

Effects of Antisense Oligodeoxynucleotides Targeting CCR3 on the Airway Response to Antigen in Rats

MARYLÈNE FORTIN,¹ NICOLAY FERRARI,¹ MARIE-ÈVE HIGGINS,¹ SERGE SÉGUIN,^{1,2}
MUSTAPHA ALLAM,² ZOULFIA ALLAKHVERDI,² CARMEN PIAGET-RODRIGUEZ,²
LUC PAQUET,¹ and PAOLO M. RENZI^{1–3}

ABSTRACT

Asthma is characterized by airway hyperresponsiveness (AHR) and inflammation, consisting predominantly of eosinophils within the airway lumen and walls. Eosinophil recruitment to the airways is mediated mainly by eotaxin and other chemokines that bind to the CC-chemokine receptor-3 (CCR3), which is highly expressed on eosinophils. This study assessed whether topical inhibition of CCR3 mRNA expression by phosphorothioate antisense oligodeoxynucleotides (AS-ODNs) modifies pulmonary eosinophilia and AHR in an antigen-induced allergic asthma model in Brown Norway (BN) rats. Results show that specific inhibition of CCR3 expression in the lungs by an AS-ODN (AS4) reduced total eosinophil infiltration and the percentage of eosinophils into the airways of ovalbumin challenged rats. Moreover, reduction in CCR3 mRNA levels was correlated with a decrease in CCR3 protein in lung tissue. In addition, AS4 treatment had no effect on circulating eosinophils or on eosinophils in the bone marrow. Finally, AHR was significantly decreased in AS4-treated rats when compared with rats treated with a mismatch AS-ODN. In conclusion, inhibition of the expression of CCR3 decreased pulmonary eosinophilia and reduced AHR after antigen challenge in rats. Topical inhibition of CCR3 expression, using an AS-ODN, could represent a novel approach for the treatment of asthma.

INTRODUCTION

ASTHMA IS A CHRONIC INFLAMMATORY DISEASE of the airways in which eosinophils accumulate and subsequently degranulate (Spry, 1971; de Monchy et al., 1995; Gleich, 1990). Recruitment and persistence of eosinophils within the airways are induced primarily by specific cytokines and chemokines released by lymphocytes, mast cells, epithelial cells, and smooth muscle cells (Baggiolini et al., 1997; Barnes, 2003; Sallusto et al., 2000). Whereas eosinophil survival is largely due to the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-5, eosinophil recruitment seems to be mediated by eotaxin

and other chemokines that bind to a single CC-chemokine receptor, CCR3, which is highly expressed on eosinophils (Combadiere et al., 1995; Yang et al., 2003; Griffiths-Johnson et al., 1993; Jose et al., 1994).

Intratracheal administration of eotaxin in mice and guinea pigs induces a rapid and substantial accumulation of eosinophils in bronchoalveolar lavage (BAL) fluid (Jose et al., 1994; Erin et al., 2002). Eotaxin can also induce eosinophil degranulation, leading to the release of proteins that damage the airway epithelium (Fujisawa et al., 2000; Kampel et al., 2000). In human asthma, eotaxin levels are increased, although expression of this chemokine is localized predominantly to airway epithelial cells (Lamkhieoued et al., 1997). Targeting eotaxin

¹Topigen Pharmaceuticals, Inc., Montreal, Quebec, Canada H1W 4A4.

²CHUM Research Center, Notre-Dame Hospital, Montreal, Quebec, Canada H2L 4M1.

³Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada, H2X 2P2.

alone has been suggested to be sufficient to inhibit the eosinophilia and airway hyperresponsiveness (AHR) that occur in animal models of asthma (Garcia-Zepeda et al., 1996; Ponath et al., 1996). However, depletion of eotaxin levels by disrupting the eotaxin gene only partially inhibits eosinophilia in mice (Rothenberg et al., 1997).

CCR3, an eotaxin receptor, is a member of the G protein-coupled seven-transmembrane receptor family and also binds MCP-3, MCP-4, RANTES, eotaxin-2, and eotaxin-3 (Fujisawa et al., 2000). All these chemokines are released by inflammatory and epithelial cells, and can contribute to eosinophil, herpes T cell type 2 (Th2), and basophil infiltration. CCR3 has several ligands, which might explain why disrupting eotaxin gene expression alone is insufficient to completely inhibit recruitment of eosinophils to the lung. Previous *in vitro* studies using BAL fluid obtained from patients with asthma showed that a combination of antibodies directed against RANTES, MCP-4, and eotaxin was more effective at inhibiting the chemoattractant effects of asthmatic BAL fluid on eosinophils than a single antibody directed against eotaxin (Lamkhieoued et al., 1997).

Focus has been given to receptor antagonists rather than ligand inhibition in manipulating CCR3 (Owen, 2001). Pyrrolidine-derived compounds have been demonstrated to be effective antagonists of CCR3 *in vitro*. However, these molecules are nonspecific because they also bind CCR5 (Bertrand and Ponath, 2000). Antibodies directed against CCR3 have been shown to cause partial inhibition of eosinophil recruitment *in vivo* (Grimaldi et al., 1999). Phosphorothioate antisense oligodeoxynucleotides (AS-ODNs) are now commonly used as a selective strategy to inhibit the expression of a variety of genes (Gao et al., 1992; Stein, 2001; Wagner, 1994). This study sought to determine the effect of an AS-ODN (AS4) targeting the CCR3 mRNA on pulmonary eosinophilia and AHR in response to leukotriene D₄ (LTD₄) in a rat model of allergen-induced lung inflammatory response.

MATERIALS AND METHODS

Design and synthesis of phosphorothioate antisense oligodeoxynucleotides targeting rat CCR3 mRNA

Four AS-ODNs (AS1 to AS4) were designed in house according to the following rules: 16- to 25-mer in length; targeting the 5' region of the mRNA; sequence with low A and GC content; and no CpG. In addition, consensus sequences such as AACGTT (interferon induction) and GGGG (nonspecific antiproliferative effect) were avoided (Stein, 2001). Antisense sequences were as follows: AS1, 5'-CAG AGA GAT ACC AAC ATG-3'; AS2, 5'-GAT CAA CAC AAC CAT CAT GT-3'; AS3,

5'-ATA ATT TGC AGC TTC CTG T-3'; and AS4, 5'-ACT CAT ATT CAT AGG GTG-3'. A mismatch ODN (MM4-ODN, 5'-CAT CAT TAT CAT GAG GTG-3') was also designed. All AS-ODNs and mismatch ODN were synthesized with a phosphorothioate backbone and cartridge (RP1) purified by SIGMA-Genosys (Oakville, ON).

