

Inflammatory and immunological cell profiles in a rat model of conjunctival immediate hypersensitivity

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Summary

A modified model of conjunctival immediate hypersensitivity in the rat is described. The advantages of this model over previously reported rat models are that it does not require invasive challenges, pre-treatment with mucolytic agents to enhance antigen penetration, or the use of haptens, and is therefore more representative of the mode of allergen challenge seen in human hay fever conjunctivitis. This model has been shown to have both early and late-phase cellular responses but only early phase clinical signs. During the early phase of the response the tarsal region accommodates a massive neutrophil infiltration and the fornix-bulbar region participates to a greater extent during the late-phase reaction, with a significant eosinophil infiltration. The development of this model has allowed us to gain a clearer insight into the mechanisms involved during conjunctival immediate hypersensitivity in the rat; and the simplicity of this model makes it attractive to use in the evaluation of new drug therapies prior to their introduction into human clinical trials.

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Introduction

Allergic conjunctivitis comprises a spectrum of clinical diseases ranging from the mild and transient to the severe and persistent. Ocular hay fever is the most common ocular allergy and manifests in a mild conjunctivitis. Vernal conjunctivitis is characterized by a number of symptoms ranging from mild to severe. The cornea is often involved with a punctate keratitis which can be threatening to vision. Atopic keratoconjunctivitis is a more serious disorder and, unlike ocular hay fever, often involves the cornea [1].

Clinical experience with steroids, disodium cromoglycate and antihistamines has shown that mast cell mechanisms are important in allergic conjunctivitis [2]. However, in humans, due to the restricted supply of experimental tissue, it is difficult to define the specific role of individual immunopathological mechanisms. Previously, rat models of ocular anaphylaxis have been described by injection of ovalbumin into ocular adnexal tissue of sensitized rats [3]; by local injection of anti-IgE in unimmunized rats [4]; by topical challenge with ovalbu-

min [5] or worm extract [6] to immunized rats after pre-treatment with a mucolytic agent; by topical application of di-DNP-lysine to eyes of rats immunized with DNP-*Ascaris* [7] and by intravenous injection of ovalbumin into rats passively immunized with anti-ovalbumin antibodies [8].

The advantages of the model described in this report are that it does not require invasive challenges, pre-treatment with mucolytic agents or the use of haptens. The aim of the present study was to investigate the pathological mechanisms involved in a model of conjunctival immediate hypersensitivity which more accurately represents the ocular component of human hay fever than previously reported rat models.

Materials and methods

Sensitization

Two groups of male Wistar rats weighing 150–200 g were sensitized as follows:

- Group A. Fifty-one rats received an intraperitoneal injection of 100 µg ovalbumin (OVA, Sigma Grade VI) with 10 mg aluminium hydroxide gel [9] in 1 ml of phosphate-buffered saline (PBS), pH 7.4; and
Group B. Contained 10 rats which were not sensitized.

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Topical challenge

Twenty-one days post-sensitization, rats were anaesthetized with pentobarbitone sodium B.P. (26.4 mg/kg) injected intraperitoneally. Each rat then received a single topical challenge of 10 μ l OVA in PBS (33.3 mg/ml) into the left conjunctival sac. The right eye received 10 μ l of PBS alone. Animals from group A were divided into six sub-groups and sacrificed by an overdose of pentobarbitone (180 mg/kg) at 15 and 30 min, 1, 4, and 24 hr, and 7 days after challenge. Negative control rats (group B) were all sacrificed 30 min after challenge.

Clinical evaluation

Left, challenged, and right, contralateral, eyes were examined under a dissecting microscope (magnification $\times 3$). Conjunctival reactivity 15 min after topical challenge was clinically evaluated on a 0–3 scale. The highest reactivity score of 3 was given to eyes which had severe oedema in conjunctival and lid tissue, tearing and vasodilatation; a score of 2 was given to eyes with moderate conjunctival oedema and erythema; a score of 1 was given to eyes with mild conjunctival oedema and erythema; and a score of 0 indicated no clinical sign of oedema, erythema or tearing. All responses were compared with negative, control eyes from challenged, unsensitized animals (group B).

Serum IgE and IgG₁

Ovalbumin-specific IgE and IgG₁ serum antibody titres were determined by the use of an enzyme-linked immunosorbent assay [10].

