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RESEARCH ARTICLE

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Immunopathologic responses of silkmoth allergen on primary and secondary lymphoid organs of mice

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Abstract

Silkmoth scales are allergens that cause several disabling hypersensitive lung diseases like asthma and allergic rhinitis in sericulture grainage industrial workers. Immunopathological responses of silkmoth allergen on primary and secondary lymphoid organs were studied in albino mice. Mice were immunized intraperitoneally and then subsequently challenged with silkmoth allergen intranasally. The mice were then evaluated for total serum IgE, eosinophil viability, and culturing of splenocytes in the presence of PHA, eosinophil peroxidase of bone marrow cells and histology of the thymus. The results indicate that the sensitized mice shows increased levels of IgE, decreased splenocyte viability and cell depletion in the thymus, and increased eosinophils in the lungs. The stimulated spleen showed a typical Th₂ pattern in all allergen sensitized animals.

Keywords: Bone marrow, eosinophils, silkmoth scales, spleen, thymus

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Introduction

Allergic rhinitis and asthma are immunological lung diseases occurring in susceptible individuals exposed to silkmoth allergens. The immune responses characteristic of these diseases includes elevated total serum IgE, lung eosinophils, and allergen-induced lymphocyte transformation. [1] If intervention is delayed, the disease may progress to central bronchiectasis, leading to pulmonary dysfunction and death. The immune system is tightly regulated network of lymphoid organs and dispersed cell population responsible for the maintenance of host resistance to foreign materials. [2] There is an increasing concern both by the public and the scientific community that exposure to environmental and biological pollutants can lead to alterations of immune system functions.

Exposure to xenobiotics and industrial pollutants that inhibit bone marrow hemopoiesis can result in immunosuppression in the host. [3] This bone marrow represents an important target site for immunotoxic responses associated with exposure to many, and its pollutants metabolites can achieve biologically active concentrations in mice. The consequence of immunotoxicity can decrease resistance to infection and development of autoimmune and allergic manifestations. [4] There is considerable evidence to show that dynamic interactions occur between biological pollutants and lymphoid organs. [5]

The possible involvement of the immune system with the industrial pollution exposure of the mice can be traced to antiquity. Attention has been directed to aspects of the immune system of affected workers by researchers in Europe and North America. [6] The purpose of this study was to assess the changes in the lymphoid organs. This study also aimed to determine the *in vitro* responsiveness of lymphoid organs to silkmoth allergen scales allergen in mice.

We have developed a model of allergic rhinitis by exposing mice to silkmoth allergen. Both intraperitoneal and intranasal administration of silkmoth allergen resulted in elevated IgE and peripheral blood lung eosinophilia. [7] It was noted that intranasal instillation of particulate allergen enhanced eosinophilia in these mice. [8]

Materials and Methods

Mice

Albino mice (Age: 4-6 weeks, Weight: about 20-25 g) were obtained from NIN, Hyderabad, Andhra Pradesh, India. The mice were tested and confirmed to be pathogen-free. The room environment was maintained at 22°C with 12 h dark/light alternatives *ad libitum*.

Preparation of silkmoth wing and abdomen scale allergen

Wing and abdomen scale allergen of silkmoth (*Bombyx mori*. L) was prepared as described previously. [9] Briefly, wings of silkmoth (*B. mori*. L) were homogenized in phosphate-buffered saline (PBS; pH 7.3). The homogenate was incubated at 4°C for overnight, centrifuged at 15,000 rpm for 30 min, and used as crude allergen. The abdomen cuticular pieces were transferred to moist filter paper after rinsing with PBS and frozen. The frozen cuticles were cut into small pieces and powdered. The powder was mixed with 50 mM Tris HCl (pH-7.5), 1 mM EDTA, 1 mM β -mercaptoethanol (ME), and 2% (w/v) sodium dodecyl sulfate (SDS) in the ratio of 1 ml per 5 mg dry weight. The suspension was allowed to stand for 1 h at 45°C and placed in a boiling water bath for 3 min. The mixture was cooled and centrifuged at 15000 rpm for 10 min. The supernatant was collected and extensively dialyzed overnight at 28°C against 0.2% SDS in 10 mM Tris HCl (pH 7.5), 1 mM EDTA, and 1 mM ME, and then freeze dried and used. [10]

