

ORIGINAL PAPER

Progesterone and environmental tobacco smoke act synergistically to exacerbate the development of allergic asthma in a mouse model

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Clinical and Experimental Allergy

Summary

Background Asthma affects males and females differently. Females have a higher incidence than males after the onset of puberty. This suggests a hormonal component to the development of the disease. Progesterone, a female hormone, has previously been shown to illicit a T-helper type 2 (TH2) immune response similar to that seen in allergic asthma. Previous studies performed by our laboratory have shown that exposure to environmental tobacco smoke (ETS) enhances the immune response to allergens.

Objective To determine if the combination of exposure to ETS and progesterone would further exacerbate the immune response in a mouse model of allergic asthma.

Methods Female mice were ovariectomized and then implanted with time-release progesterone pellets. Mice were housed in either filtered air (FA) or ETS chambers and half were exposed to aerosolized house dust mite allergen (HDMA). Bronchoalveolar lavage was performed for cell differentials; lung and spleen cells were harvested to compare IL-4 and IFN- γ production by ELISPOT.

Results Progesterone pellet implantation resulted in increased serum progesterone levels (28.3 ± 8.43 vs. 13.5 ± 7.22 ng/mL in placebo-treated mice, $P < 0.0001$). Serum total IgE levels were significantly greater in progesterone vs. non-progesterone treated animals that were also exposed to HDMA. ETS exposure enhanced total IgE levels as well. Lung homogenate cells from HDMA/progesterone-treated animals stimulated with Concanavalin A produced significantly more IL-4 compared with HDMA/placebo-treated animals (200 ± 17.6 vs. 146 ± 17.5 spots/well, $P < 0.01$ in ETS exposed animals and 221 ± 28.9 vs. 167 ± 23.4 spots/well, $P < 0.01$ in animals housed in FA). HDMA/ETS-treated animals had higher eosinophilia in lavage than all other groups.

Conclusion Increased serum progesterone levels exacerbate the allergic asthmatic phenotype in a mouse model. These effects are further exacerbated by the addition of environmental tobacco smoke. Progesterone provides a major contribution to the gender differences seen in the development and elicitation of the asthmatic response.

Keywords allergy, asthma, environmental tobacco smoke, gender, progesterone

Submitted 14 August 2006; revised 28 September 2006; accepted 27 November 2006

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Introduction

Asthma has an annual direct health cost of more than \$11.5 billion and affects approximately 20 million Americans [1]. Allergic asthma accounts for approximately 10 million of these cases [2]. In childhood males are disproportionately affected by asthma, this difference disappears in pre-adolescence, and females have a much higher incidence after puberty [3–6]. Adult females have a 35% greater prevalence of asthma than males [1]. This differ-

ence suggests a hormonal component to the development of asthma.

The two major female hormones of the oestrous cycle are oestrogen and progesterone. Progesterone is known to create a T-helper type 2 (TH2)-like environment during pregnancy. Allergic asthma is a TH2-mediated disease. The progesterone effects during pregnancy include an increase in immunoglobulins and a decrease in cell-mediated immunity [7]. Lymphocytes from pregnant females produce a protein known as progesterone-induced blocking factor

(PIBF), which results in increased levels of TH2 cytokines including IL-4 and IL-10 [8]. In a stress-induced abortion model in mice treatment with a progestin resulted in significantly fewer abortions due to increased levels of PIBF and IL-4 production [9]. It has been suggested that recurrent spontaneous abortions are often due to decreased levels of progesterone [10]. Other studies have shown that progesterone also elicits a TH2 response in non-pregnant phenotypes. Vermeulen et al. [11] showed that treatment with the progestin medroxyprogesterone acetate lead to increased antibody production following immunization. Other studies have shown that progesterone can induce a known TH1 cell line to secrete the TH2 cytokine IL-4 [12], and progesterone stimulation of peripheral blood mononuclear cell (PBMCs) results in an increase in the cytokines IL-4 and IL-13 [13]. There is also strong evidence that the immune response during the luteal phase of the ovarian cycle (when progesterone is at its highest) is skewed to TH2 [14].

There is conflicting data on the impact of sex hormones on asthma. A phenomenon known as pre-menstrual asthma (PMA) that affects up to 40% of asthmatic women and results in an exacerbation of asthmatic symptoms in the days leading up to menstruation has been described [15, 16]. There are also significantly more near-fatal asthma attacks corresponding to menstruation than at other times of the cycle [17]. Lange et al. studied the relationship of exogenously administered hormones and the development of asthma. They found no association between oral contraceptive use and the development of asthma. They did, however, find a small but significant increase in asthma risk in woman who had been on hormone replacement therapy (HRT). Similarly, Triosi et al. showed that woman on HRT for at least 10 years had twice the age-related risk of asthma as those who had never been on HRT. In terms of pulmonary function, there are numerous studies in both humans and mice that show an increase in airways hyper-reactivity and/or bronchoconstriction (two major hallmarks of asthma) when the highest levels of progesterone are present [18–20]. Other studies on post-menopausal asthmatics on HRT actually show an improvement in their forced expiratory volume [21]. It has been suggested that some of the conflicting data may be due to the need of separate out allergic asthmatics from patients with other non-atopic forms of asthma [22]. Progesterone treatment in a male mouse model of allergic asthma resulted in an increase of the TH2 cytokines IL-4 and IL-5 as well as increased airway eosinophilia [20]. Hamano et al. [23], showed that while testosterone reduced the adhesion of eosinophils, β -estradiol enhanced adhesion and the addition of progesterone led to increased degranulation of eosinophils. All of this supports the hypothesis that sex hormones effect allergic asthma.

Environmental tobacco smoke (ETS) has also been shown to play a role in allergic asthma. Our group

demonstrated in a mouse model of allergic asthma that combined exposure to ovalbumin (OVA) and ETS in OVA-sensitized mice lead to an increased and prolonged response with respect to IgE, IgG1, eosinophils, and TH2 cytokines [24]. Rumold et al. [25] reported similar findings and showed that ETS was capable of acting as an adjuvant for a TH2 response in the absence of parenteral sensitization to allergen. Our group also demonstrated gender differences in the ETS enhancement of allergic asthma from birth in OVA-sensitized mice [26]. Female mice not only had significantly increased asthmatic phenotypes over males, but this response was also exacerbated in those exposed to ETS pre- and post-natally.

