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MONOCYTE CHEMOTACTIC PROTEINS IN ALLERGEN-INDUCED RHINITIS

Pota Christodoulopoulos

Department of Pathology

McGill University, Montreal

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Abbreviations

APAAP	Alkaline phosphotase-anti-alkaline phosphotase
APC	Antigen presenting cell
CCR1-8	CC chemokine receptors 1-8
CXCR1-5	CXC chemokine receptors 1-5
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EPO	Eosinophil peroxidase
GM-CSF	Granulocyte-macrophage colony stimulating factor
ICAM-1	Intercellular adhesion molecule-1
ICC	Immunocytochemistry
IgE	Immunoglobulin E
IL-	Interleukin
ISH	*In situ* hybridization
LTC_4	Leukotriene C_4
LTD4	Leukotriene D_4
LTE4	Leukotriene E_4
MC_{TC}	Connective tissue mast cell
MC_T	Mucosal mast cell
MBP	Major basic protein
RANTES	Regulated upon activation, normal T cell expressed and secreted

TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4

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Abstract

Allergen-induced rhinitis is associated with the recruitment and activation of inflammatory cells, particularly eosinophils and CD4 + T cells into the nasal mucosa. Monocyte chemotactic proteins (MCPs) have been shown to induce chemotactic activity in these particular cell types under *in vitro* assay conditions. To assess the contribution of MCPs in the recruitment of inflammatory cells *in vivo,* we investigated the allergen-induced late response in subjects with allergic rhinitis. Using immunocytochemistry and *in situ* hybridization, we demonstrated a constitutive expression of MCP-1, -3 and -4, of which MCP-3 and -4 were significantly increased in the nasal mucosa following allergen provocation. This upregulation of MCP-3 and -4 immunoreactivity in response to allergen, was reduced in patients pretreated with topical corticosteroids. Colocalization experiments revealed that the majority of MCP-positive cells were macrophages. The results of this study suggest that allergen-induced rhinitis is associated with an increased expression of MCP-3 and -4, which may be closely related to the influx of inflammatory cells and may thus contribute to the pathogenesis of allergic rhinitis.

Résumé

La rhinite allergique est associée au recrutement et à l'activation de cellules inflammatoires (principalement éosinophiles et cellules T CD4+) dans la muqueuse nasale. *In vitro*, des protéines ont été décrites comme étant chémoattractantes pour les monocytes (MCPs). La contribution des MCPs dans le recrutement des cellules inflammatoires *in vivo* a été évaluée lors de la phase allergique tardive chez des sujets atteints de rhinite allergique. Nous avons démontré que les MCP-1, -3 et -4 s'expriment constitutivement dans la muqueuse nasale, toutefois l'expression des MCP-3 et -4 augmente significativement après provocation allergènique. Cette augmentation est moindre chez les sujets ayant reçu préalablement des corticostéroides locaux. La plupart des cellules positives pour les MCPs sont des macrophages. En conclusion, nos résultats suggèrent que la rhinite allergique est associée à une augmentation d'expression des MCP-3 et -4 qui, relativement à l'infiltrat de cellules inflammatoires, pourrait contribuer à la pathogenèse de la rhinite allergique.

1. Introduction

1.1. Allergic Rhinitis

Allergic rhinitis describes an inflammatory condition of the nasal mucosa chracterized by the anterior nasal symptoms of pruritus, sneeze, discharge and stuffiness. There may also be an associated loss of sense of smell and inability to taste. Although allergic rhinitis may have its onset at any age, the incidence of onset is greatest in children and at adolescence, with a decrease in incidence seen in advancing ages The medical history of the patient is fundamental to the diagnosis of allergic rhinitis. Important historical data include a family history of allergy and of personal atopy, age at onset of symptoms, medication use, and history of any nasal trauma (Noble 1995, Naclerio 1991, Mullins *et al.*, 1989).

Allergic rhinitis is frequently divided into two types, seasonal and perennial, based on the type of allergen the patient is exposed to, and the time of symptom onset and duration. With seasonal rhinitis, the symptoms are periodic occuring in temporal relationship to the presence of seasonal allergens in individuals who are appropriately sensitized. Pollens causing seasonal allergic rhinitis are tree pollen present in the springtime, grass pollen present in May through July, and weed pollen and mould spores which may produce symptoms in late summer and autumn. Perennial disease, which is present all year round, relates to the presence of a non-seasonal allergen. The allergens causing perennial rhinitis are frequently indoor aeroallergens, the most common being the house dust mite (*Dermatophagoides*) (Platts-Mills *et al.*, 1987, Noble 1995, Howarth & Holgate 1990).

Because allergic rhinitis is so common and the symptoms are variable, and often chronic, it is essential that the treatment is fast-acting, well tolerated, and above all, safe. Treatment of nasal allergies should be individualized, with therapeutic measures aimed at the underlying etiology (Howarth 1989).

1.1.2. Epidemiology of Allergic Rhinitis

Rhinitis is a very common disease yet little is known about its epidemiology. Allergic rhinitis is now recognized as a major cause of morbidity that significantly impairs function and quality of life (Bousquet et al. 1994). Moreover, it is now widely held that the pathophysiological mechanisms causing nasal allergy, contribute, or predispose many individuals, to the development of other airway diseases, including asthma. Allergic rhinitis may well be a factor in 24% of children with otis media with effusion, and perhaps 28% of cases of chronic sinusitis (Mygind & Dahl 1996). A large number of cross-sectional studies have demonstrated that rhinitis and asthma commonly occur together. Long-term epidemiological studies suggest that rhinitis frequently precedes asthma and that upper airway dysfunction may be a predictive factor for subsequent development of lower airway disease (Settipane 1986, Van Arsdel 1959, Settipane *et al.*, 1994). Nasal symptoms have been reported among 28% to 78% of patients with asthma, compared with approximately 20% of the general population (Blair *et al.*, 1977, Smith 1988, Pederson & Weeke 1983). Similarly, as many as 19% to 38% of patients with allergic rhinitis may have asthma, much more than 3 to 5% prevalence among the general population (Evans *et al.*, 1987).

Considerable evidence now suggests that early and appropriate intervention can improve the quality of life and productivity of patients with allergic rhinitis, and reduce the prevelance of airway complications. The goal of treatment has shifted from mere symptom alleviation to blocking the pathophysiologic mechanisms that cause chronic allergic inflammation. The earlier in a patients life that this can be accomplished, the better the anticipated consequences (Corren 1997).

1.2. Pathophysiology of rhinitis

Allergic rhinitis is an immunologically mediated disease initiated by an IgE-dependent reaction. Allergens, which generally enter the body through inhalation, interact with T and B lymphocytes to produce IgE antibodies, which attach to the surfaces of mast cells and basophils. Re-exposure to the same allergen on a mucosal surface, results in a coupling or cross-linking of the IgE molecule that leads to cellular degranulation and the release of inflammatory mediators, a process resulting in both an early- and a late- phase response (Naclerio 1991, Dvoracek *et al.*, 1984).

1.2.1. Early- phase response

Cross-linking of surface bound IgE, leads to mast degranulation, the critical initiating event of acute allergic symptoms. This explosive degranulation of mast cells induced by allergen, leads to the release of a complex cascade of mediators, which may have synergistic effects on resident cells in tissues. The mediators of the immediate-

phase response, such as, histamine, bradykinin, leukotrienes, and platelet-activating factor, generate the acute symptoms of itch, rhinorrhea, congestion, and sneezing of allergic rhinitis. Histamine is one of the most important mediators of the early phase allergic response in the nasal mucosa as its release stimulates sensory nerves leading to vasodilation and mucus hypersecretion (Kawabori *et al.*, 1995, Galli *et al.*, 1993). The release of leukotrienes and chymase also stimulates glandular exocytosis and mucus secretion. (Sommerhof et al., 1989, Holgate et al., 1996). The mediators of this early phase generate the acute symptoms, and as these mediators are metabolized and cleared from the mucosa, these symptoms wane. However, the release of cytokines and mediators, activates endothelial cells to express adhesion markers that bind circulating leukocytes, leading to a latent recruitment phase that is thought to usher in the inflammatory late-phase response (Sedgwick *et al.*, 1991, Butcher 1991).

1.2.2. Late Phase Response

The late phase response occurs 4 to 6 hours after the immediate phase. It is noted clinically by an increase in nasal mucosal thickness that can be detected as an increased nasal airflow resistance. During the LNR, inflammatory granulocytes, including eosinophils, basophils, and, less dramatically, neutrophils, are found within the mucosa. The numbers of mononuclear cells and metachromatic cells, such as mast cells, also increase. Once there, by interacting with additional stimuli, they release their own mediators. This perpetuates the inflammatory response and augments aspects of the immediate hypersensitivity reaction, such as mucosal congestion and mucus

secretion. The increase in eosinophils is reflected by large increases in eosinophil products in the nasal secretions, such as MBP. Mediators of the late-phase response include leukotrienes, histamine, and Th2 cytokines (Terada et al., 1994, Togias et al., 1988, Gosset *et al.*, 1993, Naclerio *et al.*, 1994). Histamine increases without a change in tryptase, suggesting the activation of basophils rather than a secondary degranulation of mast cells. In addition, the release of Th2 type cytokines from the leukocytes present, and from the epithelium is increased (Baraniuk 1997). It appears that the factors released from these inflammatory cells promote the expression of a combination of adhesion markers and the production of a combination of chemoattractants that promote further leukocyte infiltration, perpetuating and maintaining the inflammatory response.

1.3. The Role of Inflammatory Cells in Allergic Rhinitis

One of the hallmarks of allergic diseases is the intense accumulation of inflammatory cells in tissue locations at specific mucosal surfaces. The presence of an increased number of mast cells, basophils, T cells, macrophages, and particularly eosinophils, have been detected in nasal smears and biopsies from patients with allergic rhinitis. It has also been shown that in response to certain mediators, these inflammatory cells undergo local activation, releasing their own mediators, and thus contributing to the pathological features of this disease. These cellular changes exist to varying degrees, but have been demonstrated to occur in temporal relationship to seasonal allergen exposure. Mediator release from the primary effector cells such as

mast cells, basophls and eosinophils, can explain the symptom development in this disease through interactions with the structural elements within the nose (Howarth & Holgate1990).

1.3.1. Mast Cells

Mast cells are constitutive cells within the normal nasal mucosa and are the recognized key cells of type I hypersensitivity reactions. These cells can be subdivided into connective and mucosal phenotypes. Connective tissue mast cells (MC_{TC}) express chymase, tryptase and TNF-α (Bradding *et al.*, 1995). This cell population represents 85% of the IL-4-positive mast cells in the nasal lamina propria. During allergen exposure, there is an increase in the proportion of mast cells in the epithelial cell layer (Juluisson *et al.*, 1995). These cells produce predominantly tryptase, without chymase, and are called mucosal mast cells (MC_T). MC_Ts express IL-4, IL-5 and IL-6, and represent 15% of all of the IL-4-positive mast cells in the mucosa (Bradding *et al.*, 1995). These cells proliferate in allergic rhinitis, perhaps under the influence of Th2 cytokines (Kawabori *et al.*, 1995). Proliferation of MC_Ts appears to occur in the epithelium and most superficial layers of the lamina propria. Epithelial mast cells have a higher rate of cell division in allergic rhinitis compared with nasal mucosa taken from non-atopic individuals. Both phenotypes, MC_T and MC_{TC}, express FcεRI and may therefore participate fully in IgE-dependent allergic reactions (Church & Levi-Schaffer 1997). Cross-linking of IgE on the surface of mast cells by allergen leads to a series of intracellular events culminating in the release of preformed mediators from mast cells including histamine, tryptase, and heparin, and the generation of lipid mediators,

including PGD_2 and the sulphidopeptide leukotriene, LTC_4, and its metabolites LTD_4 and LTE_4 (Howarth & Holgate 1990). These released mediators induce nasal symptoms of itch, sneeze, discharge and blockage, through interaction with receptors present in both neural and vascular elements within the nasal mucosa with histamine being prominent in this respect (Mygind 1993).

In addition to the effects of acute mast cell degranulation on immediate symptom generation, mast cell degranulation will contribute to the eosinophilic mucosal inflammation which is evident in rhinitis. Mast cells within the nasal mucosa have been demonstrated to contain preformed cytokines, in particular IL-4, IL-5, IL-6, IL-13 and TNF-a (Bradding 1993a, 1995). Both IL-5 and TNF-α have actions relevant to eosinophil activation and recruitment (Howarth *et al.*, 1994). IL-4 potentiate the effects of TNF-α on the expression of VCAM-1 on the vascular endothelium which leads to tissue eosinophil recruitment through its interaction with eosinophil ligand VLA-4 (Thornhill & Haskard 1990, Montefort. *et al.*, 1993). In addition, IL-4 and IL-13 are involved in switching the B lymphocyte to IgE production. The ability of every sensitized mast cell to respond to stimulation with any individual allergen, as opposed to T cells, in which only a small percentage of cells specific to that allergen respond, makes mast cells potentially important cytokine-generating cells in allergy. Furthermore, the presence of preformed cytokines within mast cells, which is not the case with T cells, suggests that they are available for rapid secretion on cell stimulation (Church & Levi-Schaffer 1997).

1.3.2. Eosinophils

Eosinophils are bone marrow-derived granulocytes that are not normally prominent in either the peripheral blood or the tissues. (Weller 1997). Immunohistochemical staining of nasal mucosa biopsies has shown that eosinophils are evident. within the submucosa and epithelium, in symptomatic rhinitis (Bentley *et al.*, 1992, Bradding *et al.*, 1993a,b). The steps that lead to eosinophil recruitment into certain sites of inflammation are multiple, and require a combination of factors including enhanced expression and/or function of integrins on intravascular eosinophils and their counterligands on the vascular endothelium, as well as the actions of eosinophil chemoattractant factors (Weller *et al.*, 1996).

The influx of eosinophils in atopic allergic disease are believed to effect tissue damage through the release of their mediators (Bousquet *et al.*, 1990). The armamentarium of eosinophilic granules consists of a number of very toxic and potent mediators including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil derived neurotoxin (EDN) (Peters *et al.*, 1986, Bainton *et al.*, 1970). At the site of inflammation, these proteins can cause cellular disaggregation and epithelial desquamation. Eosinophil activation is also associated with the *de novo* generation of arachidonic acid products such as LTC_4, which contribute to nasal obstruction and to rhinorrhoea through their potent smooth muscle contractile, vasoactive, and mucous secretory activities (Volovitz, 1988).

