

Development of an animal model for allergic conjunctivitis: influence of genetic factors and allergen concentration on immune response

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ABSTRACT.

Purpose: Animal models of diseases are extremely important in the study of the physiopathogenesis of human diseases and for testing novel therapeutic interventions. The present study aimed to develop an animal model that simulates human allergic conjunctivitis and to study how allergic response may be influenced by the allergen dose used for immunization and by genetic factors.

Methods: Sixty C57Bl/6 mice and 60 BALB/c mice were immunized with placebo, or 5 µg or 500 µg of allergen derived from *Dermatophagoides pteronyssinus*. After ocular challenge, the mice were examined in order to clinically verify the occurrence or not of conjunctivitis. Material obtained from animals was used for total and specific IgE and IgG1 dosage, for assays of Der p-specific lymphocyte proliferation and supernatant cytokine dosage, and for histopathological evaluation of conjunctiva.

Results: We developed a murine model of allergic conjunctivitis induced by *D. pteronyssinus*. The model is similar to human disease both clinically and according to laboratory findings. In mouse, conjunctivitis was associated with a Th2 cytokine profile. However, IL-10 appeared to be involved with disease blockade. Mice of different strains have distinct immune responses, depending on the sensitization dose.

Conclusions: The murine model developed is suitable for the study of immunopathogenesis and as a template for future therapies. Using BALB/c and C57BL/6 mice, we demonstrated that genetic factors play a role in determining susceptibility and resistance, as well as in establishing the allergen concentration needed to induce or to block disease development.

Key words: animal models of diseases – allergic conjunctivitis – immediate hypersensitivity – mice – mucosal immunity – mites

Introduction

Allergic conjunctivitis was first recognized clinically as a disease by Bostock (1819). Subsequent studies demonstrated that this was a type 1 hypersensitivity reaction (Bonini & Ghinelli 2000), with the participation of T lymphocytes that secrete cytokines of a predominantly Th2 profile (Romagnani et al. 1991).

The estimated prevalence of allergic conjunctivitis is 20% in the Japanese population (Yasuda et al. 1999), 25% in the North American population (Calonge 1999), and 18% in children in the UK (International Study of Asthma and Allergies in Childhood [ISAAC] 1998). Although this disease is generally associated with a good prognosis, complications may arise as a consequence of chronic inflammation, with different degrees of morbidity (Tabbara 1999). In the USA, in 1996, direct expenditures on rhinoconjunctivitis treatment reached \$5.9 bn (Ray et al. 1999).

Animal models of diseases are extremely important in the study of the physiopathogenesis of human pathologies and for testing novel therapeutic interventions. The available experimental models of allergic conjunctivitis differ as to the animals used, the sensitizing allergen and the route of sensitization, among other factors (Merayo-Llôves et al. 1996;

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Magone et al. 1998; Strauss et al. 1999; Keane-Myers et al. 1999).

In the present study, we aimed to develop a murine model of allergic conjunctivitis triggered by *Dermatophagoides pteronyssinus*, a mite which is one of the main aetiological agents of ocular allergic disease in Brazil (Rosário Filho 1992). We chose to sensitize, by a subcutaneous route, two different strains of mice, C57Bl/6 and BALB/c, both in order to increase the chance of finding an 'ideal' model, and to allow us to examine the influence of genetic factors on allergic response. Studies have demonstrated differences in the immune responses of C57Bl/6 and BALB/c mice, with the former tending towards a more Th1-like profile, and the latter towards a more Th2-like profile (Matsushita & Katz 1992).

The aims of the present study were:

- (1) to develop an animal model that simulates human allergic conjunctivitis, and
- (2) to study how allergic response may be influenced by the allergen dose used for immunization and by genetic factors.

Materials and Methods

Mice were immunized with solutions prepared from *D. pteronyssinus* (Der p) extracts on day 0. After 10 days, ocular challenge was carried out in order to induce allergic conjunctivitis. Clinical analysis and the collection of material for laboratory analyses were conducted 20 mins and 24 hours, respectively, after ocular challenge.