Antisense screening in rat peritonitis model

Animal studies were conducted in two research centers (CHUM Research Center and Mispro Biotech Services, Montréal, QC, Canada) and were approved by the respective animal ethics committees. Brown Norway (BN) rats (6 to 8 weeks old) were obtained from Harlan Sprague-Dawley (Walkersville, MD). Active sensitization was performed by subcutaneous injection of 1 mL of saline containing 1 mg of chicken egg ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) and 3.5 mg of aluminum hydroxide gel (Laboratoire MAT, Beauport, QC, Canada). On day 14 postsensitization, rats (five per group) were treated with an intraperitoneal injection of either AS1, AS2, AS3, or AS4 (500 µg in 500 µL of phosphate-buffered saline [PBS]). Control rats were treated with the same dose of a mismatch control oligodeoxynucleotide (MM4) or with PBS only. Sixteen hours after AS-ODN treatment, rats were challenged with an intraperitoneal injection of OVA (200 µg in 500 µL of saline) to induce eosinophil recruitment to the peritoneal cavity. Rats were killed 8 hr after OVA challenge, and the peritoneal cavity was washed three times with 5 mL of PBS. Cells were recovered by centrifugation and total leukocyte counts were performed with a hemacytometer. Differential cell counts were performed on Cytospin slides stained with a Hema-3 stain kit (Fisher, Ottawa, ON, Canada). At least 200 cells were counted by oil immersion microscopy.

Antisense efficacy in rat model of allergen-induced airway inflammation

BN rats were sensitized with OVA, as described above. Fourteen days after sensitization, rats were injected intratracheally with either sterile saline (50 µL) or 200 µg of AS4 or MM4 (in 50 µL of sterile saline). Rats were challenged 10 min later by exposure to OVA aerosols (5% in saline) in a closed chamber for 15 min. To determine the effect of AS-ODN treatment on cellular influx to the lungs, rats were killed 15 hr after OVA challenge, and BAL was performed. Cells were recovered by centrifugation and total leukocyte counts were performed with a hemacytometer. Differential cell counts were performed on Cytospin slides stained with a Hema-3 stain kit. At least 200 cells were counted by oil immersion microscopy. Lungs were collected after BAL and processed for mRNA (right lung) or immunohistochemistry (left lung).

Assessment of the effect of AS4 on AHR in response to LTD₄ was done in separate groups of rats. On day 14 postsensitization, rats were either unchallenged (CTL), challenged with OVA (OVA), or treated with a single dose (200 µg, intratracheal) of AS4 followed by OVA challenge (OVA + AS4) or MM4 followed by OVA challenge (OVA + MM4). Fifteen hours after OVA challenge, rats were anesthetized and intubated and baseline lung resistance (R_L) was measured. Rats were then exposed intratracheally to incremental doses of LTD₄ (Cayman Chemical, Ann Arbor, MI) in 50 µL of saline and measurements of R_L were obtained immediately after the administration of each dose (Renzi et al., 1992). The concentration of LTD₄ required to double the baseline value was defined as EC₂₀₀LTD₄ and was calculated by linear interpolation between the two concentrations bounding the point at which R_L reached 200% of the baseline value.

mRNA quantification in lung tissue

The right lungs were collected after BAL and tissue was stored in RNAlater stabilization reagent (Qiagen, Mississauga, ON, Canada). CCR3 mRNA quantification was performed in lung tissue homogenates, using the Quantigene mRNA quantification kit (Genospectra, Fremont, CA). Briefly, this assay is performed in oligonucleotide-coated 96-well plates and uses specific probe sets spanning the mRNA sequence and the branched DNA technology for amplification of a luminescent signal. β_2 -Microglobulin (β_2m) mRNA levels were also quantified. β_2m was used as a reference gene for normalization of CCR3 mRNA levels in lung tissue.

Immunohistochemistry

Frozen cryosections (thickness, 7 µm) from the left lungs were fixed in methanol-acetone at -20°C for 10 min. After blocking, slides were incubated with a 10-µg/mL concentration of anti-human major basic protein monoclonal antibody (mAb) (MBP; S-20, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-CCR3 polyclonal antibody (H-52; Santa Cruz Biotechnology) overnight at 4°C. For detection of primary antibodies, fluorescence-labeled secondary antibodies (1:500) (Invitrogen, Burlington, ON, Canada) were used according to the manufacturer's guidelines. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with Prolong Antifade (Invitrogen). Slides were coded and positive cells were counted in a blinded manner. Airway sections were cut in such a way that the axial pathway could be defined and only these sections were read. Both small and large airways (with a perimeter of basement membrane between 0.1 and 2 mm) were studied. Positive cells were counted only in the subepithelial

area beneath the basement membrane (BM) to a depth of 0.5 mm, using an eyepiece graticule (0.1 mm graticule calibration).

Statistical analysis

Differences between groups were determined by analysis of variance (ANOVA). Results are expressed as mean values for the group of animals analyzed $\pm$ standard deviation (SD). Statistical significance (unpaired *t* test) was determined as $p < 0.05$.

RESULTS

Functional screening of CCR3 AS-ODN in rat model of allergen-induced peritonitis

Phosphorothioate AS-ODNs targeting the rat CCR3 gene were designed and their efficacy was first evaluated in a rat model of allergen-induced peritonitis. On day 14 postsensitization, BN rats were treated with a single injection of either AS1, AS2, AS3, or AS4 (500 µg, intraperitoneal). Control rats were treated with the same dose of a mismatch control oligonucleotide (MM4) or with PBS only. Sixteen hours after AS-ODN treatment, rats were challenged with an intraperitoneal injection of OVA to induce eosinophil recruitment to the peritoneal cavity. Rats were killed 8 h after OVA challenge, recruited cells were recovered by washing the peritoneal cavity, and total as well as differential cell counts were performed. In rats treated with AS4, the total number and percentage of eosinophils recovered from the peritoneal cavity were significantly reduced compared with control rats treated with PBS (67% inhibition, $p < 0.001$). The percentage of eosinophils recovered from rats treated with other antisenses (AS1, AS2, and AS3) or with MM4 was not significantly different from control rats. Therefore, AS4 was selected and used in all following studies.

