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Cellular recruitment and cytokine generation in a rat model of allergic lung inflammation are differentially modulated by progesterone and estradiol

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Ligeiro de Oliveira AP, Domingos HV, Cavriani G, Damazo AS, Lino dos Santos Franco A, Oliani SM, Oliveira-Filho RM, Vargaftig BB, Tavares de Lima W. Cellular recruitment and cytokine generation in a rat model of allergic lung inflammation are differentially modulated by progesterone and estradiol. *Am J Physiol Cell Physiol* 293: C1120–C1128, 2007. First published July 18, 2007; doi:10.1152/ajpcell.00286.2006.—We evaluated the role of estradiol and progesterone in allergic lung inflammation. Rats were ovariectomized (Ovx) and, 7 days later, were sensitized with ovalbumin (OA) and challenged after 2 wk with inhaled OA; experiments were performed 1 day thereafter. Ovx-allergic rats showed reduced cell recruitment into the bronchoalveolar lavage (BAL) fluid relative to sham-Ovx allergic rats, as was observed in intact allergic rats treated with ICI-182,780. Estradiol increased the number of cells in the BAL of Ovx-allergic rats, whereas progesterone induced an additional reduction. Cells of BAL and bone marrow (BM) of Ovx-allergic rats released elevated amounts of IL-10 and reduced IL-1 β and TNF- α . BM cells of Ovx-allergic rats released increased amounts of IL-10 and lower amounts of IL-4. Estradiol treatment of Ovx-allergic rats decreased the release of IL-10 but increased that of IL-4 by BM cells. Estradiol also caused an increased release of IL-1 β and TNF- α by BAL cells. Progesterone significantly increased the release of IL-10, IL-1 β , and TNF- α by BAL cells and augmented that of IL-4 by BM cells. Degranulation of bronchial mast cells from Ovx rats was reduced after in vitro challenge, an effect reverted by estradiol but not by progesterone. We suggest that the serum estradiol-to-progesterone ratio might drive cellular recruitment, modulating the pulmonary allergy and profile of release of anti-inflammatory or inflammatory cytokines. The existence of such dual hormonal effects suggests that the hormone therapy of asthmatic postmenopausal women and of those suffering of premenstrual asthma should take into account the possibility of worsening the pulmonary conditions.

asthma; eosinophils

ASTHMA IS A CHRONIC DISEASE characterized by airway reversible obstruction, inflammation, and increased responsiveness to various stimuli (15). Epidemiological data have suggested that oscillations of female sex hormone levels exacerbate the symptoms of asthma (34).

Asthma appears to be mediated by T lymphocyte-borne cytokines, and in vitro studies have shown that progesterone shifts the differentiation of naïve T helper (Th) lymphocytes toward the Th2 lineage (25). That hormone also induces the

production of IL-4 by Th2-like clones (30), through the induction of a factor that blocks Th1 responses (38).

Consistent with the hypothesis that female sex hormones influence pulmonary ventilatory efficiency, the hormonal changes at puberty in girls coincide with a late adolescent increase of the incidence of asthma, which in women becomes higher and remains so throughout the reproductive years (10). However, the literature on this issue is conflicting (3, 22, 33, 40, 43, 53), and a coherent explanation is lacking.

Compelling evidences suggest that, throughout the fertile period, an increase in asthma symptoms or a decrease in lung function shortly precedes or occurs concomitantly with the menstrual phase of the cycle, thence characterizing what has been referred to as “premenstrual asthma.” Although this entity is now well recognized, the mechanisms underlying premenstrual asthma are still far from being understood. Notwithstanding, the hormonal variations during the menstrual cycle conceivably play an important role in its pathogenesis (49).

During the menstrual cycle of patients with premenstrual asthma, estradiol is associated with a significant improvement in symptoms and in dyspnea index scores, a fact that does not appear to be related to β_2 -receptor stimulation (3). Moreover, supplemental estrogens have been used as glucocorticoid-sparing agents in asthmatic women (26). These clinical observations are in opposition to what can be observed in experimental conditions, since we (23) recently demonstrated that estradiol increases the inflammatory component of the experimental allergic lung response and that these actions seem to be mediated, at least in part, by endogenous glucocorticoids. Interestingly, the intracellular levels of active corticosterone depend on the effects of 11 β -hydroxysteroid dehydrogenase, an activity inhibited by estradiol (19). This may explain the worsening of asthma symptoms in some postmenopausal asthmatic women after estradiol treatment (6, 7). Indeed, the use of postmenopausal estrogen therapy at high doses has been reported to actually increase the subsequent risk for asthma (42). In addition, there are data indicating that progesterone induces eosinophilia in a murine model of allergic lung inflammation (17). Overall, convincing evidences for either clear beneficial or deleterious effects of estradiol and progesterone on allergic asthma are still lacking.

Mast cells are one of the major cells involved in asthma that, upon activation, release a number of mediators that share a

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wide spectrum of inflammatory and regulatory functions on the airways and lungs (14). In addition, it is worth noting that mast cells may represent the primary target responsible for the effects of sex hormones on airways (52).

Since pulmonary inflammation has been considered the major cause of bronchial hyperresponsiveness (16) and since the effects of oscillating levels of female sex hormones have not been consistently correlated to the cytokines involved with allergic asthma, we used an asthma model of ovariectomized (Ovx) rats and found a differential modulation by sex hormones of both cytokine production and allergic lung inflammation. Accordingly, in this study, we found evidence that estradiol modulates the functional activity of bone marrow cells by stimulating the release of IL-4 and inhibiting that of IL-10. On the other hand, under the influence of estradiol, bronchoalveolar lavage (BAL) cells released increased amounts of IL-1 β and TNF- α . Progesterone, in turn, was able to increase the bone marrow cells' release of IL-4 and the BAL cells' release of IL-10, IL-1 β , and TNF- α . Moreover, our data point out that female sex hormones have a dual effect on allergic lung inflammation, on the one hand being inflammatory and causing eosinophil recruitment to the lungs and on the other hand being anti-inflammatory and reducing cell migration. We can infer that fluctuations in the estradiol/progesterone level balance during the immune response would help to explain the conflicting literature data about the role played by female sex hormones in asthmatic women.