Animals

Sixty C57Bl/6 mice and 60 BALB/c mice were studied. Animals were aged 4–8 weeks. Both males and females were used. They were obtained from the animal facility of the Department of Immunology of the Institute of Biomedical Sciences, University of São Paulo and were maintained in the same facility in standard conditions. The study was carried out in accordance with the recommendations of the Association for Research in Vision and Ophthalmology.

Preparation of solutions for immunization and challenge

Lyophilized Der p extract – the allergen used in the present study – was kindly provided by the International Pharmaceutical Immunology (IPI)-ASAC Laboratory of Brazil (Laboratório IPI-ASAC do Brasil). The extract was weighed and diluted in phosphate-buffered saline (pH 7.2) to a 4.18 µg/µl concentration. After dilution, the volumes required in order for mice to receive 5 µg or 500 µg of allergen upon immunization were combined with 100 µl aluminium hydroxide, which was used as an adjuvant. A solution of 100 µl aluminium hydroxide and 120 µl saline was used as a placebo. The initial solution, at an allergen concentration of 4.18 µg/µl, was used for the ocular challenge.

Animal immunization and challenge

Mice from both strains were separated into three groups so that each group contained 20 animals from each strain. The control group (group 1) was administered with placebo and the other two groups were administered with the immunization solution containing either 5 µg (group 2) or 500 µg (group 3) of allergen. Sensitization solutions were administered subcutaneously at the base of both hind legs and into the lower abdomen. One-quarter of the dose was injected into each hind leg and the remaining half was injected into the abdomen.

Ten days after sensitization, mice (including controls) were challenged with two drops of allergen solution (4.18 µg/µl) in each eye.

Clinical evaluation

Twenty minutes after challenge, mice were examined in order to clinically verify the occurrence or not of conjunctivitis. Four clinical signs were observed: conjunctival oedema; palpebral oedema; conjunctival hyperaemia, and lacrimation. A scale of 1–3 was used for each sign, in an adaptation of the scoring system developed by Ballas et al. (1993). Thus each animal was given a total clinical score between 0 and 12, proportional to conjunctivitis intensity. The observer was not aware of the animal's status

in terms of whether it came from the placebo or immunized groups.

Laboratory evaluation

Twenty-four hours after challenge, animals were anaesthetized with ether and blood collection was carried out through cardiac puncture. Later, while still anaesthetized, animals were killed by sectioning the spinal medulla by spinal column fracture. Iliac and para-aortic lymph-node dissection and eyeball enucleation were carried out.

The material obtained was used for total and specific IgE and IgG1 dosage, for assays of Der p-specific lymphocyte proliferation and supernatant cytokine dosage, and for histopathological evaluation of conjunctiva. Serum from blood collected through cardiac puncture was used for measuring levels of total and Der p-specific IgE and IgG1 antibodies by enzyme-linked immunosorbent assay (ELISA), as described elsewhere.

For evaluation of specific proliferation, lymph node cells were cultured in flat-bottom microplates, with or without stimulation with 4.18 µg/µl Der p extract. A concentration of 3×10^6 cells/ml was maintained, with a final volume of 0.2 ml per well. Cells were cultured in standard medium for 90 hours, and were pulsed with tritiated thymidine during the final 16 hours. Radioactivity incorporation was measured in cpm using standard scintillation techniques in a β scintillation counter. Cultures were made in triplicate. Stimulation rate was calculated by dividing the mean value of the three cultures of Der p-stimulated cells by the mean value of non-stimulated cells. Rates ≥ 3 were considered as positive.

In lymphocyte cultures in which cytokines were dosed, the supernatant was collected 48 hours after the beginning of culture and cytokine levels were measured by ELISA. Interferon- γ and interleukins IL-4, IL-5, IL-6, IL-10 and IL-13 were measured.

For histopathological study, the animals' eyeballs were removed without damaging the conjunctiva, fixed for 30 mins in glutaraldehyde, and then transferred to 10% formaldehyde, where they remained for at least 24 hours. Samples were embedded in paraffin and then sectioned through the vertical plane of the central

region. About five tissue specimens containing both conjunctiva and cornea were obtained. Giemsa, periodic acid-Schiff and haematoxylin and eosin staining were used to evaluate the inflammatory infiltrate. An ophthalmologist trained in ocular pathology, blinded as to the origin of the tissues, carried out cell counts (of neutrophils, eosinophils, mast cells, lymphocytes and caliciform cells) and attributed a histopathological score to the inflammatory process, based on the scale developed by the Laboratory of Clinical Immunology, Immunology Department, Institute for Biomedical Sciences, University of São Paulo.