Airway-challenged rat model to determine the effects of AS4 on pulmonary eosinophilia

We then asked whether local blockade of CCR3 mRNA expression in lung tissue can provide protection against allergen-induced lung eosinophilia in BN rats. On day 14 postsensitization, rats were treated with a single intratracheal injection of AS4 (200 µg) or MM4 control (200 µg). Ten minutes after AS4 or MM4 treatment, rats were challenged by exposure to OVA aerosols, and killed 15 hr later. Differential cell counts were performed on cells recovered from BAL fluids. Results have shown that in untreated rats (no AS-ODN), OVA challenge resulted in a 35-fold increase in the total number of lung eosinophils when compared with unchallenged rats (Fig. 1). The

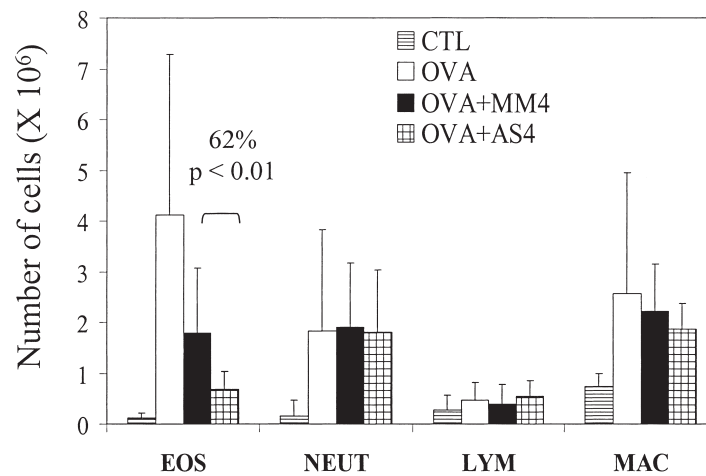


FIG. 1. Inhibition by AS4 of allergen-induced eosinophil influx into the lungs of sensitized BN rats. Fourteen days after OVA sensitization, rats were either unchallenged (CTL), challenged with OVA (OVA), or treated with a single dose (200 μ g, intratracheal) of MM4 followed by OVA challenge (OVA + MM4) or with AS4 followed by OVA challenge (OVA + AS4). Rats were killed 15 hr after OVA challenge, and differential cell counts from the BAL fluid were performed. Data represent mean total cell numbers $\pm$ SD from three separate experiments ($n = 7$ to 12 rats per group). EOS, eosinophils; NEUT, neutrophils; LYM, lymphocytes; MAC, macrophages.

OVA-induced pulmonary eosinophilia was inhibited by 62% ($p < 0.01$) in AS4-treated rats when compared with rats that received the MM4 AS-ODN (Fig. 1). The percentage of eosinophils was also markedly reduced in AS4-treated rats when compared with MM4-treated rats. BAL fluid from AS4-treated rats contained 15% eosinophils, whereas BAL fluid from MM4-treated rats contained 28% eosinophils (data not shown). The number of eosinophils recovered from the lungs of MM4-treated rats was not significantly different from that of rats challenged with OVA only. Moreover, neutrophil, lymphocyte, and macrophage counts were not significantly affected in

AS4-treated rats, which suggests that the inhibitory effect of AS4 was specific to eosinophils.

To determine whether the reduction in lung eosinophils was associated with a reduction in circulating eosinophils, we evaluated the effect of AS4-treatment on the number of eosinophils in blood and in bone marrow. In the blood of AS4-treated rats, eosinophils represented $7.4 \pm 5.2\%$ of total cells compared with $7.8 \pm 4.8\%$ in MM4-treated rats, whereas the percentage of eosinophils in the bone marrow was 7.6 ± 7.7 and $6.5 \pm 5.9\%$, respectively. We next examined the effect of AS4 on the levels of eotaxin. Eotaxin levels in BAL fluid and in the

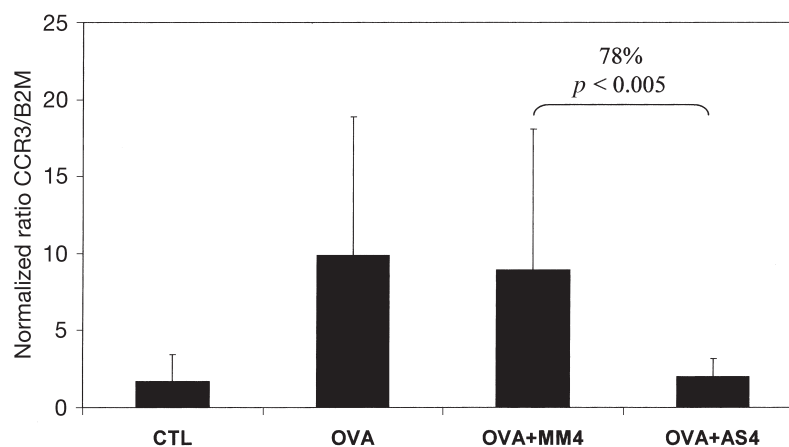


FIG. 2. CCR3 mRNA expression in airways of challenged rats treated with AS4. Rats were treated as described in Fig. 1. Lungs were collected 15 hr after OVA challenge and CCR3 mRNA expression in lung tissue homogenates was quantified by Quantigene analysis and normalized to β_2 -microglobulin (β_2 M) expression. Columns show mean CCR3/ β_2 M ratios $\pm$ SD from three separate experiments ($n = 8$ to 18 rats per group).

