# Induction of Langerhans' Cell Mitosis *in vivo* after Orchiectomy on Mice

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**We recently reported that male mice have fewer Langerhans' cells (LCs) than females and that orchiectomy resulted in an increased LC density. In this study we demonstrate that orchiectomy induces a transient increase in the number of paired LCs (PLCs) before the increase in LC density occurs. The results of double staining for adenosine triphosphatase (ATPase) and 5-bromo-2′-deoxyuridine (BrdU) showed that all examined PLCs had incorporated the intraperitoneally injected BrdU, while only 0.7 % of the unpaired LCs were BrdU-positive; that supported the notion of PLCs as being divided daughter LCs. Orchiectomy appears to induce a transient increase in mitotic activity of LCs resulting in the increased LC density.** ***Key words: Epidermis; Sex; ATPase; BrdU.***

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Epidermal Langerhans' cells (LCs) are bone-marrow-derived cells which play a pivotal role in cutaneous immune responses; they constitutively express Ia antigens and function as antigen-presenting cells (1, 2). Applying a foreign antigen, e.g., dinitrofluorobenzene, epicutaneously to the skin, usually induces immune reactions. When the same antigen is applied to skin whose LC density was greatly reduced, tolerance to the antigen occurs (3). LC density in the skin thus seems to be an important controlling factor in cutaneous immune responses.

We recently reported sex differences in the density of epidermal LCs: male mice had fewer LCs than females (4). Based on the observations that orchiectomy resulted in an increased LC density and that treatment with testosterone propionate inhibited this increase (5), it has been assumed that testicular androgens are responsible for the sex differences in LC density. An increase in the density of epidermal LCs can be explained by (*a*) LC immigration exceeding emigration, and (*b*) LCs increasing their mitotic

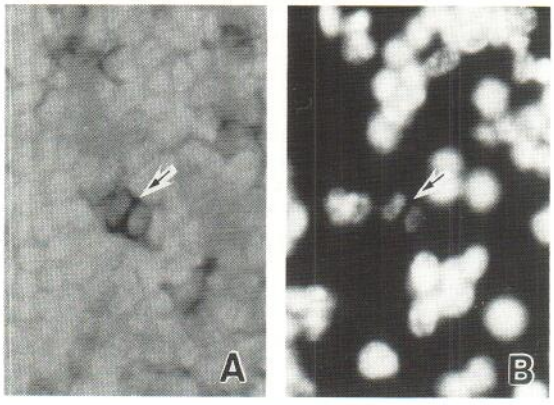


*Fig. 1.* ATPase staining (*a*) and corresponding direct immunofluorescence staining of BrdU (*b*) of epidermal LCs in the hind-limb skin of orchiectomized male mice. PLCs (*arrow*) show positive reaction to BrdU in the nucleus. ×530.

activity. Our study demonstrates that orchiectomy induces a transient increase in the number of paired LCs (PLCs), which are two closely apposed, adenosine triphosphatase (ATPase)-positive LCs. Immunohistochemical studies indicated that PLCs were divided daughter LCs and suggested an induction of epidermal LC mitosis following orchiectomy.

## MATERIALS AND METHODS

### *ATPase staining and computing of LCs*

C57BL/6 (C57BL/6NCrj) mice were purchased from Charles River Japan, Inc. Adenosine triphosphatase (ATPase) staining of LCs was carried out as described previously (5) with some minor modifications; Adenosine diphosphate (ADP) was used instead of ATP (6). One skin piece was obtained from the hind-limbs of each mouse, on which LCs and PLCs per 0.16 $mm^2$ were counted in randomly selected fields (more than 23 fields per mouse) with a reticle at ×300. PLCs were counted as two LCs. Then the number of LCs and PLCs per $mm^2$ was calculated and the PLC rate was established as (PLCs/LCs)×100 for each mouse. Six mice constituted one group; cell counting was done by blind test; data are expressed as mean ± SE (standard error of mean).

### *Orchiectomy*

Eight-week-old male mice were operated on under sodium pentobarbital anesthesia. After the hair was removed with a depilatory cream, the testes were removed through a single ventral incision. Control mice received a sham-operation.

### *BrdU treatment*

BrdU (Sigma, St. Louis, Mo) was dissolved in phosphate-buffered saline (PBS) at 10 mg/ml and 0.2 ml aliquots were injected intraperitoneally into orchiectomized male mice every 6 h for 24 h from day 6 to day 7 after orchiectomy. Treated mice were sacrificed on day 7 and subjected to ATPase staining and direct immunofluorescence staining of BrdU.

### *BrdU staining*

Epidermal sheets stained for ATPase activity were kept in acetone for 20 min at room temperature. After washing with distilled water (10 min ×2), the specimens were treated with 0.1 M NaOH for 10 min at room temperature, then washed with 0.1 M citrate–sodium citrate buffer (pH 5.5) for 15 min and subsequently with PBS (10 min ×3). Epidermal sheets were then allowed to react for 60 min with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody (Becton-Dickinson, Mountain View, Calif.) at 37°C. After washing with PBS (15 min ×4), the specimens were mounted on glass plates in PBS containing 1 mg/ml *p*-phenylenediamine and examined under a Zeiss microscope (Axiphot) with epi-illumination and filters for FITC fluorescence.

### *Statistical methods*

A two-tailed F-test was employed to assess variances. When they were significant, we used Welch's method to assess the difference of the mean, but when insignificant, an unpaired Student's *t*-test (two-tailed test) was employed. A *p*-value of less than 0.05 was considered significant.