Histopathology

Eyes were exenterated with conjunctivae and lids attached, fixed in neutral-buffered formalin, then processed and embedded in paraffin wax. Sections were stained with haematoxylin and eosin. Cell counts for neutrophils, lymphocytes, eosinophils and mononuclear cells were evaluated from four distinct areas of the conjunctiva; the upper tarsal plate (UT), mid-tarsal plate (MT), upper fornix (F) and bulbar (B). Counts were performed on two sections at least 12 μ m apart, using an eye piece field at a total magnification of $\times 400$. This provided a standardized area which allowed cell counts to be compared. Statistical analyses were performed using the two-tailed Mann–Whitney *U*-test and the Wilcoxon paired *t*-test [11].

Mast cell counts

For the determination of the percentage mast cell degranulation in the conjunctival tissue, 4 μ m paraffin sections

Table 1. Effect of topical ovalbumin challenge in group A rats

Grade of response	Number of animals animals in Group A
0	8
1	12
2	12
3	19

were stained with 1% aqueous toluidine blue. Mast cell counts were performed on conjunctival substantia propria from two sections at least 12 μ m apart. Counts were performed using an eye piece field at a total magnification of $\times 1000$. A mast cell was classified as degranulated if there were (i) five or more granules outside the cell boundary or (ii) five or more pale-staining granules within the cell. Fully degranulated mast cells were not detected by this staining procedure and this may result in an underestimation of the degree of mast cell degranulation. Statistical analyses were performed using the two-tailed Mann–Whitney *U*-test.

Results

Clinical findings

Of the rats actively sensitized by an intraperitoneal injection of ovalbumin, group A, 15.7% (8/51) did not produce any signs of a conjunctival immediate hypersensitivity reaction when challenged topically with ovalbumin 21 days after sensitization (Table 1). The response was initiated within 5 min of the instillation of antigen into the conjunctival sac, with the involvement of tarsal and bulbar regions. Oedema reached peak intensity 15–30 min post-challenge, started subsiding after 1 hr and reached a clinically normal state by 4 hr. No corneal involvement was observed. The right, contralateral eyes of the sensitized animals and both eyes from challenged, unsensitized animals showed no clinical signs of conjunctival immediate hypersensitivity.

Antibody levels

Twenty-one days post-sensitization OVA-specific IgE titres of up to 1/10 240 and IgG₁ titres of up to 1/819 200 were detected in the serum of animals from group A, providing evidence of systemic sensitization (Table 2). No such antibodies were found in serum from the negative control rats, group B.

Table 2. The relationship between ovalbumin-specific IgE and IgG₁ levels and the grade of the allergic response

Grade of response	Antibody levels (percentage of standard serum)	
	IgE	IgG ₁
0	45.9±44.9*	22.3±18.4
1	79.3±85.0	45.5±23.5
2	48.1±27.6	35.9±18.5
3	57.6±27.0	34.2±12.3

* Values are ±95% confidence limits.

Inflammatory cell counts

Cellular infiltrations were observed in the challenged and contralateral conjunctival substantia propria from group A animals; these consisted of neutrophils, lymphocytes, eosinophils and mononuclear cells. Although the design of the experiment aimed to use the contralateral eye as negative control against which the challenged tissue could be compared, it was found that cellular infiltrations in challenged tissue were accompanied by similar levels of infiltration in the unchallenged, contralateral tissue. This minimized the value of the contralateral eye as a control. Consequently, it was decided that group B animals, originally included as negative controls for OVA-specific antibody levels, would be used as the negative control.

Neutrophil infiltrations (Fig. 1a) were seen predominantly in the tarsal area of both challenged (Fig. 2a) and contralateral (Fig. 2b) tissue. In the challenged tissue these infiltrations were greater than group B levels at 30 mins ($P<0.01$), 1 hr ($P<0.05$) and 4 hr ($P<0.05$) post-challenge. Levels in the contralateral tissue were increased in MT after 30 min ($P<0.05$) and in UT after 1 hr ($P<0.01$). The elevated levels in the challenged tissue were found to be higher than the respective contralateral tissue at the 30 min ($P<0.01$) and 4 hr ($P<0.05$) time periods studied.

Lymphocyte infiltrations in challenged tarsal-fornix conjunctival tissue (Figs 1b and c) were shown to be greater than group B levels at the 30 min ($P<0.02$) and 4 hr ($P<0.05$) time intervals (Fig. 2c). Lymphocytes were also found to be increased in the contralateral UT after the 1 and 24 hr ($P<0.05$) time periods. The levels in the challenged tissue were found to be higher than the respective contralateral tissue in UT at 30 min ($P<0.05$) and MT, F and B after 4 hr ($P<0.05$).