In addition, it has been shown that human eosinophils express and synthesize a number of cytokines, including GM-CSF, IL-6, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-8,

MIP-1α, RANTES, and TNF-α (Moqbel *et al.*, 1991, Hamid *et al.*, 1992, Weller *et al.*, 1993, Kita *et al.*, 1991, Desreumaux *et al.*, 1993, Braun *et al.*, 1993, Costa *et al.*, 1993). Since IL-3, IL-5 and GM-CSF influence eosinophil differentiation, activation, and survival, many of these functions may be regulated by eosinophils at least partly in an autocrine fashion (Lopez *et al.*, 1992).

It is abundantly clear that eosinophils are major participants in the immunopathogenesis of allergic inflammation as they are characteristically recruited to such sites, releasing their cationic proteins and lipid mediators, and thus contributing to damage and dysfunction of other resident cell types.

1.3.3. T Lymphocytes

As key cells in the adaptive immune response, T lymphocytes have evolved to coordinate and amplify the effector functions of antigen specific and non-specific inflammatory cells such as B cells and eosinophils. T lymphocytes have been divided into two distinct subgroups based on their effector functions. CD4+ T cells represent the T helper cells, which are important in the regulation of antigen-driven inflammatory processes. Via antigen-specific T cell receptors, CD4 T cells are capable of recognizing processed foreign antigen in association with MHC class II on specialized antigen-presenting cells. On the other hand, CD8+ T cells which represent T suppressor cells, drive the cell-mediated response and respond to APC presenting antigen in conjunction with MHC class II molecule. The T lymphocyte represents a significant non-structural cell within the nasal mucosa. An increase in this cell

population has been described in nasal biopsies specimens in rhinitic patients. These are generally $CD4^+$ T cells displaying the activated phenotype ($CD25^+$) (Calderon *et al.*, 1994, Varney *et al.*, 1992). $CD4^+$ T lymphocytes have been shown to play a crucial role in the induction and maintenance of chronic allergic inflammation.

The presence of T lymphocytes in allergic inflammation has been well demonstrated, however, the major reason for their importance lies on the cytokine profile which they express upon activation. Although individual T cells have the capacity to produce a wide range of cytokines, restricted cytokine profiles exist in chronic inflammatory diseases. This may occur as a result of the microenvironment favoring the development of specific T cell populations at the time of induction. Cross-regulation of Th1 and Th2 cytokines occurs and in certain cases, this may lead to the polarization of the cytokine milieu towards Th1 or Th2 (Kelso 1995). A major feature of allergic diseases is the high expression of Th2-type cytokines. T lymphocytes of the T-helper 2 (Th2) subpopulation can generate IL-3, IL-4, IL-5, GM-CSF and TNF-a (Mossman & Coffman 1989). Following nasal allergen challenge, an increase in IL-4, IL-5 and GM-CSF mRNA-positive cells has been described in association with a mucosal eosinophilia (Durham *et al.*, 1992), and T cell clones derived from nasal mucosa challenged with allergen *in vivo* have a cytokine profile comparable to that of a Th2-like population (Del-Prete 1994). The Th2 phenotype is thought to influence subsequent T cell activation and IgE production by B cells in addition to promoting the attraction, activation, growth, and differentiation of specific leukocytes such as eosinophils. In this way, activated T cells can initiate and propagate allergic

inflammation and participate directly in the events responsible for allergic diseases. Steroids have been shown to improve the symptoms of allergic rhinitics by reducing the expression of Th_2 cytokines (Bentley *et al.*, 1996, Masuyama *et al.*, 1994), therefore their success in suppressing inflammation may be partly attributed to their activity in limiting Th_2 lymphocyte activation.

1.3.4. Macrophages

Macrophages are widely distributed in virtually all tissues, and their activation is a prominent feature at sites of inflammation, in particular, granuloma formation. Macrophages play an important role in the immune response as immunomodulators, APC, and effector cells. Once blood monocytes leave the circulation and enter the extravascular tissues, they differentiate into macrophages. As macrophages mature from the monocyte through to the resident mature tissue macrophage stage, they go through a series of functional changes which affect their interaction with T cells. Macrophages can also act as APCs. They take up antigen via non-specific receptors or as immune complexes, process it, and return fragments to the cell surface in association with class II molecules. It has been shown that the number of macrophages on the epithelial surface is increased 4-8 hours after allergen challenge, suggesting that macrophages may play a role in the allergic nasal late phase reaction (Bachert 1991). Macrophages are also an important source of cytokines that can manipulate the emerging T cell response. Macrophage products such as IL-1, IL-6, and TNF-α have been implicated in many inflammatory and immunologic responses elicited during

tissue injury. Th2 type cytokines, in particular IL-13, IL-4 and IL-10, may decrease the ability of activated macrophages to participate in inflammatory responses, and by inhibiting the ability of macrophages to produce IL-12, may thereby antagonize the development of Th1 cells, which can augment inflammation, (Doherty *et al.*, 1993).

1.3.5. Basophils

Basophils are only present in very low numbers in peripheral blood, and are not found in normal non-inflamed tissues, indicating they are recruited to sites of inflammation by mediators from other cell types. Basophils are evident in nasal smear samples in allergic rhinitis (Okuda *et al.*, 1985, Otsuka *et al.*, 1985) and can be demonstrated to increase in rhinitic patients following nasal allergen challenge (Bascom *et al.*, 1988). Eventhough there are no specific surface markers for its precise identification in biological fluids and tissue, evidence of basophil infiltration into the nasal mucosa during allergen challenge, is based on the mediator profile in nasal secretions (Naclerio *et al.*, 1985, Bascom *et al.*, 1988). Basophils were initially thought to function in a similar fashion to mast cells, however it is now apparent that these two cell types differ significantly from each other. Basophils, like mast cells, possess high affinity IgE receptors, are also derived from CD34-positive progenitor cells in the bone marrow (Knapp 1990). However, unlike mast cell precursors which require stem cell factor to mature in tissue, basophils are thought to be released from

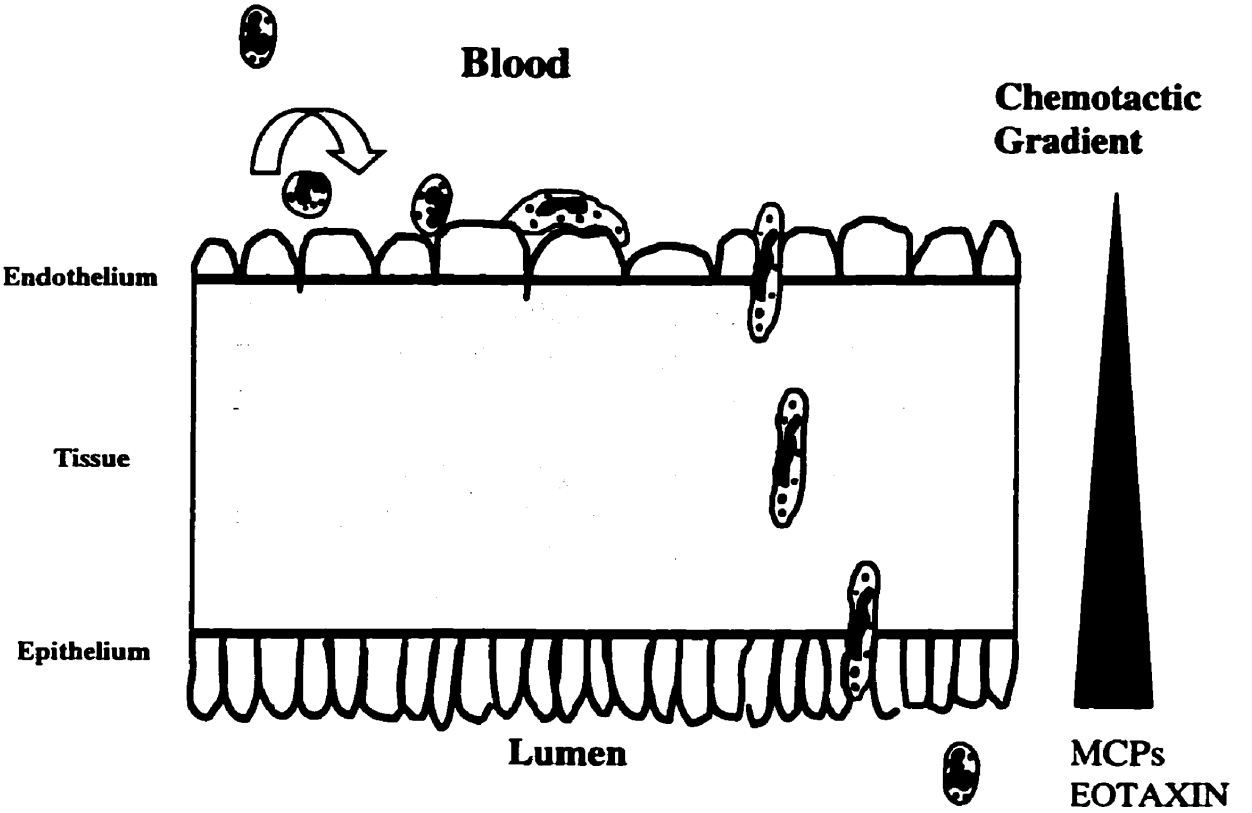
bone marrow as mature cells, with their development being dependent on the effects of IL-3 and GM-CSF as seen with eosinophils. When activated, basophils are prominent sources of inflammatory mediators found in allergic late-phase reactions, such as histamine and LTC_4. Basophils possess fewer, larger granules and differ from the mast cell in that they contain less histamine. Following IgE-dependent activation, the basophil only releases 20-30% of the histamine released from a comparable number of mast cells (Cantells *et al.*, 1987). Human basophils have also been shown to secrete cytokines, particularly IL-4 and IL-13, when activated by IgE-dependent stimuli, modulating their response and the immune responses of other cell types, that participate in allergic rhinitis (Mac Glashan et al., 1994, Schroeder *et al.*, 1994, Schroeder *et al.*, 1996). Thus, basophil-derived cytokines could potentially influence the involvement of eosinophils and lymphocytes in allergic responses by upregulating adhesion molecules, such as vascular cell adhesion molecules, and by promoting the development of Th2-type cells, both of which are processess that constitute the hallmark of allergic disease.

1.4. Leukocyte Migration

Leukocyte extravasation from the blood into the tissues is a regulated multistep process involving a series of coordinated interactions between leukocytes and endothelial cells (Butcher 1991, Springer 1994). Several families of molecular regulators, such as selectins, integrins, and chemokines, are thought to control

different aspects of this process. Initially, circulating leukocytes undergo margination, whereby they move from the centre to the periphery of the blood vessel and begin to bind reversibly to the endothelium, a process referred to as rolling. This process is mediated by the interaction of L-selectin on the leukocytes with diverse, carbohydrate containing structures such as P-selectin or E-selectin on the endothelial cells (Varki 1994). This initial weak binding may be followed, due to activation of the leukocytes by chemoattractants or binding to E-selectin (Kuijpers *et al.*, 1991), by the induction of firm adhesion. This second step is mediated by leukocyte integrins interacting with endothelial cell adhesion molecules, such as ICAM-1 and VCAM-1. (Rice *et al.*, 1990, Bochner *et al.*, 1991, Lawrence & Springer 1991). Once a leukocyte has become firmly attached to the endothelium, it must be guided to move across the endothelium to reach the site of injury. Chemokines are thought to provide the signals that convert the low-affinity, selectin-mediated interaction into the higher affinity, integrin mediated interaction that leads to extravasation of leukocytes (Luster *et al.*, 1998). In chemotaxis, cells move in the direction of increasing concentration of a chemoattractant, which typically is a soluble molecule that can diffuse away from the site of its production, where its concentration is highest (Devreotes & Zigmond 1988). Many molecular regulators have been shown to play a role in leukocyte trafficking, however, recent studies have brought our attention to the importance of chemokine involvement in this event (refer to schema on following page).

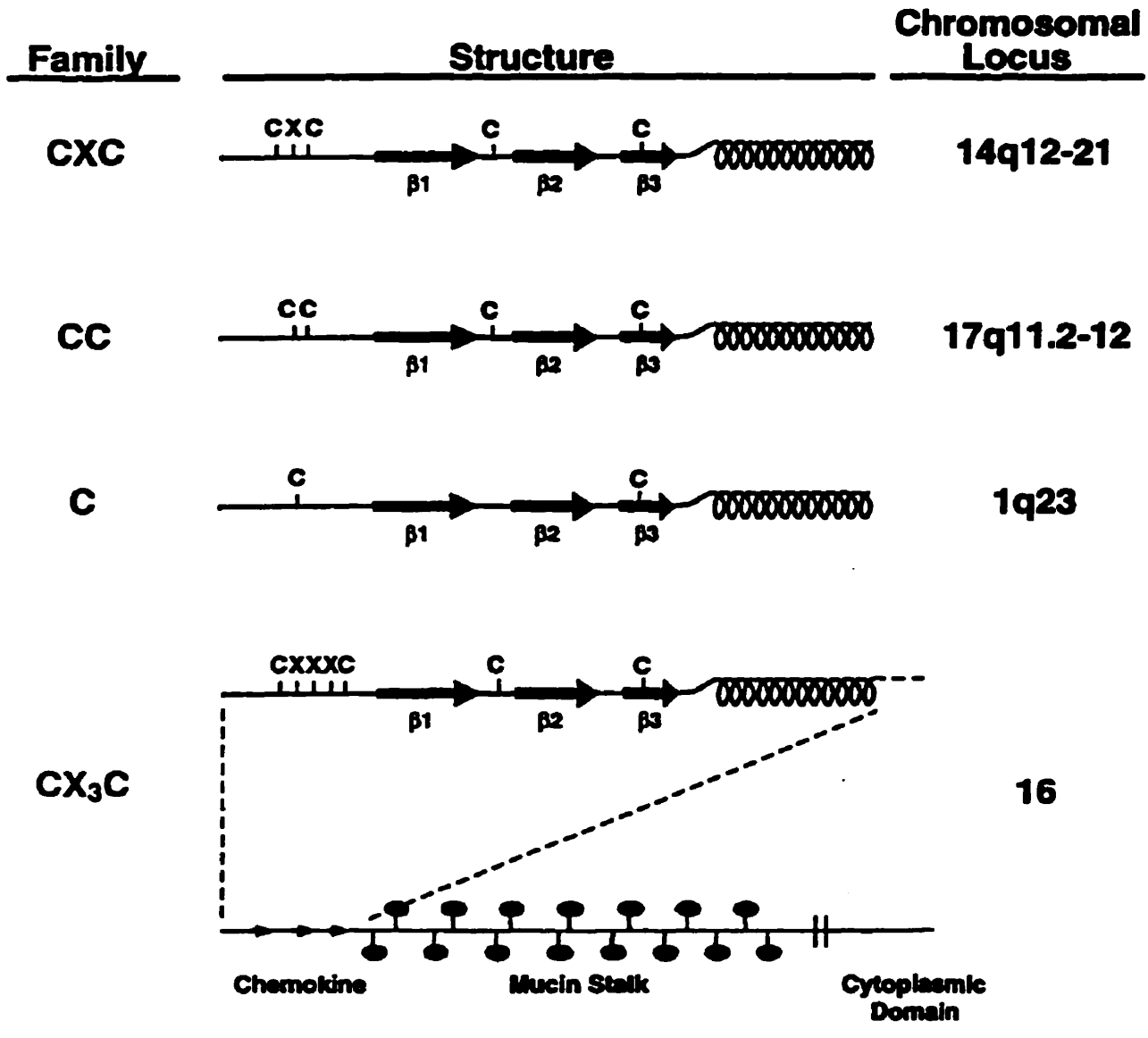
Inflammatory Cell Extravasation



Representative example of inflammatory cell extravasation. Extravasation of leukocytes during inflammation involves multiple sequential steps including (i) rolling of leukocytes along the vessel wall, which is mediated by the selectin family of adhesion molecules, (ii) tight binding of leukocytes to the endothelium resulting from activation of integrin adhesion molecules, and (iii) migration of leukocytes through the endothelial junctions and underlying tissues in response to gradients of chemotactic agents generated at the site of inflammation.

