Activated T cells in an animal model of allergic conjunctivitis

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Abstract

The aim of this study has been to determine whether the presence of lymphocytic infiltrates observed in the histology of ocular allergic conditions in humans or in the late phase of ocular anaphylactic reactions in experimental animals is a non-specific event dependent only on the degranulation of mast cells, or is conditioned by a specific response to antigen. With this in mind, responses to antigen and to a non-immunological mast cell degranulator (compound 48/80) were compared in an experimental model of allergic conjunctivitis. Rats were sensitised to ovalbumin and challenged topically in the left conjunctival sac either with ovalbumin or compound 48/80. The presence of T cells and activated T cells in the infiltrate was studied by immunohistochemical staining on conjunctival tissue obtained at 4, 24, and 48 hours after challenge. Ovalbumin sensitised and challenged rats showed increased numbers of T cells in the conjunctival infiltrate, statistically significant when compared with compound 48/80 challenged rats at 48 hours and with controls at 4, 24, and 48 hours. The number of T cells was significantly higher in compound 48/80 challenged rats only at 48 hours when compared with controls. As for the number of activated T cells, only ovalbumin sensitised and challenged rats showed significantly increased levels of these cells compared with both sensitised animals challenged with compound 48/80 and controls at 4 and 24 hours after challenge. These results suggest that the infiltration of the conjunctiva by activated T lymphocytes is, at least in part, dependent on a specific response to antigen.

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The contribution of IgE antibody mediated mechanisms to allergic eye disease is well established. Seasonal allergic conjunctivitis, vernal conjunctivitis, and atopic keratoconjunctivitis are considered to have an allergic basis. Yet, the course and the clinical and histopathological features are very different among them. The clinical signs of seasonal allergic conjunctivitis, consisting of dilatation of blood vessels and oedema of the conjunctiva, are typical of ocular type I hypersensitivity responses.¹

Vernal keratoconjunctivitis is a particularly disabling chronic manifestation of ocular allergy that, in the long term, can result in loss of sight.² It is characterised by hypertrophy of the connective tissue, resulting in the formation of cobblestone-like papillae in the superior tarsal conjunctiva and thickening of the corneal limbus, and by erosion of the corneal epithelium.³ The histological features include hyper-

trophy of the connective tissue that results in the formation of papillae; infiltration by T lymphocytes and plasma cells, some of which produce IgE⁴; hyperplasia of mast cells; infiltration by eosinophils that degranulate and cause extensive epithelial damage.⁵⁻⁷

Atopic keratoconjunctivitis is defined by chronic conjunctivitis associated with exzema. Clinical signs include blepharitis, chronic conjunctivitis with papillary hypertrophy and subconjunctival scarring, and keratitis.⁸ The histopathological findings are characterised by mast cell and eosinophilic infiltration of epithelium, proliferative changes in the epithelium, and a mononuclear cell infiltration of the substantia propria.⁹

The pathogenesis of these last two processes is poorly understood, but it is suspected that, along with an IgE mediated hypersensitivity, a cell mediated immunity with the participation of T cells may play an important role.¹⁰ We feel that this assertion is tenable only if the cellular infiltrates observed in vernal and atopic keratoconjunctivitis are specific features of the immune response.

An essential step for immune response is the activation of T lymphocytes.¹¹ The purpose of this study is to determine whether the presence of activated T cells in the conjunctiva of a rat model of conjunctival immediate hypersensitivity¹² is a non-specific event dependent only on the degranulation of mast cells or is conditioned by a specific response to antigen.

Materials and methods

ANIMALS

Male Wistar rats weighing 140-160 g were obtained from the animal house of Bristol University Medical School. All animals were kept in standard conditions. All animal handling was in accordance with the Animal Scientific Procedures Act 1986 of the United Kingdom.

REAGENTS

Ovalbumin (OVA) grade VI, crystallised and lyophilised fraction was purchased from Sigma Chemical Co. Dilutions in 0.01 M of phosphate buffered saline (PBS) pH 7.4 were prepared for use. Aluminium hydroxide [Al(OH)₃] gel was prepared according to the method of Levine and Vaz.¹³ Compound 48/80 was purchased from Sigma Chemical Co. The monoclonal antibodies purchased from Serotec Ltd against rat leucocyte markers were OX19, a mouse IgG₁ which recognises peripheral T cells and thymocytes¹⁴ and OX39, a mouse IgG₁ specific for activated T cells.¹⁵ Other immunohistochemical reagents

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employed were normal rabbit serum (Nordic), rabbit antimouse IgG polyclonal serum and monoclonal PAP/mouse complex (Dako), and 3'3' diaminobenzidine (Sigma).

