
Less Sensitivity for Late Airway Inflammation in Males than Females in BALB/c Mice

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Abstract

Several studies have investigated allergic airway inflammation, a T helper 2 (Th2)-type immune response, using a mouse model of asthma. At present, however, no reports have described sex differences in the sensitivity of late airway inflammation (LAI). The LAI induced by ovalbumin in adult BALB/c mice was compared in males and females or sham-operated males and castrated males. The males showed less severe bronchial-bronchiolar inflammation with infiltration of eosinophils and lymphocytes and lower content of such cells in bronchoalveolar fluid than the females. Moreover, interleukin-4 (IL-4) mRNA expression levels in splenic cells were lower in the males than in the females. Castrated males performed like the females. Moreover, when compared with the sham-operated males, the castrated males showed lower testosterone levels in the blood. The present results suggest that less sensitivity for LAI in the males may be because of the decreased Th2 cell responses compared with the females. Moreover the testosterone, at least in part, may be responsible for the decreased Th2 cell responses in males *in vivo*.

Introduction

Allergic asthma is characterized by airway hyperresponsiveness, eosinophil recruitment into peribronchial walls and mucus overproductions in bronchial epithelial cells [1, 2]. There are general agreements that T-helper 2 (Th2) cytokines, such as interleukin-4 (IL-4), IL-5 and IL-13 correlate with eosinophil infiltration and disease severity [3–5], mediate the allergic response and regulate the function of eosinophils [6–10]. It has been proposed that asthma may consist of an immediate phase, mediated by immunoglobulin E (IgE) and mast cells, and a late phase mediated by CD4⁺ Th2 cells and eosinophils [11, 12].

The dominance of either the Th1 or Th2 immune response in allergic airway inflammation may be influenced by genetic background [13] and environmental factors [14]. It is also well known that sex hormones influence the host immune system. For example, estrogens suppress T-cell-mediated immune responses and enhance B-cell-mediated immune responses whereas androgens suppress both types of the immune reactions [15]. Although there are many published reports using mouse asthma models, there are no studies directly examining sex differences in sensitivity in asthma models.

The present study examined the sex difference in the development of the late airway inflammation (LAI) induced by ovalbumin (OVA) in adult BALB/c mice.

Materials and methods

Mice. Specific pathogen-free, eight-week-old-sexually matured adult BALB/c mice (Japan Charles River Co., Yokohama, Japan) of either sex were used. Groups of 10 mice of the same age and approximately the same weight were used for each experiment. The animal experiments were approved by The Research Ethics Board of the Faculty of Agriculture, Yamaguchi University.

Sensitization and challenge. The sensitization and challenge procedures were performed by simple modification [13] of the methods described previously [16]. In brief, animals at the age of nine-week-old (20–25 g) were sensitized intraperitoneally (i.p) with 10 µg of OVA (OVA: Grade V; Sigma Chem. Co., MO, USA) in 0.1 ml phosphate-buffered saline (PBS; pH 7.4) of aluminium hydroxide (Alum. 1.2 mg; SERVA, Heidelberg, Germany) on day 0 and day 10. Fourteen days after the first sensitization, each mouse was challenged intranasally with 200 µg of OVA in

50 µl of PBS under anaesthesia with ketamine (45 mg/kg body weight; Sankyo Co., Tokyo, Japan) and xylazine (8 mg/kg body weight; Bayer Co., Tokyo, Japan) i.p.

Castration in male mice. Seven days before the first sensitization, castration was performed on the males under anaesthesia [17]. In brief, both testes and epididymides were excised, after a ligation of the vas deferens and spermatic vessels, through a midline incision of scrotal skin and the skin incisions were closed by wound clips (Clay-Adams, Parsippany, NJ, USA). The sham-operated mice were treated with the same method described above except for the removal of testes and epididymides.