1.5. Chemokines

The discovery of IL-8, in 1987, revealed the existence of a novel class of small chemoattractant cytokines, now called chemokines, that are seen as the stimuli that largely control leukocyte migration (Baggiolini *et al.*, 1989, Baggiolini & Dahinden 1994). While the pathologists of last century knew that leukocytes emigrated from the blood across the wall of microvessels,. the purpose of this migration remained unknown. This remained so until Elias Metchnikoff showed that leukocytes engulf and kill bacteria and recognized diapedesis as a fundamental mechanism of host defence (Metchnikoff 1901). Today, eleven years after the discovery of the first chemokine, as many as 40 to 50 human chemokines have been identified. Chemokines constitute a superfamily of small, inducible, secreted, proinflammatory cytokines that regulate diverse properties of specific leukocytes, including chemoattraction, cellular regulation and cell growth. These small proteins have four conserved cysteines forming two essential disulphide bonds (Cys1-Cys3 and Cys2-Cys4), and have thus been classified depending upon the spacing of their conserved cysteine residues. The CXC (α) chemokines have one amino acid residue separating the the two first conserved cysteine residues. In the CC (β) chemokines, the first two cysteine residues are adjacent to each other. The C (γ) chemokine, lymphotactin, has two instead of four conserved cysteines in the mature protein, and lacks one cysteine near the N terminus. CX_3C (δ) chemokines have three intervening amino acid residues between the first two cysteine residues at the N-terminal end of a mucin structure (Clark-Lewis 1995, Loetscher et al., 1996, Kennedy *et al.*, 1995, Bazan *et al.*, 1997, Pan *et al.*, 1997).



The four chemokine subfamilies subdivided based on the relative position of the cysteine residues found in the mature protein.

The α and β chemokines are the two major subfamilies that have been extensively characterized. The α chemokines are subdivided into two groups. Those containing the sequence glutamic acid-leucine-arginine near the N terminal (preceding the CXC sequence) and those that do not. Those α chemokines containing this sequence are chemotactic for neutrophils, whereas those not containing the sequence act on lymphocytes. The β chemokines, in general, attract monocytes, eosinophils, basophils, and lymphocytes with variable selectivity. Structurally the β chemokines can be subdivided into two families, the monocyte-chemoattractant-protein (MCP)-eotaxin family, containing the monocyte chemoattractant proteins and eotaxin, which are approximately 65 percent identical to each other, and all other β chemokines (Proost 1996, Taub 1995). As with the α chemokines, the N-terminal acids preceding the conserved cysteine residues of β-chemokines are critical components of the biological activity and leukocyte selectivity of these chemokines (Gong *et al.*, 1995).

Activation of leukocytes leads to shape changes which are observed within seconds after the addition of a chemoattractant to a leukocyte suspension. Polymerization and breakdown of actin filaments leads to the formation and retraction of lamellipodia, which function like arms and legs of the migrating cells. Stimulation also induces the upregulation and activation of integrins, which enable the leukocytes to adhere to the endothelial cells of the vessel wall before migrating into the tissues (Springer *et al.*, 1994). Several other rapid and transient responses are characteristic of the activation of leukocytes by chemokines, such as the rise in the intracellular free calcium concentration, the production of microbicidal oxygen radicals and bioactive

lipids, and the release of the contents of the cytoplasmic storage granules, such as proteases from neutrophils and monocytes, histamine from basophils and cytotoxic proteins from eosinophils (Baggiolini & Dahinden 1994, Baggiolini & Moser 1997).

Most chemokines are produced under pathological conditions by tissue cells and infiltrating leukocytes (Baggiolini & Dahinden 1994, Furie & Randolph 1995). Some chemokines on the other hand, seem to fulfill housekeeping functions. They may be involved in leukocyte maturation in the bone marrow, the traffic and homing of lymphocytes, and the mechanisms that ensure the renewal of circulating leukocytes (Baggiolini *et al.*, 1998). In addition, chemokines may play a broad role in cellular physiology. They have been shown to be involved in diverse processes such as hematopoiesis, angiogenesis, and inhibition of HIV proliferation (Broxmeyer et al., 1993, Strieter *et al.*, 1995, Cocchi *et al.*, 1995).

Chemokine	Target cells
CC chemokines	
MCP-1	monocytes, T cells, basophils
MCP-2	monocytes, T cells, eosinophils, basophils,
MCP-3	monocytes, T cells, eosinophils, basophils, NK cells
MCP-4	monocytes, T cells, eosinophils
RANTES	T cells, eosinophils, basophils, NK cells,
MIP-1α	monocytes, T cells, NK cells, basophils, eosinophils
MIP-1β	monocytes, T cells, dendritic cells,
Eotaxin	eosinophils
1309	monocytes
HCC-1	monocytes, hematopoietic progenitors
TARC	T cells
CXC chemokines	
IL-8	neutrophils, T cells, basophils,
IP-10	NK cells, memory T cells
MIG	T cells
NAP-2	neutrophils, basophils
ENA-78	neutrophils
GRO-α	endothelial cells
GRO-β	neutrophils, endothelial cells
GRO-γ	neutrophils, endothelial cells
C chemokines	
Lymphotactin	T cells
CX_3C chemokines	
Fractalkine	monocytes, lymphocytes

1.5.1. Chemokine Receptors

Chemokines induce cell migration and activation via seven transmembrane domain receptors (7TM) which form a distinct group of structurally related proteins within the superfamily of receptors that signal through heterotrimeric GTP-binding proteins (Baggiolini & Dahinden 1994, Murphy 1994). Eight receptors for CC chemokines (CCR1 through CCR8) and five receptors for CXC chemokines (CXCR1 through CXCR5) have been characterized (Murphy 1996). Most receptors recognize more than one chemokine, and several chemokines bind to more than one receptor, indicating that redundancy and versatility are characteristic for the chemokine system. In addition, chemokine receptors are constitutively expressed in some cells, whereas they are inducible in others. For example, CCR1 and CCR2 are constitutively expressed on monocytes but are expressed on lymphocytes only after stimulation by IL-2 (Loetscher et al., 1996). Chemokines have two main sites of interaction with their receptors, one in the N-terminal region and the other within an exposed loop of the backbone that extends between the second and the third cysteine residue. The N-terminal binding site is essential for triggering of the receptor. The receptor recognizes the loop region first, and this interaction is necessary for the correct presentation of the triggering domain (Clark-Lewis 1995).

RECEPTOR	LIGANDS
CXC Receptors	
CXCR1	IL-8
CXCR2	IL-8, GRO-α, GRO-β, GRO-γ, NAP-2
CXCR3	IP-10, MIG
CXCR4	SDF-1α
CC Receptors	
CCR1	MIP-1a, MIP-1b, RANTES, MCP-3
CCR2	MCP-1, MCP-3, MCP-4
CCR3	Eotaxin, RANTES, MCP-2, MCP-3, MCP-4
CCR4	MIP-1α, RANTES, MCP-1, TARC
CCR5	MIP-1α, MIP-1β, RANTES
CCR6	MIP-3α/LARC
CCR7	MIP-3β/ELC

1.5.2. Chemokines in Allergic Inflammation

CC chemokines attract and activate eosinophils, basophils, and lymphocytes. Because these cells are the hallmark of allergic inflammation, and CC chemokines act on these particular cells, it is believed that CC chemokines may play a role in the pathogenesis of allergic disease. The observation that RANTES and MCP-3 activate eosinophil and basophil leukocytes, inducing chemotaxis and the release of histamine and leukotrienes, was the first hint that chemokines are involved in allergy (Baggiolini & Dahinden 1994). Then eotaxin was discovered and it was shown to be a potent eosinophil chemoattractant. Eotaxin was initially described as the predominant and selective eosinophil chemoattractant in the bronchoalveolar lavage fluid from allergen-challenged guinea pigs (Griffiths-Johnson *et al.*, 1993, Jose *et al.*, 1994). It was later shown that, eotaxin is increased in tissue and BAL fluid of atopic asthmatics when compared with normal controls (Lamkhioued *et al.*, 1997). Recently, it was shown that allergen challenge of the nasal mucosa in rhinitics results in the a local upregulation of eotaxin expression (Minshall *et al.*, 1997). In addition MCP-1. –2, -3 and –4, also part of the CC chemokine subfamily, have been implicated in allergic diseases based mainly on in vitro findings, however their actual role in human allergic diseases remains to be further clarified.

1.6. Monocyte Chemoattractant Proteins

Four human MCP proteins (-1, -2, -3, -4) have been identified to date. MCPs share many structural and functional features forming a distinct subgroup within the CC chemokine family. MCPs have diverse functions, including the regulation of leukocyte chemoattraction, cellular activation, including histamine release from basophils, and the regulation of the homeostatic growth and tissue level of immune cells involved in allergy.

MCP-1 was the first CC chemokine to be characterized biologically MCP-1 was first purified from conditioned media of baboon aortic smooth muscle cells in culture on the basis of its ability to attract monocytes, but not neutrophils, in vitro (Valente *et al.*, 1988, Baggiolini & Dahinden 1994). Subsequently, MCP-2 and MCP-3 were identified. They were isolated as novel monocyte chemoattractants from the conditionned medium of MG-63 osteosarcoma cells (Van Damme *et al.*, 1992). One of the newest members, MCP-4 was described only recently. MCP-4 was identified from human fetal RNA, cloned into a baculovirus vector, and expressed in Sf9 insect cells. (Uguccioni *et al.*, 1996, Van Damme *et al.*, 1992). These chemokines are strikingly related in terms of chromosomal location, gene structure, primary protein sequence, biological activity, and receptor usage. MCPs have emerged as chemokines involved in the recruitment and acitivation of cells seen in allergy, and proposed to play a fundamental role in the development of such responses.

1.6.1. Structure and Function

The MCPs constitute a subfamily of CC chemokines that share structural and functional feature sharing approximately 65% amino acid identity (Proost *et al.*, 1996, Garcia-Zepeda *et al.*, 1996). The primary structure of the chemokines can, in general, predict function. MCPs are inactive on neutrophils and instead chemoattract monocytes, eosinophils, basophils, and lymphocytes with variable selectivity. Human MCP-1, -2, -3 and -4 have a remarkably similar intron-exon structure each gene is organized into three exons and two introns. In addition, the positions of the splice sites within the codons is conserved, suggesting that these genes arose from a common ancestral gene. In support of a recent gene duplication, the MCPs were found to be situated on the long arm of chromosome 17 (17q11.2). The MCP cDNAs encode typical secreted proteins with signal peptides that are, surprisingly, more conserved than the mature proteins. After signal peptide cleavage, the mature proteins are ~8.6 kDa. Although they do not contain potential N-linked glycosylation sites, the MCP proteins probably contain O-linked carbohydrates and sialic residues (Coillie *et al.*, 1997, Naruse *et al.*, 1996).

The structure of the mature amino-terminal acid is thought to be important for the biological activity and leukocyte selectivity of the MCP proteins. For example, the addition of a single amino acid residue before the N- terminal glutamine reduces the biological activity of MCP-1 on monocytes by 100-to 1000-fold. Furthermore, deletion of this amino terminal glutamine residue converts MCP-1 from an activator of basophils to an eosinophil chemoattractant. However if the amino terminal glutamine

of MCP-1 is replaced by an other small amino acid, such as asparagine or alanine, it still retains activity on monocytes. During the process of of generating biologically active recombinant MCP-4 in *E.coli,* it was also found that an amino-terminal extension completely inactivated these molecules.

1.6.2. Regulation and Cellular Sources

The MCPs are produced by numerous cell types, including fibroblasts, endothelial cells, and mononuclear leukocytes, in response to pro-inflammatory stimuli such as cytokines, lipopolysaccharide, or infectious agents. The main stimuli however, for secretion of the MCPs appear to be early pro-inflammatory cytokines such as IL-1 and TNF-α (Proost *et al.,* 1996, Garcia-Zepeda *et al.,* 1996). In addition, products of both Th1 and Th2 cells, IFN-γ and IL-4, respectively, can induce the production of these chemokines and also synergize with IL-1 and TNF-α to induce their secretion. IL-1, TNF-α, and IFN-γ are potent endogenous inducers of MCP-1 in mononuclear cells, fibroblasts, endothelial and whereas other cytokines such as IL-6 generally have no stimulatory effect (Van Damme *et al.,* 1989, Wang *et al.,* 1991, Sica *et al.,* 1990). Recently, it has also been shown that human eosinophils represent an important source of MCP-1 (Lin *et al.,* 1998). In addition, it is well known that epithelial cells produce these chemokines in response to cytokine stimulation. Stellato et al. have shown that MCP-1 and MCP-4 expression can be induced in airway epithelial cells upon stimulation with either TNF-α or IFN-γ.