In the present study we examined the mechanism by which this exacerbation of the asthmatic phenotype occurs. Female BALB/c mice were ovariectomized and implanted with progesterone pellets to look specifically at the hormone component of this effect. We hypothesized that the addition of ETS would result in pathogenic synergy with the progesterone. In contrast to other studies house dust mite allergen (HDMA) was the allergen used for sensitization. Studies show that 45–85% of allergic asthmatics are allergic to HDMA [27].

Materials and methods

Animals

Four-week-old female BALB/c mice were obtained from Charles River Laboratories Inc. (Wilmington, MA). Animals were housed in filtered air (FA) upon arrival and allowed to acclimate for 1 week before surgery. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis.

Experimental protocol

Ovariectomized BALB/c mice were implanted with either progesterone or placebo pellets on day – 5 (Table 1). The animals were exposed to either ETS or FA from days – 1 to 38. They were sensitized on day 0 and day 21 via an IP injection of HDMA/Alum or PBS/Alum for control animals. HDMA aerosols were performed on days 29, 31, 33 and 36, 38, 40. Animals were killed on day 42. Bronchoalveolar lavage (BAL) was performed on half of the animals from each group, and the lungs from the remaining mice were used for histological evaluation (to be presented elsewhere). Immunological analysis for allergic phenotype was evaluated using serum, bronchoalveolar lavage fluid (BALF) and cells, and via *in vitro* stimulation of cells from the lung and spleen. There were four treatment groups and each was further divided for FA or ETS exposure resulting in a total of eight groups, (see Table 2 for descriptions).

Table 1. Experimental protocol

Day	Group 1		Group 2		Group 3		Group 4	
– 4	Placebo implantation		Placebo implantation		Progesterone implantation		Progesterone implantation	
– 1	Start ETS exposure for 1/2 of the animals from each major group							
0	PBS/AIOH IP		HDMA/AIOH IP		PBS/AIOH IP		HDMA/AIOH IP	
21	PBS/AIOH IP		HDMA/AIOH IP		PBS/AIOH IP		HDMA/AIOH IP	
29, 31, 33	–		HDMA aerosol		–		HDMA aerosol	
36, 38, 40	–		HDMA aerosol		–		HDMA aerosol	
42	Lungs	BAL	Lungs	BAL	Lungs	BAL	Lungs	BAL

PBS, phosphate buffered saline; HDMA, house dust mite allergen; BAL, bronchoalveolar lavage.

Table 2. Treatment group assignments

Group	Treatment	ETS (n)	FA (n)
1	PBS/placebo	10	10
2	HDMA/placebo	10	10
3	PBS/progesterone	10	10
4	HDMA/progesterone	11	11

PBS, phosphate buffered saline; HDMA, house dust mite allergen; ETS, environmental tobacco smoke; FA, filtered air.

Ovariectomy

Mice were anaesthetized with an intraperitoneal (IP) dose of ketamine/xylazine (100 and 5 mg/kg, respectively). The shaved mouse was placed in ventral recumbency on a heated block and covered with sterile surgical drapes. Two incisions were made in the flank area (right and left). The uterine horns were visualized, brought up to the surface of the incision, and held with forceps while a battery-operated cautery was used to remove the ovary on each side. The abdominal muscle was closed with a single-interrupted suture using Coated VisorbTM polyglycolic-acid synthetic absorbable 5/0 suture (CP Medical, Portland, OR, USA). The skin was then closed with a wound clip. Mice were given 1 cm³ Lactated Ringers subcutaneously (SQ) immediately following the surgery. Once fully ambulatory, analgesic was administered (0.08 cm³; 1/30 Buprenex, Pickett Coleman Products, Richmond, VA, USA). Animals were monitored daily in accordance with UC Davis Animal Use and Care committee policies and guidelines for survival surgery in rodents. Wound clips were removed 6–7 days post-surgery.

Pellet implantation

Pellets were obtained from Innovative Research of America (Sarasota, FL, USA). Each pellet contained 12.5 mg of product (progesterone or placebo) and had a diameter of 3 mm. The pellets were designed to release their contents over a 60-day period. Two pellets were implanted into each animal. Mice were anaesthetized with an IP dose of ketamine/xylazine as in the ovariectomy surgery. Small

puncture wounds were created subcutaneously on the lateral side of neck of the animal using a number 15 scalpel. A 10° trochar device was then used to insert a 3 mm pellet approximately 2 cm beyond the incision.

Environmental tobacco smoke exposure

Mice were exposed to ETS at the UCD Center for Health and the Environment in chambers designed by Teague et al. [28]. The smoke was generated using a Model TE-10 smoke machine (Teague Enterprises, Davis, CA, USA) and type 2R4F research-reference cigarettes. The cigarettes were produced by the Kentucky Tobacco Research & Development Center (University of Kentucky, Lexington, KY, USA). Each exposure chamber has a measurement of 69 cm × 69 cm × 61 cm. Mice were exposed for 6 h a day Monday through Friday for a total of 6 weeks. The animals remained in these chambers after the smoke generation ceased and were supplied with FA. Once allergen exposure began, the HDMA animals were housed in a separate ETS exposure chamber (chamber #2). The total suspended particles were measured and had a total concentration of 1.02 ± 0.07 mg/m³ for both chambers. Nicotine concentration in chamber 1 was 217.5 ± 81.4 and 232.5 ± 34.65 µg/m³ in chamber 2. Carbon monoxide levels for the two chambers were 2.31 ± 0.77 and 2.71 ± 0.57 ppm, respectively.