SENSITISATION

Two groups of rats were injected intraperiton-

eally with 100 µg OVA plus 10 mg Al(OH)₃ in 1 ml of PBS. A third group, used as a negative control, was injected intraperitoneally with PBS alone.

CHALLENGE OF THE CONJUNCTIVAL TISSUE

Twenty one days post-sensitisation animals were anaesthetised with Valium (Roche) and Hypnorm (Jansen), and challenged topically into the left conjunctival sac. The first group sensitised with ovalbumin received an immunological challenge of 500 µg ovalbumin in 15 µl PBS, (OVA/OVA group). The other group sensitised with ovalbumin was challenged with compound 48/80, 4 mg in 10 µl PBS (OVA/48/80 group), which is a compound that degranulates mast cells by a non-immunological mechanism.¹⁶ The negative control group, injected with PBS alone, was challenged with the same dose of ovalbumin as the first group (PBS/OVA group). Clinical responses were evaluated 30 minutes after challenge without magnification and scored on a scale of 0 (no clinical signs) to 3 (maximal conjunctival redness and oedema). Animals were sacrificed at 4, 24, or 48 hours after challenge by an overdose of sodium pentobarbitone and upper conjunctivae were removed and frozen in liquid nitrogen.

ANTIBODY ASSESSMENT

This was performed to select OVA injected animals providing systemic sensitisation. Blood was obtained by cardiac puncture following sacrifice and ovalbumin specific IgE serum antibody titres were detected by using an enzyme linked immunosorbent assay (ELISA).¹⁷ In groups OVA/OVA and OVA/48/80 only those rats showing positive levels of specific antibody were included in the study. This resulted in seven or eight rat groups depending on the type of challenge and the time of sacrifice (Fig 1).

IMMUNOHISTOCHEMISTRY

An immunoperoxidase staining method, the peroxidase antiperoxidase (PAP) technique,¹⁸ was employed using the monoclonal antibodies OX19 and OX39 for the identification of peripheral T lymphocytes, activated T lymphocytes, and macrophages respectively in all upper conjunctivae. Conjunctival tissue was sectioned at a thickness of 7 µm on a cryostat. The sections were allowed to thaw at room temperature for 2 hours before being fixed in acetone and allowed to dry for 10 minutes. Endogenous peroxidase activity was removed by a 15 minute immersion in a bath of 0.3% hydrogen peroxide. This was followed by three washes in 0.05 M PBS at pH 7.6, and an incubation with normal rabbit serum 1:5 in PBS for 30 minutes at room temperature to block the non-specific binding of proteins. Sections were incubated with the appropriate monoclonal antibody overnight at 4°C. After washing, sections were incubated with polyclonal sera rabbit antimouse IgG at room temperature for 30 minutes. The sections were then washed again before being incubated with a monoclonal mouse PAP complex for 30 minutes

Figure 1 Number of T lymphocytes present in the conjunctival sections at (A) 4, (B) 24, and (C) 48 hours. (□)=total number of T cells (OX19⁺) and (■)=activated T cells (OX39⁺) in animals sensitised and challenged with ovalbumin (group OVA/OVA), sensitised with ovalbumin and challenged with compound 48/80 (group OVA/48/80), and injected with PBS alone and challenged with ovalbumin (group PBS/OVA). Results are expressed as number of cells per mm² (SEM). Significance levels using Kruskal-Wallis tests for simultaneous comparisons and two tailed Mann-Whitney U tests for pairwise comparison are indicated.

at room temperature. The chromatogenic reaction was developed by a 7 minute incubation with a 10 mg/ml 3'3'diaminobenzidine substrate solution containing 15 µl of 6% hydrogen peroxide for every 1.5 ml of solution. The reaction was stopped by washing thoroughly with distilled water followed by tap water. Finally, sections were counterstained with Harris haematoxylin and mounted in DPX for microscopic examination.