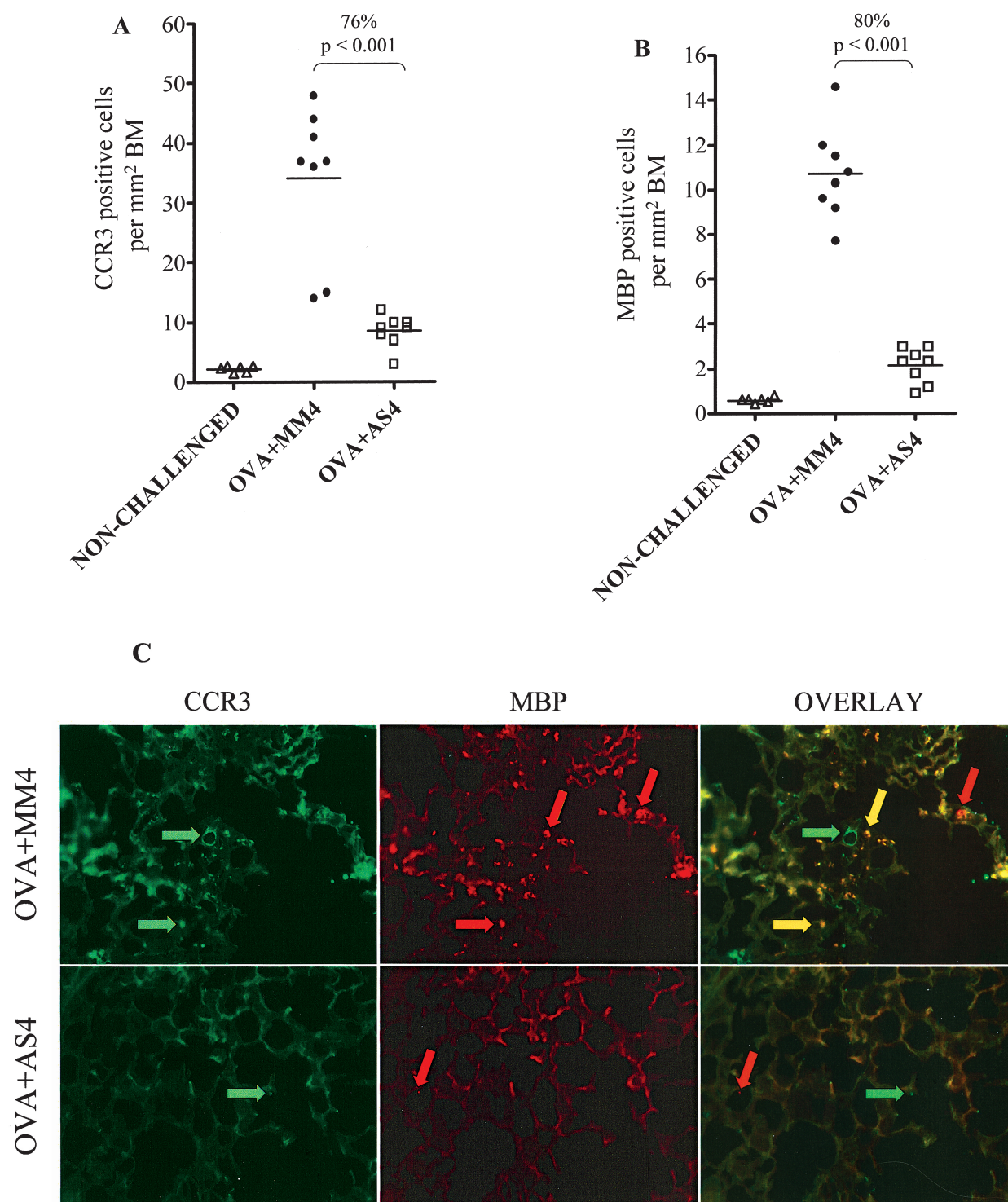


FIG. 3. Immunohistochemical analysis of CCR3- and MBP-positive cells in lung tissue sections from AS4- and MM4-treated rats. Rats were treated as described in Fig. 1. **(A)** Number of CCR3-positive cells per square millimeter of basal membrane (BM) in representative lung tissue sections. Individual data points are shown along with group mean values (horizontal lines). **(B)** Number of MBP-positive cells per square millimeter of BM in representative lung tissue sections. Individual data points are shown along with group mean values (horizontal lines). **(C)** Representative lung tissue sections from OVA-challenged rats treated with MM4 or AS4. Frozen sections were stained for CCR3 (left, green) and MBP (middle, red). CCR3-positive cells are indicated by green arrows, MBP-positive cells by red arrows. Overlay images (right) show colocalization of CCR3 and MBP (right, yellow arrows). Original magnification, $\times 200$.

blood of AS4-treated rats were not significantly altered compared with control untreated rats, indicating that AS4 had no direct effect on eotaxin (data not shown). Taken together, these results indicate that AS4 specifically inhibited the influx of eosinophils to the lungs, and suggest that this inhibition was not due to a reduction in the number of circulating eosinophils or to decreased eotaxin levels in the lungs and blood.

Modulation of lung CCR3 mRNA and protein expression after AS-ODN treatment

To determine whether the inhibitory effect of AS4 on allergen-induced eosinophilia was specific to the downregulation of CCR3 mRNA expression, we measured CCR3 mRNA levels in the lungs of OVA-challenged rats treated with the AS-ODN. As shown in Fig. 2, we observed a 6-fold increase in CCR3 mRNA expression levels in the lungs of OVA-challenged rats when compared with unchallenged rats. The allergen-induced CCR3 mRNA upregulation was completely abolished by a single intratracheal administration of AS4 before challenge with OVA. In OVA-challenged rats, AS4 pretreatment inhibited CCR3 mRNA expression by 78% as compared with MM4 pretreatment ($p < 0.005$), and reduced CCR3 expression levels to baseline levels observed in unchallenged rats. There was no significant difference in lung CCR3 mRNA expression levels between OVA and OVA + MM4 rats. These results suggest a correlation between CCR3 expression and eosinophil influx in the lungs during the allergen response.

We then assessed whether the reduction in CCR3 mRNA expression affected CCR3 protein levels *in vivo*. We measured CCR3 protein expression by immunostaining of frozen lung tissue sections from unchallenged rats or OVA-challenged rats pretreated with AS4 or MM4. Lungs from AS4-treated rats showed a significant decrease in CCR3-immunoreactive cells in the subepithelial region when compared with the airways of MM4-treated rats. As shown in Fig. 3A, there were 8.35 ± 2.4 positive cells/mm² of basal membrane in AS4-pretreated rats versus 34.3 ± 12.3 positive cells/mm² of basal membrane in MM4-pretreated rats (76% decrease, $p < 0.001$). AS4 treatment reduced the number of CCR3-positive cells near to baseline levels seen in unchallenged rats. The presence of major basic protein (MBP), an eosinophil-specific marker, was also evaluated in the lungs of unchallenged rats or OVA-challenged rats pretreated with the AS-ODN. Lung tissue from AS4-treated rats contained significantly fewer MBP-positive cells than lung tissue from MM4-treated control rats (AS4-treated rats, 2.16 ± 0.75 positive cells/mm² of basal membrane; MM4-treated rats, 10.7 ± 2 positive cells/mm² of basal membrane; 80% decrease, $p < 0.001$; Fig. 3B). AS4 treatment reduced

the number of MBP-positive cells near to baseline levels seen in unchallenged rats. These results suggest that the downregulation of CCR3 mRNA expression by AS4 correlates with a reduction of CCR3 protein in the lung tissue as shown by the decrease in CCR3 immunostaining. Furthermore, the decrease in MBP immunostaining suggests that AS4 treatment affected the number of activated eosinophils present in the lungs of OVA-challenged rats, in accordance with the differential cell count results (Fig. 1).