Immunization of mice with silkmoth allergen

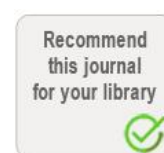
Animals were divided into the experimental and control groups. Experimental animals were exposed to silkmoth allergens intranasally, twice a week for 2 months. The mice were immunized intranasally following light anesthesia with methoxyflurane (Metofane). Silk moth allergen powder (200 μ g, with about 50 μ g of protein) in 50 μ l of PBS (1 mM conc. of protein) was instilled into the nostrils by aerosol route using the tip of a pipette. The control group was similarly challenged with PBS alone. The mice were restrained in an upright position for 2 min to regain normal breathing. The mice were finally challenged with the respective preparation and sacrificed 7 days after final exposure, followed by determination of their total serum IgE, splenocyte mitotic activity in the presence of PHA and its viability, eosinophil peroxidase of bone marrow cells, and histology of the thymus. [8],[11]

Cell isolation from spleen or spleen cell cultures

Spleen from mice sacrificed 7 days after the last exposure to allergen were collected aseptically, cut into small pieces, and minced with sterile steel-mesh screen with piston of sterile plastic syringe. The suspended cells were washed with Roswell Park Memorial Institute medium (RPMI) 1640 medium (Sigma, USA). Erythrocytes were lysed with Tris-buffered ammonium chloride, and the cells were washed in RPMI 1640 medium. The cells were counted on a hemocytometer. Spleen cells (1×10^6 cells/ml) were incubated in 96-well tissue culture plates in 200 μ l of complete RPMI (CRPMI) containing varying concentrations of PHA. Incubation was carried out for 24 h in a humidified atmosphere containing 5% CO₂ at 37°C. After 24 h of incubation, trypan blue exclusion was used to evaluate cell viability. [8]



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Total serum IgE

Total serum IgE levels were measured by enzyme-linked immunosorbent assay (ELISA) using a rat anti-mouse IgE monoclonal antibody. [12],[13] The IgE concentration in the serum was expressed in nanograms per milliliter after comparing the optical density of mouse IgE standards.

Preparation of bone marrow cells

Bone marrow cells were isolated from the femur of the mice, and the femurs were cleaned aseptically. The ends were cut off, and the cells were flushed out with Hank's balanced salt solution (HBSS) containing antibiotics with a syringe and 20-gauge needle. [14] The single cells were washed three times with HBSS and cultured in CRPMI 1640 medium.

EPO in bone marrow

Eosinophil peroxidase in the bone marrow cells was estimated using 100 μ l of bone marrow cells from mice and incubated in round-bottom microtitre plates. Cultures were set up in duplicate wells. After 48 h of incubation, the plates were centrifuged at 1000 rpm per 10 min and the medium was aspirated, followed by the addition of 100 μ l 0.1mM orthophenaldiamine in 0.05 mM Tris HCL (pH 8) containing 0.1% Tritonx-100 and 1 mM H_2O_2 to each well. After 30-min of incubation at room temperature, the color reaction was stopped by the addition of 15 μ l of 4 M sulphuric acid, and the absorbance was measured at 490 nm and determined using a microplate reader. The data are represented as optical density after subtracting the blanks. [11],[15]

Histological examination of the thymus

The thymus was slightly inflated with 10% neutral-buffered formalin and removed to prevent atelectasis. The specimens were fixed in 10% neutral-buffered formalin under a mild vacuum. The tissues were processed and embedded in paraffin and 5- μ m sections were cut and stained by hematoxylin and eosin. The slides were recorded with regard to the degree of inflammation.