1.6.3. Receptors

There have been eight human CC chemokine receptor genes cloned to date, and these are referred to as CCR-1 through CCR-8. CC chemokine receptors all appear to exhibit overlapping specificities, albeit with variable affinity for particular chemokines. Moreover, several CC chemokines can bind more than one receptor. Thus each chemokine can recruit multiple types of cells even if they express different types of receptors, whereas as each cell may respond to multiple types of chemokines even by expressing a single type of receptor (Murphy 1994, Neote 1993, Gao *et al.*, 1993, Charo *et al.*, 1994, Power *et al.*, 1995). Monocytes express the CC chemokine receptors CCR-1, CCR-2, CCR-4, and CCR-5. Eosinophils express CCR-1 and CCR-3 and basophils express CCR-3 and CCR-4. The first receptors for CC chemokines, CCR1 and CCR2, were originally designated MIP-1α / RANTES and MCP-1 receptor, respectively based on the ligands they bound with high affinity. Subsequent studies demonstrated that CCR1 and CCR2 also recognize MCP-2, MCP-3, and possibly MCP-4 (Franci *et al.*, 1995, Ben-Baruch *et al.*, 1995, Combadiere *et al.*, 1995, Gong *et al.*, 1996). CCR2 on the other hand, recognizes all of the human MCP proteins characterized to date, and is selective for the MCP proteins. CCR3, the so-called eotaxin receptor, is prominently expressed on eosinophils and is believed to be the principle chemokine receptor for the recruitment of these cells in allergy. MCP-2, -3 and-4 are also ligands for CCR3. MCP-4 completely desensitizes eosinophils toward eotaxin, suggesting that it binds with comparibly high affinity to CCR3 (Uguccioni

1996). CCR-4, another CC chemokine receptor, only recognizes MCP-1 among all the MCPs.

1.6.4. Cellular activation

In addition to promoting cellular accumulation, MCPs are potent cell activators. After binding to appropriate G-protein linked seven -transmembrane-spanning receptor, MCPs elicit a transient intracellular calcium flux, actin polymerization, oxidative burst with release of superoxide free radicals, and exocytosis of secondary granule constituents (Bischoff *et al.*, 1992, Dahinden *et al.*, 1994, Elsner *et al.*, 1996). Cellular activation is also accompanied by increased avidity of integrins for their adhesion molecules. In eosinophils, MCP-3 has been shown to differentially regulate β1 and β2 integrin avidity (Weber *et al.*, 1996). In basophils, chemokine induced cellular activation results in degranulation with the release of histamine and the *de novo* generation of leukotriene C_4 (Alam *et al.*, 1992, Alam *et al.*, 1994). Basophil activation requires cellular priming with IL-3, IL-5, or GM-CSF for maximal effect of each chemokine. Although all MCPs can induce histamine release in cytokine primed basophils, there is marked variability between individual basophil donors. The intracellular signaling pathway triggered by chemokines in eosinophils is poorly understood, but recent studies have suggested the involvement of leukotriene metabolites because chemotaxis is inhibited by leukotriene inhibitors *in vitro* and *in vivo* (Stafford & Alam 1997).

1.6.5. Chemoattraction

Although CC chemokines have diverse functions in allergic reactions, they have mainly been recognized and become the focus of interest because they are thought to provide the directional cues for the movement of leukocytes. Leukocyte extravasation from the blood into the tissues is a regulated process which consists a series of events involving a marked induction of specific chemokines. The CC chemokines, including the MCPs, have emerged as mediators involved in the recruitment of inflammatory cells seen in allergic reactions.

As their name implies, all MCPs have strong chemoattractive activity for monocytes. Although MCP-1 has generally been observed to be the most potent and efficacious chemoattractant for monocytes (Uguccioni *et al.*, 1995, Van Damme *et al.*, 1992). In contrast, they display partial overlapping chemoattractive activity on other cell types. MCP-1, -2, -3 and -4 all have chemotactic activity on basophils and T cells (Alam 1994). MCP-3 has also been shown to attract dendritic cells (Sozzani 1995). One important distinguishing feature among the MCPs, is the ability of MCP-2, -3, and -4 to attract eosinophils, whereas MCP-1 displays no activity for this particular cell type. In terms of relative chemoattractant potency, MCP-4 is very potent in attracting eosinophils, but less potent than MCP-1 in attracting monocytes or T cells (Uguccioni *et al.*, 1996). For each chemokine, cellular specificity has been demonstrated for recombinant proteins by the use of in vitro chemotaxis assays with different populations of leukocytes.

Although other CC chemokines such as RANTES and eotaxin have been investigated in human allergen-induced responses, MCPs and their role as chemoattractants in allergen-induced rhinitis has yet to be demonstrated.

1.6.6. Role of MCPs in inflammatory diseases

The secretion of MCPs has been detected in a wide variety of inflammatory diseases, and it is likely that in these diseases, chemokines cause the accumulation and activation of leukocytes in tissues (Baggiolini & Dahinden1994). The type of inflammatory infiltrate that characterizes a specific disease is thought to be controlled in part, by the subgroup of chemokines expressed in the diseased tissue. Viral meningitis is characterized by monocyte and lymphocyte infiltration of the meninges. This disease is associated with an increase in MCP-1 expression in the cerebrospinal fluid, and this increase is correlated with the extent of mononuclear-cell infiltration of the meninges (Lahrtz *et al.*, 1997). MCP-1 expression can also be detected in human atheromatous plaques, consistent with a model of atherogenesis in which MCP-1 in the vessel walls attracts monocytes that eventually become foam cells (Nelken *et al.*, 1991, Ylä-Herttuala *et al.*, 1991). In experimental autoimmune encephalomyelitis which closely mimics many of the manifestations of multiple sclerosis, the expression of MCP-1 and MCP-3 occurs immediately before the appearance of infiltrating cells in the central nervous system (Ransohoff *et al.*, 1993, 1994, Godiska *et al.*, 1995).

The in vitro activity of MCPs on inflammatory cell such as eosinophils and basophils, suggests that these molecules play a role in human allergic diseases also.

MCPs are potent eosinophil chemoattractants and histamine releasing factors, making them particularly important in allergic inflammation (Luster *et al.*, 1997). MCP-1, -3 and -4 have all been detected in the airways of patients with asthma (Sousa 1994, Humbert et al., 1997, Lamkhioued *et al.*, 1997). The expression of MCP-3 and-4 has also been detected in tissue isolated from patients with chronic sinusitis (Wright *et al.*, 1998). Although MCPs have been studied in allergic diseases such as asthma and chronic sinusitis, the role of MCPs in the pathogenesis of allergic rhinitis has not been investigated.

1.7. Treatment of allergic rhinitis

As far as we have yet to completely understand the pathogenesis of allergic rhinitis, the current therapeutic goals are purely to relieve symptoms. Whether by the use of allergen avoidance intranasal anti-histamines, or steroids. Topical steroids, however, have become the drug of choice in the management of allergic rhinitis, as they are quite effective at relieving the symptoms in this disease, and permit direct delivery to the nasal mucosa minimizing systemic effects.

1.7.1. Nasal Topical Corticosteroids

Topical corticosteroids have been extensively used therapeutically and shown to be effective drugs in the treatment of allergic rhinitis, possessing significant topical anti-inflammatory efficacy with low systemic effects (Check *et al.*, 1990, Krause 1992b). The systemic absorption of most intranasal corticosteroids is minimal at

recommended doses and their effects appear to be due to local activity on the nasal mucosa (Toogood 1991). It has been shown that intranasal corticosteroid therapy can inhibit both the early and the late inflammatory responses after allergen challenge (Welch *et al.*, 1992). Topical application of corticosteroids can achieve favorable therapeutic effects locally, without the risks of the systemic side effects normally associated with oral corticosteroids (Van As *et al.*, 1991). Long-term intranasal treatment of adults with 200-400 µg / day of these streroids, has shown that the risk of adverse effects with the use of topical corticosteroids is low (Mygind 1993).

Budesonide, the topical nasal corticosteroid used by the patients in this study, has very high topical potency in the treatment of seasonal and perennial allergic rhinitis (Vanzieleghem *et al.*, 1987, Siegel 1988, Bunnag *et al.*, 1992). Budesonide is more effective on the late-phase response than on the early phase response after allergen challenge (Gronborg *et al.*, 1993). Following intranasal administration, peak plasma concentrations are reached within 15-45 minutes. Budesonide is completely inactivated in the liver, therefore it has a low systemic potency. The half-life is 2-3 hours in adults and 1.5 hours in children after inhalation, and plasma elimination time is approximately 2 hours (Check & Kaliner 1990, Mygind 1993). Recommended dosage is 400 µg / day (50 µg twice each nostril twice a day) (Siegel 1988, Wight *et al.*, 1992).

1.7.2. Mode of action

The mechanism of action of corticosteroids involves the passive diffusion of free or unbound glucocorticoid molecules through the cell membrane in the cytoplasm,

where they bind to hormone receptors. This steroid-receptor complex migrates into the cell nucleus, where it is anchored to specific nuclear binding sites on the DNA molecules, leading to the inhibition of mRNA synthesis and protein production (Pauwels 1986, Siegel 1988, Mabry *et al.*, 1992). In allergic rhinitis, glucocorticoids reduce inflammatory cell infiltration of mast cell, basophils, T cells and eosinophils in the nasal mucosa and nasal secretions and reduce the generation of their mediators, particularly Th2 type cytokines (Gomez *et al.*, 1988, Krause 1992, Lozewicz *et al.*, 1992, Rak *et al.*, 1994). In addition, steroids have been shown to reduce capillary permeability and mucus secretion in the nasal mucosa (Pauwels 1986). These beneficial changes appear to be reversible and discontinuation of topical nasal steroids allows the return of seasonal allergic inflammation.

1.7.3. Effects of Steroids

The beneficial effects of topical application of glucocorticoids includes inhibition of both the early and late hypersensitivity response to allergens with an observed decrease in the numbers of epithelial Langerhans cells, mast cells, Th2 cells and eosinophils, infiltrating the nasal mucosa during an allergic response. This is related to the suppression of Th2 type cytokines such as IL-4. Prolonged steroid treatment has been shown to inhibit the release of other mediators such as histamine, leukotrienes, eosinophil cationic protein, and chemokines. Thus there is a substantial inhibition of the nasal secretory response (Bascom *et al.*, 1988, Pipkorn *et al.*, 1987, Sim *et al.*, 1992, Miyamasu *et al.*, 1998, Rak *et al.*, 1994). Steroids have also been

shown to inhibit chemokine expression *in vitro*. A study done by Stellato et al. demonstrated that budesonide was an effective inhibitor of the expression of MCP-4, MCP-1, and RANTES, in a cultured epithelial cell line (BEAS-2B), activated with cytokines in vitro (Stellato *et al.*, 1997, Schwiebert *et al.*, 1996). In addition, it has recently been demonstrated that corticosteroids reduce the expression of IL-16, a potent chemoattractant factor for CD4+ cells, in allergen-induced rhinitis. Therefore, one of their mechanism of action may be to reduce chemokine expression with concomitant reduction in inflammatory cell infiltration.

1.8. Hypothesis

In this study we tested the hypothesis that MCPs play a role in the recruitment of inflammatory cells into the nasal mucosa following allergen challenge. Furthermore, we hypothesized that the inhibition of the inflammatory response seen in allergen-induced rhinitis as a result of steroid therapy, may be partly attributed to the inhibition of MCPs expression.

1.9. Aims

To investigate the potential role of MCPs in allergic inflammation, we set out to characterize the expression of MCPs in allergen-induced rhinitis, and to correlate

this with the inflammatory cell infiltrate. In addition, in the context of a double-blind trial, we aimed to investigate the effects of topical corticosteroids on the cellular infiltrate and MCPs expression in the nasal mucosa after allergen provocation.

2.1. Materials and Methods

2.1.1. Study design

A total of 12 patients with allergic rhinitis were recruited from the Nasal Clinic at the Sir. Mortimer B. Davis-Jewish General Hospital. Hospital ethics committee approval was obtained prior to to patient recruitment and all participating subjects gave informed consent prior to commencement of the study. Prior to consideration for the study, patients underwent skin testing using a panel of common seasonal and perennial aeroallergens. A positive histamine control is a standard part of the study. Inclusion criteria for the subjects included: (i) history of seasonal rhinitis, (ii) a positive skin-prick test (>5mm) to ragweed extract. Patients were excluded if they had perennial allergies (e.g. dust, pets), had previously received immunotherapy or were taking oral anti-inflammatory medication. Baseline inferior nasal turbinate biopsies were taken out of season at a time when the subjects were asymptomatic. All patients were treated in a double-blind fashion with either topical nasal corticoisteroids (budesonide 200μg twice daily) or a matched placebo for 6 weeks. After the 6 weeks of treatment all subjects underwent a nasal allergen challenge using ragweed extract (1000 PNU aerosol per nostril) followed by a second nasal turbinate biopsy which was

obtained 24 hours after challenge. In order to confirm clinically the adequacy of the challenge, patient symptoms after challenge were noted, specifically the number of sneezes and the degree of nasal obstruction (rated on the scale of 4). Nasal biopsies (2.5 mm) were taken from the inferior turbinate just beyond the anterior tip. Anasthesia was obtained using a mixture of oxymetazoline and xylocaine applied topically.

2.1.2. Allergen Challenge model

The use of the nasal antigen challenge has been successfully used to monitor allergic inflammatory responses during both the early- and late- phase reactions. Importantly, observations obtained during allergen provocation have been comparable to those during natural exposure to the allergen. The nasal allergen challenge model provides evidence for a step-like progression of allergic inflammation that begins when allergen binds to IgE on mast cells and the binding leads to release of mast cell mediators. These mediators activate endothelial cells to express adhesion markers that bind circulating leukocytes that respond to chemoattractants and other activators, entering the tissue and releasing their own mediators during the late-phase response. Thus, as described, this model mimics the events occuring during natural exposure to an allergen.

2.1.3. Tissue Preparation

In preparation for *in situ* hybridization, nasal biopsies were fixed immediately in freshly prepared 4% paraformaldehyde for 2 hr, followed by three washes with 15% sucrose in DEPC treated 0.1 M phosphate- buffered saline (PBS), pH 7.4 (first two washes for 1 hr at room temperature and then overnight at 4° C). Biopsies were then placed in OCT (optimal cutting temperature) embedding medium, snap frozen in isopentane precooled in liquid nitrogen, and stored at -80° C until further use. Cryostat sections, 6 μm thick, were cut and mounted on poly-L-Lysine (PLL)-coated microscope slides, in order to maximize tissue retention on the slides throughout the various rigorous treatments involved in *in situ* hybridization. They were then air dried for 1 hr, and left to incubate at 37° C overnight. Fixation in freshly prepared paraformaldehyde maintains tissue morphology while allowing cellular penetration of the probe and thus efficient hybridization.

In preparation for immunocytochemistry, the nasal biopsy tissue was immersed in 15% PBS on ice for 15-20 minutes. After the tissue was blocked in OCT medium, cryostat sections were cut at a thickness of 5 μm, mounted on microscope slides, air dried for 1 hour. The sections were subsequently fixed by immersion in a mixture of acetone-methanol (60:40) for 7 minutes at room temperature, air dried for 1 hr, and stored at -20°C until further use.

