]	240,635 ± 9,309[b]	42,510 ± 4,300[b]	123,181 ± 2,414[a]
Pituitary-grafted dwarf	1,721 ± 202[c]	188,852 ± 7,269[c]	27,053 ± 3,061[c]	106,189 ± 3,000[b]

Note. The values (cpm) represent mean ± SEM of six determinations in 3-month-old animals. Columns lacking a common letter differ ($P < 0.05$).

Table V. Blastogenic Proliferation of Splenocytes from 11-Month-Old Sham-Operated and Pituitary-Grafted Ames Dwarf Mice Versus Normal Mice

	Medium	+Con A (2.5 μg/ml)	+PHA (5 μg/ml)	+LPS (10 μg/ml)
Normals	2,120 ± 144[a]	233,103 ± 12,531[a]	25,588 ± 1,841[a]	73,695 ± 5,612[a]
Sham-operated dwarf	2,971 ± 277[b]	188,525 ± 6,667[b]	41,278 ± 3,884[b]	117,935 ± 6,876[b,*]
Pituitary-grafted dwarf	3,223 ± 155[c,*]	194,436 ± 6,398[b]	26,273 ± 1,284[a]	74,372 ± 2,916[a]

Note. The values (cpm) represent mean ± SEM of six determinations in 11-month-old animals. Columns lacking a common letter differ ($P < 0.05$; $^*P < 0.01$).

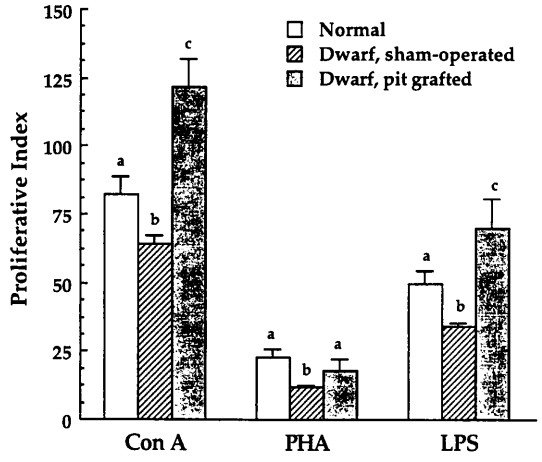

Figure 3. Proliferative indices of 3-month-old normal, sham-operated dwarf and pituitary grafted dwarf mouse splenocytes following stimulation with concanavalin A (Con A), phytohemagglutinin (PHA), and lipopolysaccharide (LPS). Bars represent means ± SEM. Columns within each mitogen lacking a common letter differ ($P < 0.05$).

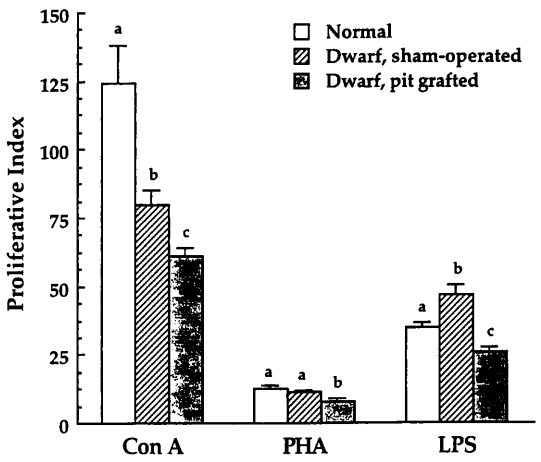

Figure 4. Proliferative indices of 11-month-old normal, sham-operated dwarf and pituitary grafted dwarf mouse splenocytes following stimulation with concanavalin A (Con A), phytohemagglutinin (PHA), and lipopolysaccharide (LPS). Bars represent means ± SEM. Columns within each mitogen lacking a common letter differ (Con A, $P < 0.01$; PHA, LPS, $P < 0.05$).

Ames and Snell dwarf mice are comparable, the difference between the results of these studies may have been due to different age of weaning of the animals (approximately 60 days in our study versus 21 days in the experiments of Baroni *et al.* [8]).

