

Experimental Model of Pulmonary Allergic Inflammation Induced by Latex in Balb/c

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Abstract

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Introduction: The prevalence of allergic airway diseases has dramatically increased in recent years all over the world. Murine models of allergic airway inflammation have provided helpful information about treatment and cellular and molecular mechanisms of the disease. Previous published works using murine models to investigate latex allergy did not introduce a complete characteristic eosinophilic allergic airway inflammation. Latex allergy is important due to serious health impacts and widespread use of its products. Thus, the aim of this study was to establish a new mouse model of latex allergic airway inflammation using aerosol inhalation.

Material and Methods: Initially, four groups of mice were injected intraperitoneally (IP) with 0, 10, 50, or 200 µg of latex extract and their serum anti-latex IgE titers were compared using ELISA to find out the optimum dose for IP injection.

In the second stage, a standard protocol of inhalation was designed and three doses of latex extract solutions including 1%, 0.1%, and 0.01% were used to induce allergic airway inflammation. Characteristics of this model were shown by studying different parameters including bronchoalveolar lavage (BAL), cytokines (Interleukin-5 [IL-5] and interleukin-13 [IL-13]) and serum anti-latex IgE and IgG1 titers by ELISA, specific histologic changes in the lung and eosinophilia of the bone marrow, peripheral blood, BAL, and lung inflammatory foci.

Results: The aerosol inhalation of 1% latex allergens solution and pre-sensitization with 50 µg of latex in this study resulted in the development of characteristic allergic airway inflammation in BALB/c mice. These features included elevated allergen specific IgE and IgG1, peripheral blood, BAL and bone marrow eosinophilia and characteristic inflammatory response in lung with eosinophil infiltration. Elevated levels of IL-5 and IL-13 can be a sign of this type of inflammation.

Conclusion: This paper describes a latex aerosol inhalational challenge model of eosinophilic airway inflammation in latex pre-sensitized BALB/c mice.

Key words: Eosinophilic allergic airway inflammation, Latex, Model, Mouse.

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Introduction

Allergic diseases and asthma are among major health issues increasing steadily worldwide. Asthma is an airway chronic disease leading to reversible obstruction, inflammation, hyperreactivity and remodelling of the airways. There are three basic pathways that lead to hyperreactivity of airways. One pathway is dependent on eosinophils and interleukin-5 (IL-5), another on immunoglobulin E (IgE) and mast cells, and the last on interleukin-

13 (IL-13) (1). The eosinophils are important in asthma, since they are known as the most important inducer cells of hyperreactivity. Eosinophils applied these effects by cytokine production and release of other mediators such as specific enzymes (2).

Repeated exposure to low dose allergen induces anti-allergen IgE production. More challenges cause secondary humoral response and

inflammation of lung tissue (3).

Latex products have been used widely in last decades. As a consequence, latex allergy has become an important increasing health problem worldwide. Latex allergic reactions are seen as contact dermatitis, asthma, anaphylaxis, urticaria, rhinitis, and conjunctivitis (4, 5). Different studies on latex allergy in various populations reported prevalence rates ranging between 1.1% and 7.6%. In some cases such as children with spina bifida, the risk increased dramatically up to 65%. Healthcare workers are frequently exposed to gloves and other latex products. The prevalence of latex related asthma in sensitized healthcare workers may increase up to 4.5% (6). Rubber industry workers have an increased prevalence of chronic respiratory symptoms and 50% of sensitized cases may show asthma signs (7).

Treatment of latex allergy seems to be problematic, as preventive measures such as allergen avoidance are not always feasible due to its widespread use. For instance, tires are manufactured with different concentrations of natural latex. Urban traffic causes release of tremendous small latex-containing particles. Some epidemiologic and clinical studies have shown the effects of air pollutants on immune system, particularly on induction and augmentation of allergic inflammation (8, 9). Furthermore, analysis of airborne particles and dust has shown the presence of different allergens in these samples (10). Extractable latex and other cross-reactive allergens in ambient samples of sedimented freeway dust and airborne particulate matter have reported too (11, 12). Latex allergens have cross-reactivity with some fruits (13-16), trees, and pollen allergens (17-19). Exposure to these allergens can occur through different routes, including ingestion, inhalation, and cutaneous and mucous membrane contact. These exposures affect the sensitization and explain the high prevalence of latex allergy.