2.1.4. Immunocytochemistry

The technique of immunocytochemistry allows us to identify a protein by means of antigen-antibody imteraction. This concept of using antibodies raised in the laboratory to localize antigen within tissue has been used since the 1940's (Coons et al., 1941). Many methods are available for immunocytochemistry, but the choice of technique is usually based on the sensitivity of the method. In general, three principle methods are available: the direct method, indirect method and the indirect antibody-enzyme method. In this study, we used an indirect-enzyme method, the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique which utilizes an enzyme-anti-enzyme complex appraoch. This method consists of a primary unlabelled antibody, an unconjugated bridging secondary antibody, raised to the immunoglobulin of the species providing the primary antibody, and the label detection reagent, in the form of an enzyme-anti-enzyme complex, which is a labelled antibody raised against the species in which the second antiserum has been raised. Although a complex procedure, this indirect enzymatic method allows a higher sensitivity to be obtained (Bullock & Petrusz, 1982). Thus, this method is extremely important when trying to detect secreted proteins which act locally, and are often only produced in small quantities.

2.1.4.1. *Immunocytochemistry Methodology*

The APAAP technique was used to phenotype the cells present within the nasal biopsies, by use of specific monoclonal mouse anti-human antibodies directed against human T helper lymphocytes (CD4; Becton Dickinson, Cowley, U.K.), human

eosinophils (anti-MBP, BMK-13, a gift from Dr. Redwan Moqbel, University of Alberta), and macrophages (anti-CD68, Dako Diagnostics). MCP-1, -3 and -4 immunoreactivity was detected by the use of specific monoclonal antibodies, anti-MCP-1 and anti-MCP-3 (Cedar Lane, Canada) and anti-MCP-4 (supplied by Dr. Andrew Luster, Harvard University, Boston). For negative control preparations, the primary antibody was replaced by a nonspecific inappropriate mouse immunoglobulin, at the same dilution. An antibody to MCP-2 was unable to be procured either through commercial or non-commercial sources.

Slides were retrieved from -20° C and allowed to reach room temperature before beginning the experiment. Subsequently, a water resistant marker (dimethylpolysiloxane 20% in propan-1-ol with 1% concentrated sulphuric acid) was used to encircle, and thus isolate each individual section on each slide. Slides were immersed in TBS (150mM NaCl, 50 mM Tris-HCl, pH7.4) for 5 min, and incubated with a Universal Blocking Solution for 10 min in order to reduce non-specific staining. The slides were then incubated with 60 µl of the primary monoclonal antibody (specified above) in a humid chamber at 4° C overnight. The next day, slides were washed in tris-buffered saline (TBS), three times for 5 mins, and then the biopsy samples incubated with 60 µl of the secondary antibody for 30 mins at room temperature. An uncongujated rabbit-anti-mouse Ig (Dako) in antibody diluting buffer (ADB) at 1:60 dilution was used. After the 30 min incubation, the slides were washed in TBS as before. The slides were then incubated with 60 µl of murine monoclonal antibody to alkaline phosphatase, an APAAP conjugate (Dako), for 30 min. Following

a further washing of the slides with TBS, to develop the specific immunostaining, each biopsy specimen was saturated with Fast Red (Sigma), the chromogen, dissolved in the alkaline phosphatase substrate. Prior to use, this mixture was filtered through a 0.2μm filtre. After 15 - 20 mins, the slides were immersed in TBS for 5 mins and then in water for 2 mins, in order to stop the reaction. The intensity of the immunostaining reaction which depends on the duration of the fast red incubation, and was adjusted by examining the slides frequently under a light microscope until an optimal signal was observed.. Positive immunoreactivity was observed as a red staining The slides were then counterstained in Mayer's haematoxylin for 40-60 seconds to reveal the histology of the biopsy section. After rinsing the slides in tap water, they were immersed in lithium carbonate for 20 secs to enhance the contrast of the blue nuclear staining. Again, the slides were well rinsed in water and finally, a thin layer of crystal mount (Dako) was applied on each slide. The slides were then dried in the oven at 37°C overnight, and the following day coverslipped for purposes of light microscopy examination.

2.1.4.2. *Double Immunocytochemistry*

To confirm the phenotype of MCP immunoreactive positive cells, we undertook double sequential immunocytochemistry. Specific monoclonal antibodies (anti-MBP, anti-CD68, anti-CD3) were used to phenotype eosinophils, macrophages and T cells, respectively. Each of these monoclonal antibodies was used in combination with a polyclonal rabbit anti-human MCP-3 antibody or an anti-MCP-4

antibody. Endogenous peroxidase was blocked using 1% H_2O_2 (plus 0.02% sodium azide in TBS) for 30 min. After a brief wash in TBS, sections were incubated in 1% BSA (Sigma Chemical Co.) in TBS for 30 min to prevent any possible background reaction. Each of the monoclonal antibodies used to phenotype the inflammatory cells was combined with the polyclonal antibody and left to incubate overnight in a humid chamber at 4°C. After the overnight incubation, slides were washed in TBS and then incubated with a secondary layer of a mixture of rabbit anti-mouse IgG and a peroxidase conjugated swine anti-rabbit IgG. After a 30 min incubation, slides were washed in TBS, and then incubated with a tertiary layer of a mixture of streptavidin peroxidase (Amersham) and murine APAAP conjugate. Sections were developed sequentially in Fast Red followed by diaminobenzidine (DAB) (Sigma Chemical Co). The slides then were washed in TBS and counterstained lightly with Meyer's haematoxylin. MCP-3 and MCP-4 producing cells stained brown, eosinophils, macrophages and T cells stained red and eosinophils, macrophages, and T cells producing MCP-3 stained reddish brown.

2.1.5. *In Situ* Hybridization (ISH)

In situ hybridization, which is the cellular localization of specific nucleic acid sequences (DNA or RNA) using a labelled complementary strand, was first introduced in 1969 by Pardue & Gall. More recently, ISH has been applied to localize mRNA (Hamid *et al.*, 1987). The demonstration of mRNA within a cell provides valuable information about gene expression and indicates possible synthesis of the

corresponding protein. The general principle of ISH is based on the fact that labelled single-stranded RNA or DNA containing sequences (probes) are hybridized intracellularly to mRNA under appropriate conditions, thereby forming stable hybrids (Hamid 1993). Different types of probes are available to detect mRNA, including double- and single-stranded DNA probes, oligonucleotides, and single-stranded RNA probes (Singer *et al.*, 1986, Herrington & McGee 1990a, Denny *et al.*, 1989, Cox *et al.*, 1984). Single-stranded RNA probes, also known as complementary RNA (cRNA) probes produced by the process of *in vitro* transcription using an mRNA expression vector, have been used extensively in recent years for the detection of mRNA. The use of RNA probes has a number of advantages over other types of probes. These include the ability to synthesize probes of relatively constant size and with no vector sequences, the high thermal stability and affinity of RNA-RNA hybrids. All these favor high specificity and sensitivity for RNA probes (Herringhton *et al.*, 1990b, Hamid 1993). Two types of labelling can be used for RNA probes: isotopic and non-isotopic labelling. Although highly sensitive, certain problems are encountered with radiolabelled probes such as the hazards of radioactivity and the slow speed of visualization. These problems have prompted the development of non-isotopic labels to be used for RNA hybridization. Biotin was one of the first non-isotopic labels to be used for RNA hybridization (Giaid *et al.*, 1989). More recently, digoxigenin-11-UTP, a very sensitive and efficient label has been employed in labelling RNA probes (Yap *et al.*, 1992, Ying *et al.*, 1993, 1994). The RNA hybrids obtained by using non-radiolabelled probes are usually detected by immunocytochemical methods. The

cellular resolution obtained with non-isotopically labelled probes is usually excellent and the signals are developed in a very short time when compared with radiolabelled probes. Thus, in this study, in situ hybridization using digoxigenin labelled riboprobes was used to localize mRNA for MCP-4. Only MCP-4 mRNA was examined in this study, given that riboprobes for MCP-1, -2 and -3 were not available to us.

2.1.5.1. *Preparation of Complementary RNA (cRNA) probes*

Cox et al. were the first to describe an *in situ* hybridization technique which used antisense RNA probes generated from specially constructed plasmid vectors. The desired cDNA fragment is inserted in reverse orientation downstream from a bacteriophage promoter. In the presence of a specific RNA polymerase, labelled antisense RNA (cRNA) can be synthesized and used to generate RNA-RNA hybrids (cRNA-mRNA) in the individual cells (Cox et al., 1984).

The production of cRNA probes begins with the insertion of template DNA, which encodes the cRNA sequence in question, into an RNA expression vector (e.g. pGEM, pBluescript). These special transcription vectors contain two promoters that are recognized by specific polymerases which, depending on the specific polymerase used, will transcribe labeled antisense (complementary RNA strand to target mRNA) or sense (identical RNA strand to target mRNA) probes from the included DNA template. In our study, MCP-4 DNA template was inserted into a pcDNA1.1/Amp (Invitrogen) transcription vector, 4.8 kb pairs in length, with SP6 and T7 polymerase promoter sites allowing transcription of the insert in alternate directions. To obtain

transcripts of the right size the vector must be linearized immediately downstream of the insert or within the insert with the use of enzymes. The linearization of the pcDNA1.1 vector was performed using the restriction enzymes EcoRI (sense) and Hind III (antisense).

2.1.5.2. *In vitro transcription*

To synthesize a single-stranded, digoxigenin RNA probe, human MCP-4 cDNA was used as a template. We incubated 1 µg of this linearized plasmid template in a 20 µl reaction mixture containing 5X transcription buffer (4 µl), 0.5µl of RNase inhibitor (Rnasin), 8 µl of the following nucleotide triphosphate mixture (3.5 µl of Dig UTP, 6.5 µl of normal UTP, and 10 µl of each ATP, GTP, and CTP), 4.5 µl of DEPC H_2O, 2 µl of RNA polymerase making up a total volume of a 20 µl mixture. The mixture was then incubated for 2 hours at 37 °C. Transcription was terminated by the addition of 1 µl RNase-free DNase and incubated for 15 minutes at 37 °C to destroy the template. 2.5 µl of 4M NaCl and 70 µl of 100% ethanol was added to this mixture before allowing to precipitate overnight at -20°C. The next day, the mixture was spun down at 12 000 rpm at 4°C for 10 mins. After carefully discarding the supernatant, approximately 100 µl of ice cold 70% ethanol was added to the mixture inorder to precipitate the RNA probe, spun at 12 000 rpm at 4°C for 15 mins, and again the supernatant was discarded. The RNA pellet was then dried in a speed vacuum for 5

mins. Once dry, the pellet was resuspended in 10 µl of DEPC H_2O and stored at -20°C until further use.

2.1.5.3. *In Situ Hybridization Methodology*

In order to facilitate the hybridization of the digoxigenin-labelled probe to the mRNA within the cells, the tissues must first undergo a stage of prehybridization. Once removed from the -80° C freezer, the slides were brought to room temperature before beginning the experiment. Initially, the slides were immersed in 4% paraformaldehyde/PBS for 5 mins at room temperature in order to further fix the biopsy specimens. Subsequently, the sections were treated with 0.3% Triton X-100 in PBS for 10 minutes. After a brief wash in PBS, sections were exposed to proteinase K solution (1 µg/ml in 20 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 7.2) for 15 minutes at 37°C. After a brief rinse in PBS, slides were immersed in 4% paraformaldehyde/PBS for 5 minutes, transferred to PBS for a further wash and then allowed to air-dry for 1 hr. Once dry, the MCP-4 digoxigenin-labelled probe was applied to each section. The concentration of MCP-4 digoxigenin-labelled riboprobe, both antisense and sense control, were adjusted to approximately 100 to 500ng per section in hybridization buffer composed of 50% formamide, 5X Denhards solution, 5X standard saline citrate buffer (SSC), and 500 µg/ml denatured salmon sperm DNA (Sigma) denatured at 100° C which is added right before use. A volume of 25 µl of this hybridization mixture was added to each section. The sections were covered with a coverslip and incubated overnight at 40 C in a humid chamber. The next day, slides

were washed sequentially in 4xSSC at 42 C for 10 mins twice, incubated with 20μg/ml RNase A in 2xSSC for 30 mins at 37 C to remove any unhybridized RNA, and washed in 2xSSC at 42° C for 10 mins followed by 0.1xSSC for 10 min at room temperature. After a brief wash in Tris-buffered saline (TBS), sections were incubated with 3% bovine serum albumin/TBS for 10 minutes to reduce nonspecific background, then incubated with 1:500 to 1:5000 dilutions of <Dig>-AP congugate (sheep polyclonal antidigoxigenin antiserum conjugated with alkaline phosphotase) in TBS containing 0.1% Triton X-100 in a humid box, for 4 hrs, at room temperature. After the 4 hr incubation, the slides were washed three times in TBS, each for 15 mins, followed by an incubation with equalization buffer (0.1 mol/L Tris-HCL, 0.1 mol/L NaCL, 50 mmol/L $MgCl_2$, pH 9.5) for 5 mins at room temperature. Color development was achieved by immersing the slides in a freshly prepared substrate solution consisting of BCIP (X-phosphotase-5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium salt) and equalization buffer for approximately 50 mins at room temperature. The reaction is usually monitored by by examiming the intensity of the staining under light microscopy. Once the reaction is complete, slides were transferred to TBS and washed in tap water, counterstained with hematoxylin for 5 seconds, and dipped briefly in lithium carbonate. The slides were then mounted with crystal mount and left to dry overnight in the incubator at 37°C, and coverslipped the following day.

2.1.5.4. *Controls*

Some sets of tissue sections were hybridized with the sense probes identical to

the coding strand of the mRNA of human MCP-4 following the same procedure as above. A further control procedure was performed by treating a separate set of slides with an RNase A solution (20 μg/ml at 37 °C for 60 min) before the prehybridization step. Hybridization was then performed with the labeled antisense cRNA probes as previously described. The absence of specific hybridization in both cases, confirmed the specificity of the signals obtained using the antisense probes.

2.1.6. Quantitation

For immunocytochemical analysis and *in situ* hybridization, sections were coded and counted in a blinded, random fashion with an Olympus microscope with an eyepiece graticule at x 200 magnification. At least two sections were immunostained or hybridized from which six to eight fields were evaluated. Epithelial staining was assessed using morphometry, and the results were expressed as the percentage of epithelium that expressed mRNA or immunoreactivity on the basis of a semiquantitative score from 0 - 4 (0, no staining; 0.5, <12.5%; 1, 12.5% to 25%; 1.5, 25% to 37.5%; 2, 37.5% to 50%; 2.5, 50% to 62.5%; 3, 62.5% to 75%; 3.5, 75% to 87.5%; 4, 87.5% to 100%). Subepithelial immunostaining or hybridization was presented as the mean counts of cells expressing protein or mRNA per high-power field (0.202 mm^2). For co-localization studies, the results were expressed as a percentage of the MCP-3 and -4 immunoreative cells which coexpressed either CD3, CD68 or MBP.