Mononuclear cell infiltrations in the challenged tissue (Figs 1b and c) were shown to be greater than group B levels in MT at 30 min ($P<0.02$), 24 hr ($P<0.01$) and 7 days ($P<0.01$), F at 24 hr ($P<0.02$) and B at 1 hr ($P<0.002$) (Fig. 2d). Mononuclear cells were also increased in contralateral MT after 30 min and 7 days ($P<0.05$), in F after 1 hr ($P<0.02$) and 24 hr ($P<0.05$) and in B after 1 hr ($P<0.05$). The levels in the challenged tissue were higher than the respective contralateral tissue in MT and B areas 7 days after challenge ($P<0.05$).

Eosinophil infiltrations in the challenged tissue (Fig. 1d) were shown to be higher than group B levels in MT after 4 hr, 24 hr and 7 days ($P<0.01$) and in MT, contralateral tissue after 1 hr ($P<0.02$). The levels in the challenged tissue were found to be greater than the respective contralateral tissue in the MT after 1 hr ($P<0.01$) and F after 1 and 4 hr ($P<0.05$).

When only moderately (grade 2) and severely (grade 3) responding animals were selected for immunological and inflammatory cell counts, the infiltration profiles were similar to those constructed using all group A animals with one major exception, the eosinophil infiltration. In challenged tissue from grade 2 and 3 responding animals, eosinophil levels were shown to be greater than group B levels in UT after 24 hr ($P<0.01$) and 7 days ($P<0.05$), MT after 4 hr, 24 hr and 7 days ($P<0.002$), F after 4 hr ($P<0.02$), 24 hr ($P<0.01$) and 7 days ($P<0.02$), and B after 4 hr ($P<0.02$) and 24 hr ($P<0.01$) (Fig. 2e). Increased levels of eosinophils were also found in contralateral MT tissue after 1 hr ($P<0.01$) and B after 4 hr ($P<0.02$).

Degranulated mast cell counts

Mast cells were found to be present in the conjunctival substantia propria region but absent in conjunctival epithelium. Using tissue obtained from all group A animals, the level of mast cell degranulation in the challenged tissue was shown to be increased, to a level of 20%, after 15 min ($P<0.05$) only, with a significantly elevated level of 15% recorded in the contralateral tissue 30 min post-challenge ($P<0.05$). However, the levels in the challenged tissue, reaching a maximum of 34%, were found to be greater throughout the 15 min to 4 hr period ($P<0.05$) than the respective contralateral tissues.

When moderately (grade 2) and severely (grade 3) responding animals were selected for study, the levels of mast cell degranulation were found to be increased, reaching between 40% and 50%, with greater degrees of significance. Using these grade 2 and 3 responders, the level of mast cell degranulation (Fig. 3) in the challenged tissue was shown to be higher than group B levels during the 15 min to 1 hr period post-challenge ($P<0.02$).

