CCR4 and CCR5 expression in conjunctival specimens as differential markers of T_H1/ T_H2 in ocular surface disorders

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Background: Accurate inflammatory mechanisms in chronic ocular surface diseases (OSDs) cannot routinely be assessed. New techniques for investigating ocular surface inflammatory pathways are of major importance.

Objective: To investigate the expressions of CCR4 and CCR5, known to be related to the T_H2 and T_H1 systems, respectively, and HLA-DR in conjunctival impression cytology specimens from patients with chronic OSDs.

Methods: In this case-controlled study, impression cytology specimens were taken in a series of patients with vernal keratoconjunctivitis (n = 21), giant papillary conjunctivitis (n = 6), or keratoconjunctivitis sicca (KCS; n = 17), or receiving topical antiglaucoma treatments (n = 31), and from 20 normal subjects. Conjunctival cells were incubated with mAbs to CCR4, CCR5, CD45, and HLA-DR to quantify conjunctival inflammation in a masked manner using flow cytometry.

Results: HLA-DR was higher in the glaucoma and KCS groups than in allergic and normal eyes. CCR4 was overexpressed in allergy and glaucoma, whereas CCR5 was higher in the KCS and glaucomatous groups. CD45 was expressed by only few cells in all groups, with almost no significant differences. CCR4 expression was negatively correlated with CCR5 and HLA-DR, whereas CCR5 was positively correlated with HLA-DR.

Conclusion: This study confirms the overexpression of chemokine receptors by the conjunctival epithelium in OSDs. CCR4 and CCR5 expression may vary according to the immune pathway involved. Accurate mechanisms in ocular surface inflammatory reactions—that is, those related to the T_H1 or T_H2 systems—could be differentiated by CCR4/CCR5 profiles. Our results also suggest that long-term use of topical treatments may stimulate both systems. (J Allergy Clin Immunol 2005;116:614-9.)

Key words: *CCR4, CCR5, conjunctiva, flow cytometry, glaucoma, HLA-DR, impression cytology, Sjögren syndrome, T_H1, T_H2*

Allergic eye diseases (AEDs) constitute a heterogeneous group of clinicopathological disorders that can cause a large variety of clinical manifestations, from simple intermittent symptoms of itching, tearing, or redness to severe sight-threatening corneal impairment.[1,2] These diseases may involve quite different mechanisms, cytokines, and cellular populations as central actors. Mast cell degranulation, histamine release, and eosinophils thus play key roles in seasonal and perennial conjunctivitis, whereas vernal keratoconjunctivitis (VKC) or atopic keratoconjunctivitis (AKC) are characterized by cellular infiltrates mainly composed of T lymphocytes.[3-5] In severe, chronic allergy-related conditions, 2 main inflammatory pathways may be differentiated: the T_H1 and T_H2 inflammatory cascades, which involve different cytokines and are roughly considered inhibitory of each other when activated.[6] In previous reports based on conjunctival biopsies in patients with allergy, cytokines belonging to the T_H1 or T_H2 systems were investigated, resulting in the recognition of T_H2 activation in VKC, whereas both T_H1 and T_H2 cascades could be found in AKC.[4] Similar results were obtained by flow-cytometry investigation of lymphocyte populations released in the tear flow in allergic disorders[3] or cytokine expression assessed by RT-PCR in brush conjunctival cytology specimens.[6] The necessarily invasive character of conjunctival biopsies, which cannot be routinely proposed to patients with AEDs, as well as the major difficulty of collecting enough lymphocytes from the tear film among cell debris, desquamated epithelial cells, or polymorphonuclear cells, render these techniques highly difficult for routinely exploring allergic patients. Moreover, clinically well-defined allergic disorders are only a part of the wide range of inflammatory ocular surface diseases (OSDs), which also include keratoconjunctivitis sicca (KCS), meibomian gland dysfunction, ocular rosacea, and eyedrop toxicity. Clinical symptoms and signs lack specificity, and most of them leave physicians and patients with unanswered questions and

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Abbreviations used
AED: Allergic eye disease
AKC: Atopic keratoconjunctivitis
GPC: Giant papillary conjunctivitis
KCS: Keratoconjunctivitis sicca
OSD: Ocular surface disease
PC5: Phycoerythrin-cyanin 5
VKC: Vernal keratoconjunctivitis

unsatisfactory responses concerning the accurate mechanisms involved, appropriate treatments, clinical behavior, and symptom relief.

This dilemma emphasizes the need for simple, noninvasive or minimally invasive methods to achieve mechanism identification of chronic inflammatory OSDs and discriminate between allergic and nonallergic pathways. In the last few years, our group has developed the flow-cytometry technique in impression cytology specimens, which has provided extended knowledge of inflammation-related or apoptosis-related membrane antigens[7-9] in OSDs, mainly those in relation with dry eye or antiglaucoma treatments. We therefore used the same technique in a series of patients with clinically well-defined allergic or nonallergic OSDs, with markers belonging to the chemokine receptor family and considered to be related to the T_H1 and T_H2 systems,[10] CCR5 and CCR4, respectively, together with HLA-DR class II antigens as a hallmark for inflammatory assessment, and CD45, a molecule expressed on hematopoietic cells, used for identifying immune cells. The aim of this pilot exploratory case-controlled study was thus to explore new, promising markers and try to provide a useful tool for investigating OSDs and discriminating between the inflammatory pathways involved.