Statistical analysis

Differences between groups were analysed by Student's two-tailed test with paired samples, in order to determine statistical significance. Groups 2 and 3 were compared with group 1 (controls) and with each other. Differences with p -values < 0.05 were considered statistically significant.

Results

Clinical and histopathological scores

We succeeded in inducing allergic conjunctivitis triggered by *D. pteronyssinus* in both strains of mice. The ideal dose for sensitization differed between the two strains, at 5 μ g for C57Bl/6 mice and 500 μ g for BALB/c mice. That is, C57Bl/6 mice showed a greater frequency and intensity of allergic conjunctivitis when immunized with the lower dose, whereas the BALB/c mice showed greater intensity and frequency with the higher dose. With C57Bl/6 mice, a possible blockade of allergic response was observed when the quantity of allergen was increased. BALB/c mice, by contrast, required an increased sensitization dose in order to achieve a greater allergic reaction.

Both clinical and histopathological scores were compatible with the findings mentioned above (Fig. 1). The mean clinical scores were 0 (control), 6.75 (group 2) and 0.67 (group 3) in C57Bl/6 mice and 0 (control), 0.2 (group 2) and 2.3 (group 3) in BALB/c mice. The mean histopathological scores were 0 (control), 3.1 (group 2) and 0.3 (group 3) in

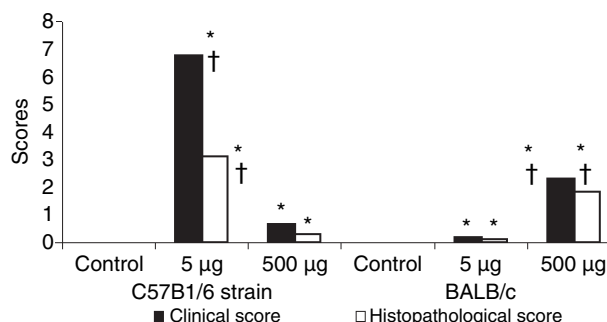


Fig. 1. Clinical and histopathological scores for groups 1 (control group), 2 (5 μ g group) and 3 (500 μ g group) * $p < 0.05$ for comparison with control group; † $p < 0.05$ for comparison between groups 2 and 3.

C57Bl/6 mice and 0 (control), 0.15 (group 2) and 1.85 (group 3) in BALB/c mice.

Histopathological study showed an inflammatory process in the conjunctiva and cornea, with epithelia lesions, oedema and infiltrates with a predominance of neutrophils and eosinophils (Fig. 2). We also found an important presence of lymphocytes and a discrete increase in mast cell presence in the substantia propria of conjunctiva.

Total and specific IgE dosage

Greater concentrations of both total and Der p-specific IgE were found in immunized animals than in controls. However, the increase in total IgE was only statistically significant in BALB/c mice immunized with 500 μ g allergen. The increase in specific IgE in group 2 (5 μ g) and group 3 (500 μ g) mice and the difference in antibody levels between these two groups were statistically significant in both BALB/c ($p < 0.01$) and C57Bl/6 ($p < 0.05$) mice.

Regarding specific IgE, it was found that, in line with clinical and histo-

pathological scores, responses were more or less intense depending on the dose used for immunization (Fig. 3). Whereas the C57Bl/6 strain produced higher levels of specific IgE when sensitized with 5 μ g Der p, the BALB/c strain produced higher levels when sensitized with 500 μ g. A comparison between both strains of control animals showed that mean blood concentrations of total IgE were at least five- to 10-fold higher in BALB/c animals.

Total and specific IgG1 dosage

There was a slight increase in IgG1 concentration in immunized animals when compared with controls. Mean antibody levels were approximately twice as high in BALB/c mice as in C57Bl/6 mice. However, the increase in total IgG1 was statistically significant in C57Bl/6 mice only. Der p-specific IgG1 was detected in sensitized animals of both strains, in concentrations roughly 10- to 20-fold higher than in controls.