MATERIALS AND METHODS

Reagents. Ovalbumin (OA; chicken egg albumin, grade II), crystal violet, May-Grünwald-Giemsa dye, Paraplast, paraformaldehyde, xylene solution, acetic acid, methacholine (acetyl- β -methylcholine), RPMI-1640 medium culture, progesterone, and 17 β -estradiol were purchased from Sigma Chemical (St. Louis, MO). ICI-182,780 was from Tocris (Ellisville, MO). Aluminum hydroxide (Pepsamar) was from Sanofi-Synthelabo (Rio de Janeiro, Brazil); chloral hydrate was from Vetec Química (Rio de Janeiro, Brazil). Xylazine was from König (São Paulo, Brazil); ketamine and Pentantibiotic were from Fort Dodge (Fort Dodge, IA). Actinomycin D was from Amresco (Solon, OH).

Animals. Female Wistar rats (180–220 g) from our departmental animal facilities were used throughout. They were housed in groups of five rats per cage in a light- and temperature-controlled room (12:12-h light-dark cycle, $21 \pm 2^\circ\text{C}$) with free access to food and water. Experiments were approved (Report no. 076/99) by the local Animal Care Committee, following guidelines that comply with those of the Canadian Council on Animal Care (28).

Ovariectomy. Rats were anesthetized with an intraperitoneal injection of ketamine-xylazine (100 and 20 mg/kg, respectively). An incision was made on the lower part of the abdomen; the ovaries were identified, held tightly, and removed free from adherent tissue (8). Sutures were performed, and animals received a single dose of Pentantibiotic (570 mg/kg) by the subcutaneous route. The effectiveness of Ovx was assessed by analysis of the morphological features of cells in vaginal smears, by quantification of the uterine weight, and by determinations of the circulating levels of estradiol and progesterone. Rats subjected to similar manipulations except for the ovary removal were used as the sham-operated controls and labeled as "sham Ovx" animals. Normal basal values of all parameters studied were obtained from a nonmanipulated group of female rats (basal group). The per se effect of ovaries removal was evaluated in nonsensitized Ovx rats. To investigate the interaction of circu-

lating sex hormones with the profile of allergic lung inflammation, only estrous sham Ovx-allergic rats and diestrous Ovx-allergic rats were used in the experiments.

Estradiol and progesterone circulating levels. Blood samples were collected from the orbital plexus of anesthetized rats before immunization (*day 0*, corresponding to 7-day Ovx), at the booster (*day 7*, corresponding to 14-day Ovx), at the challenge (*day 14*, corresponding to 21-day Ovx), and 24 h after antigen challenge (*day 15*, corresponding to 22-day Ovx). In a parallel set of experiments, estradiol and progesterone levels were also quantified in blood samples collected from Ovx-allergic rats treated subcutaneously with estradiol and progesterone 12 h before the antigen challenge. Aliquots of blood were centrifuged (170 g, 10 min), and the resulting sera were stored at -70°C . Estradiol and progesterone were determined using ELISA kits (Diagnostic Products, Los Angeles, CA). The detection limit for estradiol was 0.011–0.025 pg/ml and that for progesterone was 0.009–0.02 ng/ml.

Sensitization and antigen challenge. Ovx and sham Ovx rats (7 days after operations) were sensitized by an intraperitoneal injection of a suspension of 10 μg OA with 10 mg aluminum hydroxide (*day 0*). One week later (*day 7*), rats were boosted subcutaneously with 10 μg OA dissolved in saline and labeled as "allergic." At *day 14*, rats were subjected to a single 15-min exposure of aerosolized OA (1% in PBS) using an ultrasonic nebulizer device (Icel, São Paulo, Brazil) coupled to a plastic inhalation chamber ($18.5 \times 18.5 \times 13.5$ cm). According to the manufacturers, the nebulizer produces aerosol particles with a mean size of 0.5–1.0 μm . Experiments were performed 24 h after antigen challenge, and animals were exsanguinated by sectioning the abdominal aorta under deep chloral hydrate anesthesia (>400 mg/kg ip).

BAL and cell countings. BAL was performed in killed Ovx-allergic rats and their respective controls (i.e., sham Ovx-allergic rats). For this purpose, a total volume of 40 ml (2 times, 20 ml) of PBS was injected into the lungs by the tracheal route (9). The BAL fluid was collected and centrifuged (170 g, 10 min); the cellular pellet was resuspended in 1 ml PBS. Aliquots of cells suspensions (90 μl) were stained with 10 μl of 0.2% crystal violet to quantify total cells. Differential cell countings (neutrophils, eosinophils, and mononuclear cells) were carried out using standard morphological criteria after cytospin processing and staining with Rosenfeld's dye (23).

Femoral lavage. The femurs of killed rats were removed, and the epiphyses were cut transversely (11). Bone marrow cells were flushed out with PBS (5 ml), and the recovered lavage fluid was centrifuged (170 g, 10 min). Cell pellets were resuspended in PBS (1 ml), stained with crystal violet (0.2%), and quantified microscopically as described above.

Determination of cytokines (TNF- α , IL-1 β , IL-4, and IL-10). The total cells recovered from BAL or femoral lavage (FL) were suspended in RPMI-1640 culture medium enriched with 10% FBS. Cell viability was determined by the trypan blue exclusion test. Aliquots (500 μl) containing 2×10^6 cells/ml were harvested into 24-well plastic microplates (NUNC, Naperville, IL) under a 5% CO_2 -95% O_2 atmosphere at 37°C . Aliquots of supernatants were collected 4 and 24 h later and stored at -80°C until analyzed.