Airway-challenged rat model to determine the effect of AS4 on airway hyperresponsiveness to LTD₄

We have previously shown that blocking the allergen-induced eosinophilia in the lungs by inhibiting expression of the common β chain of the IL-3/IL-5/GM-CSF receptor reduced AHR to LTD₄ (Allakhverdi et al., 2002). To evaluate whether AS4 could affect AHR, we determined the effect of pretreatment with AS4 (200 μ g, intratracheal) on the cumulative dose-response of LTD₄ 15 hr after OVA challenge. As shown in Fig. 4, OVA challenge resulted in a significant decrease in the LTD₄ effective dose required to double the resistance baseline value ($EC_{200}LTD_4$) when compared with unchallenged

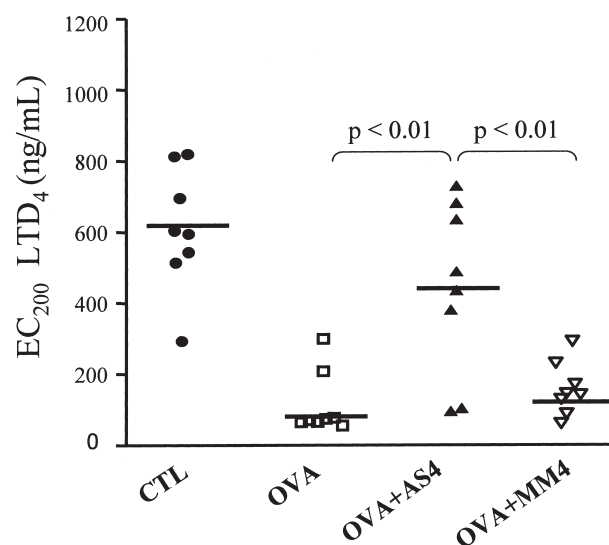


FIG. 4. Evaluation of AS4 efficacy on allergen-induced AHR. On day 14 postsensitization, rats were either unchallenged (CTL), challenged with OVA (OVA), or treated with a single dose (200 μ g, i.t.) of MM4 (OVA + MM4) or AS4 (OVA + AS4) AS-ODN followed by OVA challenge. AHR to LTD₄ was measured 15 hr after challenge by determining the $EC_{200}LTD_4$ to double the baseline R_L value. Data represent the distribution of individual $EC_{200}LTD_4$ values for each group along with group mean values (horizontal lines) for two separate experiments.

rats. MM4 pretreatment had no effect on the EC₂₀₀LTD₄ whereas pretreatment with AS4 significantly increased the LTD₄ effective dose required to double the resistance baseline value (438 ± 86.4 ng/ml for AS4 and 113 ± 31.7 ng/ml in rats pretreated with MM4, $p < 0.01$). Pretreatment of rats with lower doses of AS4 (50 or 100 μ g) had no significant effect on LTD₄ responsiveness (data not shown). Collectively, these results indicate that downregulation of CCR3 mRNA by AS4 reduced allergen-induced lung eosinophilia and reduced AHR.

DISCUSSION

In humans, asthma and several other inflammatory or allergic diseases are associated with the aberrant accumulation of eosinophils and other inflammatory cells (Walsh, 1997; Mohri et al., 1998). Key to this reaction is the dendritic cell, a professional antigen-presenting cell found in the airway epithelium and in peripheral lymphoid tissue. The interaction of T cells with dendritic cells leads to the activation of CD4⁺ cells and to a lesser extent CD8⁺ cells. This interaction is usually associated with proliferation and differentiation of cells that synthesize and secrete IL-4, IL-5, IL-6, IL-10, and IL-13, the profile reminiscent of Th2-type CD4⁺ cells that act on various cells including B cells, eosinophils, macrophages, and epithelial cells. Such response mediates IgE synthesis and an eosinophilic inflammation through a coordinated action of several chemoattractant molecules. However, there is an important influence of species and strain of animal models used to induce a Th2-type response (Martin et al., 2004). The present study used BN rats because these animals exhibit inflammatory responses after antigen challenge that are similar to human allergic asthma, including high levels of specific IgE after sensitization with OVA, a high prevalence of both early and late airway responses, pulmonary eosinophilia, high IL-4 and IL-5 expression, low interferon (IFN)- γ secretion, and increased airway responsiveness (Eidelman et al., 1998; Renzi et al., 1993, 1996; Sapienza et al., 1991; Allakhverdi et al., 2000).

In rodents and in humans, tissue eosinophilia has been related to the "overexpression" of CCR3 and its ligands eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 (Humbert et al., 1997; Ying et al., 1999). Data from the present study support this observation. Indeed, we have shown that at the time of peak eosinophil influx into the airways after OVA challenge, CCR3 mRNA expression levels were increased when compared with levels observed in the lungs of unchallenged rats.

Eotaxin has received considerable attention because of its role in causing selective eosinophil recruitment to the site of allergic inflammation and also eosinophil differentiation from progenitors (Lamkhioed et al., 1997,