METHODS

Seventy-five patients with OSDs and 20 normal subjects underwent impression cytology collection for this case-controlled study conducted in compliance with the Declaration of Helsinki, Scotland amendment, 2000. Patient characteristics are given in Table I. Seventeen patients had aqueous-deficient KCS as assessed by Schirmer values less than 7 mm at 5 minutes and a positive fluorescein test (at least 2 on the Oxford scheme), associated (n = 10) or not (n = 7) with documented Sjögren syndrome. All of them had received only tear substitutes, with no steroid or topical cyclosporine, for at least 3 months. Twenty-seven patients were also diagnosed with chronic AEDs, 21 with VKC and 6 with giant papillary conjunctivitis (GPC) resulting from poorly tolerated contact lens wear. All patients with VKC had a history of atopy and characteristic features of the disease: limbal and/or tarsal involvement with papillae, follicles, and Trantas dots, and typical symptoms. Again, none of them at the time of impression cytology collection had received any steroid or topical cyclosporine, but only mast cell stabilizers and/or anti-H1 antagonists. All were highly inflammatory at the time of examination, demonstrating a poor effect of topical treatment in relieving allergic manifestations. A series of 31 glaucoma patients with chronic primary open-angle glaucoma was also investigated. They had been treated for at least 1 year with the same treatment, but they were included because they showed at least mild symptoms and/or signs of poor tolerance of their eye drops: repeated stinging or ocular discomfort upon and between instillations; pronounced conjunctival hyperemia; a break-up time less than 8 seconds; a positive fluorescein staining of the cornea that could not be explained by another mechanism, such as aqueous deficiency; active blepharitis related to rosacea; or atopic manifestations. Patients with glaucoma were divided into 2 groups: 13 treated with a monotherapy, either a prostaglandin analogue or a β-blocker, all containing a preservative, and 18 receiving at least 2 topical treatments, including the latter 2 families and/or carbonic anhydrase inhibitors or α2-agonists. Finally, 20 normal subjects who had no history of ocular disease or clinical ophthalmic abnormality, assessed after slit-lamp examination, and who had not received any topical treatment for at least 3 months, were also investigated, after approval by the Ethics Committee of Dijon University. Concerning the patients with OSDs, including symptomatic patients with glaucoma, the Ethics Committee of Paris 6 University had stated that the exploration of OSDs by using impression cytology collection did not require specific approval. Nevertheless, all patients received specific explanations on impression cytology principles and the study aims, and gave informed consent for the procedure and subsequent use of conjunctival specimens.

Impression cytology specimens

Only 1 eye was examined to avoid any bias related to immune between-eye dependency, especially in atopic patients. In most cases, the worse eye was chosen for impression cytology collection. In the case of total symmetry, the right eye was selected for impression cytology analyses. As previously described,[7,9] specimens were collected in the superior bulbar conjunctiva by using 0.20-μm polyether sulfone filters (Supor Membranes; Gelman Sciences, Ann Arbor, Mich) after instillation of 1 drop of 0.4% oxybuprocain. Two pieces of filters, measuring 13 mm × 6.5 mm, were applied to the superior bulbar conjunctiva in areas covered by the upper eyelid to avoid cells exposed to desiccation and environment in the palpebral fissure. As we had previously validated this procedure,[9,11] specimens were taken at least 15 minutes after the use of fluorescein eye drops. Membranes were immediately suspended and fixed in cold PBS containing 0.05% paraformaldehyde. Conjunctival cells were further extracted by gentle agitation and then centrifuged at 1600 rpm for 5 minutes before processing for flow cytometry.

Flow cytometry

Expressions of HLA-DR, CCR4, CCR5, and CD45 were assessed by using flow cytometry and indirect or direct immunofluorescence procedures. Purified anti–HLA-DR (α-chain, clone TAL.1B5) was purchased from DakoCytomation (Glostrup, Denmark); phycoerythrin-conjugated anti-CCR4 (mouse IgG1, clone 1G1), fluorescein isothiocyanate–conjugated anti-CCR5 (mouse IgG2a, clone 2D7) from Pharmingen BD Biosciences (San Diego, Calif); and phycoerythrin-cyanin 5 (PC5)–conjugated anti-CD45 (clone Immu 19.2) from Beckman Coulter (Miami, Fla). Anti–HLA-DR was used at a 1:25 dilution and incubated 30 minutes in the dark at room temperature before being counterstained with a 1:25 dilution of fluorescein isothiocyanate–conjugated goat antimouse immunoglobulin (Dako Cytomation) for 30 minutes. Direct immunostainings were performed for CCR4, CCR5, and CD45 for 30 minutes. Antibodies were used in a 1:50, 1:25, and 1:25 dilution, respectively. The corresponding isotypic negative controls were used, mouse IgG1 (Beckman Coulter) for HLA-DR, mouse IgG1-PE for CCR4, mouse IgG2a-PE for CCR5 (Pharmingen BD Biosciences), and mouse IgG1-PC5 for CD45 (Beckman Coulter). Cells were processed on a flow cytometer EPICS XL (Beckman Coulter). As previously described,[9,11] cells were gated

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