LYMPHOCYTE CELL COUNTS

Slides stained with the PAP technique for each monoclonal antibody were counted blind on a binocular microscope using an eyepiece grid at 400× magnification. All positively staining cells in the field, consisting of both epithelial and subepithelial areas, were counted. The grid was moved from the proximal to the distal extreme of the tissue and all fields were examined. Data were expressed as the average number of cells per mm² for each section studied.

Fig 2A

Figure 2 Microphotographs of two histological sections of the same mid-tarsal conjunctiva stained with the PAP technique and counterstained with haematoxylin. Positive cells stain brown while negative cells and nuclei appear blue. (A) T cells (OX19⁺) infiltrating the substantia propria and the epithelium. (B) Activated T cells (OX39⁺) infiltrating mainly the substantia propria.

Fig 2B

STATISTICAL METHODS

The Kruskal-Wallis test, a non-parametric analysis of variance, was used for simultaneous comparisons between groups and, where significant, pairwise comparisons were performed by two tailed Mann-Whitney U tests. Values of $p < 0.05$ were considered significant.

Results

CLINICAL SIGNS

Positive responses consisted of hyperaemia and oedema of the lids and the conjunctiva, all clearly evident at 10 minutes, reaching their maximal intensity at 30 minutes, and almost completely subsiding 1 hour after topical challenge with drops of OVA or compound 48/80. Animals from group OVA/48/80 exhibited responses of a more marked intensity with more prominent oedema of lid and conjunctiva than that of animals from group OVA/OVA. Non-sensitised animals, group PBS/OVA, did not display inflammatory signs following the challenge with ovalbumin.

ANTIBODY LEVELS

In 89% of animals injected intraperitoneally with ovalbumin, detectable levels of ovalbumin specific serum IgE antibody could be proved by the ELISA technique, providing evidence of systemic sensitisation and only these animals were included in the study. The serum of negative control rats contained no ovalbumin specific antibody.

LYMPHOCYTE COUNTS

Cryostat sections stained by the PAP technique for peripheral T lymphocytes with the monoclonal antibody OX19 showed positive cells as a brown colour due to the chromagen, while negative cells and nuclei appeared blue due to the haematoxylin counterstain (Fig 2A). Similarly, staining for activated T lymphocytes with the monoclonal antibody OX39 gave positive cells in brown and negative cells and nuclei in blue (Fig 2B).

The number of peripheral T cells (OX19⁺) present in the conjunctival infiltrate of challenged eyes at 4 hours was found to be significantly greater in both the OVA/OVA and the OVA/48/80 groups, when compared with the PBS/OVA group ($p < 0.05$ for both). There were no significant differences between the OVA/OVA and OVA/48/80 groups. The number of activated T cells (OX39⁺) at 4 hours post challenge was found to be significantly greater in the OVA/OVA group when compared with both the OVA/48/80 group and the PBS/OVA group ($p < 0.01$ for both). The number of activated T cells in the OVA/48/80 and PBS/OVA groups was not significantly different (Fig 1).

At 24 hours post challenge, the number of OX19⁺ cells in group OVA/OVA was significantly greater than in group PBS/OVA ($p < 0.05$), which did not differ from group OVA/48/80. There was no significant difference between the OVA/48/80 and PBS/OVA groups. Similarly the number of OX39⁺ cells was significantly

increased in the OVA/OVA group when compared with the OVA/48/80 ($p < 0.05$) and PBS/OVA groups ($p < 0.01$). The numbers of OX39⁺ cells in the OVA/OVA and PBS/OVA did not differ (Fig 1).

In tissue taken at 48 hours post challenge, the OVA/OVA group exhibited significantly increased levels of OX19⁺ cells when compared with the OVA/48/80 group ($p < 0.05$) and the PBS/OVA group ($p < 0.01$). The number of activated T cells was again significantly greater in the OVA/OVA group when compared with the OVA/48/80 group and the PBS/OVA group ($p < 0.001$ for both). No significant differences in the numbers of peripheral T cells or activated T cells were found between the OVA/48/80 and PBS/OVA groups (Fig 1).

The percentage of activated T lymphocytes with respect to the total number of T cells (activated T cells $\times$ 100/total number of T cells) present in the conjunctiva was significantly increased in groups sensitised and challenged with ovalbumin (OVA/OVA groups) compared with groups sensitised with ovalbumin and challenged with compound 48/80 (OVA/48/80 groups) and negative control groups (PBS/OVA) at 4 and 24 hours but not at 48 hours after challenge (Fig 3).