Preparation of bronchoalveolar lavage fluids and blood sampling. Four days after the challenge, mice ($n=10$ in each group) were anaesthetized as described above and whole blood was obtained from the heart puncture using a heparinized syringe. The number of eosinophils was counted after staining with Hinkelmann's solution. Bronchoalveolar lavage (BAL) fluids were obtained by cannulating the trachea through a small incision with 3 ml of 0.9% sterile saline. BAL cells were centrifuged once with Hank's balanced salt solution (HBSS) (HBSS; Gibco, Gaithersburg, MD, USA) containing 2% fetal calf serum at 400 g at 4 °C. The pelleted BAL cells were resuspended in HBSS and the total number of leucocytes was counted by a haemocytometer after staining with Turk's solution. Five hundred cells in cytocentrifuged preparation of BAL fluids stained with Giemsa were differentiated. The total number of each cell type was determined by multiplying the percentage by the total number of cells. The results were expressed as the number of each cell population in 1 ml of BAL fluids.

Histology. Samples of the left lung were fixed in 10% neutral buffered formalin (pH 7.4) and embedded in paraffin blocks. Sections of five-µm thickness prepared from embedded tissue were stained with hematoxylin and eosin (HE), and toluidine blue or periodic acid-Schiff. The severity of lesions in each transversely sectioned bronchus-bronchiole was evaluated by light microscopy and scored as follows: no lesions, 0; mucus or desquamated epithelial cells in the lumen, 1; a few goblet cells in the epithelial layer, 1; many such cells, 2; thickened basement membrane, 1; mild infiltration of eosinophils and lymphocytes in the mucosa, 1; severe infiltration in the mucosa, 2; hypertrophy of smooth muscle cells in mucosa, 1. In each mouse the score index (SI) was calculated by dividing the total score by the number of bronchi-bronchiole examined. The average SI was then determined blindly for each group ($n=10$ in each group) by two different observers.

mRNA expression for IL-4 and interferon- γ by splenic cells. A coupled reverse transcriptase polymerase-chain reaction (RT-PCR) was used to quantify the RNA levels [18] in splenic cells ($n=4$ in each group). The total RNA was extracted using RNasinTM kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. About

10 µg aliquots of the RNA sample was reverse transcribed in 25 µl final volume with Avian Myeloblastosis Virus (AMV) reverse transcriptase first-strand cDNA synthesis kit (Life Sciences Inc., St. Petersburg, USA) according to the manufacturer's instructions. The PCR amplification was performed in the total volume of 25 µl (1.25 U of LA Taq polymerase; Takara Shuzo, Shiga, Japan), 0.2 µM sense and antisense primers, and 200 µM dATP, dTTP, dGTP and dCTP and thereafter overlaid with 20 µl of mineral oil (Sigma, St. Louis, MO, USA). The following oligonucleotide primers (the number in parentheses refers to the nucleotide region of the sequence) were used: specific forward primer for IL-4, 5'-GACGGCACAGAGCTATTGATGGGTC-3' (70–94) and specific reverse primer for IL-4, 5'-TAGGCTTTCCAGGAAGTCTTTCAGTGTGATG TG-3' (450–480) [19]; specific forward primer for interferon- γ (IFN- γ), 5'-TTCTCCTCCTGCGGCCTAGCTCTG-3' (41–64) and specific reverse primer for IFN- γ , 5'-GAATGCTT GCGCTGGACCTGTGGG-3' (438–462) [20]. Thermal cycling was begun according to the following profile; 1 min at 94 °C for denaturation and 3 min at 68 °C for annealing and extension. The optimal number of cycles for PCR amplification was determined experimentally and defined as the number of cycles that would achieve a detectable but less than saturating product concentration. The results were individually normalized to the signal of the 'house keeping gene', hypoxanthine-guanine phosphoribosyl transferase (HPRT): specific forward primer for HPRT, 5'-GTTGGATACAGGCCAGACTTTGTTG-3' (601–625) and specific reverse primer for HPRT, 5'-GATTCAACTTGCGCTCATCTTtaggc-3' (739–763) [21], which was amplified in the same tubes for cytokine cDNA amplification. Specifically, PCR was started with primers for a given cytokine species alone and then the primers for HPRT were added to the reaction tube during the appropriate round of cycling so that the fixed 22 cycles for HPRT and the predetermined optimal cycles for the cytokine (IL-4; 31, IFN- γ ; 30) would be carried out at the end of the amplification reaction. Aliquots of PCR reaction products (approximately 10 µl) were electrophoresed through 2% agarose gels (FMC Bio-products, Rockland, ME, USA) contained 0.2 µg/ml of ethidium bromide. Gels were illuminated with ultraviolet light and the amounts of amplified products were determined from the densities of the corresponding bands using a Gel Doc 1000 system (Bio-Rad, Hercules, CA, USA). The specificities of amplified products were confirmed by direct cycle sequencing based on the dideoxy-mediated chain termination method, using a Taq dye deoxyterminator cycle sequencing kit (Applied Biosystems, CA, USA) after the amplified bands were cut out and extracted with glass milk powder (GeneClean II; Bio-101, La Jolla, CA, USA).