TNF- α activity was evaluated by a cytotoxicity assay using L-929 cells (12). In brief, TNF- α activity was assayed in 50- μl samples, serially diluted (2-fold dilutions) in 96-well plates harvested with L-929 cells (2.5×10^4 cells/well), in the presence of actinomycin D (final concentration, 5 $\mu\text{g}/\text{ml}$). After an overnight incubation, the degree of cell lysis was assessed by staining with a 0.05% crystal violet solution in 10% methanol for 15 min, followed by rinsing the plates with distilled water and drying. Methanol (10%) was then added to each well to dissolve the crystals generated in stained cells; the absorbance was measured at 620 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT). The TNF- α titer (U/ml) was

Table 1. Serum levels of estradiol and progesterone before and after Ovx and the effects of treatments with estradiol or progesterone before ovalbumin challenge

	Estradiol, pg/ml	Progesterone, ng/ml
<i>Sham Ovx</i>		
Estrus	15.6 ± 3.0	10.6 ± 1.2
Diestrus	8.3 ± 1.5	21.3 ± 1.2
Metaestrus	15.2 ± 1.0	21.0 ± 0.2
Proestrus	34.3 ± 4.6	4.0 ± 0.2
<i>Ovx</i>		
At sensitization (7-day Ovx)	3.8 ± 0.7	10.0 ± 1.5
At booster (14-day Ovx)	2.9 ± 0.9	5.6 ± 1.4
At challenge (21-day Ovx)	2.0 ± 0.8	5.1 ± 0.9
At experiment (22-day Ovx)	2.0 ± 0.5	4.8 ± 1.2
+Estradiol	68.1 ± 11.2	5.6 ± 0.6
+Progesterone	10.3 ± 1.2	23.2 ± 1.1

Values are means ± SE of radioimmunoassay data from 5 rats/group. Orbital plexus blood was taken under deep anesthesia from sham ovariectomized (Ovx)-allergic and Ovx-allergic rats at various time points corresponding to the experimental steps; estradiol and progesterone (single doses) were administered 24 h before the experiments (see MATERIALS AND METHODS).

defined as the reciprocal of the dilution that induces 50% of lysis of L-929 cells.

IL-1 β , IL-4, and IL-10 were quantified in samples of incubates of BAL and FL cells using ELISA kits purchased from R&D Systems (Minneapolis, MN); the detection limits were 62.5, 15.6, and 62.5 pg/ml, respectively. IL-1 β , IL-4, and IL-10 levels were obtained using standard curves.

Ex vivo antigen challenge and morphometric analysis of bronchial mast cells. Intrapulmonary bronchial (IB) segments were isolated from sensitized sham Ovx and Ovx rats and from nonmanipulated (basal) rats. IB segments were isolated and dissected out of the surrounding tissues. Segments were harvested into 24-well plastic microplates containing 1 ml RPMI-1640 culture medium. Microplates were kept in a humid atmosphere containing 95% O₂-5% CO₂ and allowed to equilibrate for 60 min. Thereafter, the ex vivo antigen challenge was performed by adding 100 μ g/ml (final concentration) OA and standing for 24 h. Incubated IB segments were then processed to quantify the mast cell degranulation. In brief, IB segments were fixed in 4% paraformaldehyde in Sørensen phosphate buffer (pH 7.4) for 24 h at 4°C. The fragments were washed in the same buffer, dehydrated through a graded series of ethanol, clarified in xylene solution, and embedded in Paraplast medium. Sections (5 μ m thick) of the samples were cut, placed on glass slides, and stained with 1% toluidine blue in 1% borax solution for subsequent analysis. Morphometric analyses for degranulated mast cell quantification were carried out in an Axioskop II mot plus (Zeiss) equipped with a digital camera using Axiovision software (Zeiss).

Hormone treatments. Groups of Ovx-allergic rats were treated, 24 h prior to the antigen challenge, with a single subcutaneous injection of 17 β -estradiol (280 μ g) or progesterone (200 μ g). Control groups consisted of Ovx-allergic rats injected with the corresponding volumes of the hormone vehicles (corn oil for 17 β -estradiol or distilled water for progesterone). One group of otherwise intact allergic rats was treated, 24 h before the antigen challenge, with a single injection of ICI 182,780 (500 μ g ip), an estradiol receptor antagonist (20, 29) devoid of agonist effects (18).

Statistical analysis. Data are presented as means ± SE. Comparisons between groups were made by ANOVA followed by Duncan's post test. The 4.0 version (1990–1993) of GraphPad InStat software was used for this purpose. Values of $P < 0.05$ were considered significant.

RESULTS

Serum estradiol and progesterone levels. Table 1 shows the fluctuations of estradiol and progesterone levels in the serum of physiologically cycling sham Ovx-allergic rats and in diestrus Ovx-allergic rats before and after single-dose estradiol or progesterone treatments. Levels of estradiol were the least at diestrus, intermediate at estrus and metaestrus, and highest at proestrus; those of progesterone were the least at proestrus, intermediate at estrus, and highest at diestrus and metaestrus. The profile of sex hormone levels of Ovx-allergic rats reflected the expected decay, which was faster for estradiol. As observed, the treatment of Ovx-allergic rats with estradiol or progesterone before the antigen challenge significantly increased the systemic levels of these hormones relative to their Ovx untreated counterparts. Moreover, Ovx resulted in a significant loss of uterine weight and in vaginal cell morphological changes compatible with the diestrus phase (data not shown).