The increase in antibody levels was statistically significant in groups 2 and

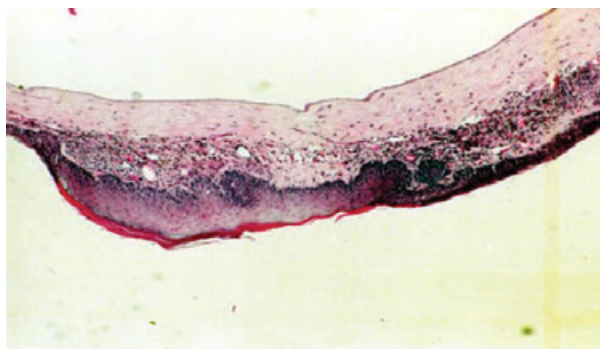


Fig. 2. Histopathological evaluation of the conjunctiva inflammatory process in the conjunctiva and cornea, with lesions in the epithelia of structures, oedema and infiltrates with a predominance of neutrophils and eosinophils.

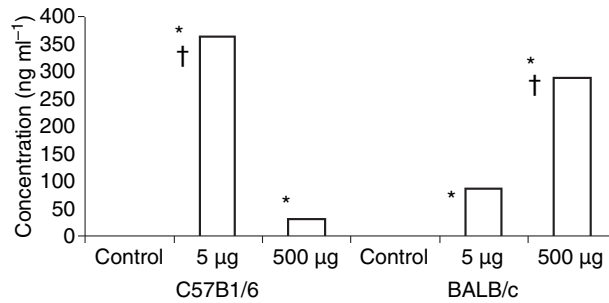


Fig. 3. Specific IgE dosage in groups 1 (control group), 2 (5 µg group) and 3 (500 µg group) *p < 0.05 for comparison with control group; †p < 0.05 for comparison between groups 2 and 3.

3 of BALB/c mice (p < 0.05) and in group 2 of C57Bl/6 mice (p < 0.01). The difference in the levels of specific IgG1 between C57Bl/6 groups 2 and 3 was also statistically significant.

Der p-specific lymphocyte proliferation

As expected, greater proliferation was observed in immunized animals than in control groups. This difference was statistically significant for both sensitized groups of C57Bl/6 mice (p < 0.01) but only for group 3 BALB/c mice (immunized with 500 µg) (p < 0.01). Blastic proliferation in the BALB/c strain was directly proportional to the amount of allergen used in sensitization. There was

no correlation between immunization dose and proliferation rate in C57Bl/6 mice.

Cytokines produced by lymphocytes after Der p stimulation

Cytokine concentrations in lymphocyte culture supernatants were significantly higher in sensitized animals than in controls (Figs 4 and 5). Cytokine production tended to be correlated with immunization dose. IL-4, IL-5, IL-6 and IL-13 production was inversely proportional to the immunization dose in C57Bl/6 mice, with the greatest production detected in animals sensitized with 5 µg. The opposite relationship was observed in

BALB/c mice, with the greatest production in animals immunized with 500 µg. Thus the animals with greater frequency and intensity of allergic conjunctivitis according to clinical criteria also had greater secretion of the above mentioned cytokines, characterizing a Th2 profile.

By contrast, the analysis of IL-10, a mediator commonly detected in Th2-type immune reactions, showed the opposite relationship in C57Bl/6 mice, with higher levels of this cytokine detected in mice immunized with greater amounts of allergen. Thus the greatest concentrations of IL-10 were detected in group 3 (500 µg), in which ocular disease was less frequent and intense, and in which the lowest levels of Th2-profile cytokines IL-4, IL-5, IL-6 and IL-13 – except for controls – were detected. Increased IL-10 production was also detected in sensitized BALB/c animals, but this increase was not dependent on immunization dose and was not correlated with clinical and histopathological scores.

IFN-γ production was increased in immunized mice, with greater levels in group 3 for both strains (p < 0.05).