Results



Spleen cell viability

Insect borne allergen is generally proteins and act as active metabolites. Presently, we demonstrated the effect of these allergens on a variety of immune functions. *In vivo* administrations of high doses of allergen to albino mice have been identified to inhibit splenic cell mitogenic activity. Experiments were also extended on spleen cell viability; mice splenocytes were treated with the allergen in the presence of PHA and trypan blue was used to evaluate cell viability. Allergen decreased spleen cell viability at a concentration greater than 0.1 mM [Figure 1].

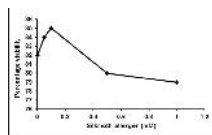


Figure 1: Effect of silkmoth allergen on spleen cell viability: Albino mice splenocytes were treated in the presence of PHA and trypan blue exclusion was used to evaluate cell viability. The immunosuppressive activity of silkmoth allergen on spleen cells was studied. Splenocytes were incubated with various allergen concentrations for 24 h

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Immunosuppressive effects of fresh intact silkmoth scales on splenocytes could be accounted for by the toxic effects of silkmoth scales at high concentrations. To examine this possibility, the mice were fed with scales for 15 days at 3-day interval. At the end of booster challenging, mice splenocytes were incubated with PHA-containing CRPMI media for about 24 h, and the viability was determined by trypan blue exclusion at the end of 48 h. Intact fresh silkmoth scales had no effect on mice splenocyte viability [Figure 2].

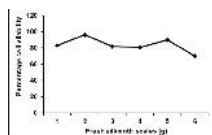


Figure 2: Effect of fresh silkmoth scales on PHA-stimulated mice spleen cell viability and viability was determined by trypan blue exclusion. The immunosuppressive activity of intact silkmoth wing and abdominal scales on spleen cell viability was studied. Intact scales were artificially infused into mice for 24 h

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Total serum IgE-specific IgG

All mice were exposed to silkmoth allergens showed increase in the total serum IgE levels as compared with the preimmunization levels. Silkmoth scales immunized mice were treated with anti-IgE antibody. The spleen were harvested from the mice 1 week after antibody administration and cultured to determine if the effect on IgE synthesis persisted *in vitro*. After 96 h, the IgE levels of 20 ng/ml were measured in the splenocyte cultures from the IgG-treated mice. In contrast, IgE levels were detectable in the cultures prepared from the anti-IgE-treated mice [Figure 3].

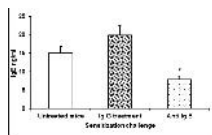


Figure 3: Anti-IgE treatment *in vivo* suppresses immunoglobulin synthesis by splenocytes *in vitro*

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Eosinophil peroxidase in bone marrow

All animals challenged with silkmoth allergen, demonstrated an increase in eosinophil peroxidase in their bone marrow cells. This increase was maximum after 2 day of the last exposure, whereas intact fresh scales have not shown much significant difference [Figure 4].

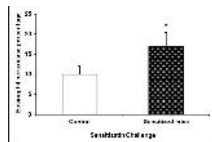


Figure 4: An increase in eosinophil peroxidase in the bone marrow cells of mice after treatment with silkmoth allergen

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Histological changes of the thymus

In the experimental design, a 8-10-week-old albino mice received a single intravenous injection with 150 mg/kg and followed for a subsequent for 24 days after injection. There was weight gain to the original value at 24 days after injection. The thymus showed complete cortex depletion and extensive vasculature of epithelial cells, which is considered as a secondary effect.

Changes in the cortex were identified with cellular depletion at day 4 and increased proportions at day 21. The cell depletion persisted until day 24 and then medulla was also disappeared [Figure 5].