2.1.7. Statistics

Data analysis was performed using a statistical package (Systat v6.0, Evanston, IL). To compare the number of cells expressing MCP-1, -3 and -4 mRNA and immunoreactivity, and the number of MBP, CD68 and CD4 +ve cells at baseline and after allergen challenge, we used a paired student's t test. The mean Δ values for the steroid and placebo treated patients were compared with the use of an unpaired student's t test. Correlation coefficients and significance values were obtained by the use of Pearson's moment correlation with a subsequent Bonferroni correction factor. Results were considered statistically significant for values of $p<0.05$.

3. Results

3.1. Clinical Observations

Patients were requested to note the number of sneezes and the degree of nasal blockage during the 24 hour period following nasal ragweed challenge. While steroids were not effective in suppressing the early response as assessed by the number of sneezes, there was a significant reduction in the degree of nasal obstruction between the time points of 1 hour and 24 hours. Thus there was a clearly observable difference between the placebo and steroid treated groups, which validated the adequacy of challenge and the efficacy of our topical corticosteroid (Fig. 1).

3.2. MCP-1 Immunoreactivity

At baseline, MCP-1 immunoreactive cells were identified at high levels in both epithelial and subepithelial areas. Within the epithelium, MCP-1 immunoreactivity was localized predominantly in the apical layer. In the placebo treated subjects, there was no significant increase either in the epithelial or subepithelial MCP-1 immunoreactivity 24 hours after antigen provocation compared with baseline values. Within the budesonide-treated group, there was a significant decrease in the MCP-1 immunoreactive positive cells after challenge ($p<0.005$), both in the epithelium and subepithelium of the nasal mucosa as compared to baseline levels (Fig. 2a and 2b).

3.3. MCP-3 Immunoreactivity

MCP-3 immunoreactivity was identified at baseline in all rhinitic subjects and statistically increased 24 hours following allergen challenge both in the epithelium ($p<0.001$) and subepithelium ($p<0.05$) of the nasal mucosa in subjects given placebo. At baseline, there was no significant difference in the number of MCP-3 positive cells between patients treated either with steroid or placebo. However, within the steroid treated group, an increase in cells expressing MCP-3 immunoreactivity after allergen challenge was not observed. In contrast, within this group, there was a significant decrease in epithelial MCP-3 immunoreactivity ($p<0.05$) and in the number of MCP-3 immunoreactive cells in the subepithelium ($p<0.05$) after challenge, when compared to baseline values (Fig. 3a and 3b).

3.4. MCP-4 Immunoreactivity

At baseline, the MCP-4 immunoreactivity was identified predominantly within the epithelium, with some staining detected in the subepithelium of the nasal mucosa. In subjects treated with placebo, immunohistologic examination of nasal biopsy specimens that were obtained 24 hours after antigen challenge revealed a substantial augmentation in MCP-4 immunoreactivity focally distributed along the epithelial cell layer ($p<0.001$). In addition, there was a statistically significant increase in the number of cells expressing MCP-4 after challenge in the submucosal layer. At baseline, there was no significant difference in the number of MCP-4 positive cells between patients treated either with corticosteroid or placebo. In patients treated with corticosteroid, MCP-4 immunoreactivity was decreased following challenge both in the epithelium and subepithelium ($p<0.05$), although epithelial counts failed to reach statistical significance (Fig. 4a and 4b).

3.5. MCP-4 mRNA expression

MCP-4 mRNA expression was identified at baseline in all rhinitic subjects, predominantly within the epithelium of the nasal mucosa. In subjects treated with placebo, MCP-4 mRNA expression was significantly increased 24 hours following allergen challenge both in the epithelium ($p<0.05$) and subepithelium ($p<0.001$) of the nasal mucosa when compared with baseline values. In the budesonide-treated group, MCP-4 mRNA was decreased following challenge both in the epithelium and subepithelium, however this failed to achieve statistical significance (Fig 5a and 5b).

3.6. Assessment of Inflammatory Cell Infiltrate

In patients treated with placebo, there was a statistically significant increase in the number of MBP +ve cells in the nasal submucosa following nasal allergen challenge as compared with baseline biopsies ($p<0.05$). There was no significant difference at baseline in the number of MBP-positive cells between patients subsequently treated with either steroid or placebo. However, the increase in cells expressing MBP after allergen challenge in the placebo-treated group was not observed when patients were pretreated with topical corticosteroids. Actually, there was a significant difference between the delta values (difference between post and pre challenge) for MBP immunoreactivity between the placebo and steroid treated groups ($p<0.05$) (Fig. 6).

The number of CD4 +ve cells also increased significantly following allergen challenge in the placebo-treated group ($p<0.05$). There was no difference in the baseline expression of CD4+ T cells between the placebo and steroid treated groups, however, the increase in CD4 immunoreactivity observed 24 hours after challenge was reversed by pretreatment with topical corticosteroids ($p<0.05$). Comparison of the mean differences between the two groups indicated that the placebo-treated patients had significantly more CD4+ cells after allergen challenge when compared with the steroid-treated group ($p<0.05$) (Fig. 7).

Finally, the expression of CD68 +ve cells in the subepithelium after allergen challenge showed no significant difference when compared to baseline biopsies in the

placebo treated group, however, the number of positive cells was significantly decreased after allergen provocation in the steroid treated group ($p<0.05$) and comparison of the mean differences for the two groups indicated that the placebo-treated group had significantly more CD68 +ve cells when compared with the steroid treated groups ($p<0.05$) (Fig. 9).

To determine if the expression of the MCPs in the epithelial or subepithelial cell layers was associated with the infiltration of inflammatory cells following challenge, we performed correlation analyses. There were no significant correlation between MCP-1 and any of the inflammatory cells and although both MCP-3 and -4 moderately correlated with MBP, CD4 and CD68 +ve cells, this failed to achieve statistical significance.

3.7. Phenotype of Cells Expressing MCP-3 and MCP-4

Colocalization studies were performed in nasal biopsies from three rhinitic subjects given placebo after challenge using double immunocytochemistry. The majority of MCP-3 and MCP-4 within the nasal mucosa was associated with CD68-positive macrophages (mean ± SD; n=3, 64.8 ± 10.2% ; 67.3 ± 6.4% respectively). Significant numbers of cells expressing MCP-3 and MCP-4 also expressed eosinophil and T cell markers (mean SD; n=3, MBP-positive eosinophils, 14.2 ± 3.05% expressing MCP-3 and 12 ± 2.8% expressing MCP-4; CD3-positive T lymphocytes, 15.83 ± 6.6% expressing MCP-4 and 15.5 ± 3.9% expressing MCP-4) (Table 1 and

2). The remainder of the MCP-3 and -4 positive cells within the submucosa (approximately 5%) did not colocalize to any of the inflammatory markers used.

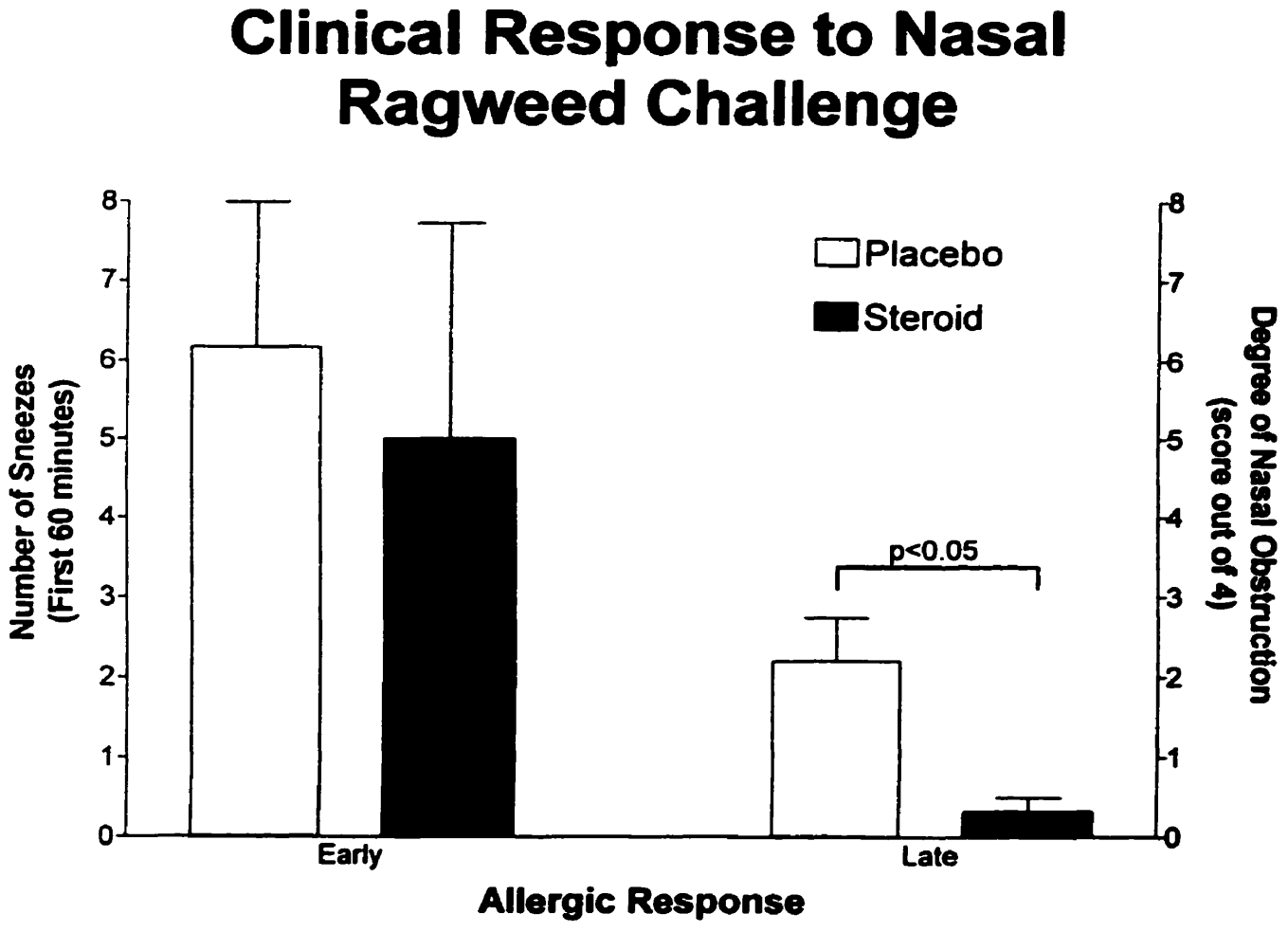


Figure 1: Clinical responses to nasal ragweed challenge. Early response estimated by recording the number of sneezes during the first 60 minutes post-challenge. The late response was estimated by the degree of nasal blockage (subjective score out of 4) from 1-24 hours post challenge. The late response was significantly suppressed by steroid pre-treatment.

Epithelial MCP-1 Immunoreactivity

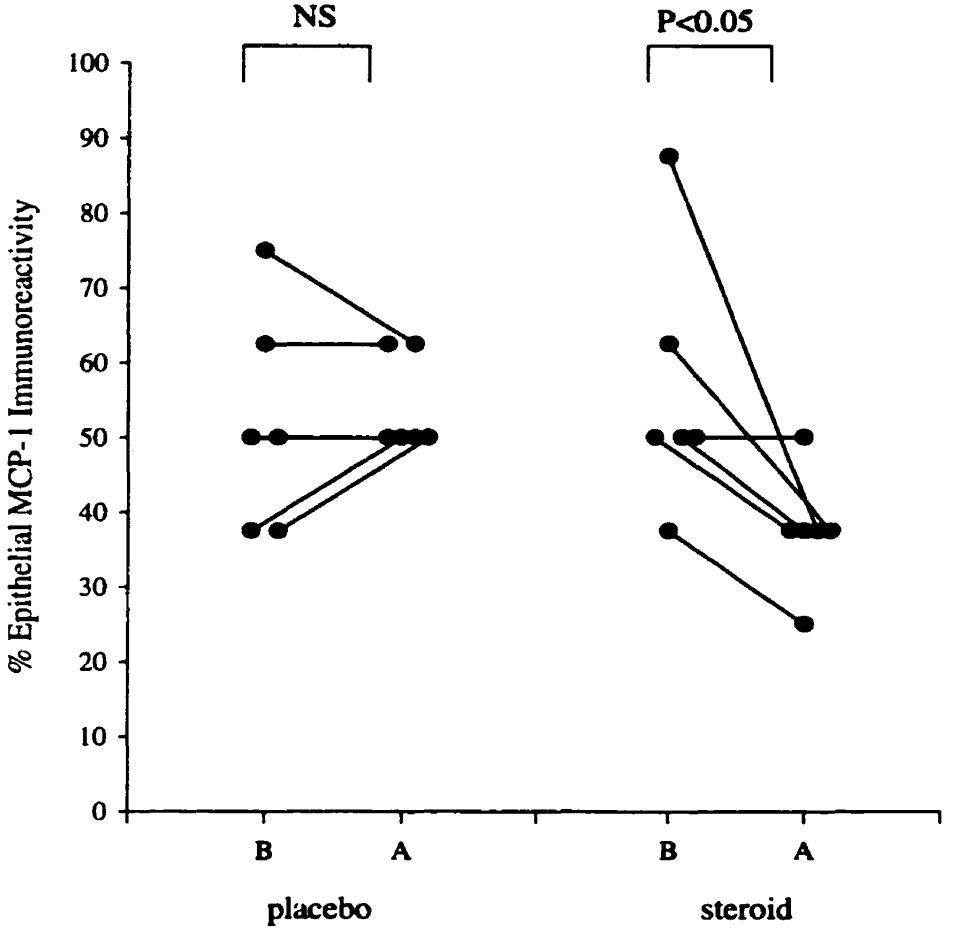


Figure 2a: Epithelial MCP-1 immunoreactivity in nasal biopsy specimens obtained before (B) and 24 hours after (A) allergen challenge in patients with allergic rhinitis receiving either placebo or steroid treatment. Results are expressed as percentage of epithelium showing positive signal/total epithelium.