Sensitization injections

Each animal received two IP sensitization injections given 3 weeks apart. The injections contained 5 µg of the HDMA *Dermatophagoides farinae* (Greer Laboratories, Inc., Lenoir, NC, USA) and 2 mg of aluminium hydroxide adjuvant in a total volume of 0.5 mL. Non-sensitized control animals received the adjuvant alone in sterile saline.

House dust mite aerosolization

Mice were exposed to *D. farinae* using a Tri-R Airborne Infection Apparatus (Tri-R Instruments, New York, NY,

USA) first described by Middlebrook [29]. Briefly, the mice were placed in a divided wire cage within the exposure unit. A HeartTM nebulizer (Westmed Inc., Tucson, AZ, USA) was used to aerosolize a 1 mg/mL solution of HDMA, which then passed through a venturi nebulizer before entering the exposure chamber. Animals were exposed for 30 min. The amount of HDMA aerosolized was 8.6 ± 1.62 mL for an average aerosol output of 0.29 ± 0.054 mL/min. The chamber was exhausted for 20 min following exposure. Animals were then removed from the chamber and returned to FA.

Serum progesterone extraction and enzyme-linked immunosorbent assay

Serum progesterone levels were measured using a commercially available ELISA kit (Neogen Corporation, Lexington, KY, USA). Before performing the assay, progesterone was extracted from the serum according to manufacturer's directions. Briefly, serum was added to petroleum ether and vortexed. The phases were then allowed to separate and the organic phase containing the extracted progesterone was removed. The liquid was then evaporated using a stream of nitrogen gas produced by an N-EVAP Analytical Evaporator (Organomation Associates Inc., South Berlin, MA, USA). The residue was then dissolved in extraction buffer provided with the kit. The ELISA was a competition assay between a progesterone enzyme conjugate and the progesterone in the sample. A standard curve was run with all samples with a detectable range of 0–40 ng/mL. Data is presented in terms of ng/mL.

Necropsy, bronchoalveolar lavage and tissue preparation

Animals were killed via an overdose with sodium pentobarbital. Animals were first bled from the orbital venous sinus and finally exsanguinated from the axillary artery. Spleens were harvested for cell culture. The tracheas of all animals were cannulated. BAL was performed on half of the animals using the method described by Medin et al. [30]. Three 1 cm³ instillations of Hanks balanced salt solution (HBSS) were used to lavage the lungs. The lungs were then harvested for cell culture. The lungs of the other animals were tied off at the hilus following cannulation. The left lobes were fixed with 4% paraformaldehyde at 30 cm pressure for 1 hour and stored in 4% paraformaldehyde until they were processed into paraffin (data presented elsewhere). The right lobes were harvested for cell culture.

Cell preparation

Total BAL cells were quantified using a Coulter particle counter (Beckman Coulter, Fullerton, CA, USA). Cells were spun down onto slides using a cytocentrifuge and stained with a modified Wright–Geimsa stain (Hema 3, Fisher

Scientific, Hampton, NH, USA). Two hundred cells were counted and differential cell counts were performed.

Splenocytes were extracted from harvested spleens and red blood cells were lysed. Cells were washed in HBSS and cell counts performed. CD4⁺ cells were negatively selected for using a mixture of magnetic beads that were coupled with rat antibodies against CD45R, CD11b, Ter-119, CD16/32 and CD8 (DynaL Biotech, Oslo, Norway). Cell counts were performed on the negatively selected cells resuspended in culture media.

Harvested lungs were finely chopped and homogenized in media containing 30 µg/mL of DNase 1 and 150 units/mL of collagenase. Digestion took place for 1 h at 37 °C. Following digestion the red blood cells were lysed. The homogenate was passed over a cell strainer and the single cell suspension was washed in HBSS. Cells were resuspended in culture media and counted.

Cytokine producing cells

Cytokine producing cells were enumerated by enzyme-linked immunospot assay (ELISPOT). IL-4 and IFN-γ production was assayed in lung homogenate cells and in CD4⁺ splenocytes using commercially available kits (R&D Systems, Minneapolis, MN, USA). Cells were added to polyvinylidene difluoride (PVDF)-backed microplates coated with cytokine-specific antibodies at a concentration of 1×10^5 cells/well. Cells were then stimulated in duplicate with either media alone, Concanavalin A (ConA) (5 µg/mL), or HDMA (0.5 mg/mL) for 23 h in a 37 °C CO₂ incubator. Unbound cells were washed away and a biotinylated antibody specific for the given cytokine was added. Next streptavidin conjugated alkaline-phosphatase was added and subsequently the spots were developed using a substrate that caused a blue–black precipitate to form. Each spot represents a single cytokine producing cell. The spots were enumerated using a Zeiss KS Elispot Reader (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

Total immunoglobulin E enzyme-linked immunosorbent assay

Total IgE in serum was determined using a commercially available ELISA kit (Bethyl Laboratories Inc., Montgomery, TX, USA). The assay was performed with minimal changes to the manufacturers instructions. Briefly, 96-well plates were coated with a 1 : 100 dilution of goat anti-mouse IgE antibody in carbonate/bicarbonate buffer pH 9.6 for 1 h at room temperature followed by overnight incubation at 4 °C. Following extensive washing with PBS-Tween, the plate was blocked with 1% bovine serum albumin and incubated for 30 min at 37 °C. The plate was washed again and the standards and samples were added and allowed to incubate for 1 h at 37 °C. The standard curve had a range from 3.9 to 250 ng/mL. Serum samples were diluted 1 : 150 in PBS. The bound antibody was

detected using a goat anti-mouse IgE antibody conjugated to horse radish peroxidase at a dilution of 1 : 10 000. Data is presented in ng/mL.

House dust mite allergen-specific immunoglobulin E enzyme-linked immunosorbent assay

HDMA-specific IgE was determined by ELISA. Briefly, 96-well plates were sensitized with 10 µg/well of HDMA in carbonate/bicarbonate buffer pH 9.6 overnight at 4 °C. Plates were then blocked with 1% rabbit serum albumin. Following extensive washing with PBS-Tween, pooled serum samples and controls were diluted 1 : 4 and plated at 50 µL/well and incubated for 2 h at 37 °C. The bound antibody was detected using a horse radish peroxidase-conjugated goat anti-mouse IgE antibody at a dilution of 1 : 5000 (Bethyl Laboratories Inc., Montgomery, TX, USA). Data was corrected for interplate variation and is presented as corrected absorbance values.