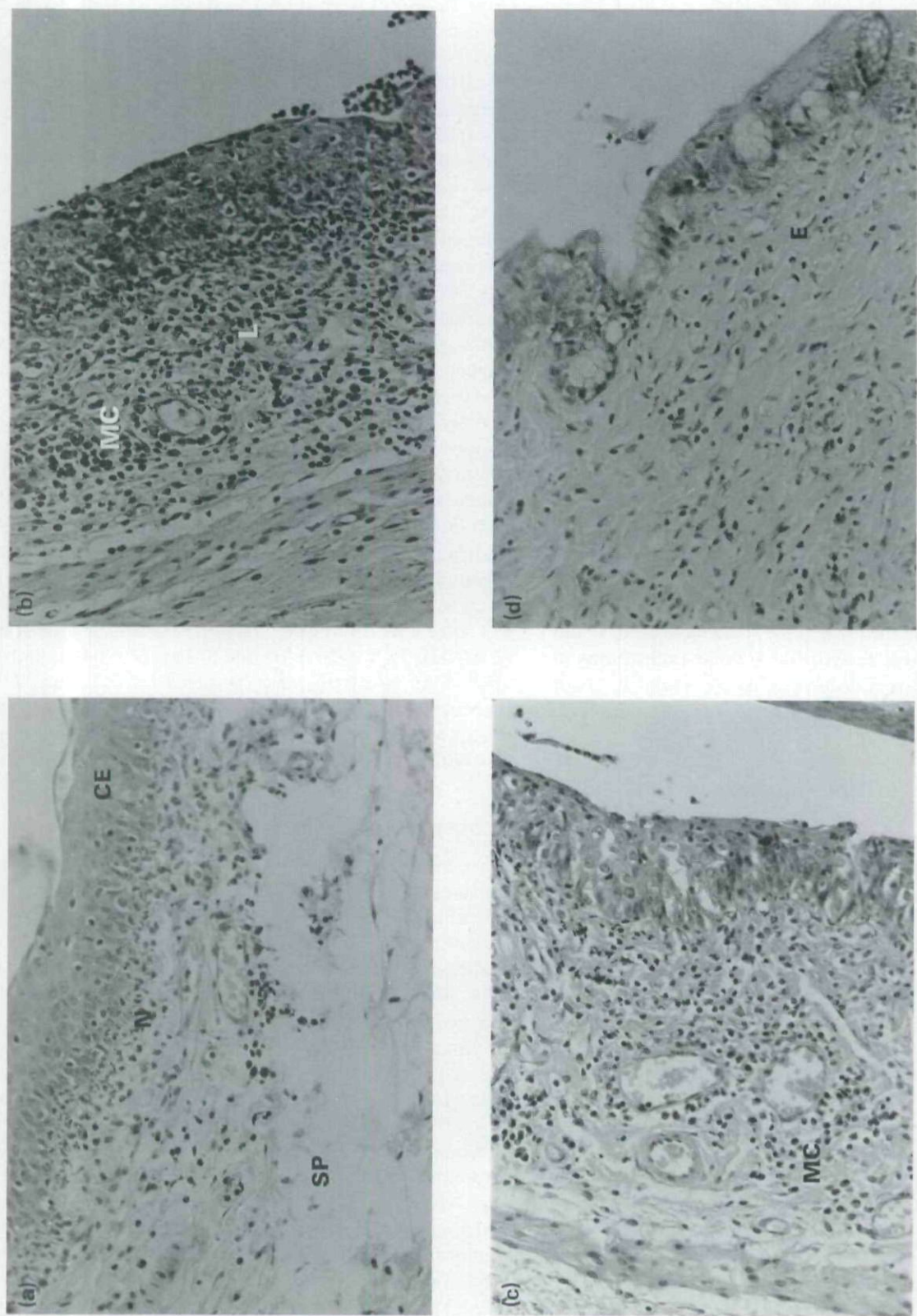
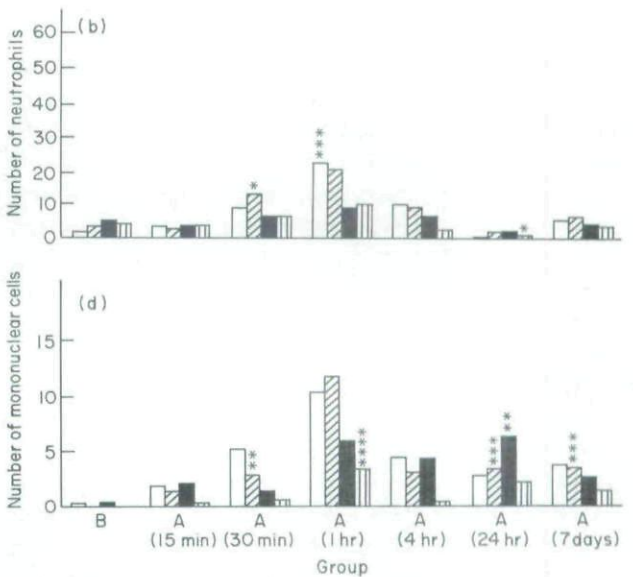
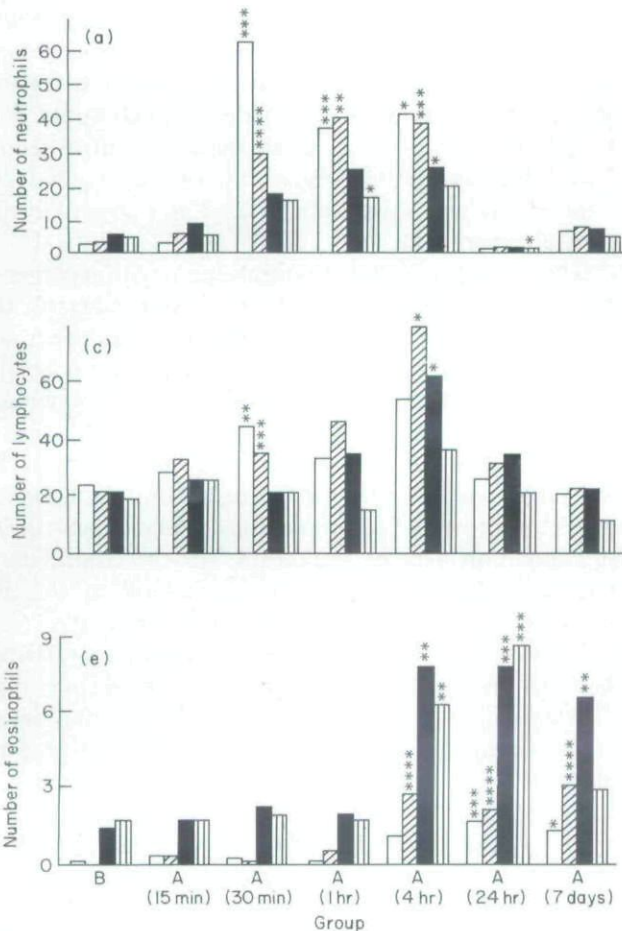