Discussion

In the present study we developed a model of allergic conjunctivitis triggered by *D. pteronyssinus* in C57Bl/6 and BALB/c mice. The disease induced in the model is similar to its human counterpart both clinically and histopathologically. Clinically, we observed that mice rubbed their eyes and presented with palpebral and conjunctival oedema, ocular secretion and conjunctival hyperaemia, corresponding to the immediate phase of allergic process, as described in humans (Friedlaender 1993). Histopathological analysis revealed neutrophil and eosinophil infiltrates in the conjunctiva of studied animals, corresponding to the late phase of the allergic process, similarly to that observed in human conjunctivitis (Bonini et al. 1988). In the present study, we introduce a murine model that is similar to other models of allergic conjunctivitis that have been developed recently, but which has the advantage of employing an allergen that is relevant worldwide (Nakamura et al. 2003; Stern et al. 2005; Ohbayashi et al. 2007). Our

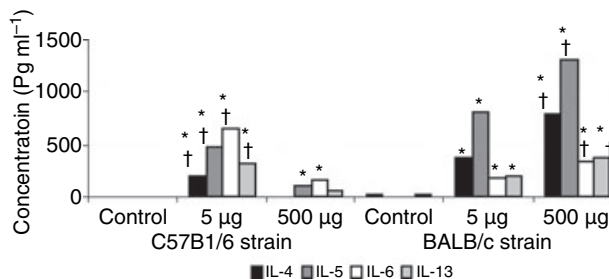


Fig. 4. Cytokine concentrations in the lymphocyte culture supernatants in groups 1 (control group), 2 (5 µg group) and 3 (500 µg group) *p < 0.05 for comparison with control group; †p < 0.05 for comparison between groups 2 and 3.

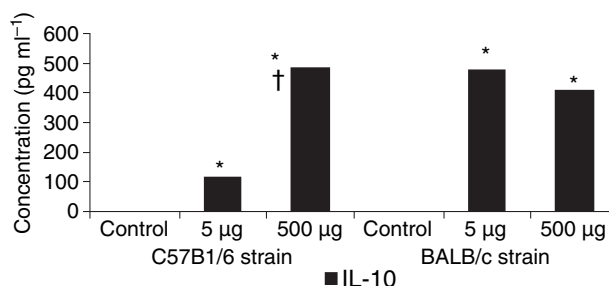


Fig. 5. Cytokine concentrations in the lymphocyte culture supernatants in groups 1 (control group), 2 (5 µg group) and 3 (500 µg group) *p < 0.05 for comparison with control group; †p < 0.05 for comparison between groups 2 and 3.

model, like most models in the literature, mimics only the acute form of allergic conjunctivitis as our subject material did not undergo repeated challenges.

In addition to ocular disease, there was also a systemic immune response induced by the mite, characterized, in laboratory terms, by the production of total and specific IgG1 and IgE and by specific lymphocyte proliferation with production of Th2 cytokines IL-4, IL-5, IL-6 and IL-13.

Our results indicate that genetic characteristics may not only determine susceptibility or resistance to allergic disease, but may also define the ideal dose of allergen necessary for triggering it. Depending on the amount of antigen used in stimulation, the threshold for triggering disease may not be reached. By contrast, if the dose is excessive, it may cause a blockade of the pathological response. High doses of antigen at immunization may induce cellular tolerance or induce an immune response dominated by the production of immunoglobulins of other types, thus suppressing IgE production.

As described in the literature (Murray et al. 1989), in addition to major histocompatibility complex (MHC), the affinity with which antigen is presented influences the determination of the immune response pattern. High-affinity presentation tends to trigger a Th1 response, whereas low-affinity presentation tends to trigger a Th2 response. Ultimately, this affinity is determined by the three-molecule complex formed by antigen, MHC and T-cell receptor (TCR). Therefore, an immune response may vary between strains if the same sensitizing antigen is maintained and within the same strain if different antigens are used for immunization (Levine & Vaz 1970).

In the C57Bl/6 strain, mice immunized with 5 µg Der p showed the highest frequency and severity of allergic conjunctivitis, as determined by clinical and histopathological criteria. These animals also presented higher proliferation rates and greater secretion of specific IgG1 and IgE, and of IL-4, IL-5, IL-6 and IL-13. IL-10 and IFN-γ secretion levels, by contrast, although higher than those of controls, were lower than those of animals immunized with 500 µg.