Direct radioimmunoassay of testosterone. The concentration of testosterone in plasma was measured by a

nonextraction procedure [22] using a total testosterone kit (Nippon DPC Co., Tokyo, Japan). Fifty μ l of the plasma and 1 μ l of PBS containing ^{125} I-labelled testosterone were added to a rabbit antitestosterone serum-coated tube. After 3 h of incubation at 37 °C the mixture was decanted and the radioactivity of each tube was counted with a gamma counter (Aloka, Tokyo, Japan).

Statistics. Data are expressed as the mean $\pm$ standard error (SE) for each group. Statistical analysis was performed with unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant.

Results

Comparison of late airway inflammation and mRNA expression for IL-4 and IFN- γ in males and females or sham-operated males and castrated males

The number of blood eosinophils was lower in the males ($269 \pm 64/\mu$ l) than that in the females ($385 \pm 72/\mu$ l). The total number of cells, eosinophils and lymphocytes in BAL fluids was significantly lower in the males than that in the females ($P < 0.05$; Fig. 1A). On the other hand, no significant differences were observed in the number of macrophages and neutrophils between the two groups (Fig. 1B).

The number of blood eosinophils in the castrated males was higher ($382 \pm 80/\mu$ l) than that in the sham-operated

males ($251 \pm 76/\mu$ l) ($P < 0.05$). The total number of cells (Fig. 1C) and the number of eosinophils and lymphocytes (Fig. 1D) in BAL fluids in the castrated males was higher than that in the sham-operated males ($P < 0.05$). There was no difference of total numbers of macrophages and neutrophils between the two groups (Fig. 4B).

IL-4 mRNA expression levels in the males were significantly lower than those in the females ($P < 0.05$; Fig. 2A). On the other hand there was no statistical difference of IFN- γ mRNA expression levels between the two groups (Fig. 2B).

The castrated males showed a significant high IL-4 mRNA expression levels when compared with the sham-operated males ($P < 0.05$; Fig. 3).

Comparison of histology in males and females or sham-operated males and castrated males

The severity of bronchial-bronchiolar lesions in the males (SI; 2.3 ± 0.4) was significantly lower than that in the females (SI; 3.5 ± 0.7) ($P < 0.05$). In general, bronchial-bronchiolar lesions in males (Fig. 4A,B) were much less severe than those in females (Fig. 4C,D). As shown in Fig. 4C,D in the females there were prominent goblet cell metaplasia and hyperplasia of the hypertrophic bronchial-bronchiolar epithelial cells with mucous secretion at a certain degree in bronchial lumens, though those changes