Figure 5: Histology of the thymus at 0, 4, and 21 days after intravenous injection of moth allergen extract (150 mg/kg) into albino mice. A = 0 days; B = 4 days; C = 21 days. Changes in the cortex are shown at a higher magnification. Cellular depletion at day 4 and increased proportions of mitotic figures indicated by an arrow at 21 days $\times$ 280

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Discussion

The intranasal instillation of silkmoth allergens in the present study resulted in the development of a characteristic allergic response in mice. Elevated levels of total serum IgE was observed in mice challenged with silkmoth allergen as compared to the control. This indicates that the associated proteins such as sericins and fibroins are the causative agents of allergy during the development of silkmoth. This also suggested that silk allergy is caused not only by occupational but also by environmental exposure to insects, which favors the growth of allergy-causing organism like *Aspergillus fumigatus*. The feature included elevated the total serum IgE levels in viability and proliferation lymphoid organs, peripheral blood eosinophilia, and characteristic inflammatory responses in the lung with eosinophil infiltration. These features are typical of Th₂-mediated response and are the salient features of human allergy. [16] Evaluation of immunological and airway responses in these animals after exposure to silkmoth allergen proteins revealed increased airway and immunological resistance. This response is similar to human allergic asthma, where patients challenged with specific allergen develop smooth muscle constriction that lead to airway spasm. Generally, mice possess an intact immune system to silkmoth allergen and Th₂ response is characteristic of allergic response. [17] The lung inflammation was greater; the interstitial inflammation and perivascular and bronchial infiltration observed in this model resembled allergen-induced reactions reported in other animal models. [8],[17]

Whether a common factor is responsible for the suppression of the lymphocyte response and altered proportion of cells needs to be investigated. It would appear that such changes are a reflection of immune suppression or of a direct effect of various pathogenic microorganisms take an advantage over immune system. The mitogen PHA stimulated splenocyte activity in the control group, whereas PHA was unable to stimulate splenocytic mitotic activity in silkmoth scales-challenged mice. The result of our study suggests that the mitogen stimulated splenocyte of silkmoth scale-challenged mice have reduced mitogen response. This response is a trait marker of depression due to inhalation of silkmoth scales. It is interesting to speculate that the factors involved in the various aspects of immune malfunction may also play a role in causing changes in hematopoiesis and immune cells status in the silkmoth scales-inhaled mice. [17]

Histology in mice models clearly reveal that Th₂ cells are at the center of the immunopathology of inflammation and airway hyper reactivity induced by silkmoth allergens. Strong Th₁ response can also cause immunopathology like inflammation. It is possible that B cells and antibodies as well as eosinophils play a more important role in other aspects of the relationship between immune system and the allergen. In human patients, genetic traits and the differences in the nature of priming and sensitization may be important determinants of pathology and pathophysiology. Data obtained from mice models point to T cells as major targets for immunological interventions.

The present data supports that elevated immune responses to the exposure to silkmoth scales were minimal and that a lung disease due to immune change is unlikely following allergen inhalation. In addition, it is possible that memory cells produced by allergen stimulation of the lung-associated lymph nodes may migrate to distant lymphoid tissues, including the spleen. The presence of memory cells in the spleen could be evaluated with additional allergen challenge. The data from this study indicate that the lung-associated lymph nodes accumulated silkmoth scale particulates, which cleared in the lung lymphatics to result in an increased cellularity in these tissues. However, these tissues retained normal allergen retention and immune functions and showed only slightly elevated immune responses.

The immune response is largely determined by the availability of antigen to the lymphoid organs, and the pathological condition of the host tissue results in altered immune responses. These alterations may in turn affect the distribution of inhaled allergens like silkmoth scales. ^[17] Studies until date on the effects of dose response of purified allergen or with intact scales on immune function that is on splenocytes have not dealt with tissue toxicity. Due to high concentration of allergen percentage of splenocyte, viability was not reduced, suggesting that cell death was not an issue. Furthermore, the present result cannot be explained by a mechanism that does not involve spleen cell death or the general shutdown of all cellular biochemical pathways. A dose-related effect would suggest a receptor-mediated event. The present results also indicate an enhanced number of eosinophils. Presence of elevated eosinophil levels in the bronchoalveolar lavage fluid and the peripheral blood has been shown to correlate with allergic inflammatory diseases such as asthma. ^{[18],[19]} Activated eosinophils produce a variety of proinflammatory mediators such as eosinophil cationic protein major basic protein eotoxin, eosinophil peroxidase, and eosinophil neurotoxin. ^[20]