Effects of estradiol and progesterone on allergic lung inflammation. Ovx-allergic rats showed reduced lung inflammation, as indicated by decreased numbers of total cells recovered

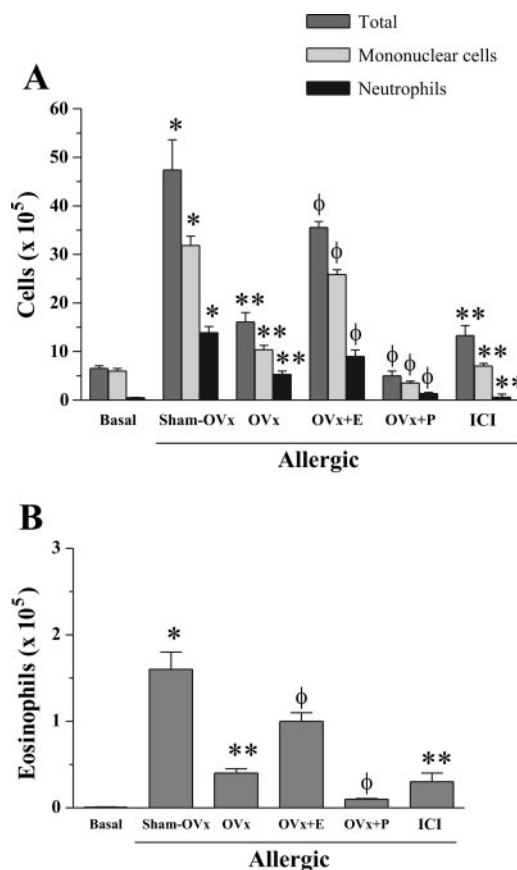


Fig. 1. Total mononuclear cells and neutrophils (A) and eosinophil counts (B) in bronchoalveolar lavage (BAL) fluid of allergic rats [sham ovariectomized (Ovx) and Ovx]. Groups of Ovx-allergic rats were treated with estradiol (E) or progesterone (P) before antigen challenge. A parallel group of otherwise intact, allergic rats was treated with the estrogen receptor antagonist ICI-182,780 (ICI). Basal values were obtained from nonmanipulated rats. Data are means ± SE from 5–8 experiments. * $P < 0.05$ compared with the basal group; ** $P < 0.05$ compared with the sham Ovx-allergic group; $\phi P < 0.05$ compared with the untreated Ovx-allergic group.

in the BAL fluid compared with the sham Ovx-allergic group (Fig. 1A). Ovx-allergic rats had a reduced number of mononuclear cells and neutrophils as well as diminished eosinophil counts relative to sham Ovx-allergic rats (Fig. 1B). The same alterations were elicited by treating otherwise intact allergic rats with the estrogen receptor antagonist ICI-182,780 (Fig. 1, A and B). Moreover, serum levels of estradiol and progesterone of ICI-182,780-treated rats were similar to those observed during the estrous cycle (data not shown).

The treatment of Ovx-allergic rats with estradiol prior to the antigen challenge led to a full recovery of the cellular influx into the lungs compared with counts in the BAL fluid of sham Ovx-allergic rats. The full compartmentation of mononuclear cells, neutrophils, and eosinophils was also achieved by the treatment (Fig. 1, A and B). In contrast, progesterone exacerbated the reduction of inflammatory cell recruitment to the lungs compared with untreated Ovx-allergic rats (Fig. 1, A and B).

Influence of sex hormones on the release of cytokines by BAL and bone marrow cells. Ovx did not modify basal levels of cytokines released by BAL and bone marrow cells of nonallergic rats (Figs. 2–4). Incubated (4 h) BAL cells from sham

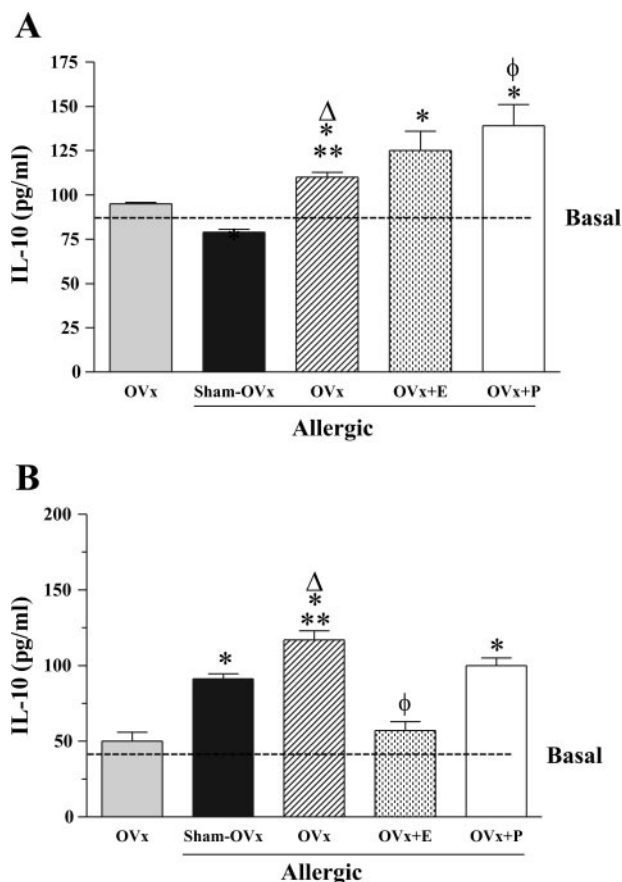


Fig. 2. IL-10 released by cultured BAL fluid (A) and bone marrow cells (B) 24 h after in vivo antigen challenge of allergic rats (sham Ovx and Ovx). Rats of Ovx-allergic groups were treated with estradiol or progesterone before the antigen challenge. Basal values were obtained from nonmanipulated rats. Data are means $\pm$ SE from 5–8 experiments. $*P < 0.05$ compared with the basal group; $**P < 0.05$ compared with the sham Ovx group; $\phi P < 0.05$ compared with the untreated Ovx-allergic group; $\Delta P < 0.05$ compared with the Ovx-nonallergic group.