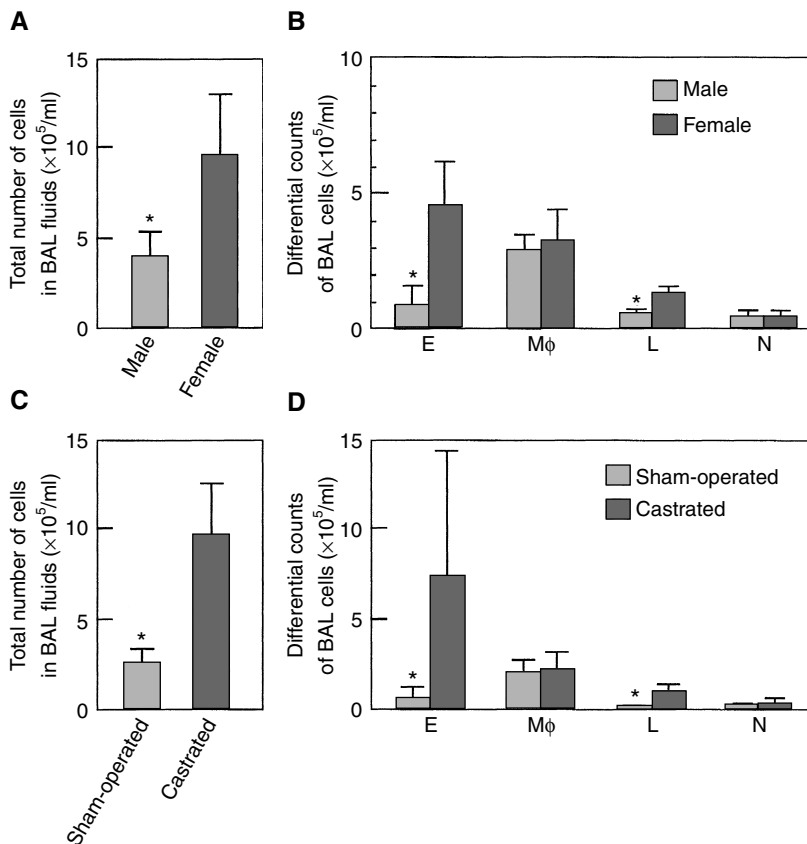


Figure 1 Sex differences in total number of cells (A) and differential counts (B) of bronchoalveolar lavage (BAL) cells in the males and females. Adult BALB/c mice were sensitized twice intraperitoneally (i.p.) with 10 μ g of ovalbumin (OVA) with Alum on day 0 and day 10, and thereafter challenged intranasally with 200 μ g of OVA in phosphate-buffered saline four days after second sensitization. BAL cells were collected four days after challenge. Effect of castration on total number of cells (C) and differential counts (D) of BAL cells in sham-operated males and castrated males. Castrations were performed seven days before the first sensitization. Data shown are mean $\pm$ SE. * $P < 0.05$ between two groups. E, eosinophil; M ϕ , macrophage; L, lymphocyte; N, neutrophil.

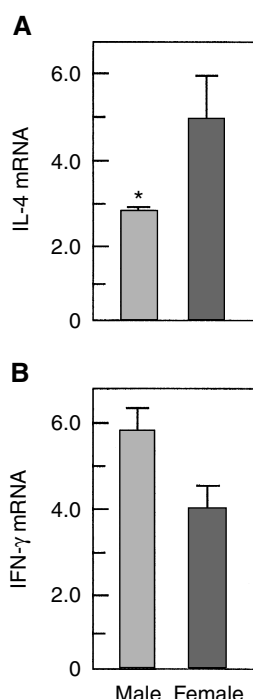


Figure 2 Sex differences in expression levels of the interleukin-4 (IL-4) or interferon- γ (IFN- γ) mRNA by splenic cells in the males and females. A protocol of OVA sensitization and airway challenge is detailed in Fig. 1. Expression of cytokines was analysed by reverse transcriptase-polymerase chain reaction (RT-PCR). All data were individually normalized by dividing by the value for hypoxanthine-guanine phosphoribosyl transferase (HPRT) cDNA. Data shown are mean $\pm$ SE of the mean. * $P < 0.05$ between the two groups.

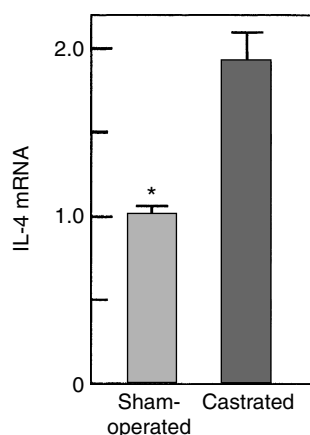


Figure 3 The effect of castration on interleukin-4 (IL-4) mRNA expression levels by splenic cells in the sham-operated males and the castrated males. Castrations were performed seven days before the first sensitization. A protocol of OVA sensitization and airway challenge is detailed in Fig. 1. Expression of cytokines was analysed by reverse transcriptase-polymerase chain reaction (RT-PCR). All data were individually normalized by dividing by the value for HPRT cDNA. * $P < 0.05$ between the two groups.