Much of our knowledge about the mechanisms and treatment of asthma originates from animal studies, particularly murine models. Most of allergic asthma murine models are allergic airway inflammation models (1). There are different exposure routes to induce allergy in respiratory tract. Three usual methods include intratracheal instillation, intranasal administration, and aerosol exposure (20). The latter is the most similar to human exposure to low mass concentration of the allergen. Pathologic signs of the models induced by these methods are more comparable to human asthma (21). The first study on mouse and latex allergy was reported in 1994 (Kurup et al.). They used intranasal instillation method to induce respiratory allergy in mice (22). There are some further reports investigating the mechanisms and different aspects of respiratory latex allergy in mice, but there is not any report about a complete and reliable model. Furthermore, previous works did not use the aerosol inhalation method. Thus the aim of the present study was to establish a mouse model of pulmonary latex allergy using aerosol inhalation as sensitization method. For this purpose, a sensitization protocol was designed based on our previous experiences. Immunization times were fixed in all experiments.

Various injection and inhalation doses of latex allergens were used to find the optimum doses. To study and confirm the establishment of the model, the hallmark immunologic and histopathologic parameters of allergic airway inflammation were characterized.

Material and Methods

Animals

Six to eight weeks old, male BALB/c mice were purchased from Pasteur Institute of Iran, Tehran. Once arrived, the mice were quarantined for at least 1 week before use. They were kept in a controlled environment regarding temperature, humidity and light, with filtered airflow, sterilized instruments, and free access to standard rodent autoclaved chow and water ad libitum.

Nitrile gloves were worn while handling mice and washing the cages during all procedures to prevent unexpected latex exposure. Animals were killed under euthanasia standards.

Allergens

Ammoniated latex was obtained from Gillan gloves factory, Rasht, Iran. We used the following sequential procedure to separate latex allergens: it was centrifuged five times at 20000 Xg and 4°C for 50 min. The latex was separated into two phases at the end of each centrifugation: a solid rubber phase and an aqueous fluid, the latter was collected.

The aqueous fluid was then sequentially filtered through 200, 100, 60, and 22 µm filters to discard residual solid rubber. The clear fluid was dialyzed using saline solution and then using water with a 14000 Dalton cut-off membrane, then lyophilized, aliquoted, and kept at -20°C for long time storage. It was diluted and filter-sterilized before use. The protein content was analyzed using a modified Lowry assay (ASTM, 1998; Standard D5 712-95). The antigen profiles were prepared using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In order to determine the endotoxin contamination level, we showed that it could not stimulate proliferation of naive BALB/c splenocytes in a standard titrated thymidine incorporation assay, which is a functional endotoxin assay.

Animal Immunization

Determination of Injection Dose

Four groups of mice (n=4 each group) were immunized by two intraperitoneal injections on days 0 and 14. Each group received 10, 50, or 200 µg of latex allergens in saline adsorbed to alum adjuvant or saline with alum in a total volume of 100 µl. On day 24, the mice were killed and their blood was collected for serum antibody analyses.

Immunization Protocol and Determination of Inhalation Dose

Three groups of 8 mice were intraperitoneally immunized on days 0 and 14 with 50 µg of latex allergen extract adsorbed to alum adjuvant in a total volume of 100 µl. Subsequently, each group was exposed to aerosolized latex antigens for 30 min/day on days 24, 26, 28, and 30. They were challenged with latex allergens by exposure to an

aerosol of either 1%, 0.1%, or 0.01% (wt/vol) latex extract in saline administered with constant pressure through an ultrasonic nebulizer (NE-UO7, Omron Co, Tokyo, Japan) in a chamber of 5500 cm³ volume. A fourth group of 8 mice was sham. They were sensitized with intraperitoneal administration of alum in saline, followed by sterile saline aerosol inhalation using the same protocol as negative control group. Sampling and analyses were performed 24 hours after the last inhalation.