2003; Palframan et al., 1998). Eotaxin has been identified in BAL fluid collected from sensitized guinea-pigs post antigen challenge (Griffiths-Johnson et al., 1993). The demonstration of eotaxin-induced recruitment of eosinophils led to the suggestion that downregulation of eotaxin gene expression would prevent eosinophil recruitment and reduce the changes associated with allergic disease. However, eosinophil recruitment has been demonstrated in eotaxin deficient mice (Yang et al., 1998), indicating that eotaxin is not the only mediator for recruiting eosinophils. Eotaxin deficient mice develop both eosinophilia and AHR after allergen challenge (Tomkinson et al., 2001), and experiments performed in sensitized mice showed that antibodies against eotaxin reduce antigen-induced AHR by only 50% (Gonzalo et al., 1998). These findings suggest that eotaxin accounts for only part of the chemotactic activity of asthmatic BAL fluid towards eosinophils. The other mediators acting through CCR3 may play a role in the recruitment of eosinophils in response to antigen challenge supporting our approach that blocking the receptor may prove to be more effective than blocking a single ligand. Results from our *in vivo* experiments showed that AS4 reduced the percentage of eosinophils in BAL fluid more than that reported for eotaxin deficient mice (Yang et al., 1998), again, supporting our hypothesis. Moreover, our data are in accordance with a recent study showing that CCR3-deficient mice had a profound decrease in OVA-induced BAL eosinophilia (Pope et al., 2005). In addition, our results demonstrate that this effect is unlikely to be due to AS4 affecting eotaxin protein levels as we found that AS4 did not affect eotaxin concentration in BAL fluid or in blood. Administration of a monoclonal antibody (mAb) targeting CCR3 has been demonstrated to partially decrease eosinophilia (Grimaldi et al., 1999), but multiple doses of mAb were required for this effect and no effects on airway responsiveness were reported. In another study several concurrent administrations (systemic/local) of a rat anti-mouse CCR3 antibody were required to deplete eosinophils from the lungs of OVA-sensitized/challenged mice (Justice et al., 2003). Results from the present study demonstrate that a single topical administration of AS4 was sufficient to significantly reduce pulmonary eosinophilia following antigen challenge. Differences in the percent decrease in pulmonary eosinophilia found in the present study compared to others may be due to different animal models employed or mechanism involved (AS-ODN-mediated inhibition of CCR3 mRNA expression versus eotaxin deficiency or mAb-mediated inhibition of CCR3).

OVA challenge in sensitized rats has been reported to increase the number of eosinophils in the blood and bone marrow, but this phenomenon may take at least 24 hours (Banner et al., 1996). Results from the present study indicate that the effects of AS4 were present as early as 15

hours after antigen challenge (Fig. 1). No significant differences were found in the number of eosinophils in the blood or bone marrow between control and AS4 treated rats post antigen challenge. These results suggest that AS4 affects local recruitment of eosinophils that are already present within lung tissues or its capillaries. A study utilizing a rat lung explant model provides evidence to support this (Eidelman et al., 1996). Eosinophils were observed to appear within lung explants after *in vitro* OVA challenge, even though no blood circulation was present to convey eosinophils from their more common sites of origin in the bone marrow to the airways. In addition, eosinophil progenitors have been reported to differentiate within the airways after an appropriate stimulus (Cameron et al., 2000; Robinson et al., 1999). AS-ODNs delivered topically to the lungs may act locally on eosinophils that are marginated in lung vessels. Eosinophil margination within lung capillaries of the rabbit has been reported (Ermer et al., 1993). Therefore, it is possible that AS-ODNs targeting CCR3 inhibit the infiltration of sequestered eosinophils into the airways. However, whether the inhibition of eosinophil infiltration is only due to downregulation of CCR3 on eosinophils is not clear. Our immunostaining (CCR3 and MBP) results may provide clues to answer that question. Indeed, we observed eosinophils (positive for MBP) but negative for CCR3 in lung tissue of AS4 treated rats suggesting that we have been able to decrease CCR3 expression in these cells. However, our data also showed CCR3 immunostaining of what appears to be other cells types such as endothelial and epithelial cells. Although this study is preliminary and incomplete, it raises the question about the role of CCR3 and the effect of inhibiting CCR3 expression in cell types other than eosinophils.

A growing body of evidence links eosinophils and CCR3 to AHR (Ma et al., 2002). Therefore, we examined whether CCR3 inhibition affected the increased AHR to LTD₄ after antigen challenge. Our results suggest that CCR3 downregulation significantly decreased the AHR to LTD₄ that occurs after OVA challenge supporting the role of CCR3 and eosinophils for AHR.

AS-ODNs reduce target mRNA and protein levels both *in vitro* and *in vivo*, and many are being developed as therapeutic agents against various diseases (Crooke, 2004). However, some class related toxicity (phosphorothioate) has been described with parenteral delivery of AS-ODNs, which limits the therapeutic application of these agents. AS-ODNs, of the phosphorothioate class in particular, can bind with low affinity to a large number of proteins. This feature contributes to the organ distribution, accumulation and toxicity associated with systemic delivery of AS-ODNs. Thus, intratracheal administration of AS-ODNs, the method used in the present study, is one approach to limiting the systemic availability of AS-ODNs. Moreover, the fact that AS4 can block CCR3 in

sequestered eosinophils suggest that AS4 might be effective in a clinical setting where the treatment would be on established disease.

In summary, results from this study demonstrate that inhibition of CCR3 mRNA expression in the lungs can be achieved by topical delivery of an AS-ODN. Furthermore, inhibition of CCR3 modifies the inflammatory (eosinophilia) and physiological (AHR) responses to antigen challenge in BN rats. These data show the role of CCR3 for the modulation of the allergic response and that its inhibition by topical delivery of an AS-ODN could be a new promising therapeutic approach for the treatment of asthma.

ACKNOWLEDGMENT

This study was funded in part by CIHR grant MOP 53101.

REFERENCES

- ALLAKHVERDI, Z., LAMKHIOUED, B., OLIVENSTEIN, R., HAMID, Q., and RENZI, P.M. (2000). CD8 depletion-induced late airway response is characterized by eosinophilia, increased eotaxin, and decreased IFN- γ expression in rats. *Am. J. Respir. Crit. Care Med.* **162**, 1123–1131.
- ALLAKHVERDI, Z., ALLAM, M., and RENZI, P.M. (2002). Inhibition of antigen-induced eosinophilia and AHR by antisense oligonucleotides directed against the common β chain of IL-3, IL-5, GM-CSF receptors in a rat model of allergic asthma. *Am. J. Respir. Crit. Care Med.* **165**, 1015–1021.
- BAGGIOLINI, M., DEWALD, B., and MOSER, B. (1997). Human chemokines: An update. *Annu. Rev. Immunol.* **15**, 675–705.
- BANNER, K.H., PAUL, W., and PAGE, C.P. (1996). Ovalbumin challenge following immunization elicits recruitment of eosinophils but not bronchial hyperresponsiveness in guinea-pigs: Time course and relationship to eosinophil activation status. *Pulm. Pharmacol.* **9**, 179–187.
- BARNES, P.J. (2003). Cytokine-directed therapies for the treatment of chronic airway diseases. *Cytokine Growth Factor Rev.* **14**, 511–522.
- BERTRAND, C.P., and PONATH, P.D. (2000). CCR3 blockade as a new therapy for asthma. *Expert Opin. Investig. Drugs* **9**, 43–52.
- CAMERON, L., CHRISTODOULOPOULOS, P., LAVIGNE, F., NAKAMURA, Y., EIDELMAN, D., MCEUEN, A., WALLS, A., TAVERNIER, J. MINSHALL, E., MOQBEL, R., and HAMID, Q. (2000). Evidence for local eosinophil differentiation within allergic nasal mucosa: Inhibition with soluble IL-5 receptor. *J. Immunol.* **164**, 1538–1545.
- COMBADIÈRE, C., AHUJA, S.K., and MURPHY, P.M. (1995). Cloning and functional expression of a human eosinophil CC chemokine receptor. *J. Biol. Chem.* **270**, 16491–16494.
- CROOKE, S.T. (2004). Progress in antisense technology. *Annu. Rev. Med.* **55**, 61–95.

