Expression of T lymphocyte chemoattractants and activation markers in vernal keratoconjunctivitis

A M Abu El-Asrar, S Struyf, S A Al-Kharashi, L Missotten, J Van Damme, K Geboes

Background/aims: T lymphocytes are present in increased numbers in the conjunctiva of patients with vernal keratoconjunctivitis (VKC) and their activation has a central role in the pathogenesis of the chronic allergic inflammatory reactions seen in VKC. The aims of this study were to examine the expression of three recently described potent T lymphocyte chemoattractants, PARC (pulmonary and activation regulated chemokine), macrophage derived chemokine (MDC), and I-309, the MDC receptor CCR4, and T lymphocyte activation markers, CD25, CD26, CD62L, CD71, and CD30, and to correlate them with the counts of CD3+ T lymphocytes in the conjunctiva of patients with VKC.

Method: Conjunctival biopsy specimens from 11 patients with active VKC, and eight control subjects were studied by immunohistochemical techniques using a panel of monoclonal and polyclonal antibodies directed against PARC, MDC, I-309, CCR4, CD25, CD26, CD62L, CD71, and CD30. The numbers of positively stained cells were counted. The phenotype of inflammatory cells expressing chemokines was examined by double immunohistochemistry.

Results: In the normal conjunctiva, vascular endothelial cells in the upper substantia propria showed weak immunoreactivity for CD26. There was no immunoreactivity for the other antibodies. VKC specimens showed inflammatory cells expressing PARC, MDC, and I-309. The numbers of PARC+ inflammatory cells were higher than the numbers of MDC+ and I-309+ inflammatory cells and the mean values of the three groups differed significantly (17.0 (SD 10.1); 9.5 (9.9), and 4.3 (7.9), respectively, p = 0.0117, ANOVA). The numbers of PARC+ inflammatory cells had the strongest correlation with the numbers of CD3+ T lymphocytes. Few CCR4+ inflammatory cells were observed in only three specimens. Double immunohistochemistry revealed that all inflammatory cells expressing chemokines were CD68+ monocytes/macrophages. The numbers of CD25+ T lymphocytes were higher than the numbers of CD26+, CD62L+, CD71+, and CD30+ T lymphocytes and the mean values of the five groups differed significantly (46.2 (27.9), 30.7 (16.0), 20.1 (8.6), 7.8 (7.7), and 6.5 (4.0), respectively; p < 0.001, ANOVA). The numbers of CD25+ T lymphocytes had the strongest correlation with the numbers of CD3+ T lymphocytes.

Conclusion: These results suggest a potential role for PARC, MDC, and I-309 in attracting T lymphocytes into conjunctiva in VKC. T lymphocytes in VKC are activated and express several activation markers which might contribute to the pathogenesis of VKC.
In addition, we examined the expression of T cell activation markers, CD25 (the IL-2 receptor α-chain), CD26 or dipeptidyl peptidase IV (DPP IV), CD62L (L-selectin), CD71 (transferrin receptor), and CD30. Furthermore, we examined the correlations between the numbers of T lymphocytes and the numbers of inflammatory cells expressing these CC chemokines and activation markers.

PATIENTS AND METHODS

Patients

Eleven consecutive Saudi patients with active VKC seen at the outpatient clinic of King Abdulaziz University Hospital were included in the study. The patients were seven males and four females. The mean age was 12.6 (SD 2.3) years (range 8–15 years). The symptoms mentioned by all the patients were itching, redness, photophobia, and tearing. Each patient underwent complete ophthalmic examination, and the corneal and conjunctival changes were noted and recorded. All patients had the limbal form of the disease characterised by broad gelatious infiltrates of the limbus. Limbal conjunctival biopsy specimens were obtained from each patient. None of the patients was on topical therapy before obtaining the biopsy. In addition, eight limbal conjunctival biopsy specimens were obtained from patients undergoing strabismus surgery without obvious inflammation and served as controls. None of the controls had a history or signs of VKC. The controls were from the same age group, and were five males and three females. All the controls were Saudis, and were operated in King Abdulaziz University Hospital. This study was approved by the Research Center, College of Medicine, King Saud University, and the patients admitted to the study gave their informed consent.