Data analysis

All data analysis was done using the graphing/statistical programme Prism (GraphPad Software Inc., San Diego, CA, USA). Significant differences between the groups were determined using one-way ANOVA followed by Tukey's test. Differences were considered significant if the *P*-value was 0.05 or lower.

Results

Effects of house dust mite allergen, progesterone and environmental tobacco smoke on cytokine production in lung homogenate

To examine the effects of progesterone treatment and ETS exposure on cytokine profiles, cells were isolated from both the lung and the spleen. Cytokine production was determined by ELISPOT for both IL-4 and IFN-γ under both ConA and HDMA stimulation. The greatest difference seen between the groups was in IL-4 production in ConA-stimulated cells from the lung (Fig. 1a). The HDMA/progesterone-treated animals had significantly greater production of IL-4 in both ETS and FA over all other groups. The specific effect of progesterone can be seen by comparing the HDMA/progesterone-group to the HDMA/placebo-group (200 ± 17.6 vs. 146 ± 17.5 spots/well, $P < 0.01$) in the ETS group and (221 ± 28.9 vs. 167 ± 23.4 spots/well, $P < 0.01$) in the FA group, respectively. No progesterone effect was seen in the HDMA-stimulated cells (Fig. 1b). HDMA-treated animals had significantly more IL-4 production in both ConA and HDMA-stimulated cells over the non-HDMA-treated animals. In contrast, IFN-γ production in ConA-stimulated cells was the highest in the HDMA/placebo-treated animals (Fig. 1c). Both the ETS and FA animals in this group

had significantly more IFN-γ production than the HDMA/progesterone/ETS group (58 ± 9.6 and 47 ± 14.3 vs. 18 ± 5.9 spots/well, $P < 0.001$ and < 0.01 , respectively). There was a non-significant trend of these also having greater IFN-γ production than the HDMA/progesterone/FA group. There was no significant production of IFN-γ in the HDMA-stimulated cells (Fig. 1d).

Effects of house dust mite allergen, progesterone and environmental tobacco smoke on cytokine production in CD4⁺ splenocytes

Splenocytes were enriched for CD4⁺ cells by negative selection. As with the lung homogenate, the splenocytes were stimulated with both HDMA and ConA to evaluate IL-4 and IFN-γ production. All HDMA-treated groups had comparable numbers of IL-4 spots per ELISPOT well in the ConA-stimulated cells and all were significantly higher than the non-HDMA-treated groups (Fig. 2a). House dust mite stimulation of CD4⁺ (Fig. 2b) cells resulted in the greatest production of IL-4 in the HDMA/placebo/FA group (35 ± 9.6 spots/well); a significant increase over the HDMA/progesterone/FA group (12 ± 4.2 spots/well, P -value < 0.01) as well as the non-HDMA-treated groups. The HDMA/placebo/ETS group also had significantly more IL-4 producing cells than the non-HDMA-treated groups. IFN-γ production in the CD4⁺ splenocytes was very limited. The only slightly significant result was seen in the ConA stimulation of the HDMA/placebo/FA group (9 ± 4.6 spots/well, P -value < 0.05 vs. both FA/placebo groups and the FA/progesterone/ETS group, Fig. 2c).

Eosinophilia in the airways of sensitized mice

One of the major hallmarks of allergic asthma is the development of airway eosinophilia. In this experiment BAL was performed on mice to determine the cellular accumulations present in their airways. All HDMA-treated animals had significantly increased levels of eosinophils over non-HDMA-treated animals (Fig. 3). The HDMA/ETS groups had the highest levels of absolute cells/mL ($1.8 \times 10^6 \pm 0.8 \times 10^6$ cells/mL in the progesterone group and $1.8 \times 10^6 \pm 1 \times 10^6$ cells/mL in the placebo group). The group with the next highest level of eosinophilia was the HDMA/progesterone/FA group with a count of $1.6 \times 10^6 \pm 1 \times 10^6$ cells/mL. The HDMA/placebo/FA group had a count of $0.6 \times 10^6 \pm 0.2 \times 10^6$ cells/mL. None of the PBS-treated groups had any eosinophils present in their lavage.

Effects of environmental tobacco smoke exposure and progesterone treatment on the protein content of bronchoalveolar lavage fluid

Protein concentration was measured in BALF using a standard Bradford protein assay (Fig. 4). The HDMA/