Subepithelial MCP-1 Immunoreactivity

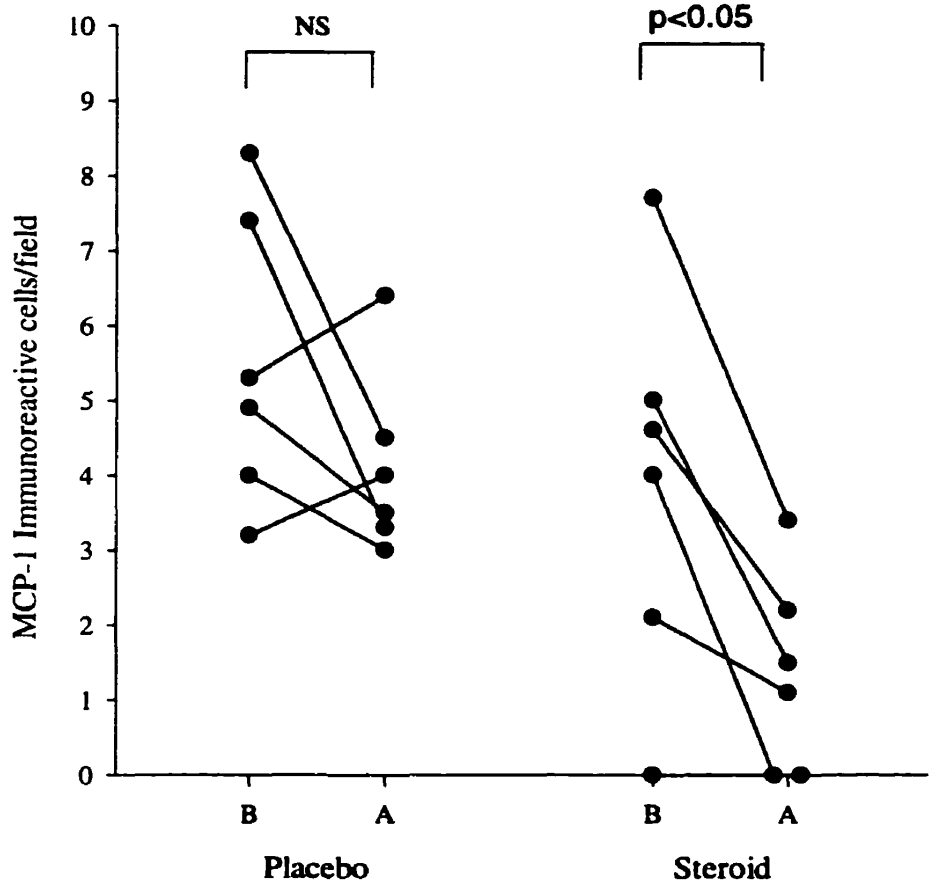


Figure 2b: Number of MCP-1 immunoreactive cells in the subepithelium of nasal biopsy specimens obtained before (B) and 24 hours after (A) allergen challenge in patients with allergic rhinitis receiving either placebo or steroid treatment. Results are expressed as the mean number of MCP-1 positive cells per field.

Epithelial MCP-3 Immunoreactivity

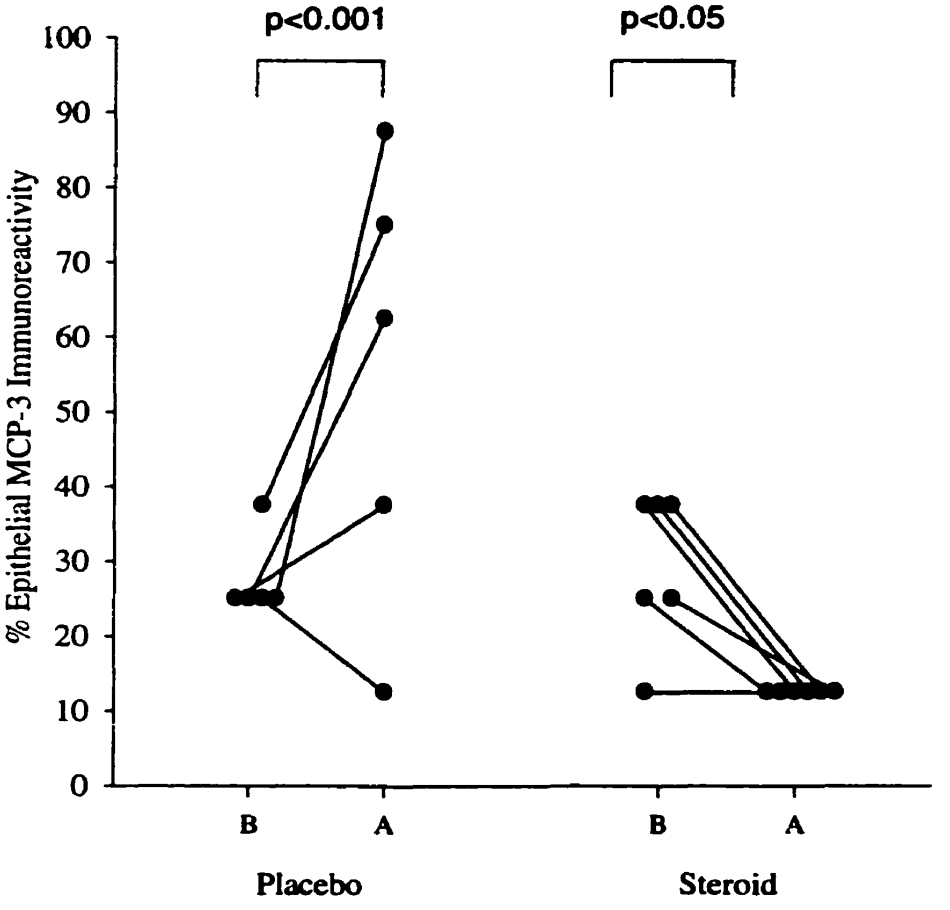


Figure 3a: Epithelial MCP-3 immunoreactivity in nasal biopsy specimens obtained before (B) and 24 hours after (A) challenge in patients with allergic rhinitis receiving either placebo or steroid treatment. Results are expressed as percentage of epithelium showing positive signal/total epithelium.

Subepithelial MCP-3 Immunoreactivity

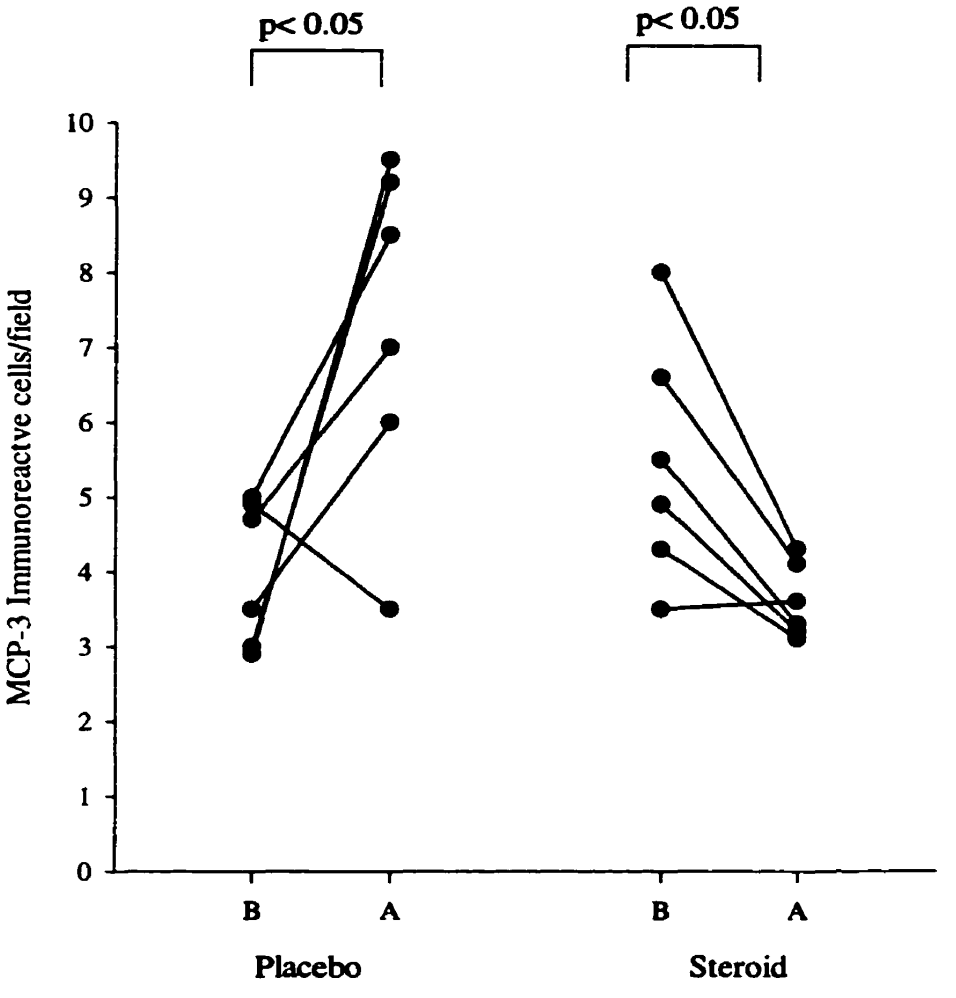


Figure 3b: Number of MCP-3 immunoreactive cells in the subepithelium of nasal biopsy specimens obtained before (B) and after (A) allergen challenge in patients with allergic rhinitis receiving either placebo or steroid treatment. Results are expressed as the mean number of MCP-3 positive cells per field.

Epithelial MCP-4 Immunoreactivity

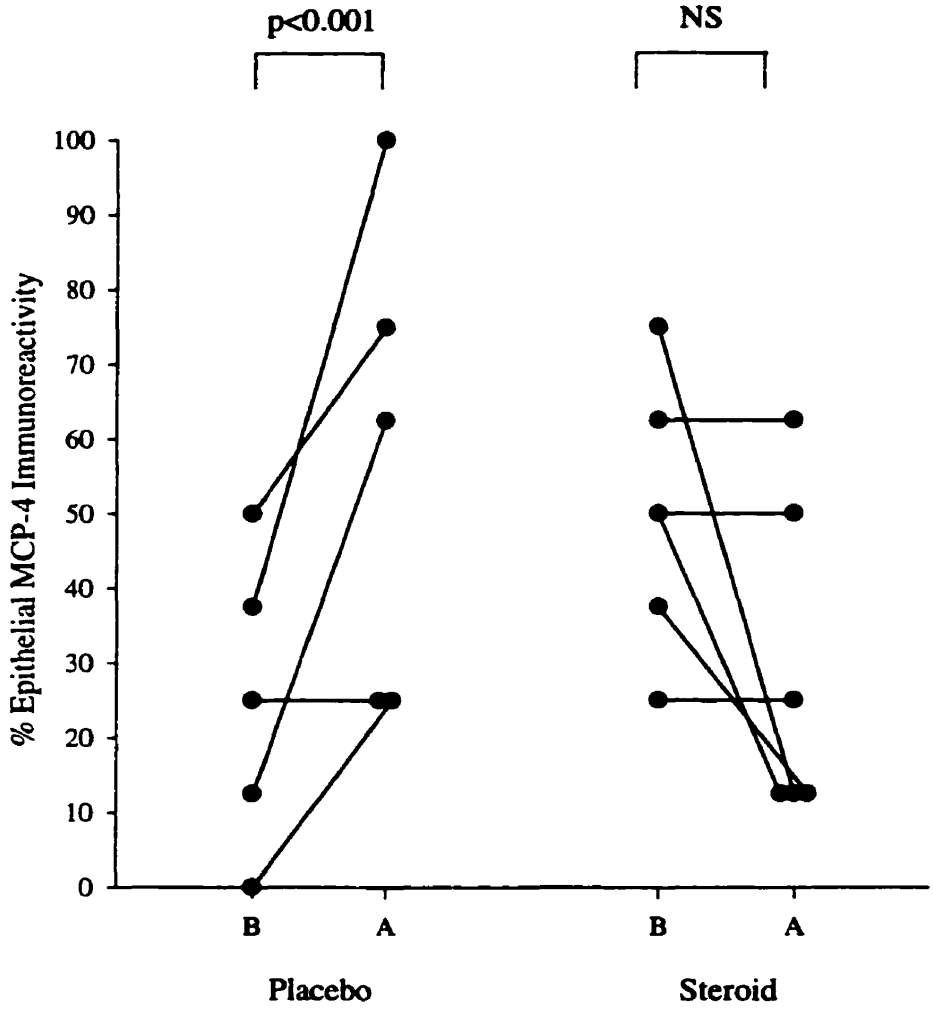


Figure 4a: Epithelial MCP-4 immunoreactivity in nasal biopsy specimens obtained before (B) and 24 hours after (A) challenge in patients with allergic rhinitis receiving either placebo or steroid treatment. results are expressed as percentage of epithelium showing positive signal/total epithelium.

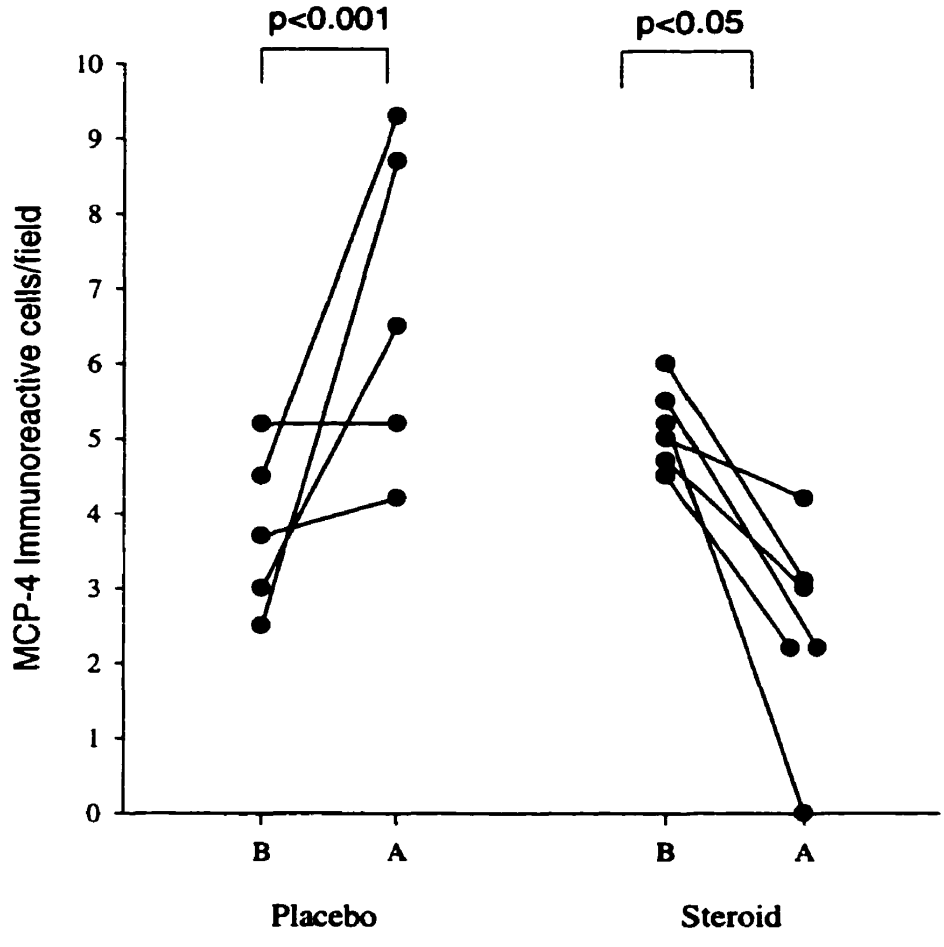


Figure 4b: Number of MCP-4 immunoreactive cells in the subepithelium of nasal biopsy specimens obtained before (B) and 24 hours after (A) challenge in patients with allergic rhinitis either given placebo or steroid treatment. Results are expressed as the mean number of MCP-4 positive cells per field.