Sampling

Mice were anesthetized with ether. After blood smear preparation by tail bleeding and then blood collection, their sera were obtained from whole blood by centrifugation for 15 minutes at 2000 Xg and stored at -20°C before antibody analysis. Then the mice were sacrificed and divided into two groups: A group of 4 mice for bronchoalveolar lavage (BAL) preparation and a second group to collect the lung tissue for histopathologic studies. In the former, the trachea was cannulated and BAL fluid (BALF) containing cells was recovered by flushing the lungs with four aliquots of 0.5 ml of fresh PBS using a syringe. A mean of 80% of the instilled PBS was retrieved. BAL cell slides were prepared using a Cytospin (Shandon 3, Pittsburgh, PA) and air-dried. The remaining BAL preparation was centrifuged at 350 Xg for 5 minutes and the BALF was collected, aliquoted, and stored at -20°C for subsequent cytokine analysis. In the second group, the lower lobe of left lung was cut into three slices and fixed in 10% buffered formalin and paraffin-embedded. Tissues were cut in 5 mm sections and stained with hematoxylin-chromotop 2R for evaluation of eosinophilia and Periodic acid Schiff (PAS) to visualize mucous production and goblet cells. The femurs from all mice were flushed with 2 ml of 0.1% BSA (Sigma) in fresh PBS by a syringe to collect the bone marrow cells. The bone marrow cells were then undergone cytospot and air dried.

BAL, Bone marrow and blood cell slides were stained with Wright-Geimsa. At least 200 cells were counted per mouse and cells were identified by morphologic criteria to determine the percentage of eosinophils in each sample.

Histopathology

Each lung lobe was divided into three horizontal slices and they were paraffin-embedded in the same block. After serial sectioning, there were 2-4 sections from 3 different lengths of the lung lobe in each slide. The total lung sections were scored at 100x or 400x final magnification. In order to evaluate the criteria of eosinophilic inflammation in the lung, the following semiquantitative scoring systems were used to grade the histopathologic lung changes. Peribronchial inflammatory cell infiltrates was graded as follows: 0 = lack of any infiltrate; 1+ = most bronchioles with scattered infiltrates; 2+ = most bronchioles with an infiltrate of up to 2 cells thick; 3+ = most bronchioles with an infiltrate of up to 5 cells thick; and 4+ = most bronchioles with an infiltrate of more than 5 cells thick. The sum of the airway scores from lung slide was divided by the number of airways examined

(20-30 per mouse), and expressed as peribronchial inflammatory score in a semiquantitative unit. The same scoring method was used for perivascular inflammation grading.

Alveolar septa cellularity was graded as follows: 0 = no infiltrate of inflammatory cells nor widening of septa; 1+ = minimally increased cellularity without significant widening of septa; 2+ = obvious cellular infiltrates with moderate widening of septa; and 3+ = markedly increased cellularity with thickened septa. Interstitial alveolar space cellularity was scored as follows: 0 = no infiltrate; 1+ = scattered infiltrates of inflammatory cells in alveolar spaces; and 2+ = the alveolar spaces full of inflammatory cells. The sum of the alveolar scores from each lung section was divided by the number of sections examined and expressed as septal or interstitial alveolar inflammatory scores in semiquantitative units.

The proportion of goblet cells within the airway epithelium was scored at 400x final magnification as follows: 0=< 5% goblet cells; 1= 5-25%; 2= 25-50%; 3= 50-75%, 4=> 75%. The sum of the airway scores from each lung slide was divided by the number of airways examined (20-30 per mouse) and expressed as mucous score in a semiquantitative term.

Eosinophils were visualized at 1000x final magnification and counted using a rectangular eyepiece (Nikon, HWF10X-F) calibrated with stage micrometer slide. For each slide, at least 10-20 fields of view around the airways were counted. The airways with inflammatory cells were randomly selected from all sections in the slide. The eosinophils were counted within an area of 0.1 mm² of epithelium and subepithelium and multiplied by 10. The ratio of total number of eosinophils to total area for each mouse lung was calculated and expressed as the number of eosinophils per mm².