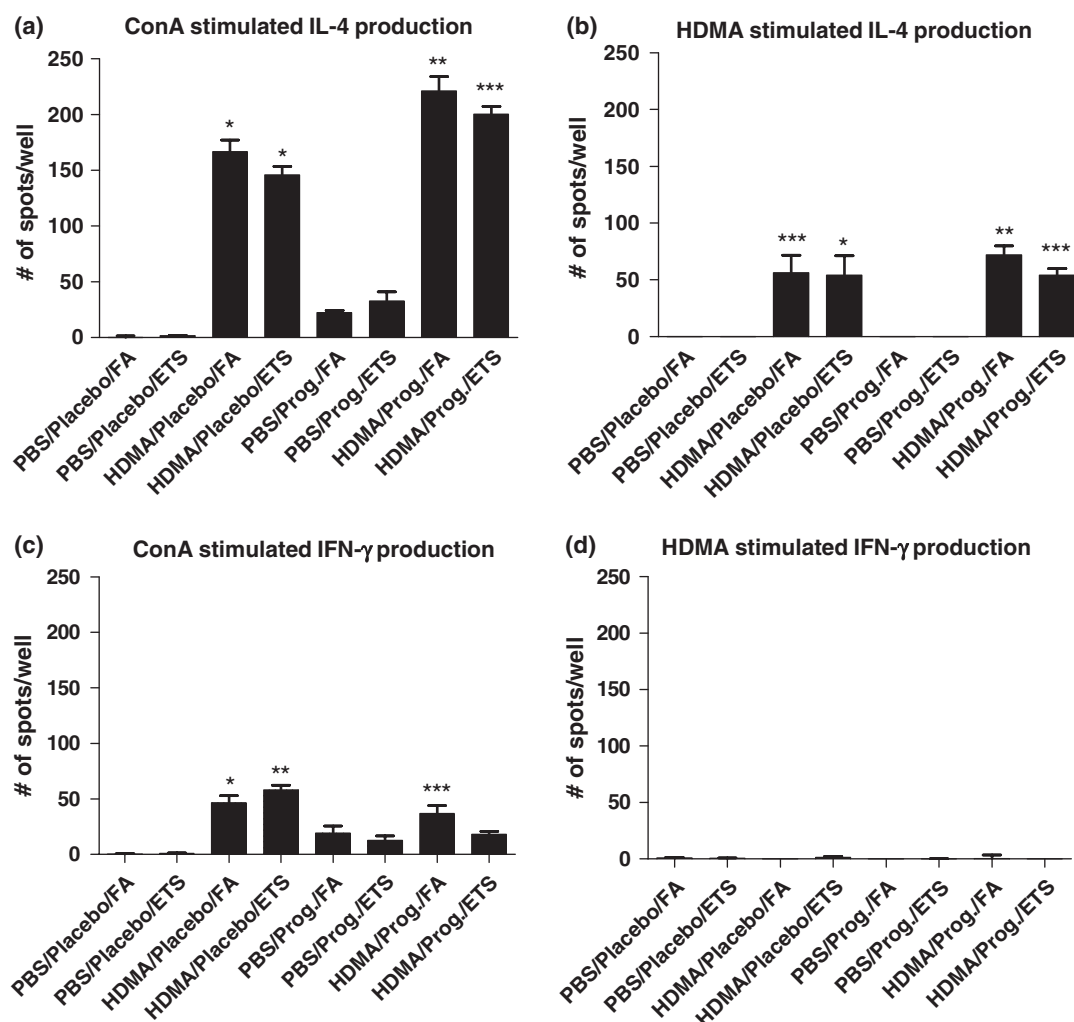


Fig. 1. Cytokine production in lung homogenate cells. (a) ConA-stimulated IL-4 Production: ****P*-value < 0.001 vs. Groups 1 and 3 [environmental tobacco smoke (ETS) and filtered air (FA)], < 0.01 vs. Group 2 (ETS). ***P*-value < 0.001 vs. Group 1 and 3 (ETS and FA) & Group 2 (ETS), < 0.01 vs. Group 2 (FA). **P*-value < 0.001 vs. Group 1 and 3 (ETS and FA). (b) House dust mite allergen (HDMA) stimulated IL-4 production: ****P*-value < 0.05 vs. Groups 1 and 3 (ETS and FA), ***P*-value < 0.01 vs. Groups 1 and 3 (ETS and FA), **P*-value < 0.05 vs. Group 3 (ETS) and Group 1 (FA). (c) ConA-stimulated IFN- γ production: ****P*-value < 0.01 vs. Group 1 (ETS and FA), ***P*-value < 0.001 vs. Group 4 (ETS), Group 1 and 3 (ETS and FA), **P*-value < 0.001 vs. Group 1 (ETS and FA), < 0.01 vs. Groups 3 and 4 (ETS), < 0.05 vs. Group 3 (FA). (d) HDMA-stimulated IFN- γ production: no statistical significance.

progesterone-treated animals had the highest level of protein in the BALF [0.82 ± 0.133 mg/mL (ETS) and 0.86 ± 0.077 mg/mL (FA)]. Both of these levels are significantly higher than all non-HDMA-treated animals as well as the HDMA/placebo/FA animals (0.57 ± 0.076 mg/mL, *P*-value < 0.001).

Total and house dust mite allergen-specific immunoglobulin production in serum

Figure 5a shows that both of the HDMA/progesterone-treated groups had the highest mean total IgE levels in serum [$24\,900 \pm 11\,060$ ng/mL (ETS) and $25\,760 \pm 6892$ ng/mL (FA)]. Serum concentrations from these two

groups are statistically significantly greater than those seen all other six treatment groups. The next highest concentration of total IgE is seen in the HDMA/Placebo-treated groups [$15\,570 \pm 3\,072$ ng/mL (ETS) and $13\,720 \pm 4\,143$ ng/mL (FA)]. Serum concentrations from these two groups are statistically significantly greater than those seen in all four non-HDMA-treated groups. While not statistically significant, there was a trend that the PBS/progesterone-treated groups had higher total IgE than the PBS/placebo-treated groups (3260 ± 2236 vs. 1155 ± 1005 ng/mL in ETS groups and 3563 ± 1757 vs. 648 ± 542 ng/mL in FA-treated groups).

Figure 5b shows that all of the HDMA-sensitized animals had significantly greater levels of serum HDMA-