varied amongst mice. In some cases desquamated epithelial cells mixed with some eosinophils, lymphocytes and macrophages in bronchial-bronchiolar lumens were observed. A severe peri-bronchial and -bronchiolar infiltration of lymphocytes and eosinophils with oedema and hyperaemia in the bronchial-bronchiolar walls and hypertrophy of the bronchial smooth muscles were observed in most cases. There were thickening of the bronchial epithelial basement membranes in some cases. A large number of eosinophils and lymphocytes infiltrated around small arteries with degeneration of walls in most cases. A small number of eosinophils and lymphocytes accumulated within the lumen with swelled proliferated endothelial cells in some cases. There was a few or no infiltration of mast cells in the bronchial wall.

The bronchial-bronchiolar lesions in the castrated males (SI; 1.8 ± 0.3) were severer than those in the sham-operated males (SI; 0.5 ± 0.3) ($P < 0.01$).

Comparison of plasma testosterone levels in sham-operated males and castrated males

The testosterone levels in the castrated males were lower than those in the sham-operated males (Fig. 5) ($P < 0.05$).

Discussion

We demonstrated that the males had less severe LAI, and this was paralleled with the low IL-4 mRNA expression levels in splenic cells as compared with the females suggesting that LAI reduced because of the systemic decreased Th2 cell responses in the males [13].

The decreased LAI in the males may be induced by the systemic suppression of Th2 cell responses by testosterone. It seems likely because when compared with the sham-operated males, the castrated males showed low-levels of testosterone in the blood and they showed more severe LAI with the higher expression levels of IL-4 mRNA similar to the females. It is possible because *in vitro* study has suggested that testosterone may regulate directly allergic inflammation by reducing eosinophil adhesion to endothelial cells and eosinophil viability being responsible for eosinophil accumulation in inflammatory areas [23]. In addition, females produced significantly higher levels of antigen-specific IgE, which is regulated by Th2 cells [24], than males in CBA/J mice sensitized with phospholipase A₂ (PLA₂) and castrated males produced significantly higher amounts of PLA₂-specific IgE, and castrated males treated with testosterone decreased PLA₂-specific IgE production as compared with sham-operated males [25]. By contrast, however, *in vitro* study has demonstrated that testosterone acts directly on CD4⁺ T cells to secrete Th2 cytokines (e.g. IL-4 and IL-10) [26]. Also it has been reported that the relative resistance of female mice to platelet-activating factor induced immediate hypersensitivity