Discussion

All rats sensitised with OVA that showed anti-OVA IgE antibodies and challenged with OVA and all rats challenged with compound 48/80 responded with oedema and hyperaemia that was evident shortly after topical challenge. The peak of the intensity of the reaction occurred approximately 30 minutes after injection. From the rapidity of this response, there is no doubt that it can be called an immediate reaction. There were no differences either in the time of appearance and duration of the reaction or in the clinical signs between the challenge with compound 48/80 and the challenge with OVA in OVA immunised rats. Compound 48/80 produces mast cell degranulation by a non-immunological mechanism.¹⁶ The coupling of

two molecules of IgE with antigen also produces mast cell degranulation.⁸ Therefore, we can assume that the response in the OVA/OVA group was initiated by the coupling of OVA to specific IgE antibody in conjunctival mast cells. The differences in intensity of the clinical reaction may be due to a higher number of mast cells and a higher degree of degranulation involved by compound 48/80, which acts non-specifically, than by specific anti-OVA IgE antibody. Indeed, there is evidence in other reports¹² that the conjunctival reaction elicited by topical challenge with OVA in OVA immunised Wistar rats is associated histologically with mast cell degranulation. We cannot discount that other hypersensitivity mechanisms are not adding up later to the response, but only immediate hypersensitivity, mediated by mast cell degranulation, has been reported within 30 minutes after challenge.

The presence of a late phase reaction after exposure to airborne allergens has been reported since 1873, and successive reports have documented its relation to IgE mediated mechanisms and the participation of different cell types that conform to the histological picture of this kind of reaction.¹⁹

Mononuclear cell infiltrations have been observed 6 to 8 hours after challenge with airborne antigens, but generally have not been considered as important.^{20,21} However, Frew *et al*²² reported the presence of T lymphocytes in an animal model of bronchial asthma, which supports the view that T lymphocytes may contribute to the pathogenesis of allergic inflammation.

The infiltration by lymphocytes in the late phase reaction (LPR) has been described in relation to mediator containing cells degranulating after exposure to stimuli such as IgE or compound 48/80. Rat mast cells have been found to contain chemoattractant factors for lymphocytes and this could explain the appearance of lymphocyte infiltrates.²³ In our experiments these factors very probably contributed to the significant T cell infiltration seen at 4 hours in the immunologically challenged group and could

Figure 3 Percentage of activated T cells in respect of the total number of T cells at specific intervals post-challenge. (■)=groups sensitised and challenged with ovalbumin (OVA/OVA); (□)=groups sensitised with ovalbumin and challenged with compound 48/80 (OVA/48/80); (▨)=groups injected with PBS alone and challenged with ovalbumin (PBS/OVA). Results are expressed as percentage of cells per mm² (SEM). Significance levels using Kruskal-Wallis tests for simultaneous comparisons and two tailed Mann-Whitney U tests for pairwise comparisons are indicated.

also explain the increased presence of T cells in the 48/80 challenged animals compared with the negative control group.

At 24 hours the T cell infiltrate in the OVA/48/80 group had diminished and had reached control levels by 48 hours after challenge. Significantly raised levels of activated T cells in the conjunctiva were observed in the immunologically challenged group only and not in the group challenged with 48/80, this cell population being similar to that found in the negative control group. Therefore, the late infiltration by T cells and the presence of significantly raised levels of activated T cells at 4 and 24 hours after the topical challenge cannot be explained by the degranulation of mast cells alone and appears to be related to the presence of specific antigen.

The activation and subsequent proliferation of $T4^+$ lymphocytes is a two step process. The first signal requires the interaction between antigen and the T cell antigen receptor. The second signal is supplied by a variety of growth factors such as interleukin 2 (IL-2), which triggers cell proliferation. The advent of the first step is designated by the appearance on the cell surface of IL-2 receptors, seen as early as 4 to 8 hours. Also, monoclonal antibodies specific for the T cell antigen receptor or its associated structures and some lectins can reproduce the effect of antigen activation.²⁴ In this report, T cell activation can be due to the interaction between ovalbumin and the T cell antigen receptor.