Fig. 1. Histological sections of haematoxylin and eosin stained, mid-tarsal tissue from the eye of grade 3 responders at specific intervals post-challenge. (a) Thirty minutes after challenge. Neutrophils (N) accumulating just below conjunctival epithelium (CE) with oedema in the substantia propria (SP) ($\times 250$). (b) One hour after challenge. Mononuclear cells (MC) and lymphocytes (L) infiltrating the substantia propria ($\times 250$). (c) Four hours after challenge. Mononuclear cells (MC) and lymphocytes accumulating around vessels ($\times 250$). (d) Twenty-four hours after challenge. Eosinophils (E) infiltrating the substantia propria. Neutrophils seen to have decreased below control level, with the lymphocyte population approaching normal levels ($\times 250$).



Elevated levels were also recorded in the contralateral tissue after 1 hr ($P < 0.02$). The levels of mast cell degranulation in the challenged tissue were found, using the Wilcoxon paired *t*-test, to be greater at the 30 min and 1 hr period ($P < 0.05$) than respective contralateral tissues.

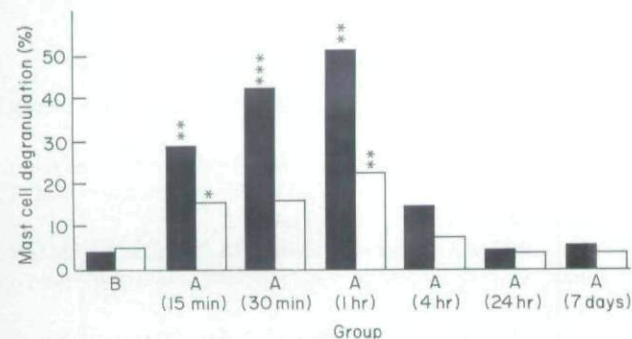


Fig. 3. Percentage of degranulated mast cells from challenged (■) and contralateral (□) tissue, from grade 2 and 3 responding animals, at specific intervals post-challenge. Significance levels, obtained using two-tailed Mann-Whitney *U*-tests, are indicated: * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$.

Fig. 2. Profiles of cellular infiltrations in four conjunctival substantia propria areas at specific intervals post-challenge, including all group A animals unless otherwise indicated. Significance levels, obtained using two-tailed Mann-Whitney *U*-tests, are indicated: * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$ and **** $P < 0.002$. (a) Neutrophil levels in challenged tissue. (b) Neutrophil levels in contralateral tissue. (c) Lymphocyte levels in challenged tissue. (d) Mononuclear cell levels in challenged tissue. (e) Eosinophils levels in challenged tissue from grade 2 and 3 responding animals from group A. Conjunctival regions: upper tarsal (□), mid-tarsal (▨), fornix (■), bulbar (▩).