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Figures

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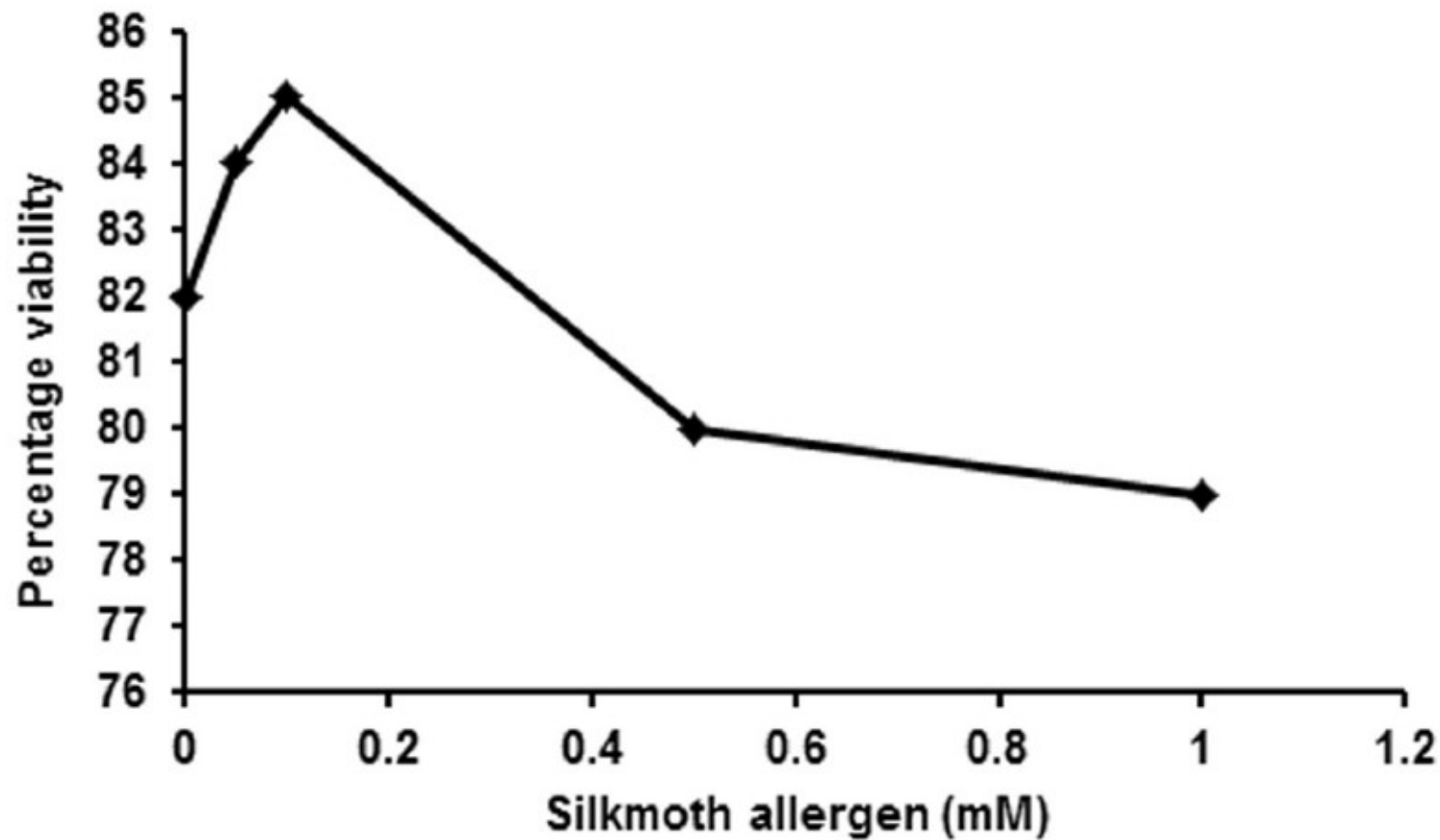
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IgE ng/ml