Immunohistochemical staining

The conjunctival biopsy specimens were immediately snap frozen in Tissue-Tek optimum cutting temperature (OCT) compound (Miles Laboratories, IN, USA) and maintained at −80°C until use. For immunohistochemistry, 5 µm serially cut cryostat sections were dried overnight at room temperature, fixed in absolute acetone for 10 minutes, and then treated with 2% hydrogen peroxide in methanol for 3 minutes to block endogenous peroxidase activity. After rinsing three times in phosphate buffered saline (PBS) at pH 7.2 for 15 minutes, the slides were incubated for 30 minutes with the monoclonal and polyclonal antibodies listed in Table 1. Optimal concentrations of all antibodies used were determined in pilot experiments. After a wash with PBS, the sections were incubated for 30 minutes with Envision+, Peroxidase, Rabbit, or EnVision+, Peroxidase, Mouse (Dako, CA, USA) and washed again with PBS. Then, the reaction product was visualised by incubation for 10 minutes in 0.05M acetate buffer at pH 4.9, containing 0.05% 3-amino-9-ethylcarbazole and 0.01% hydrogen peroxide, resulting in red immunoreactive staining. Afterwards the sections were rinsed with PBS, washed with distilled water and incubated for 30 minutes with the chemokine antibodies. After a wash with PBS, the sections were incubated for 30 minutes with a rabbit anti-mouse alkaline phosphatase labelled antibody (Sigma-Aldrich). The blue reaction product was developed using fast blue BB salt (4-benzoylamino-2.5-diethoxybenzene-diazonium chloride) (Sigma-Aldrich) for 5 minutes.

Quantitation

Cells were counted in five representative fields that were chosen on the basis of the presence of an adequate number of inflammatory cells. We ignored fields in which no positively stained cells were present. We used an eye piece calibrated grid with 40X magnification. With this magnification and calibration, we counted the cells present in an area of 0.33 × 0.22 mm.

Statistical analysis

All data are presented as mean (SD). The data were analysed using one way analysis of variance (ANOVA), non-parametric one way ANOVA based on the Kruskal-Wallis test, correlation analysis, and ridge regression. Programs 4R, 7D, and 3S from the BMDP Statistical Package were used. The differences were considered significant if the p value was < 0.05.

RESULTS

There was no staining in the negative control slides. In normal conjunctiva, weak CD26 immunoreactivity was observed on vascular endothelial cells in the upper substantia propria. There was no immunoreactivity for PARC, MDC, I-309, CCR4, CD25, CD62L, CD71, and CD30. In VKC specimens, a heavy inflammatory infiltrate of CD3+ T lymphocytes was noted in the epithelium and in the

<table>
<thead>
<tr>
<th>Table 1: Monoclonal and polyclonal antibodies used in this study</th>
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<tr>
<td>Primary antibody</td>
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</tr>
<tr>
<td>Anti-CD3 (UCHT1) (mc)</td>
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<td>Anti-PARC (64S07) (mc)</td>
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<td>Anti-PARC (pc)</td>
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<td>Anti-MDC (pc)</td>
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<td>Anti-I-309 (33305.11) (mc)</td>
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<td>Anti-CCR4 (H-48) (pc)</td>
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<td>Anti-CD25 (ACT-1) (mc)</td>
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<td>Anti-CD26 (MA261) (mc)</td>
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<td>Anti-CD62L (M7084) (mc)</td>
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<td>Anti-CD71 (MA712) (mc)</td>
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<td>Anti-CD30 (BerH2) (mc)</td>
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<td>Location of manufacturers: Dako, CA, USA; R &amp; D Systems Europe Ltd, Abingdon, UK; PeproTech Inc, Rocky Hill, NJ, USA; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA; PharMingen, San Diego, CA, USA.</td>
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substantia propria just beneath the epithelium in all specimens. Inflammatory mononuclear cells expressing granular cytoplasmic PARC, MDC, and I-309 were noted in the upper substantia propria at sites of heavy CD3+ T lymphocyte accumulation (Fig 1). PARC+ inflammatory cells were noted in all specimens, whereas MDC+ inflammatory cells were observed in nine specimens and I-309+ inflammatory cells were observed in six specimens. Both PARC antibodies that we used generated the same labelling pattern on inflammatory cells. Membranous immunoreactivity for CCR4 was noted on inflammatory mononuclear cells in the upper substantia propria in three specimens.

Double immunohistochemistry to confirm the phenotype of chemokine positive inflammatory cells showed that all the mononuclear cells expressing chemokines were CD68 positive monocytes/macrophages (Fig 2). Many of the PARC+ inflammatory cells had a dendritic morphology.