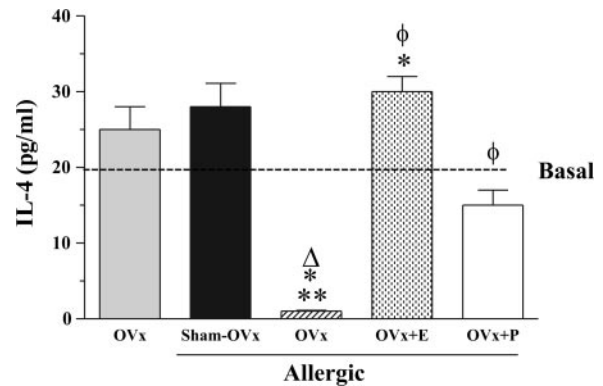


Fig. 3. IL-4 released by bone marrow cells 24 h after in vivo antigen challenge of allergic rats (sham Ovx and Ovx). Rats of Ovx-allergic groups were treated with estradiol or progesterone before the antigen challenge. Basal values were obtained from nonmanipulated rats. Data are means $\pm$ SE from 5–8 experiments. $*P < 0.05$ compared with the basal group; $**P < 0.05$ compared with the sham Ovx group; $\phi P < 0.05$ compared with the untreated Ovx-allergic group; $\Delta P < 0.05$ compared with the Ovx-nonallergic group.

Ovx-allergic rats released less amounts of IL-10 compared with basal controls (Fig. 2A), but the release of IL-10 by BAL cells from Ovx-allergic rats was higher than that of cells from their sham Ovx counterparts, but was essentially the same as that by the basal group cells. Estradiol treatment did not alter the levels of released IL-10 relative to untreated Ovx-allergic rats, but these levels were increased compared with the basal group (Fig. 2A). On the other hand, the treatment of Ovx-allergic rats with progesterone augmented the increased production of IL-10 over that observed in the untreated Ovx-allergic group and in the control (basal) group.

Figure 2B shows the levels of IL-10 released by bone marrow cells after 4 h of incubation. In this compartment, cells of sham Ovx-allergic rats showed enhanced production of IL-10 compared with basal values. Cells of Ovx-allergic rats had an additional production of IL-10, which was significantly decreased by estradiol but not by progesterone treatment. Noticeably, the IL-10 release by bone marrow cells of Ovx-allergic rats was not modified by progesterone. After 24 h of incubation, IL-10 levels in supernatants of cultured BAL and FL cells of Ovx-allergic rats were undetectable.

The production of IL-4 by cultured BAL cells from every group was virtually absent. As such, only FL cell activity is shown in Fig. 3. After 4 h of incubation, bone marrow cells from Ovx-allergic rats had strongly reduced levels of IL-4 secretion compared with sham Ovx-allergic rats, which, in turn, were elevated compared with basal values. Both estradiol and progesterone treatments of Ovx animals effectively stimulated IL-4 production, but only estradiol recovered the production to the sham Ovx level. Upon 24 h of incubation, IL-4 in the supernatants of BAL cells were no longer detectable.

Figure 4A shows that the concentrations of IL-1 β in the supernatants of 4-h cultured BAL cells from Ovx-allergic rats were significantly reduced compared with those from sham Ovx-allergic rats, which expressed IL-1 β levels much higher than the basal group. Both estradiol and progesterone treatments increased levels of IL-1 β in the incubates of BAL cells from Ovx-allergic rats, but this cytokine was no longer detected in 24-h incubates. TNF- α levels in supernatants of 4-h cultured BAL cells from sham-Ovx allergic rats were signifi-

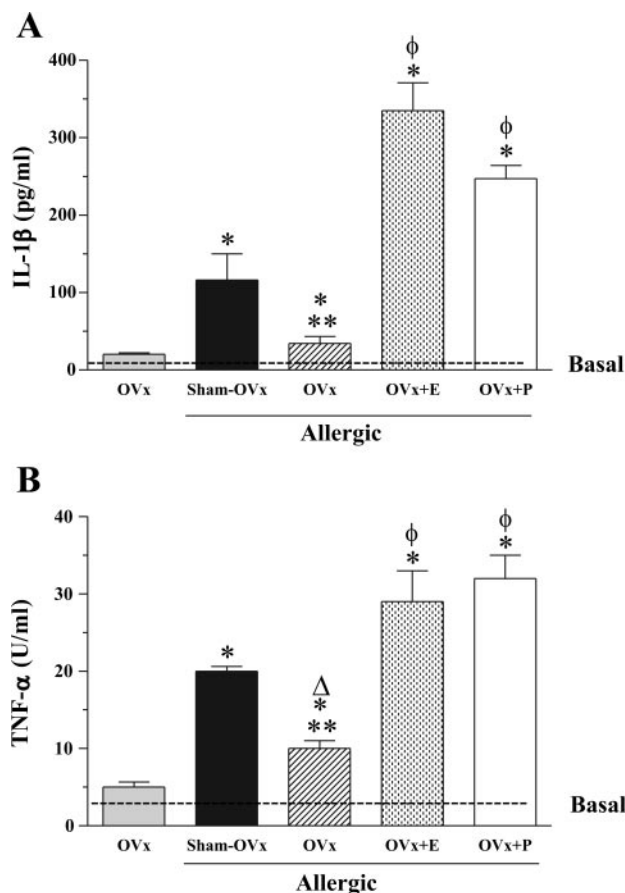


Fig. 4. IL-1 β (A) and TNF- α (B) released by cultured BAL fluid cells 24 h after antigen challenge of allergic rats (sham OvX and OvX). Rats of OvX-allergic groups were treated with estradiol or progesterone before the antigen challenge. Basal values were obtained from nonmanipulated rats. Data are means $\pm$ SE from 5–8 experiments. * P < 0.05 compared with the basal group; ** P < 0.05 compared with the sham OvX group; ϕP < 0.05 compared with the untreated OvX-allergic group; ΔP < 0.05 compared with the OvX-nonallergic group.