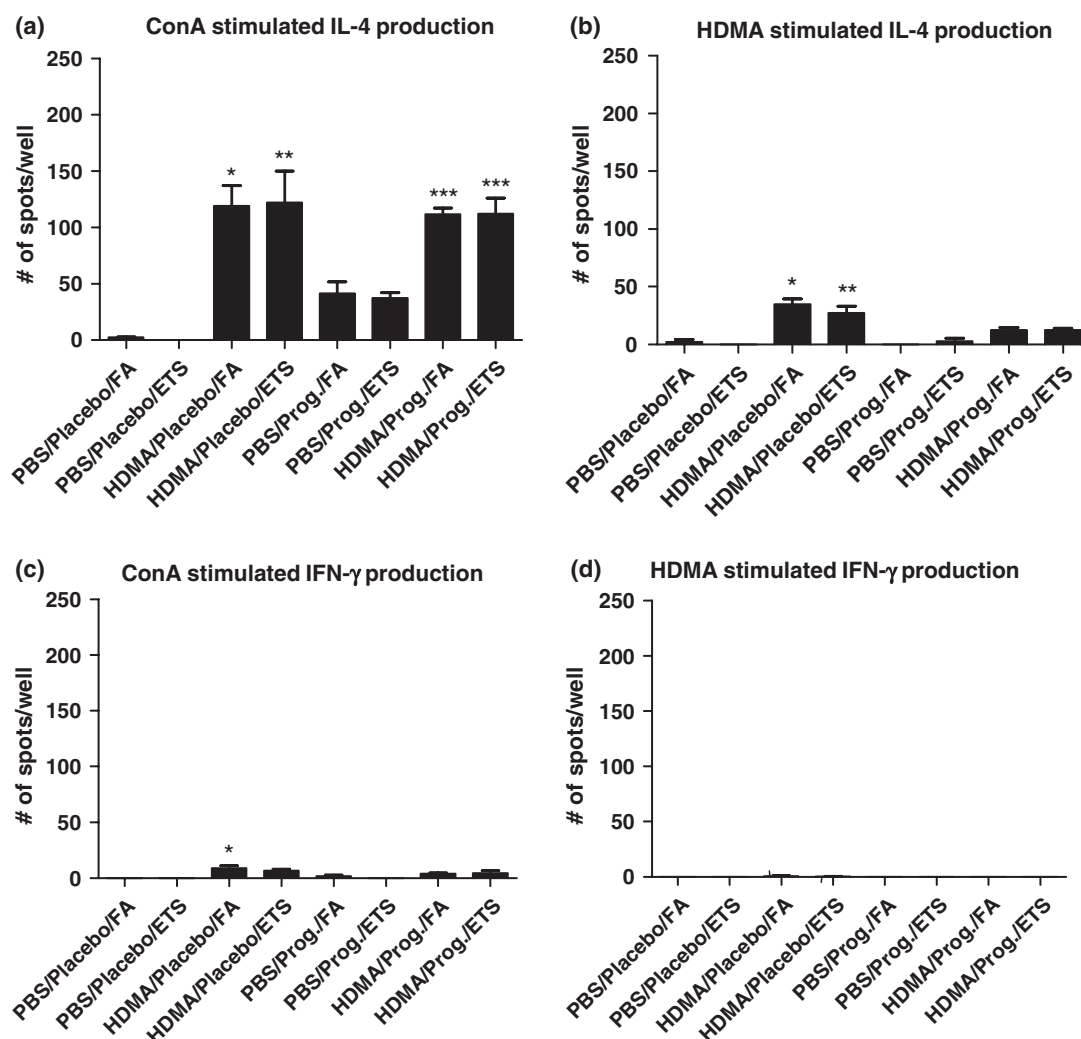


Fig. 2. Cytokine production in CD4⁺ splenocytes: (a) ConA-stimulated IL-4 production: ****P*-value < 0.01 vs. Group 1 [environmental tobacco smoke (ETS) and filtered air (FA)], ***P*-value < 0.01 vs. Group 1 (ETS and FA), < 0.05 vs. Group 3 (ETS and FA), **P*-value < 0.01 vs. Group 1 (ETS and FA), < 0.05 vs. Group 3 (FA). (b) House dust mite allergen (HDMA)-stimulated IL-4 production: ***P*-value < 0.001 vs. Group 3 (FA) and Group 1 (ETS), < 0.01 vs. Group 3 (ETS) and Group 1 (FA), **P*-value < 0.001 vs. Group 4 (ETS) and Group 1 and 3 (ETS and FA), < 0.01 vs. Group 4 (FA). (c) ConA stimulated IFN-γ production: **P*-value < 0.05 vs. Group 3 (ETS) and Group 1 (ETS and FA). (d) HDMA stimulated IFN-γ production: no statistical significance.

specific IgE than that seen in the non-sensitized controls. Neither progesterone nor ETS exposure had any apparent effect on serum HDMA-IgE levels.

Serum progesterone levels

Implanted progesterone pellets resulted in an increase in serum progesterone levels over the placebo pellets. Progesterone-treated animals had a mean serum progesterone level of 28.3 ± 8.42 vs. 13.5 ± 7.22 ng/mL in the placebo-treated animals, *P*-value < 0.0001.

Discussion

There has been much debate about how specific hormones affect asthma. One of the greatest difficulties in separating

out the specific effects of progesterone or oestrogen is the fact that the two hormones do not normally exist in an isolated state *in vivo*. This study aimed to ratify that problem by eliminating the production of endogenous hormones via ovariectomizing mice and then treating the animals with exogenous progesterone via pellet implantation resulting in increased serum levels of the hormone. A previous study in male mice looked at the effects of instilling a progestogen directly into the trachea on the development of an allergic phenotype [20]. To our knowledge this is the first study of its kind to evaluate the effect of elevated serum progesterone levels on the development of allergic asthma in a mouse model. This study corroborates the findings of Hellings et al. [20]. It clearly demonstrates that elevated levels of serum progesterone exacerbated the allergic phenotype of the animals.

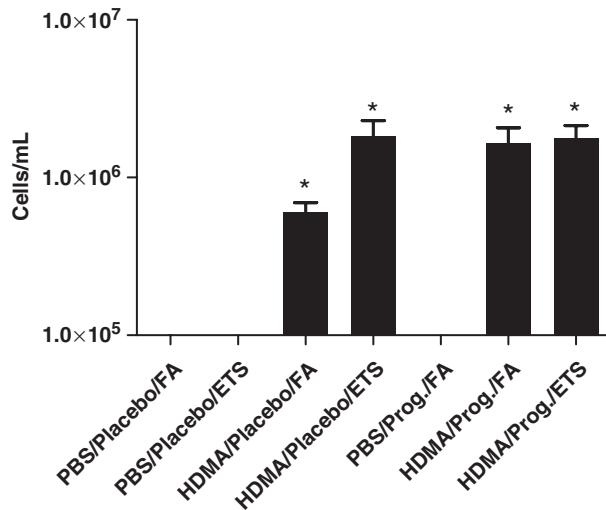


Fig. 3. Eosinophils in bronchoalveolar lavage: **P*-value < 0.05 vs. Group 3 [filtered air (FA)] and Group 1 [environmental tobacco smoke (ETS) and FA] data from Group 3 (ETS) is unavailable due to technical error.