## RESULTS

In normal male and female mice, only a few PLCs (Fig. 1 A) were observed, while they were abundant in males after orchiectomy. LC density and PLC rate

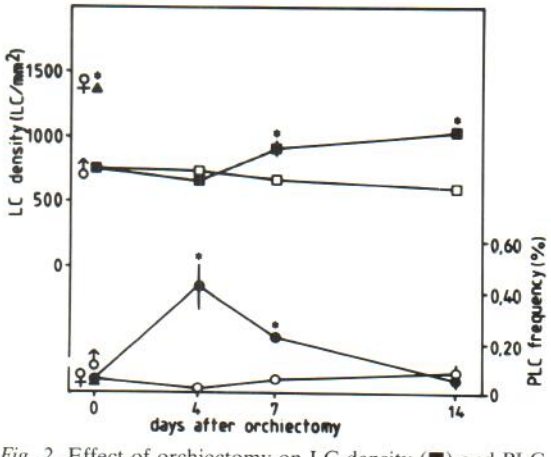


*Fig. 2.* Effect of orchiectomy on LC density (■) and PLC frequency (●). Control mice were sham-operated (□, ○). LC density and PLC frequency on day 0 indicate figures of normal male (■, ●) and female (▲) mice. *$p<0.05$.

in normal and castrated mice are summarized in Fig. 2. PLC figures for normal 8-week-old mice did not reveal any sex difference (male 0.06±0.01 %; female 0.04±0.01 %), but LC density was significantly lower in males (758±24/mm$^2$) than in females (1359±44/mm$^2$).

On day 4 after orchiectomy, LC density did not differ significantly between sham-operated males (728±12/mm$^2$) and orchiectomized (673±30/mm$^2$) males. The number of PLCs, on the other hand, was significantly greater in orchiectomized (0.43±0.08 %) than in sham-operated males (0.02±0.01 %).

On day 7, LC density in orchiectomized males had increased significantly (916±45/mm$^2$) above that in sham-operated males (669±8/mm$^2$). PLC figures for orchiectomized males (0.23±0.01 %) were also significantly higher than for control males (0.06±0.03 %).

The increase in PLC frequency after orchiectomy was transient, however; on day 14, the number of PLCs in the orchiectomized group (0.06±0.03 %) was no longer significantly different from that in the control group (0.09±0.03 %). The increase in LC density, in contrast, was not transient: LC density in orchiectomized males (1057±35/mm$^2$) remained higher than in those sham-operated (612±16/mm$^2$).

We carried out double staining for ATPase activity and intraperitoneally injected BrdU, which is a thymidine analogue and is incorporated into the nuclei of S phase cells (7). Because some keratinocytes in the basal layer incorporated BrdU and exhibited positive staining, we were unable to identify the nuclei of some LCs and PLCs by fluorescence microscopy. However, all observable PLCs (67/67) showed positive staining against BrdU in the nucleus (Fig. 1 B), while only 0.7 % of the unpaired LCs (7/600) were BrdU-positive. This indicates that PLCs are divided daughter LCs.

## DISCUSSION

Epidermal LCs are bone-marrow-derived, antigen-presenting cells (1, 2). The mechanisms controlling their differentiation, proliferation and migration are not yet entirely understood. The density of epidermal LCs is determined by (*a*) the balance of LC immigration and emigration and (*b*) their mitotic activity. Epidermal repopulation by bone-marrow-derived LCs was demonstrated in the construction of bone marrow chimeras (8). The migration of LCs may be controlled by other cell types, such as keratinocytes (9) or endothelial cells of the dermal vessels (10). The proliferation of LCs has been observed repeatedly, an indication that LCs constitute a self-reproducing population (11–15). LCs commence mitosis by stripping the skin (13) and treating it with tumour promoter (13) or contact sensitizer (14). The precise control mechanisms of LC mitosis remain obscure, however.

Miyauchi & Hashimoto (11) reported that PLCs were often observed in the early recovery phase after ultraviolet-B irradiation and suggested that PLCs were divided daughter LCs. We defined PLCs in our study as two closely apposed, ATPase-positive LCs. Miyauchi & Hashimoto (11) described large and round LCs as dividing LCs and symmetrically distributed LCs with a small interval between them as separated daughter LCs. In our study, however, these LCs were not included in the PLCs because it was difficult to distinguish between them and non-dividing LCs under conditions of high LC density and short dendrites in normal females and orchiectomized males (4, 5). Our findings indicate that orchiectomy induces a transient increase in mitotic LC activity and suggest that LC density is determined, at least in part, by this mitotic activity. We cannot exclude the possibility that orchiectomy also induced the immigration of dermal LCs into the epidermis. It should be noted that the induction of LC mitosis was transient and that PLC figures revealed no sex differences between normal male and female mice. Therefore, the sex difference in LC density may partly be preserved by a mitotic LC activity of a very similar degree in both sexes.

A hormonal control mechanism of LC mitosis may exist because androgens inhibited the increase in LC density after orchiectomy (5). However, the mitotic activity of LCs does not seem to be proportional to the concentration of circulating androgens, as there was no significant difference in PLC figures between normal males and females and between orchiectomized males and those sham-operated on, as of 14 days after the operation. Various factors are apparently involved in the control of LC mitosis, with the precise mechanisms waiting further elucidation.

## ACKNOWLEDGEMENT

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