cantly enhanced compared with basal cells (Fig. 4B); in contrast, TNF- α secretion by BAL cells from OvX-allergic rats was much less than that of their sham OvX-allergic controls. This effect of OvX was exceedingly reverted by estradiol and progesterone treatments. Supernatants of cultured bone marrow cells from OvX-allergic rats and sham OvX-allergic rats had significantly and similarly increased IL-1 β levels (sham OvX-allergic = 355 ± 27 pg/ml; OvX-allergic = 300 ± 14 pg/ml) compared with values of the basal group (150 ± 10 pg/ml). Similar levels of TNF- α were found in the supernatants of cultured bone marrow cells from OvX-allergic rats (6.2 ± 1 U/ml) and sham OvX-allergic rats (11 ± 1 U/ml), both of which were significantly higher than those in the basal group (3.5 ± 0.3 U/ml).

Role of sex hormones on antigen-induced bronchial mast cell degranulation. The effects of OvX and estradiol or progesterone treatments on in vitro bronchial mast cell degranulation are shown in Table 2 and Fig. 5. The ex vivo antigen challenge caused a significant increase of degranulation of mast cells of bronchial segments taken from sham OvX-allergic rats, whereas those from OvX allergic animals had significantly less degranulation. After the in vivo treatment of OvX-allergic animals with estradiol, bronchial segments showed increased

mast cell degranulation, whereas progesterone had no such effect. It was noticeable that sham OvX and OvX manipulations were not able to change mast cell integrity.

DISCUSSION

In this study, we showed that the magnitude of the allergic lung inflammation in OvX rats is significantly less intense than that of their matched controls (Fig. 1). We found that the OvX-induced depletion of sex hormones augmented the release of IL-10 by BAL and bone marrow cells of allergic rats. Moreover, bone marrow cells from OvX animals reduced the generation of IL-4, whereas their BAL cells decreased IL-1 β and TNF- α release (Figs. 2–4). Of interest was that removal of the ovaries did not modify basal levels of cytokines of nonallergic rats (Figs. 2–4) but reduced bronchial mast cell degranulation after ex vivo allergen challenge (Table 2 and Fig. 5). In our study, treatment of OvX rats with estradiol before the allergen challenge reverted the reduced cellular recruitment into the lungs, whereas progesterone induced an additional reduction of the cellular influx (Fig. 1A). Thus, we inferred that estradiol and progesterone exert opposite effects on allergic lung inflammation.

Although the issue is debatable, estradiol replacement therapy is regularly prescribed for postmenopausal women (44, 47). In this context, data showing both inflammatory and anti-inflammatory effects of estradiol are available (36, 37). In the present study, our view is that the intensity of allergic lung inflammation might be related to the estradiol-to-progesterone level ratio at the moment of immune sensitization and antigen challenge. In fact, when the estradiol-to-progesterone ratio favored estradiol (estradiol/progesterone = 1.5, see sham OvX data in estrus; Table 1), a more intense inflammatory reaction was detected (see the sham OvX-allergic group in Fig. 1A); the opposite was observed when progesterone was favored (estra-

Table 2. Involvement of ovarian hormones on bronchial mast cell degranulation induced by ex vivo antigen challenge

	Numbers of Mast Cells, cells/mm ³		Percent Degranulation
	Intact	Degranulated	
Control groups			
Basal	27.7 $\pm$ 5.1	0.0	0
Sham OvX	35.9 $\pm$ 3.1	0.0	0
OvX	35.2 $\pm$ 3.8	0.0	0
Experimental groups			
Sham OvX-allergic	12.5 $\pm$ 2.5*	50.0 $\pm$ 4.1	80
OvX-allergic	31.1 $\pm$ 6.5	12.6 $\pm$ 1.2†	29
OvX-allergic + estradiol	47.5 $\pm$ 6.9	29.1 $\pm$ 4.2‡	38
OvX-allergic + progesterone	50.4 $\pm$ 6.1	16.5 $\pm$ 4.4	25

Data are means $\pm$ SE of numbers of mast cells (in cells/mm²) of 10 serial sections of 5 rats/group. Mast cell degranulation was taken as a measure of the ex vivo allergic reaction in bronchial tissues isolated from ovalbumin-sensitized OvX rats. The control groups were basal, sham OvX, and nonsensitized OvX female rats. The experimental groups were sensitized rats subjected or not subjected to OvX (OvX-allergic and sham OvX-allergic, respectively) as well as groups of OvX-allergic rats treated with estradiol (280 μ g/kg) or progesterone (200 μ g/kg). Bronchial segments were isolated in rats and processed with ex vivo allergen challenge as described in MATERIALS AND METHODS. * P < 0.01 compared with intact mast cells of sham OvX rats; † P < 0.001 compared with degranulated mast cells of sham OvX-allergic rats; ‡ P < 0.05 compared with OvX-allergic rats.