In BALB/c mice, animals immunized with 500 µg Der p showed the highest frequency and severity of allergic conjunctivitis according to clinical and histopathological criteria. These animals also presented higher rates of lymphocyte proliferation and greater secretion of specific IgG1 and IgE, and of IL-4, IL-5, IL-6, IL-13 and IFN-γ.

BALB/c mice – controls and sensitized animals alike – showed a greater trend towards secreting total IgE and certain Th2 cytokines (IL-4, IL-5, IL-13) than C57Bl/6 animals, which is in agreement with data from the literature. However, in BALB/c mice, allergic conjunctivitis was less frequent and less severe according to clinical and histopathological criteria. Furthermore, the ideal sensitization dose was higher (500 µg), which may characterize the presence of a threshold for triggering allergic response. The concept of a threshold for triggering certain allergic responses also exists for humans (Bjorgsten et al. 1996).

We may speculate that MHC and TCRs of C57Bl/6 animals have high affinity for the epitopes of *D. pteronyssinus* and, therefore, tend to mount a Th1-profile response against these epitopes. This trend might be counteracted if the antigen to be presented were present in minimal amounts in the environment (5 µg). The opposite would occur with BALB/c mice, in which an extremely low affinity of the MHC–epitope–TCR triad would require large amounts of allergen in order to reach the threshold for triggering the immune response. This speculation leads us to question whether any individual can develop an allergic reaction once optimal conditions, given his or her genetic background, are established.

Cytokine secretion by lymphocyte of C57Bl/6 mice was inversely proportional to sensitization dose, with the exception of IL-10 and IFN-γ. We may therefore hypothesize that, in this strain, excessive immunization doses may lead to blockade of the allergic reaction, perhaps through the action of cytokines IL-10, which may be produced by regulatory cells. IL-10 production in C57Bl/6 mice sensitized with 500 µg, which showed a lower frequency and intensity of allergic ocular disease, was statistically higher

than that of animals receiving 5 µg and of control animals. Monocytes and B cells may also contribute to cytokine production in the lymphocyte proliferation assays. We did not perform intracellular cytokine staining to find out which cells were producing cytokines.

In BALB/c mice, IL-10 secretion was significantly increased in sensitized groups in comparison with control groups, but no relationship was found between cytokine level and immunization dose. However, it is possible that blockade was not observed in these animals because the necessary blockade dose was not reached.

Data in the literature suggest that IL-10 is one of the controlling factors in the development and suppression of specific immunity, and that it is able to inhibit the proliferation and secretory activity of both subtypes of T lymphocytes (Del Prete et al. 1993; Akdis & Blaser 2001; Hawrylowicz & O'Garra 2005).

Both C57Bl/6 and BALB/c mice immunized with 500 µg Der p produced larger amounts of interferon-γ. In C57Bl/6, IFN-γ could be associated with immune deviation from Th2 to Th1 profile with less severe conjunctivitis. Otherwise, in BALB/c, IFN-γ, like other Th1 cytokines, may participate in the development of allergy response, as shown in the literature (Rizzo et al. 1995; Stern et al. 2005). It has also been demonstrated that, in the physiopathogenesis of more severe and chronic ocular diseases, in addition to a greater participation of cells such as eosinophils and Th2 lymphocytes, there may also be a role for the Th1 branch of the immune response (Metz et al. 1997).

We conclude that the murine model of allergic conjunctivitis triggered by *D. pteronyssinus* developed in the present study is capable of simulating human disease, is practical, and may be easily reproduced by other researchers. It may therefore be used in the study of the physiopathogenesis of allergic ocular disease, in the development of therapeutic interventions, and in the evaluation of the effects of such interventions on immune response.

We have demonstrated that genetic factors determine not only the susceptibility or resistance to atopic disease, but also influence the ideal antigen dose for triggering the process. The

data generated in the present study indicate the existence of a threshold dose for sensitization that must be reached in order to trigger an allergic response, and of a blockade dose, whereby an excessive quantity of allergens blocks any allergic reaction.

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