The basal proliferative capacity of lymphocytes did not change with age in dwarf mice and was increased compared with that observed in normal mice. This unexpected effect might be related to the lack of circulating PRL *in vivo* in the dwarf, and presence of low amounts of PRL in the fetal bovine serum, which is a component of the media used for *in vitro* blastogenic proliferation. Perhaps PRL activity of the media leads to a rebound effect of lymphocytes while in normal mice the presence of circulating PRL *in vivo* precludes this effect *in vitro*. Increased proliferation of lymphocytes in dwarf as compared with normal mice was seen also after exposure to Con A, but not PHA. The differential effects observed for Con A– and PHA-stimulated proliferation might be related to altered glycosylation of antigen presenting receptors in T cells. On the other hand, when considering the proliferation index, which accounts for differences in basal proliferation, an inhibitory effect on the proliferative capacity of lymphocytes stimulated by Con A and PHA was observed in young dwarf animals. In old animals, a similar effect was observed for Con A while no differences in PHA-stimulated proliferation were found, suggesting age-related changes of immune activity in dwarf mice as has been described for other species (13). Although age-related decline in immunological capability was documented previously (8), in the present study only the proliferation stimulated with Con A and the relative number of splenocytes per

gram body weight followed this pattern, while stimulation with PHA was followed by an increased rather than reduced proliferative response in older animals. The latter of which is also confirmed by the lack of changes in the number of cells following the adjustment for body weight. This effect might be related to the lack of circulating PRL in the dwarf mice (17) or to the age-related increase in plasma PRL which has been observed in other species (18). Pituitary grafting reduced the absolute values of the proliferative capacity of T and B cells. However, in terms of the proliferation index, the proliferative capacity of Con A–stimulated T cells from dwarf mice was increased as compared with normal animals. This observation concurs with previous results obtained in Snell dwarf mice (8). After PHA, a stimulatory effect of the grafts was also observed with proliferation reaching values similar to those observed in normals. From these observations we can conclude that the hormone(s) secreted by the transplants (presumably PRL) exerts differential effects on the mechanisms of mitogen-induced proliferation.

In old dwarf animals, pituitary grafts inhibited the proliferative index of lymphocytes, thus suggesting a suppressive effect of high PRL levels on lymphocyte proliferation capacity. Immunosuppressive effects of PRL are consistent with previous studies from our laboratory performed in rats (13) and with effects of this hormone *in vitro* (14).

The effects of pituitary transplants on the proliferative index of B lymphocytes were opposite in the two age groups examined. In young animals, the B cell proliferative index was increased by the transplants, while in old animals it was decreased, suggesting age-dependent effects of PRL on B lymphocytes. Other investigators did not observe effects of PRL on peripheral B cells (19, 20) perhaps because PRL alone is not able to modify lymphocyte activation in vivo as has been shown previously in the Ames dwarf (11), but is stimulatory when combined with GH. The presence of an ectopic pituitary gland in dwarf mice resembles the administration of both hormones at the same time, as we have demonstrated that the ectopic gland secretes mainly PRL, it can also release GH (Esquifino, Villinua, Bartke, unpublished observations).

Ames dwarf mice display a deficiency in T progenitor cells, which was expressed in this study by age-related changes in the number of peripheral CD_4+ and CD_8+ cells (10). Pituitary transplants decreased the relative percentage of CD_4+ cells in young dwarf mice and normalized their percentage in old animals, supporting the existence of an age-dependent PRL effect on peripheral lymphocytes (13, 21). However, pituitary grafts failed to normalize the relative percentage of CD_8+ cells in old sham-operated dwarfs indicating selective effects of PRL through the different subpopulations of T cells.

In young dwarf animals pituitary transplants normalized the relative percentage of CD_8+ cells, which could explain the increase in NK activity shown in previous studies (10). However, in old dwarf animals pituitary grafting did not reduce the increased (versus normals) percentage of CD_8+ cells. Perhaps the increases in the percentage of CD_8+ cells might be related to life span in dwarfs (10). These increases agree with the previously reported increase in NK activity in dwarf mice after pituitary grafting (10). All of the observations discussed above correspond with recent reports that PRL exerts it major effects on the mature lymphocytes (22).

In summary, the Ames dwarf mouse, an animal model of PRL deficiency, was employed to demonstrate the immunomodulatory ability of PRL. In 3-month-old animals, pituitary grafting increased the relative number of splenocytes per gram body weight when compared with sham-operated dwarfs. Lymphocyte proliferation in response to both T and B cell mitogens was either normalized or enhanced following pituitary grafting in young dwarfs. In contrast, no differences were observed following pituitary grafting in the relative number of splenocytes per gram body weight in the 11-month-old dwarf animals. Furthermore, pituitary grafting suppressed proliferative responses to T and B cell mitogens in the older dwarfs. Therefore, the recovery of immunocompetence following PRL replacement is age dependent.

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