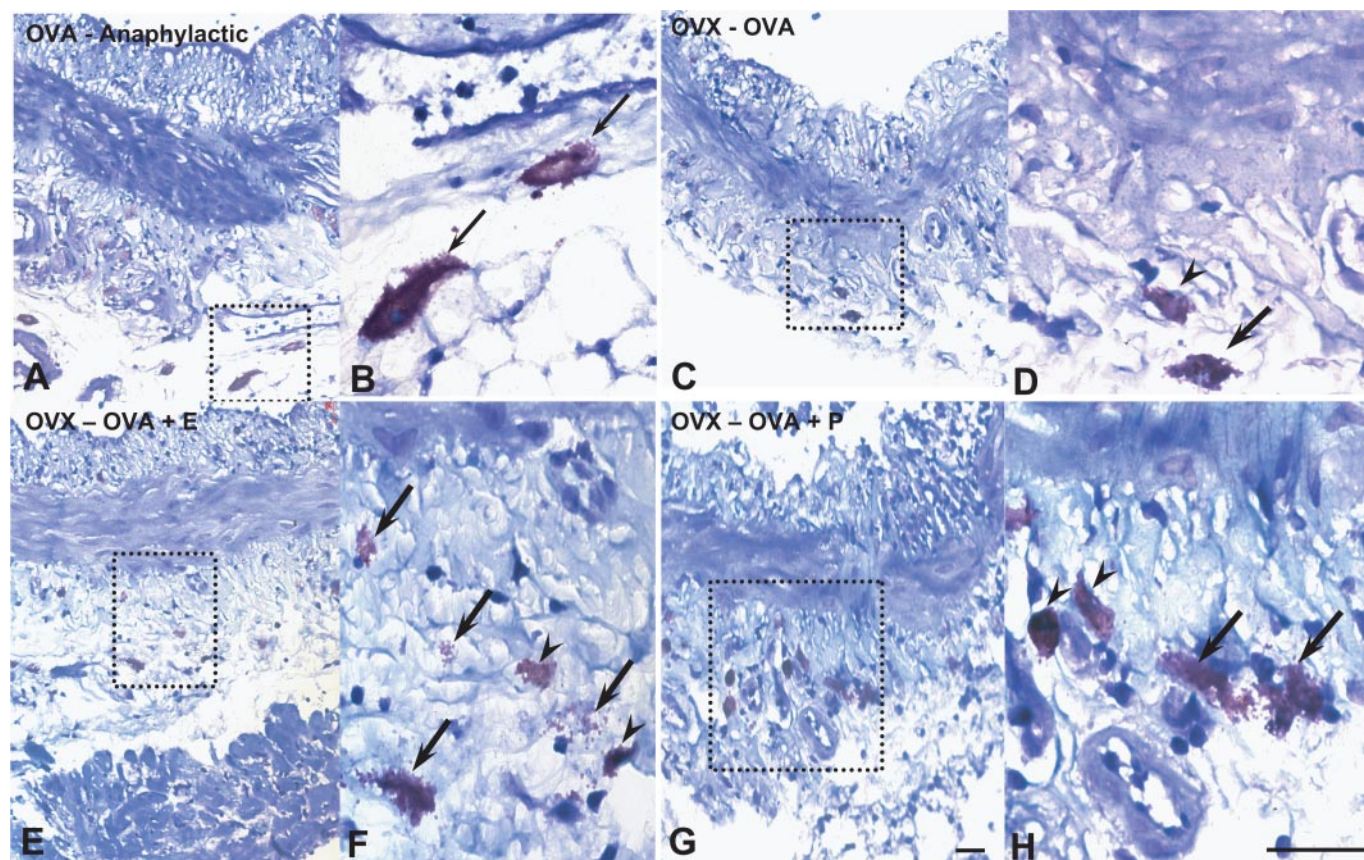


Fig. 5. Mast cell degranulation of bronchial tissue after ex vivo antigen challenge. *A* and *B*: intense mast cell degranulation (arrows) was observed after antigen challenge of bronchial tissue of sham Ovx-allergic rats. *C* and *D*: bronchial tissue of Ovx-allergic rats significantly reduced the mast cell degranulation (arrowhead). Treatment of Ovx-allergic rats with estradiol (*E* and *F*) but not with progesterone (*G* and *H*) markedly increased the mast cell degranulation (arrows) due to the ex vivo antigen challenge. The integrity of mast cells of bronchial segments of basal, sham Ovx, and Ovx rats was not modified. *B*, *D*, *F*, and *H* are higher-magnification images of the dashed boxes in *A*, *C*, *E*, and *G*. OVA, ovalbumin. Tissues were stained with toluidine blue. Bars = 30 μ m.

diol/progesterone = 0.4, see 22-day Ovx-allergic group data in Table 1 and Fig. 1A).

Taking the preceding points into account, we then raised a question concerning the role of sex hormones on the functional activity of mast cells and of the cells recovered from the pulmonary and bone marrow compartments. Thus, cells that did reach the lung were investigated in terms of their functional activity, evaluating the cytokine generation. In the present study, we demonstrated that the bronchial tissue of Ovx-allergic rats had reduced mast cell degranulation after ex vivo antigen challenge (Table 2 and Fig. 5A). This reduced degranulation would result in a decreased release of inflammatory mediators and might explain, at least in part, the decreased lung inflammatory component of Ovx-allergic rats (Fig. 1). On the other hand, such events could not be accounted for by the observed profiles of cytokine release by cultured BAL and bone marrow cells of Ovx-allergic rats (Figs. 2–4).

Our study demonstrated that bronchial mast cell degranulation is enhanced by estradiol and not so by progesterone (Table 2 and Fig. 5). Indeed, sex hormones affect mast cells function, since estradiol facilitates histamine release by these cells after antigen challenge (4, 5). In addition, the estradiol α -receptor mediates the enhancement of mast cell/basophil cell line degranulation (51). Mast cells express estrogen receptors that, once activated by estradiol, cause an augmentation of mast cell

secretion. Interestingly, lipophilic pollutants sharing the effects of estradiol induce an increase of mast cell degranulation (27). In addition, progesterone inhibits histamine secretion from mast cells upon antigen challenge (46). Overall, our data suggest that estradiol and progesterone could display opposed effects in allergic lung inflammation.