The numbers of inflammatory cells expressing PARC were higher than the numbers of inflammatory cells expressing MDC and I-309 (Table 2). The mean values of the three groups differed significantly (p = 0.0117, ANOVA). Furthermore, post-ANOVA pairwise comparisons based on the Bonferroni method showed that the numbers of inflammatory cells expressing PARC+ were significantly higher than the numbers of inflammatory cells expressing I-309 (p = 0.0032, t test).

Membranous immunoreactivity for CD25, CD26, CD62L, CD71, and CD30 was noted on mononuclear inflammatory cells in the epithelial and stromal inflammatory infiltrate (Fig 3). Inflammatory cells expressing CD25, CD26, CD62L, and CD30 were noted in all specimens, whereas CD71+ cells were observed in seven specimens. A marked upregulation of CD26 expression was noted on superficial and deep stromal vascular endothelial cells (Fig 4). CD30 expression was confirmed by staining with two anti-CD30 monoclonal antibodies. The activation markers CD25 and CCR4 were shown to be expressed on CD3+ T lymphocytes by serial sections (Fig 3).

The numbers of inflammatory cells expressing CD25 were higher than the numbers of inflammatory cells expressing CD26, CD62L, CD71, and CD30 (Table 2). The mean values of the five groups differed significantly (p <0.001, ANOVA done by non-parametric Kruskal-Wallis test). Furthermore, post-ANOVA pairwise comparisons showed that the numbers of inflammatory cells expressing CD25 were significantly higher than the numbers of inflammatory cells expressing CD71 (Z* = 4.24) and CD30 (Z = 4.44). The numbers of inflammatory cells expressing CD26 were significantly higher than the numbers of inflammatory cells expressing CD71 (Z = 3.59) and CD30 (Z = 3.79). (The critical Z value was Z ≥2.81 for five groups at a 5% level of significance (Kruskal-Wallis test).)
Correlations between the numbers of CD3+ T lymphocytes and the numbers of inflammatory cells expressing chemokines

A significant positive correlation was observed between the numbers of CD3+ T lymphocytes and the numbers of inflammatory cells expressing PARC (r = 0.6709, p = 0.0238), MDC (r = 0.7453, p = 0.0085), and I-309 (r = 0.6389, p = 0.0343). Ridge regression analysis indicated that the numbers of inflammatory cells expressing PARC had the strongest correlation (79% explained variation) with the numbers of CD3+ T lymphocytes.

Correlations between the numbers of inflammatory cells expressing chemokines and the numbers of inflammatory cells expressing activation markers

A significant positive correlation was observed between the numbers of inflammatory cells expressing CD3+ T lymphocytes and the numbers of inflammatory cells expressing CD25 (r = 0.7789, p = 0.0047), CD62L (r = 0.6464, p = 0.0316), and CD71 (r = 0.6259, p = 0.0394). Ridge regression analysis indicated that the numbers of inflammatory cells expressing CD25 had the strongest correlation (46.1% explained variation) with the numbers of CD3+ T lymphocytes.

Correlations between the numbers of inflammatory cells expressing chemokines and the numbers of inflammatory cells expressing activation markers

A significant positive correlation was observed between the numbers of inflammatory cells expressing PARC and the numbers of inflammatory cells expressing CD25 (r = 0.8782, p = 0.0004). In addition, there was a significant positive correlation between the numbers of inflammatory cells expressing MDC and the numbers of inflammatory cells expressing CD26 (r = 0.6354, p = 0.0357).

DISCUSSION

In vitro, PARC mRNA is expressed by monocyte derived dendritic cells, and activated monocytes. However, Hieshima et al. demonstrated that PARC is chemotactic for both activated (CD3+) T cells and non-activated (CD14−) lymphocytes. PARC binds to a yet unknown receptor expressed on naive T cells. The specific expression of PARC by dendritic cells at the site of initiation of an immune response, combined with its chemotactic activity for naive T cells, suggests that PARC has an important role in the induction of immune responses.