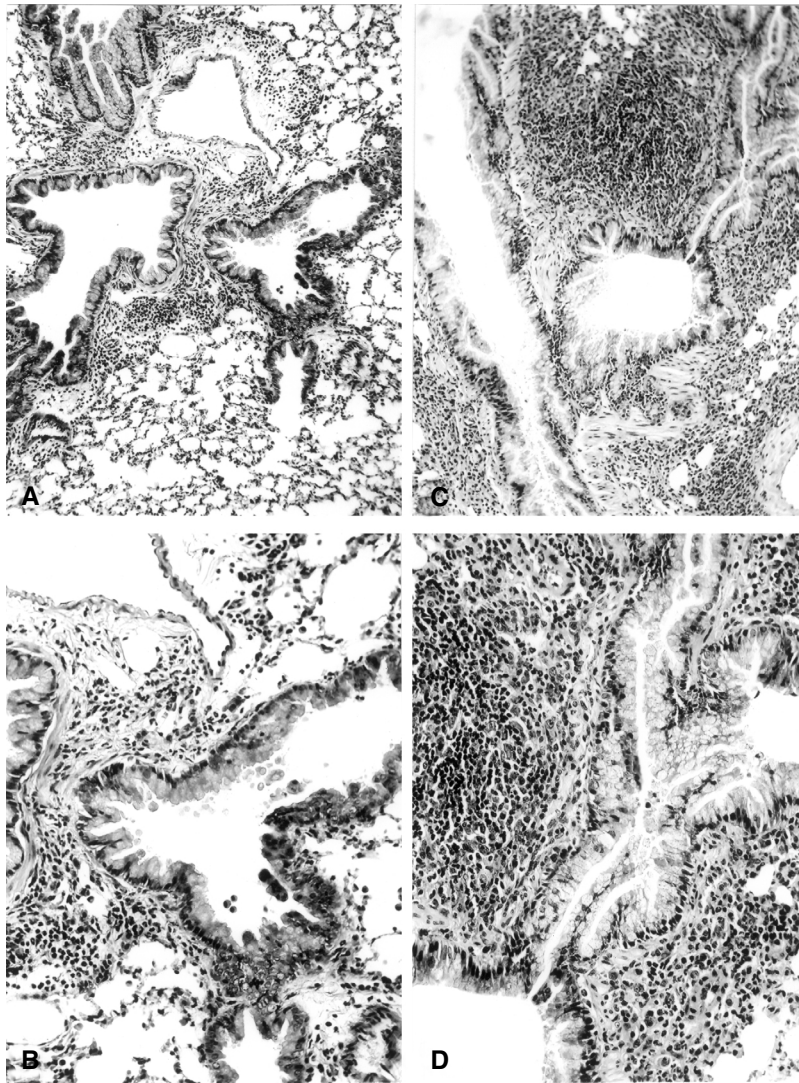


Figure 4 Representative histology from the lung four days after OVA challenge in a male (A and B, higher magnification of A) and a female (C and D, higher magnification of C). When compared with the male mice, the lung from a female mouse demonstrated a prominent goblet-cell metaplasia and hyperplasia with a remarkable peribronchial infiltration of lymphocytes and eosinophils in the bronchial mucosa. A protocol of OVA sensitization and airway challenge is detailed in Fig. 1. A and C, $\times 50$; B and D, $\times 100$. Hematoxylin and eosin (HE).

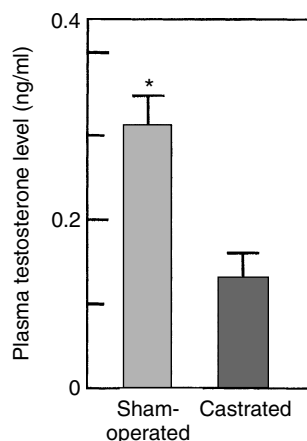


Figure 5 The effect of castration on plasma testosterone levels in the sham-operated males and castrated males. Castrations were performed seven days before the first sensitization. A protocol of ovalbumin (OVA) sensitization and airway challenge is detailed in Fig. 1. Data shown are mean $\pm$ SE of the mean. * $P < 0.05$ between the two groups.

(Th2-cell-type response) [27]. Moreover in humans, boys are more susceptible to asthma than girls before the age of 10, and thereafter girls become more susceptible to asthma than boys up to the age of sexual maturity and male hormones could account for the striking reduction in the incidence of asthma in males during an active sexual life [28]. On the other hand, it has been reported that genetic (also as discussed below) and environmental factors affect the development of asthma rather than sex difference or age-dependency in humans [14]. Thus at present, these contradicting sex differences in sensitivity of asthma observed in animal models *in vivo* and *in vitro* and in human asthma seem to be more complicated events. Further studies are needed to clarify the mechanisms of sex difference in this model.

It has been suggested that different strains of mice have different responses to exogenous antigens and that genetic differences in the immune response may, at least in part,

be mediated by either Th1 or Th2 cytokines [29]. For example, BALB/c mice or C57BL/6 mice may have Th2- or Th1-like responses to exogenous antigens, respectively [29]. Interestingly enough, even within BALB/c mice, there was a different late LAI owing to different Th2 cell responses by sex-difference and this may be because of the suppression of Th2 cell response by testosterone rather than the systemic polarization into Th1 responses owing to mutual Th1/Th2 inhibitory effect [24] in males as there was no significant difference of IFN- γ (Th1 cytokine) expression levels between females and males. In conclusion, we would like to stress the less sensitivity for LAI in the males than the females in sexually matured adult BALB/c mice, which are often used as an experimental animal model in asthma.

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