Treatment with estradiol reverted the increased release of IL-10 by bone marrow cells, whereas it had the opposite effect on that of IL-4. On the other hand, progesterone did not affect the IL-10 production by bone marrow cells but significantly increased the levels of IL-4 (Figs. 2B and 3). Overall, our data might suggest that upon allergic lung inflammation, estradiol and progesterone exert a relevant modulator role on cytokine release. Administered estrogen or progesterone was sufficient to have a systemic effect and might be associated to cytokine modulation (Table 1). Furthermore, the cytokines quantified in this study are involved directly or indirectly with cell recruitment to the lung and with bronchial hyperresponsiveness observed in asthma (45). For these reasons, we suggest an association between oscillations of sex hormones with a modulation of cytokine generation in the worsening of asthma.

It is known that IL-4 and IL-10 are involved in allergic asthma (13). Indeed, experimental IL-4 neutralization suppresses IgE antibodies and reduces bronchial hyperresponsiveness. Moreover, IL-10, an antiinflammatory cytokine, is a

deviator of the immune system in allergic disease, also suppressing mast cell and eosinophil activity. IL-10 is also involved with the reduction of IgE synthesis (35, 39). Moreover, IL-4 production by CD⁺ T cells is affected by cyclical variations of circulating estradiol levels (48). Yet, estradiol increases the generation of IL-4 by CD⁺ T cells (21). Recently, we (31) demonstrated that estradiol modulates IL-5 and eosinophil recruitment in the murine asthma model. Overall, the interaction between cytokines and sex hormones is confirmed by the fact that sex hormones significantly modulated Th1/Th2-type cytokine production. Our study shows, for the first time, the complex regulatory role of estradiol and progesterone in an asthma model. In agreement, the production of cytokines by bone marrow cells was more sensitive to the modifications imposed by the sex hormones, mainly by estradiol (compare the magnitude of changes of IL-10 and IL-4 levels after estradiol and progesterone treatments in Figs. 2B and 3). Notwithstanding, the functional activity of BAL cells did not follow such changes. Indeed, the release of IL-4 by BAL cells of allergic animals was not changed by Ovx, whereas IL-10 levels were significantly increased after progesterone treatment but were unaffected by estradiol (Fig. 2A).

Estradiol and progesterone were also effective in stimulating the release of IL-1 β and TNF- α by BAL cells, a fact that might suggest the importance of sex hormones as a systemic component of asthma. Since these hormones had a dual effect on cell migration (Fig. 1) and since they also stimulated IL-1 β and TNF- α release by BAL cells (Fig. 4), it is suggested that the reduced inflammation of Ovx-allergic rats coexisting with enhanced production of proinflammatory cytokines IL-1 β and TNF- α might be explained by the surmounting antiinflammatory effect of IL-10. This assumption is based on the fact that treatment of Ovx-allergic rats with estradiol led altogether to increased cell migration and cytokine production, whereas progesterone strongly inhibited cellular influx and enhanced cytokine production (see Figs. 1 and 4). It is of interest that previous data from our laboratory showed that oophorectomy does not modify the synthesis of IgE upon antigen sensitization (23). Therefore, it is likely that the type-1/type-2 cytokine balance, rather than a change in a single cytokine, accounts for the profile of allergic lung inflammation. Thus, we assume that the relative change in sex hormones may predispose to an increased/decreased balance of cytokines in Ovx-allergic animals.

Progesterone showed anti-inflammatory effects (Fig. 1A), and, in certain cases, severe premenstrual exacerbation of asthma has been successfully treated with progesterone, presumably due to smooth muscle relaxation and control of the microvascular leakage (2). However, the risk of serious side effects such as allergic bronchial hyperresponsiveness (17) limits the use of progesterone for this purpose (41). In addition, low physiological doses of progesterone have been shown to increase the production of IL-1 β and TNF- α by human and rat macrophages, whereas higher doses suppressed cytokine release and IL-1 β mRNA expression (24), thus suggesting that the hormonal profile is an important factor for the release of inflammatory mediators.

Considering the present results and those of the literature showing that 17 β -estradiol reduces the proliferation of splenic dendritic cells (50), it is likely that acquired cell immunity is affected by estradiol. Interestingly, recent evidence has indi-

cated that estradiol stimulates the production of Th2-derived anti-inflammatory cytokines such as IL-10, IL-4, and TGF- β (32), which might explain the observed profile of pulmonary inflammation in Ovx-allergic rats. Our previous observations with tamoxifen (23) have suggested that estradiol positively modulates allergic lung inflammation; in the present study, this was confirmed by using ICI-182,780, an estrogen receptor antagonist devoid of agonist activity (18). Reinforcing this view, non-Ovx-allergic rats with ICI-182,780 treatment did not have significantly altered profiles of estradiol and progesterone in serum during the estrous cycle.

The results presented herein identify what appears to be a complex interaction of sex hormones and lung allergic phenomena, whereby estradiol and progesterone modulate the pulmonary influx of inflammatory cells and their functional activity and estradiol mediates mast cell degranulation. Therefore, a putative balance involving the circulating estradiol-to-progesterone ratio might switch the allergic response to an anti-inflammatory response characterized by reduced pulmonary recruitment of eosinophils and increased IL-10 generation. Alternatively, estradiol could exert a proinflammatory effect, as evidenced by the increased generation of IL-4, IL-1 β , and TNF- α (Figs. 3 and 4, A and B). Such dual hormonal effects on the allergic lung inflammatory response may provide an explanation for the conflicting clinical data and suggest that the hormone therapy of postmenopausal women as well as of those suffering of premenstrual asthma would deserve a critical evaluation of the functional status of lung cells and, in more severe conditions, of bone marrow cells.

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