Different cell types were defined as follows: small mononuclear cells = lymphocytes; larger mononuclear cells with moderate basophilic cytoplasm and large coarse nucleoli = transformed cells; the largest cells with generous eosinophilic cytoplasm and small nucleoli = histiocytes; cells with segmented nuclei and red cytoplasm on chromotop 2R stain = eosinophils; and cells with segmented nuclei without red cytoplasm on chromotop 2R stain = neutrophils.

Detection of anti-latex antibody levels in the serum

Anti-latex IgE and IgG1 levels were detected using ELISA. In brief, ELISA plates (Nunc-Maxisorb, Nunc, Roskilde, Denmark) were coated with 100 µl of latex extract solution (50 µg of latex extract in 0.05 M NaHCO₃, pH = 9.06) overnight at 4°C.

The plate was washed with PBS-Tween 20 (0.05%) and blocked (200 µl of 1% BSA in PBS) for 2 hours at 37°C and washed again. 100 µl of serum sample per well was incubated for 2 hours at 37°C to measure the IgE level. For IgG1 detection, a 1:20 dilution of each serum sample was used. After washing, 100 µl of biotin-conjugated rat anti-mouse IgE monoclonal antibody (Clone: R35-118, Pharmingen, 3 µg/ml) or rat anti-mouse IgG1 heavy

chain (MCA 33613, Serotec, 125 ng/ml) was added to each well and incubated for 2 hours at 37°C. The plates were then washed again and 100 µl of a 1:1000 dilution of streptavidin-conjugated horse radish peroxidase (554066, Pharmingen, San Diego, CA) was added and incubated for 30 minutes at room temperature. The wells were washed, treated with 100 µl of substrate (tetramethylbenzidine) for 20-30 minutes at room temperature in a dark room. Then the reaction was stopped with H₂SO₄ and the color developed was visualized at 450 nm with a wavelength correction of 630 nm in a Titertec multiscan spectrophotometer (Stat Fax 2100). As reference for the baseline (cut off) and negative control, pooled serum of 10 naive mice which had not received latex was used. A pooled serum of 10 immunized mice (50 µg of latex extract per mouse, weekly for 8 weeks) was used as positive control. All measurements were performed as duplicates of no more than 5% difference. The results were reported in terms of optical density (OD).

Quantitation of Cytokine Levels in BALF

Cytokines were analyzed in BALF samples using standard sandwich ELISA. IL-5 ELISA was performed using the OPTeia kit (BD Biosciences Pharmingen, San Diego, CA). IL-13 measurement kit was purchased from R&D systems (R&D systems Inc., Minneapolis, USA). The plates were blocked using Block ACE (Serotec Ltd, Oxford, UK). The ELISA was performed according to the manufacturer's protocol. Cytokine concentrations were calculated from the standard curves using a Titertec multiscan spectrophotometer (Stat fax 2100). The detection thresholds were 30 pg/ml and 10 pg/ml for IL-13 and IL-5, respectively.

Statistical Analysis

The data were expressed as means ± SEM. They were considered statistically significant when $p < 0.05$. Differences were determined using the Kruskal-Wallis test and for comparing of the pairs with non-parametric comparison test.

Results

Histopathology and Eosinophils

Control mice showed normal lung architecture. Histologic analysis of tissue sections revealed no inflammatory signs in interstitial alveolar septa and spaces in none of the groups. Significant peribronchial and perivascular infiltrates were seen in all of latex sensitized mice. Increased number of mucous-producing goblet cells was shown in the airways of 1% and 0.1% latex inhalation groups, as compared with negative control mice. There were no significant differences between 1% and 0.1% latex inhalation groups (Table 1). The same results were seen in the peripheral blood eosinophil percentage (Table 2). Although the peribronchial eosinophil density was increased in 1% and 0.1% latex extract inhalation groups compared to negative controls, it was higher in 1% latex inhalation group (Table 1). BAL and bone marrow eosinophils increased in 1% latex inhalation group compared to other groups (Table 2).

The inflammatory infiltrates of lung tissues consisted of lymphocytes, histiocytes, eosinophils, neutrophils, and plasma cells.