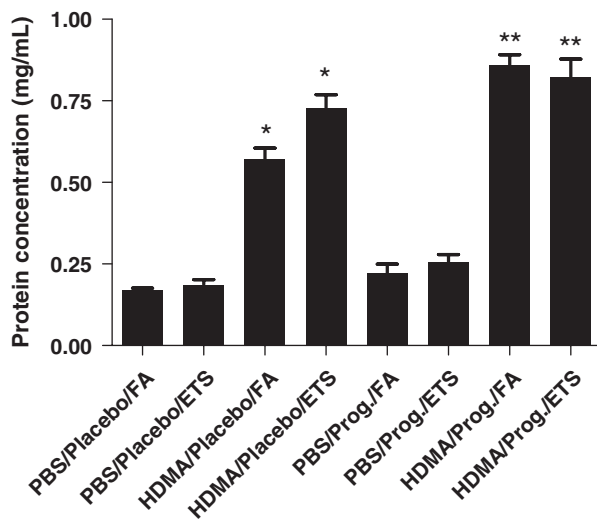


Fig. 4. Protein concentration in bronchoalveolar lavage: ***P*-value < 0.001 vs. Groups 1 and 3 [environmental tobacco smoke (ETS) and filtered air (FA)] and Group 2 (FA), **P*-value < 0.001 vs. Groups 1 and 3 (ETS and FA).

A TH2 cytokine response is characteristic of allergic asthma. A major TH2 cytokine involved in this response is IL-4. HDMA-sensitized animals treated with progesterone had a significant increase in IL-4 production when lung homogenate cells were stimulated with a T cell mitogen over the equivalent non-progesterone-treated animals. These same animals had significantly lower IFN- γ production in the same cell population. This demonstrates a clear shift towards a TH2 phenotype as one would expect in a model of allergic asthma. Progesterone treatment in

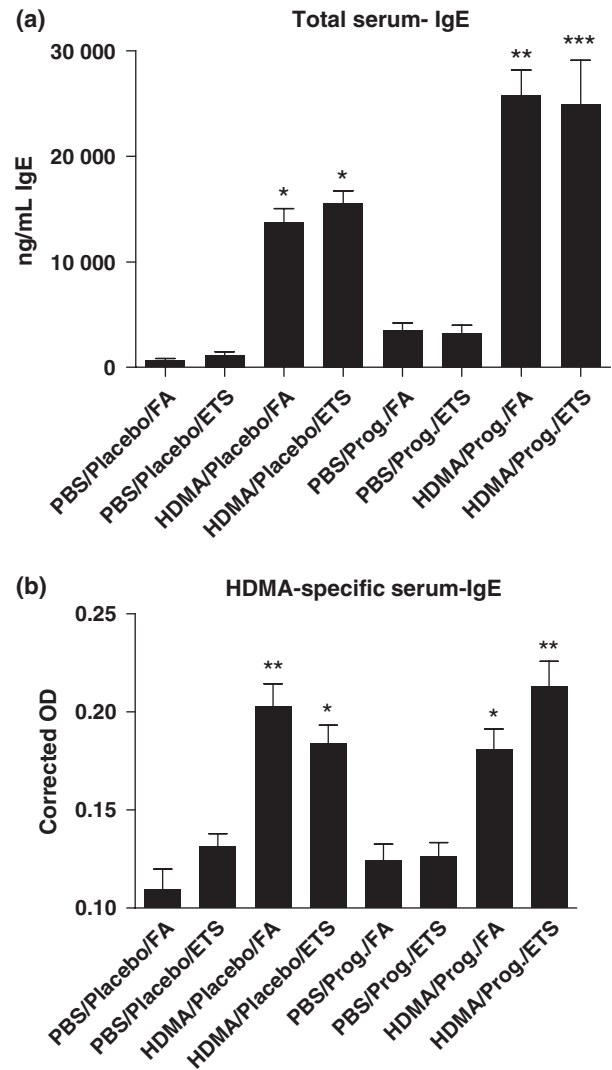


Fig. 5. (a) Total IgE in serum: ****P*-value < 0.001 vs. Groups 1 and 3 [environmental tobacco smoke (ETS) and filtered air (FA)] and Group 2 (FA), < 0.05 vs. Group 2 (ETS), ***P*-value < 0.001 vs. Groups 1 and 3 (ETS and FA) and Group 2 (FA), < 0.01 vs. Group 2 (ETS), **P*-value < 0.001 vs. Groups 1 and 3 (ETS and FA). (b) House dust mite allergen (HDMA)-specific IgE in serum: ***P*-value < 0.001 vs. Groups 1 and 3 (ETS and FA), **P*-value < 0.01 vs. Groups 3 (ETS and FA), < 0.05 vs. Group 1 ETS, < 0.001 vs. Group 1 (FA).

the non-HDMA-sensitized animals did not have this same effect. Levels were slightly increased in the progesterone- vs. non-progesterone-treated animals (Fig. 1) but this was not a statistically significant difference. This observation indicates that the progesterone alone was not enough to cause the TH2 shift; it merely exacerbated the reaction in the already allergen-primed animals. This is demonstrated further by the effects of HDMA stimulation on the same cell populations. Here the progesterone affect is not apparent because the effects of HDMA stimulation account for only the portion of IL-4 production caused specifically in response to the sensitization and

aerosolization of the HDMA. In contrast, ConA is a T cell mitogen and therefore amplifies the cytokine production seen in response to all other stimuli including the progesterone and ETS. ETS exposure did not have a noticeable effect on cytokine production.