- DE MONCHY, J.G., KAUFFMAN, H.F., VENGE, P., KOETER, G.H., JANSEN, H.M., SLUITER, H.J., and DE VRIES, K. (1995). Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am. Rev. Respir. Dis.* **131**, 373–376.
- EIDELMAN, D.H., MINSHALL, E., DANDURAND, R.J., SCHOTMAN, E., SONG, Y.L., YASRUEL, Z., MOQBEL, R., and HAMID, Q. (1996). Evidence for major basic protein immunoreactivity and interleukin 5 gene activation during the late phase response in explanted airways. *Am. J. Respir. Cell Mol. Biol.* **15**, 582–589.
- EIDELMAN, D.H., BELLOFIORE, S., and MARTIN, J.G. (1998). Late airway responses to antigen challenge in sensitized inbred rats. *Am. Rev. Respir. Dis.* **137**, 1033–1037.
- ERIN, E.M., WILLIAMS, T.J., BARNES, P.J., and HANSEL, T.T. (2002). Eotaxin receptor (CCR3) antagonism in asthma and allergic disease. *Curr. Drug Targets Inflamm. Allergy* **1**, 201–214.
- ERMERT, L., SEEGER, W., and DUNCKER, H.R. (1993). Computer-assisted morphometry of the intracapillary leukocyte pool in the rabbit lung. *Cell Tissue Res.* **271**, 469–476.
- FUJISAWA, T., KATO, Y., NAGASE, H., ATSUTA, J., TERADA, A., IGUCHI, K., KAMIYA, H., MORITA, Y., KITAURA, M., KAWASAKI, H., YOSHIE, O., and HIRAI, K. (2000). Chemokines induce eosinophil degranulation through CCR-3. *J. Allergy Clin. Immunol.* **106**, 507–513.
- GAO, W.Y., HAN, F.S., STORM, C., EGAN, W., and CHENG, Y.C. (1992). Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: Implications for antisense technology. *Mol. Pharmacol.* **41**, 223–229.
- GARCIA-ZEPEDA, E.A., ROTHENBERG, M.E., OWNBEY, R.T., CELESTIN, J., LEDER, P., and LUSTER, A.D. (1996). Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat. Med.* **2**, 449–456.
- GLEICH, G.J. (1990). The eosinophil and bronchial asthma: Current understanding. *J. Allergy Clin. Immunol.* **85**, 422–436.
- GONZALO, J.A., LLOYD, C.M., WEN, D., ALBAR, J.P., WELLS, T.N., PROUDFOOT, A., MARTINEZ, A., DORF, M., BJERKE, T., COYLE, A.J., and GUTIERREZ-RAMOS, J.C. (1998). The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyper-responsiveness. *J. Exp. Med.* **188**, 157–167.
- GRIFFITHS-JOHNSON, D.A., COLLINS, P.D., ROSSI, A.G., JOSE, P.J., and WILLIAMS, T.J. (1993). The chemokine, eotaxin, activates guinea-pig eosinophils *in vitro* and causes their accumulation into the lung *in vivo*. *Biochem. Biophys. Res. Commun.* **197**, 1167–1172.
- GRIMALDI, J.C., YU, N.X., GRUNIG, G., SEYMOUR, B.W., COTTREZ, F., ROBINSON, D.S., HOSKEN, N., FERLIN, W.G., WU, X., SOTO, H., O'GARRA, A., HOWARD, M.C., and COFFMAN, R.L. (1999). Depletion of eosinophils in mice through the use of antibodies specific for C-C chemokine receptor 3 (CCR3). *J. Leukoc. Biol.* **65**, 846–853.
- HUMBERT, M., YING, S., CORRIGAN, C., MENZ, G., BARKANS, J., PFISTER, R., MENG, Q., VAN DAMME, J., OPDENAKKER, G., DURHAM, S.R., and KAY, A.B. (1997). Bronchial mucosal expression of the genes encoding chemokines RANTES and MCP-3 in symptomatic atopic and nonatopic asthmatics: Relationship to the eosinophil-active cytokines interleukin (IL)-5, granulocyte macrophage-colony-stimulating factor, and IL-3. *Am. J. Respir. Cell Mol. Biol.* **16**, 1–8.
- JOSE, P.J., GRIFFITHS-JOHNSON, D.A., COLLINS, P.D., WALSH, D.T., MOQBEL, R., TOTTY, N.F., TRUONG, O., HSUAN, J.J., and WILLIAMS, T.J. (1994). Eotaxin: A potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J. Exp. Med.* **179**, 881–887.
- JUSTICE, J.P., BORCHERS, M.T., CROSBY, J.R., HINES, E.M., SHEN, H.H., OCHKUR, S.I., MCGARRY, M.P., LEE, N.A., and LEE, J.J. (2003). Ablation of eosinophils leads to a reduction of allergen-induced pulmonary pathology. *Am. J. Physiol. Lung Cell Mol. Physiol.* **284**, L169–L178.
- KAMPEN, G.T., STAFFORD, S., ADACHI, T., JINQUAN, T., QUAN, S., GRANT, J.A., SKOV, P.S., POULSEN, L.K., and ALAM, R. (2000). Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood* **95**, 1911–1917.
- LAMKHILOU, B., RENZI, P.M., ABI-YOUNES, S., GARCIA-ZEPEDA, E.A., ALLAKHVERDI, Z., GHAFAR, O., ROTHENBERG, M.D., LUSTER, A.D., and HAMID, Q. (1997). Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J. Immunol.* **159**, 4593–4601.
- LAMKHILOU, B., SOUSSI GOUNNI, A., HAMID, Q., MANSOUR, N., DELESPESE, G., and RENZI, P.M. (2003). The CCR-3 receptor is involved in eosinophil differentiation and is upregulated by T_H2 cytokines in CD34⁺ progenitor cells. *J. Immunol.* **170**, 537–547.
- MA, W., BRYCE, P.J., HUMBLE, A.A., LAOUINI, D., YALCINDAG, A., ALENUS, H., FRIEND, D.S., OETTGEN, H.C., GERARD, C., and GEHA, R.S. (2002). CCR3 is essential for skin eosinophilia and airway hyper-responsiveness in a murine model of allergic skin inflammation. *J. Clin. Invest.* **109**, 621–628.
- MARTIN, G.J., SUZUKI, M., RAMOS-BARBON, D., and ISOGAI, S. (2004). T cell cytokines: Animal models. *Paediatr. Respir. Rev.* **5**, S47–S51.
- MOHRI, H., MOTOMURA, S., and OKUBO, T. (1998). Unusual leukocytosis with eosinophilia by an allergic disease. *Am. J. Hematol.* **57**, 90–91.
- OWEN, C. (2001). Chemokine receptors in airway disease: Which receptors to target? *Pulm. Pharmacol. Ther.* **14**, 193–202.
- PALFRAMAN, R.T., COLLINS, P.D., WILLIAMS, T.J., and RANKIN, S.M. (1998). Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow. *Blood* **91**, 2240–2248.
- PONATH, P.D., QIN, S., RINGLER, D.J., CLARK-LEWIS, I., WANG, J., KASSAM, N., SMITH, H., SHI, X., GONZALO, J.A., NEWMAN, W., GUTIERREZ-RAMOS, J.C., and MACKAY, C.R. (1996). Cloning of the human eosinophil chemoattractant, eotaxin: Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J. Clin. Invest.* **97**, 604–612.