Discussion

Rat models of ocular immediate hypersensitivity have been described in which the responses have been elicited by local injection of antigen into ocular adnexal tissue of immunized rats [3], and by local injection into the eye lid tissue of anti-IgE in unimmunized rats [4]. However, these models do not represent true conjunctival immediate hypersensitivity, nor do they represent the mode of allergen challenge which occurs in human hay fever conjunctivitis. Models of conjunctival immediate hypersensitivity have also been reported in which sensitized rats were challenged with the appropriate antigen [5,6], but these responses were dependent on pre-treatment of the conjunctival tissue with a mucolytic agent, dithiothreitol (DTT). After DTT pre-treatment, it is unlikely that such a model would be suitable for immunological and pharmacological modulation studies due to the possible interaction of DTT with topically applied drugs or with tear immunoglobulins [7]. More recently another model has been described which does not require DTT pre-treatment and in which the allergenic stimulus is the hapten,

di-DNP-lysine [7]. However, hapten-induced conjunctival immediate hypersensitivity responses, though useful in investigating the humoral component of the reaction, may not simulate the complete ocular component of human hay fever as closely as a simple antigen-induced model. This study describes a modified model of conjunctival immediate hypersensitivity produced in the Wistar rat which has many advantages over previously reported rat models, in that it does not require: (i) invasive challenges, (ii) pre-treatment with mucolytic agents to allow antigen penetration, or (iii) the use of haptens.

This rat model has been shown to have both immediate and late-phase cellular responses, but only immediate clinical signs. The cellular infiltrations were, in general, characterized to specific conjunctival areas with an apparent distinction between the involvement of tarsal and fornix-bulbar conjunctival tissue. During the immediate phase of the response the tarsal region accommodated a massive neutrophil infiltration, and during the late-phase reaction the fornix-bulbar region participated more extensively with a significant eosinophil infiltration.

Enzyme-linked immunosorbent assay studies have indicated that both OVA-specific IgE and IgG₁ antibodies are produced in this model, and it is possible that a combined involvement of both antibodies leads to the clinical and histological observations described (Doherty, *et al.*, in preparation). The apparent lack of correlation between the OVA-specific IgE and IgG₁ antibody levels and the grade of clinical response induced may be due to variability within the mechanics of the challenge procedure, which in turn determines the amount of antigen available to induce the immediate hypersensitivity response. This may include 'blinking-out' some of the challenge solution or high normal levels of tear production, which would have the effect of diluting the applied challenge solution.

The immunopathological events identified in this study can be attributed at least in part, to the activity of mast cell mediators which are chemotactic for neutrophils [12], lymphocytes [13], eosinophils [14] and mononuclear cells [15]. The combined infiltrations of neutrophils and mononuclear cells may be responsible for the release of pro-inflammatory products [15]; their concentration determined by the degree of activation of the early infiltrating cells. This in turn may be related to mast cell-associated mediators produced as a result of the original antigen challenge.

In designing the experiment, it was planned to include the contralateral eye as control. However, during the analysis of the results, it was found that the contralateral eye became infiltrated in the absence of any clinical signs. This prevented its use as an adequate control with which to compare histological features in the experimental eye.

Group B animals were therefore included as negative controls for OVA-specific antibody levels enabling comparisons to be made between reactive and non-reactive groups; however, each time period studied could then only be compared to the 30 min negative control group. The important point arising out of this is that the contralateral eye should not be used as a control in this type of experiment.

The presence of cellular infiltrations in both experimental and contralateral conjunctival tissue suggests the activity of a network system in which messages, generated in the challenged tissue, are transferred to the contralateral tissue. Similar activity has been described previously [16], where the induction of an inflammatory response in one hind paw produced similar changes in the contralateral hind paw; Levine *et al.* [16] called this phenomenon reflex neurogenic inflammation. Reflex neurogenic inflammation may be responsible for the contralateral conjunctival tissue involvement described in this rat model of conjunctival immediate hypersensitivity.

It has been shown that there are many similarities between this rat model and observations made in human allergic conjunctivitis [1, 17, 18]. These include the clinical signs of conjunctival oedema and erythema with tearing, elevated allergen-specific IgE levels, cellular infiltrations into the affected tissue and mast cell degranulation.

The simplicity of this Wistar rat model, along with its close similarity to human allergic conjunctivitis, makes it attractive for use in investigating the precise immune mechanisms involved in conjunctival allergic disease, and has proved useful in determining the therapeutic value of new drugs prior to their introduction into human clinical trials [McGrath *et al.*, in preparation].

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