The progesterone effect seen in the HDMA-exposed animals in the lung was absent from the splenocytes. Mitogen stimulation resulted in equivalent IL-4 production in all HDMA treatment groups. The animals with the most IL-4 production under HDMA stimulation were actually in the non-progesterone HDMA-treated groups. This is suggestive that the progesterone effects are targeted to the lung. It is possible that this is due to progesterone receptor distribution in tissues. It has been shown that the lung has high-affinity binding sites for progesterone receptor complexes while the spleen does not [31]. Additionally, progesterone receptors have often been detected in different forms of lung cancer [32, 33]. Another reason for the difference in IL-4 production between the two tissues is the type of cells that are being stimulated. In the lung whole lung homogenates were studied vs. CD4⁺ cells in the spleen. Lung homogenates contain multiple cell types that could potentially produce IL-4 including lymphocytes and mast cells. Expression of progesterone receptors on mast cells has been demonstrated in human upper airways [34]. Clearly, CD4⁺ cells do not produce IL-4 in isolation from all other cell types. The lack of help from other cell populations such as antigen presenting cells explains why the total overall IL-4 production is reduced in the spleen. It has also been shown that PIBF stimulated CD4⁺- and CD8⁺-enriched cell populations had equal amounts of IL-3, IL-4 and IL-10 production [8]. Histological evaluation of fixed lung tissue is currently underway and is to be presented in a later publication. Progesterone receptor distribution will be evaluated to help elicit the mechanism by which progesterone affects the pathogenesis of allergic asthma.

Immunoglobulin E is the antibody involved in allergies and asthma. The major symptoms of allergy are mediated by IgE cross-linking with its specific allergen, which results in mast cell degranulation and the release of multiple mediators. IL-4 released from TH2 cells causes class switching of B cells to generate IgE producing plasma cells. Animals in this study were sensitized and exposed to HDMA. A successful model of allergy requires the development of allergen-specific IgE. The data presented (Fig. 5b) demonstrates that the model successfully induces specific IgE production. Neither Progesterone nor ETS had a noticeable effect on HDMA-specific IgE production.

The results from the total IgE data show a significant progesterone effect. Figure 5a shows that the HDMA/progesterone groups have significantly more total IgE production than the HDMA/placebo groups. While ETS alone does not significantly increase total IgE it does synergize with progesterone in the HDMA-treated animals to increase total IgE. This can be seen when comparing the

HDMA/progesterone/ETS group with both the HDMA/placebo/FA and HDMA/Placebo/ETS groups. While the HDMA/progesterone/ETS group has significantly more total IgE than both of the HDMA/placebo groups, the difference is more significant ($P < 0.001$) vs. the HDMA/placebo/FA group than in the HDMA/Placebo/ETS group ($P < 0.05$). This same trend is seen in the HDMA/Progesterone/FA group vs. the HDMA/Placebo/FA ($P < 0.001$) and the HDMA/Placebo/ETS group ($P < 0.01$). Numerous studies have shown that an increase in total IgE is closely related to airway hyper-responsiveness [35] and reported asthmatic symptoms [36] irrespective of allergen-specific IgE. This is suggestive that other components beyond allergens can result in increased IgE production and asthmatic symptoms in asthmatics. When comparing the three different stimuli in this study it is clear that HDMA has the greatest effect on total IgE production, followed by progesterone and then ETS. Previous studies by this laboratory have demonstrated the ability of ETS to increase Total IgE [24]. This effect is most likely muted in the current study due to the fact that HDMA is such a potent allergen that its effects overshadow the effects of the ETS. The current study shows that progesterone has a clear effect on total IgE levels in the induced allergic asthmatic phenotype and this effect is exacerbated by exposure to ETS indicating pathogenic synergy.

Airway inflammation is another major hallmark of allergic asthma. The most common cell type seen in airway inflammation is the eosinophil. This study clearly demonstrated the development of allergen-induced airway inflammation. While there was not a significant difference in the absolute cell counts among the HDMA-treated groups, the greatest level of eosinophilia was seen in the HDMA/ETS-treated groups demonstrating an ETS effect. There was also a trend that the HDMA/progesterone/FA group had increased levels of eosinophils over the HDMA/placebo/FA group indicating a progesterone effect. Interestingly, comparison of the percent of eosinophils in the lavage shows some statistical significance. The HDMA/progesterone/ETS group has statistical significance over both the HDMA/progesterone/FA group and the HDMA/placebo/FA group ($P < 0.01$). This is once again suggestive of a progesterone effect and pathogenic synergy between progesterone and ETS.

Increased protein concentration in the bronchial lumen is also a known indicator of airway inflammation that occurs in the late-phase of the asthmatic response [37]. The protein levels seen in the BALF demonstrates that the progesterone treatment does have an effect on protein levels in BALF (Fig. 4). The fact that the progesterone treatment alone did not produce a significant increase over the HDMA/placebo/ETS group suggests that ETS also accounts for some of the increased protein concentration.

Future studies are required to further define the roles of progesterone and ETS on the development of allergic

asthma. Pulmonary function testing would provide important information on changes to bronchial function – an important measurement of the allergic response.

In summary, we have demonstrated the ability of progesterone to exacerbate the allergic asthmatic phenotype in a mouse model. Furthermore, we have shown that ETS exposure has a synergistic effect with progesterone on the model. We conclude that progesterone provides a major contribution to the gender differences seen with respect to development and elicitation of the asthmatic response.

Acknowledgements

The authors thank Brian Tarkington, Tim Duvall and Louise Olsen for their assistance with maintenance of the Tri-R Airborne Infection Apparatus. We would also like to thank Michael Goldsmith and Dale Uyeminami for their technical assistances with the cigarette smoke generator system and general helpfulness of Dr Kent Pinkerton at the Toxic Pollutant Health Research Laboratory. We acknowledge Shanie McCarty for excellent care of the animals throughout the experiment. We thank Londa Berghaus, Abira Bandyopadhyay and Marie Suffia for their technical expertise. This research was supported by funds from the California Tobacco-Related Disease Research Program of the University of California, Grant number 14IT-0081.

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