In the present study, we detected cytoplasmic expression of PARC by CD68 positive monocytes/macrophages. Many of these cells had a dendritic morphology. In agreement with our data, previous studies showed that PARC mRNA was restricted to CD68 positive macrophages in human atherosclerotic plaques. In addition, high PARC expression was noted in alveolar macrophages and dendritic cells in the germinal centres of regional lymph nodes. MDC is constitutively produced by dendritic cells, macrophages, and thymic medullary epithelial cells, whereas monocytes, B lymphocytes, natural killer cells, and CD4+ T lymphocytes produce MDC only upon appropriate stimulation. More recently, MDC was shown to be
preferentially produced by activated Th2 cells.\textsuperscript{23} MDC is chemotactic for activated T lymphocytes, IL-2 activated natural killer cells towards Th1 cells, and dendritic cells.\textsuperscript{24,25} In a mouse model of allergic inflammation in the lung, MDC is produced by alveolar macrophages.\textsuperscript{26} In this animal model, Lloyd et al\textsuperscript{32} showed that the CCR4/MDC pathway has a dominant role in effector Th2 recruitment under conditions of chronic, repeated antigen stimulation. The expression of I-309 by monocytes/macrophages in the conjunctiva from patients with VKC is consistent with in vitro studies that demonstrated I-309 expression and secretion by appropriately stimulated human peripheral blood monocytes.\textsuperscript{27} Surprisingly, few cells in VKC conjunctiva expressed CCR4. Our finding of poor CCR4 expression might be due to CCR4 internalisation after stimulation with its ligands.\textsuperscript{28}

The expression of PARC and MDC by monocytes/macrophages in VKC conjunctiva might be explained by upregulation by inflammatory cytokines. Recently, several studies demonstrated that the Th2 cytokines, IL-4, and IL-13 induced PARC expression in macrophages\textsuperscript{33} and MDC expression in monocytes.\textsuperscript{34}

In this study, we found that the T cells infiltrating the conjunctiva of patients with VKC express several activation markers. CD25 cells were the most numerous followed by CD26\textsuperscript{+} cells and CD62L\textsuperscript{+} cells. CD71\textsuperscript{+} cells and CD30\textsuperscript{+} cells were few. In addition, the numbers of CD25\textsuperscript{+} cells had the strongest correlation with the numbers of CD3\textsuperscript{+} T lymphocytes. T cells express CD25 upon activation\textsuperscript{35} and CD25 expression is promoted by the Th2 cytokine, IL-4.\textsuperscript{36} Stimulation of CD4\textsuperscript{+} T cells secreting Th2 type cytokines by dendritic cells elicits T cell proliferation, production of cytokines, and upregulates CD25 expression.\textsuperscript{37} Recently, Suto et al\textsuperscript{38} demonstrated that CD4\textsuperscript{+} CD25\textsuperscript{+} T cells modulate the Th1 and Th2 cell balance in Th2 cells and thus upregulate Th2 cells. Subsequently, MDC was shown to act selectively on chronically activated Th2 lymphocytes by interacting with the CCR4 receptor,\textsuperscript{39} which is preferentially expressed by this T cell effector subset.\textsuperscript{40} More recently, MDC was also found to be able to induce human eosinophil chemotaxis in a CCR3 and CCR4 independent manner.\textsuperscript{41}

The conjunctiva from patients with VKC showed cytoplastic expression of MDC, and I-309 by CD68 positive monocytes/macrophages. Previous studies have reported expression of MDC by dendritic cells in skin biopsy specimens from patients with atopic dermatitis, a Th2 oriented disorder.\textsuperscript{23,25} In a mouse model of allergic inflammation in the lung, MDC is produced by alveolar macrophages.\textsuperscript{26} In this animal model, Lloyd et al\textsuperscript{32} showed that the CCR4/MDC pathway has a dominant role in effector Th2 recruitment under conditions of chronic, repeated antigen stimulation. The expression of I-309 by monocytes/macrophages in the conjunctiva from patients with VKC is consistent with in vitro studies that demonstrated I-309 expression and secretion by appropriately stimulated human peripheral blood monocytes.\textsuperscript{27} Surprisingly, few cells in VKC conjunctiva expressed CCR4. Our finding of poor CCR4 expression might be due to CCR4 internalisation after stimulation with its ligands.\textsuperscript{28}

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In conclusion, PARC and MDC expressed by CD68 positive monocytes/macrophages may function to recruit naive and activated T lymphocytes in VKC. PARC could play a major part in the recruitment of naive T cells which might initiate the immune responses. PARC and MDC might further attract activated T cells. T lymphocytes infiltrating the conjunctiva of patients with VKC express several activation markers which might contribute to the pathogenesis of VKC.

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