BAL Cytokines

To characterize the allergic phenotype further, IL-5 and IL-13 levels were measured in BALF. Significantly increased levels of these two cytokines were detected in 1% latex extract inhalation group compared to other groups (Figure 2).

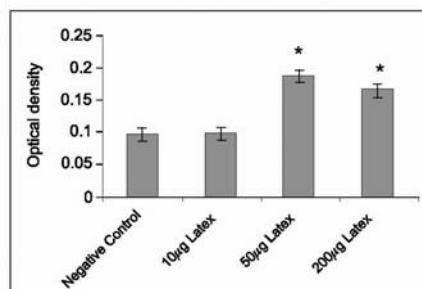


Fig. 1. Anti-latex IgE antibody levels in the sera of mice sensitized intraperitoneally with different doses of latex, data expressed as Mean ± SEM (n=4, $p < 0.05$).

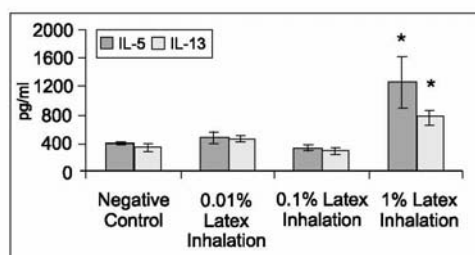


Fig. 2. IL-13 and IL-5 levels in bronchoalveolar lavage fluid of mice. The results are expressed as Mean ± SEM, n=4, $p < 0.05$.

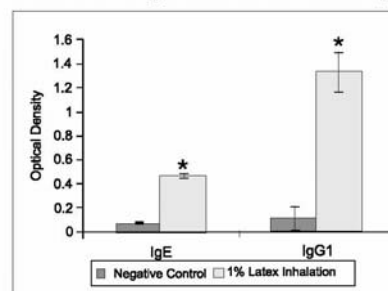


Fig. 3. Anti-latex IgE and IgG₁ antibody levels in the sera of 1% latex inhalation and negative control mice. The results are expressed as Mean ± SEM, n=4, $p < 0.05$.

Serum Antibodies

The serum levels of two hallmark antibodies of allergic inflammation were analyzed. Allergen-specific IgE and IgG₁ levels were expressed as optical density and shown in Figure 3.

The serum levels of above antibodies in 1% latex inhalation group were higher than negative control mice.

Table 1. Mean ($\pm$ SEM) of inflammatory changes in the lung.

Groups	Goblet Cells	Perivascular Infiltrates	Peribronchial Infiltrates	No. of Eosinophils/mm ² (1)	Alveolar Septa Cellularity	Alveolar Spaces Cellularity
Negative Control	0.50 $\pm$ 0.50	0.40 $\pm$ 0.18	0.50 $\pm$ 0.18	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
0.01% Latex Inhalation	0.58 $\pm$ 0.25	1.08 $\pm$ 0.08**	1.18 $\pm$ 0.03**	0.00 $\pm$ 0.00	0.05 $\pm$ 0.05	0.00 $\pm$ 0.00
0.1% Latex Inhalation	3.13 $\pm$ 0.13*	1.78 $\pm$ 0.12**	1.90 $\pm$ 0.14**	5.30 $\pm$ 0.14**	0.03 $\pm$ 0.03	0.00 $\pm$ 0.00
1% Latex Inhalation	3.25 $\pm$ 0.25*	1.90 $\pm$ 0.14**	1.95 $\pm$ 0.03**	11.80 $\pm$ 0.14**	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00

Stained sections were scored as described in materials and methods and visualized by staining with periodic acid schiff and Hematoxylin-Chromotop 2R. n=4 each group; the significance is indicated by *= p <0.05, **= p <0.01 compared to negative control group. (1); Number of eosinophils in 1 mm² area of bronchiolar epithelium and subepithelium with inflammatory infiltrates.