In our present study, the increase of activated T cells elicited by antigen suggests the participation of cellular mechanisms of immunity in the conjunctival allergic response following immunological challenge and supports the notion that activated T cells may play an important role in the pathogenesis of some human ocular allergic diseases. As T cells regulate immune responses and mediate many characteristics of inflammatory reactions, the presence of activated T cells in the conjunctiva may provide the link for the development of distinct clinical pictures of chronic conjunctivitis associated with allergy. A great increase of T cells and activated T cells has been shown in biopsy specimens from the conjunctiva of atopic keratoconjunctivitis patients.²⁵ Based on reports on the activity of T cells provided by the literature, we can speculate on possible mechanisms for the role of activated T lymphocytes in the local response.

It has been reported that activated T lymphocytes produce and release factors such as interleukin 5 (IL-5) and granulocyte macrophage colony stimulating factor (GM-CSF), which induce differentiation, activation, and migration of the eosinophils to the inflammatory focus²⁶⁻²⁸ where they degranulate causing extensive epithelial damage.²⁹ Activated T cells also produce and release factors, such as IL-3, which induce the growth and differentiation of mast cells, and this can result in hyperplasia of these cells in the peripheral tissue, as shown in rodents.³⁰ IL-4, IL-5, and IL-6 are responsible for the activation, proliferation of B cells, and their subsequent differentiation into plasma cells.³¹ It has also been shown that IL-4 induces IgE production by B cells.³² Activated T cells also produce factors that lead to the production

and deposition of collagen by fibroblasts, resulting in hyperplasia of connective tissue.^{33,34} Epithelial damage, eosinophil infiltration, hyperplasia of mast cells, infiltration by plasma cells, and hyperplasia of connective tissue are the main characteristics of the typical histological picture of vernal conjunctivitis.

In conclusion, it appears that activated T cells are attracted to allergic conjunctiva reaction sites in response to specific antigen. The data presented here, together with the above mentioned observations, suggest that activated T lymphocytes may play an important role in the pathological changes which characterise the histological features of vernal conjunctivitis.

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History of ophthalmology

Hermann von Helmholtz

Hermann von Helmholtz contributed to ophthalmology by inventing the ophthalmoscope in 1850, on similar principles to those of Galileo's telescope, 250 years previously (pity Galileo wasn't an ophthalmologist).

But remembering Helmholtz only for this achievement is as unjust as remembering Einstein solely for $E=mc^2$, as his range of interest was vast.

Helmholtz trained as a Prussian army surgeon, which must have involved many less subtle techniques than those employed in eye surgery. While living in Potsdam barracks he wrote his first dissertation, on the physiology of nerve fibres in crab ganglia, which by itself implies a certain eclecticism.

Essays followed on the first law of thermodynamics – that is, that energy can change its form but cannot disappear or be created, and the second – that is, that energy goes from concentration to dissipation but never the other way round.

His physiological research included measurement of the speed of nerve impulses, demonstration that muscle energy is a major source of animal heat, and work on the mechanism of accommodation.

A talented musician, he was fascinated by acoustics, and did much work on the ossicles and tympanum of the ear. When singing in front of his piano one day he noted that the noise made the strings vibrate, and this led him to consider how sound waves travel through the 'ether.' He also formulated the theory that different regions of the basilar membrane resonate to different frequencies. At different times he held chairs of physics, physiology, pathology, and anatomy, but always asserted that his first love was medicine.

Helmholtz first visited Britain in 1856, but was only moderately impressed by the scientific meeting he attended, noting that 'some papers were important contributions, and some the tomfoolery of the crack-brained.' Probably a fair comment.

At the banquet for his seventieth birthday, he was asked how he got his ideas. He replied that 'after investigating a problem, happy thoughts come unexpectedly, without effort. Never when at my desk, but during the slow ascent of wooded hills on sunny days.' Darwin would have agreed – his inspiration on the origin of species came to him during a carriage drive. Budding researchers could use this as a novel justification for not being at their desks.

Helmholtz's original ophthalmoscope was subsequently modified by others. Zehender showed that it was possible to visualise one's own fundus, and in 1863 Heymann invented the ingenious 'auto-ophthalmoscope', which allowed the left eye to visualise the retina of the right. One would think that this could be used to advantage by medical students. In 1870, De Wecker went one step further, he introduced a 'demonstration ophthalmoscope,' which allowed the fundus to be viewed by two observers simultaneously. Maybe this could also be resurrected for teaching purposes?

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