- POPE, S.M., ZIMMERMANN, N., STRINGER, K.F., KAROW, K.L., and ROTHENBERG, M.E. (2005). The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *J. Immunol.* **175**, 5341–5350.
- RENZI, P.M., SAPIENZA, S., WASERMAN, S., DU, T., OLIVENSTEIN, R., WANG, N.S., and MARTIN, J.G. (1992). Effect of interleukin-2 on the airway response to antigen in the rat. *Am. Rev. Respir. Dis.* **146**, 163–169.
- RENZI, P.M., OLIVENSTEIN, R., and MARTIN, J.G. (1993). Inflammatory cell populations in the airways and parenchyma after antigen challenge in the rat. *Am. Rev. Respir. Dis.* **147**, 967–974.
- RENZI, P.M., AL ASSAAD, A.S., YANG, J., YASRUEL, Z., and HAMID, Q. (1996). Cytokine expression in the presence or absence of late airway responses after antigen challenge of sensitized rats. *Am. J. Respir. Cell Mol. Biol.* **15**, 367–373.
- ROBINSON, D.S., DAMIA, R., ZEIBECOGLOU, K., MOLET, S., NORTH, J., YAMADA, T., KAY, A.B., and HAMID, Q. (1999). CD34⁺/interleukin-5R α messenger RNA⁺ cells in the bronchial mucosa in asthma: Potential airway eosinophil progenitors. *Am. J. Respir. Cell Mol. Biol.* **23**, 9–13.
- ROTHENBERG, M.E., MACLEAN, J.A., PEARLMAN, E., LUSTER, A.D., and LEDER, P. (1997). Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J. Exp. Med.* **185**, 785–790.
- SALLUSTO, F., MACKAY, C.R., and LANZAVECCHIA, A. (2000). The role of chemokine receptors in primary, effector, and memory immune responses. *Annu. Rev. Immunol.* **18**, 593–620.
- SAPIENZA, S., DU, T., EIDELMAN, D.H., WANG, N.S., and MARTIN, J.G. (1991). Structural changes in the airways of sensitized Brown Norway rats after antigen challenge. *Am. Rev. Respir. Dis.* **144**, 423–427.
- SPRY, C.J. (1971). Mechanism of eosinophilia. VI. Eosinophil mobilization. *Cell Tissue Kinet.* **4**, 365–374.
- STEIN, C.A. (2001). The experimental use of antisense oligonucleotides: A guide for the perplexed. *J. Clin. Invest.* **108**, 641–644.
- TOMKINSON, A., DUEZ, C., CIESLEWICZ, G., and GELFAND, E.W. (2001). Eotaxin-1-deficient mice develop airway eosinophilia airway hyperresponsiveness. *Int. Arch. Allergy Immunol.* **126**, 119–125.
- WAGNER, R.W. (1994). Gene inhibition using antisense oligodeoxynucleotides. *Nature* **372**, 333–335.
- WALSH, G.M. (1997). Human eosinophils: Their accumulation, activation and fate. *Br. J. Haematol.* **97**, 701–709.
- YANG, M., HOGAN, S.P., MAHALINGAM, S., POPE, S.M., ZIMMERMANN, N., FULKERSON, P., DENT, L.A., YOUNG, I.G., MATTHAEI, K.I., ROTHENBERG, M.E., and FOSTER, P.S. (2003). Eotaxin-2 and IL-5 cooperate in the lung to regulate IL-13 production and airway eosinophilia and hyperreactivity. *J. Allergy Clin. Immunol.* **112**, 935–943.
- YANG, Y., LOY, J., RYSECK, R.P., CARRASCO, D., and BRAVO, R. (1998). Antigen-induced eosinophilic lung inflammation develops in mice deficient in chemokine eotaxin. *Blood* **92**, 3912–3923.
- YING, S., ROBINSON, D.S., MENG, Q., BARATA, L.T., McEUN, A.R., BUCKLEY, M.G., WALLS, A.F., ASKENASE, P.W., and KAY, A.B. (1999). C-C chemokines in allergen-induced late-phase cutaneous responses in atopic subjects: Association of eotaxin with early 6-hour eosinophils, and of eotaxin-2 and monocyte chemoattractant protein-4 with the later 24-hour tissue eosinophilia, and relationship to basophils and other C-C chemokines (monocyte chemoattractant protein-3 and RANTES). *J. Immunol.* **163**, 3976–3984.

Address reprint requests to:

Dr. Paolo Renzi

CHUM Research Center

Notre-Dame Hospital

2065 Alexandre de Sève, Room Z-8905

Montreal, Quebec, Canada, H2L 2W5

E-mail: renzip@earthlink.net

Received August 2, 2004; accepted in revised form February 9, 2006.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.