Table 2. Percentage of eosinophils in different samples

Groups	Peripheral Blood Eosinophils ¹	Bone Marrow Eosinophils ¹	Bronchoalveolar Lavage Eosinophils ²
Negative Control	0.70 $\pm$ 0.15	2.60 $\pm$ 0.60	0.00 $\pm$ 0.00
0.01% Latex Inhalation	2.38 $\pm$ 0.47	2.00 $\pm$ 0.42	0.25 $\pm$ 0.25
0.1% Latex Inhalation	4.75 $\pm$ 0.50*	2.38 $\pm$ 0.94	0.00 $\pm$ 0.00
1% Latex Inhalation	4.00 $\pm$ 0.54*	6.63 $\pm$ 0.93**	7.25 $\pm$ 3.43**

Data presented as Mean $\pm$ SEM.

1, n=8 each group; 2, n=4 each group; *, p <0.001 compared to negative control group; **, p <0.01.

Discussion

In this study, a mouse model of eosinophilic allergic airway inflammation was established using latex allergen aerosols. Eight to ten weeks old BALB/c mice housed in clean air room, were immunized intraperitoneally on days 0 and 14 with 50 μ g of alum adsorbed latex allergens in a total volume of 100 μ l. Subsequently, they were challenged with 10 ml of 1% latex allergens in saline (wt/vol) administered as nebulized aerosols for 30 min/day on days 24, 26, 28, and 30 in a chamber of 5500 cm² volume. Twenty four hours after the last inhalation, sampling and assessment of the model characteristics was performed.

In 1994, Kurup and colleagues showed latex antigens were able to induce allergic airway inflammation in mice. They immunized BALB/c and C57BL/6 mice intraperitoneally or intranasally for 8 weeks and administered another intranasal allergen dose finally (22). Their studies demonstrated moderate to severe interstitial inflammatory infiltrates accompanied by granuloma. Although it was shown the development of perivascular and peribronchial infiltration of inflammatory cells, eosinophilia, and allergen-specific antibodies, it could not be a reliable asthma model, as the parenchymal inflammation could lead to pulmonary granulomas and/or fibrosis not resembling human asthma features (27). They reported the role of IL-4 in airway reactivity using a mouse model in 1999. In this study, they immunized the mice intranasally twice a week for 4 weeks. Alveolar inflammation was shown in this study too (25).

Another published article presented a similar model (three immunizations a week for 4 weeks) as an airway hyperreactivity induction model, but the parenchymal inflammation and granulomas were reported again (26). Although airway hyperreactivity was shown in the last two studies, alveolar inflammation which is not the asthma-specific

affects the pulmonary hyperreactivity. Repeated and chronic intranasal bolus delivery of allergens is likely to cause this problem.

In our model, inhalation of low mass concentration of aerosolized latex allergens minimized the parenchymal alveolar inflammation. Hardy et al (2003) describes a mouse model of pulmonary allergic inflammation to a major latex allergen Hev b 5 (23). They sensitized the mice intratracheally four times every 2nd day. Howell and colleagues (2004) used another murine model to investigate the immunomodulatory effect of endotoxin on the development of latex allergy. They sensitized the mice intranasally and intratracheally 5 days a week for 72 days (24). These two latter works studied some of asthma features such as perivascular and peribronchial infiltration of inflammatory cells, eosinophilia of inflammatory lung tissues, and allergen-specific antibodies, but the authors did not mention if they had considered the alveolar parenchymal inflammation too.

Conclusion

We are presenting here a latex induced eosinophilic allergic airway inflammation model in mice with hallmark characteristics of asthma, including eosinophilia of inflammatory lung tissue and bronchoalveolar lavage, peribronchial and perivascular infiltration of inflammatory cells, and mucous hypersecretion (increased proportion of goblet cells in airway epithelium). No marked alveolar inflammation developed in this model. Allergen-specific immune response was shown by increasing levels of anti-latex IgE and IgG1. Furthermore, the levels of IL-5 and IL-13, which are effective hallmark cytokines in human and murine models of allergic airway inflammation, increased in this model. An aerosol inhalation method, which is the most similar to human allergen exposure, was used in this work using a complex mixture of crude

latex allergens. This model is a useful tool to investigate the cellular and molecular mechanisms of allergic airway inflammation, particularly those induced by latex, as well as treatment of this disease.

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