Epithelial MCP-4 mRNA Expression

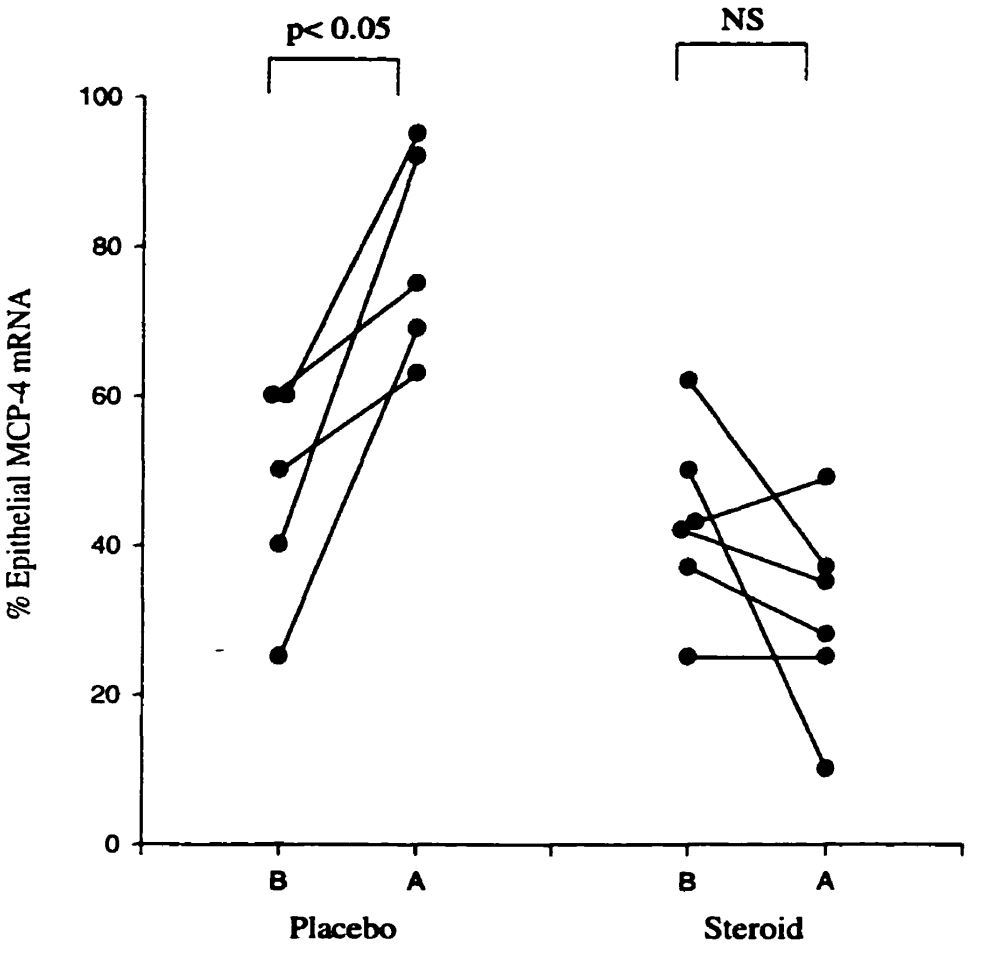


Figure 5a: Epithelial expression of MCP-4 mRNA in nasal biopsy specimens obtained before (B) and 24 hours after (A) allergen challenge in patients with allergic rhinitis receiving either placebo or steroid treatment. Results are expressed as percentage of epithelium showing positive signal/total epithelium.

Subepithelial MCP-4 mRNA Expression

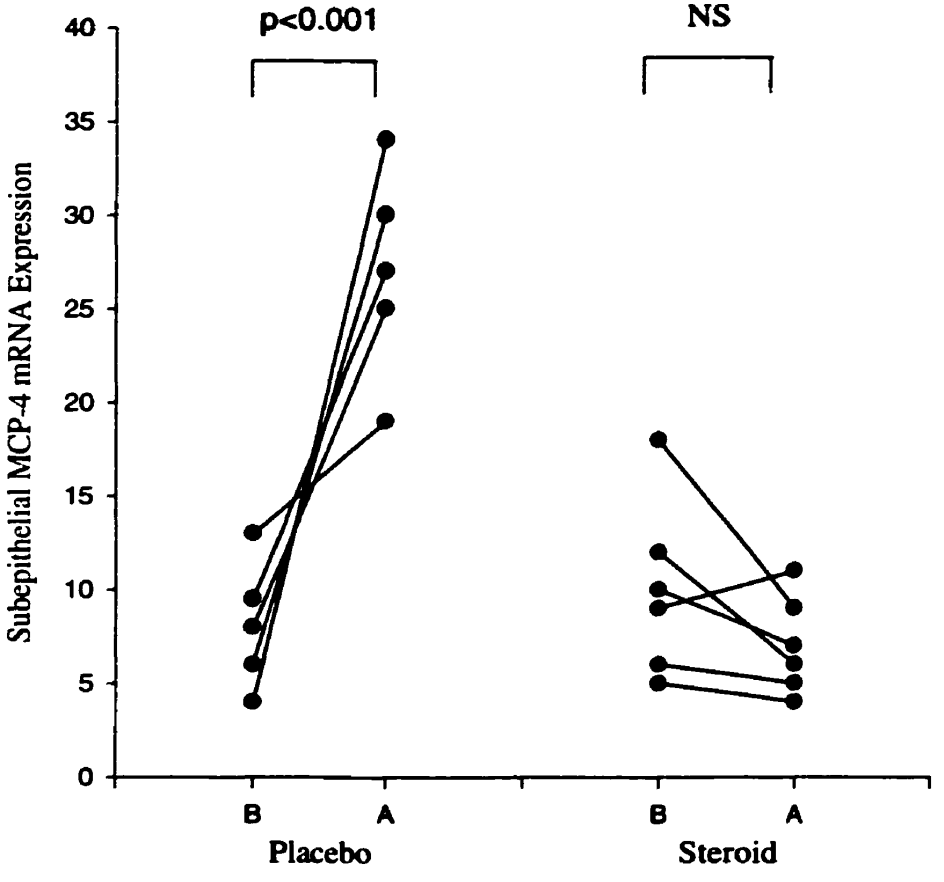


Figure 5b: Subepithelial expression of MCP-4 mRNA in nasal biopsy specimens obtained before (B) and 24 hours after (A) allergen challenge in patients with allergic rhinitis receiving either placebo or steroid treatment. Results are expressed as number of positive cells per field.

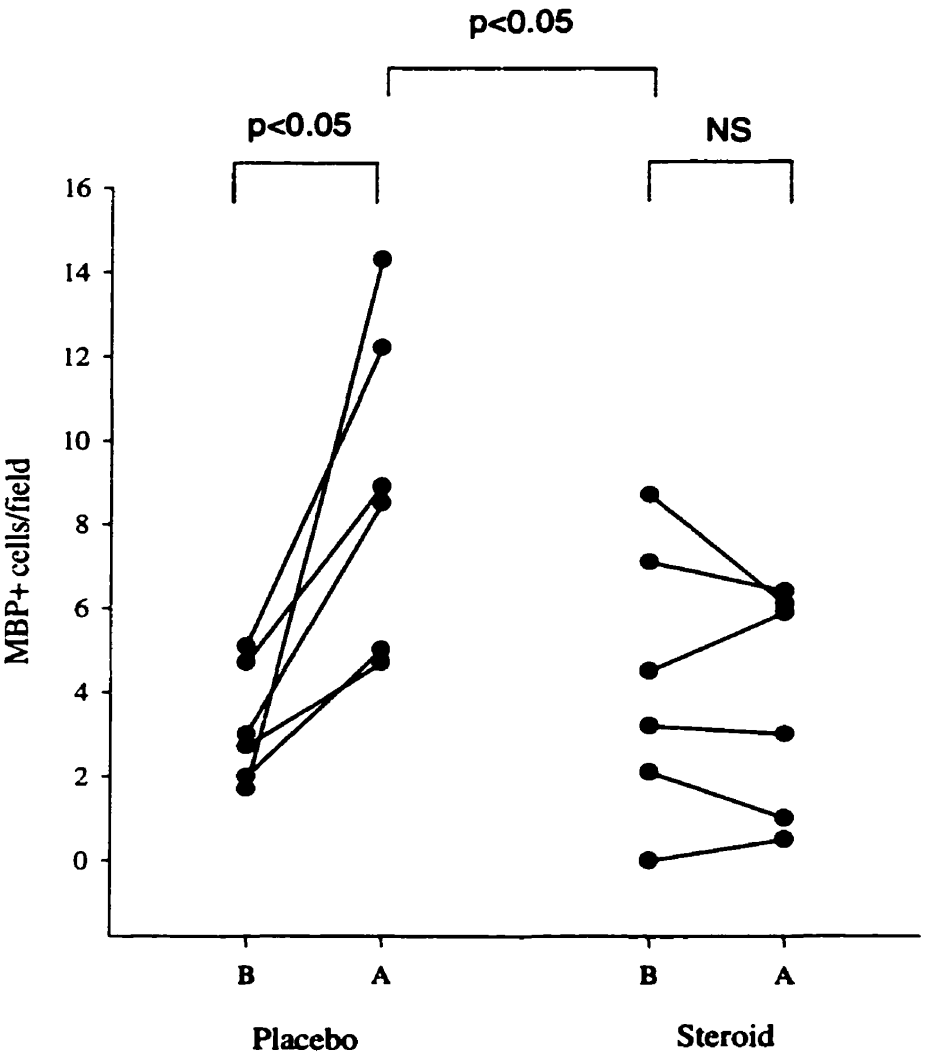


Figure 6: Number of MBP+ cells (eosinophils) within the submucosa of nasal biopsy specimens obtained before (B) and 24 hours after (A) challenge in patients with allergic rhinitis either given placebo or treated with steroids. Results are expressed as the mean number of positive cells per field.

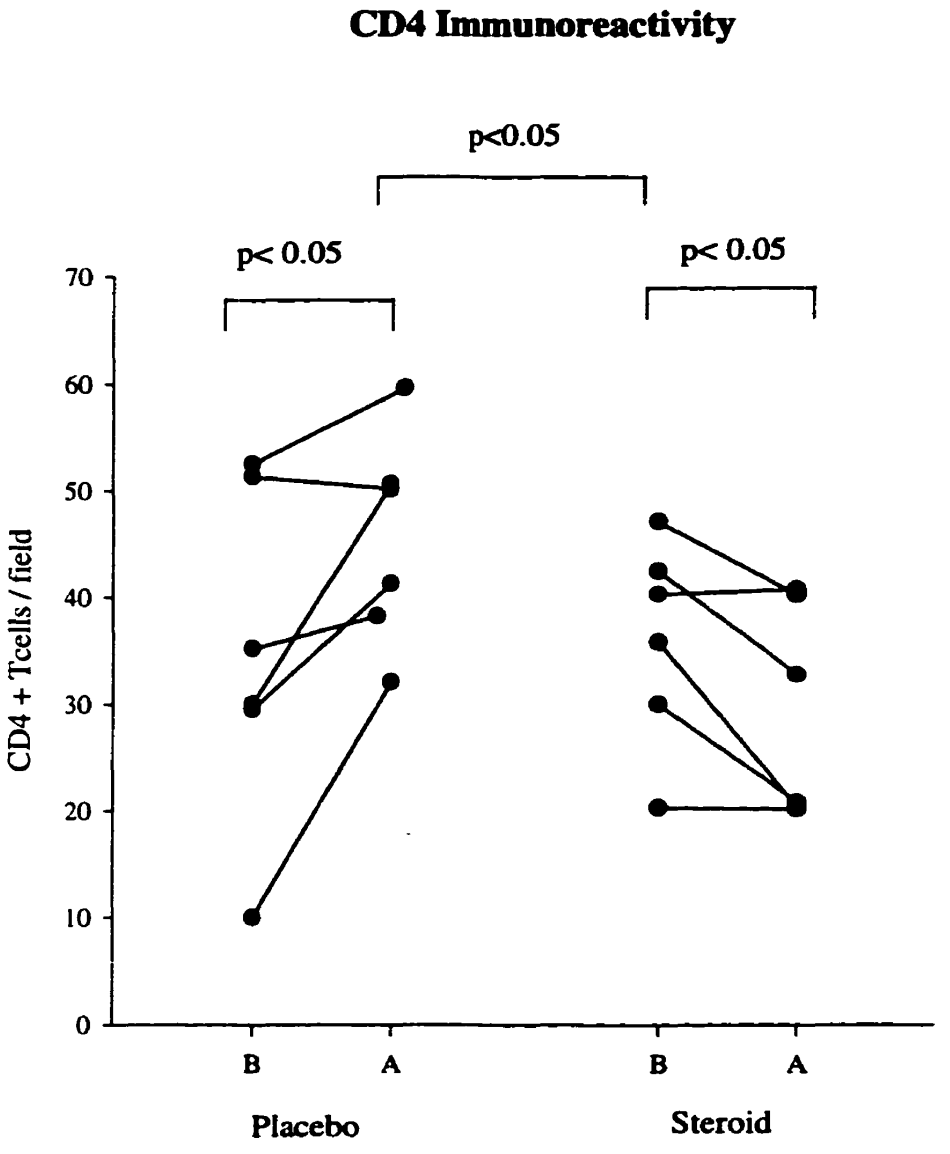


Figure 7: Number of CD4+ cells (T helper cells) within the submucosa of nasal biopsy specimens obtained before (B) and 24 hours after (A) challenge in patients with allergic rhinitis either given placebo or treated with steroids. Results are expressed as the mean number of CD4+ cells per field.

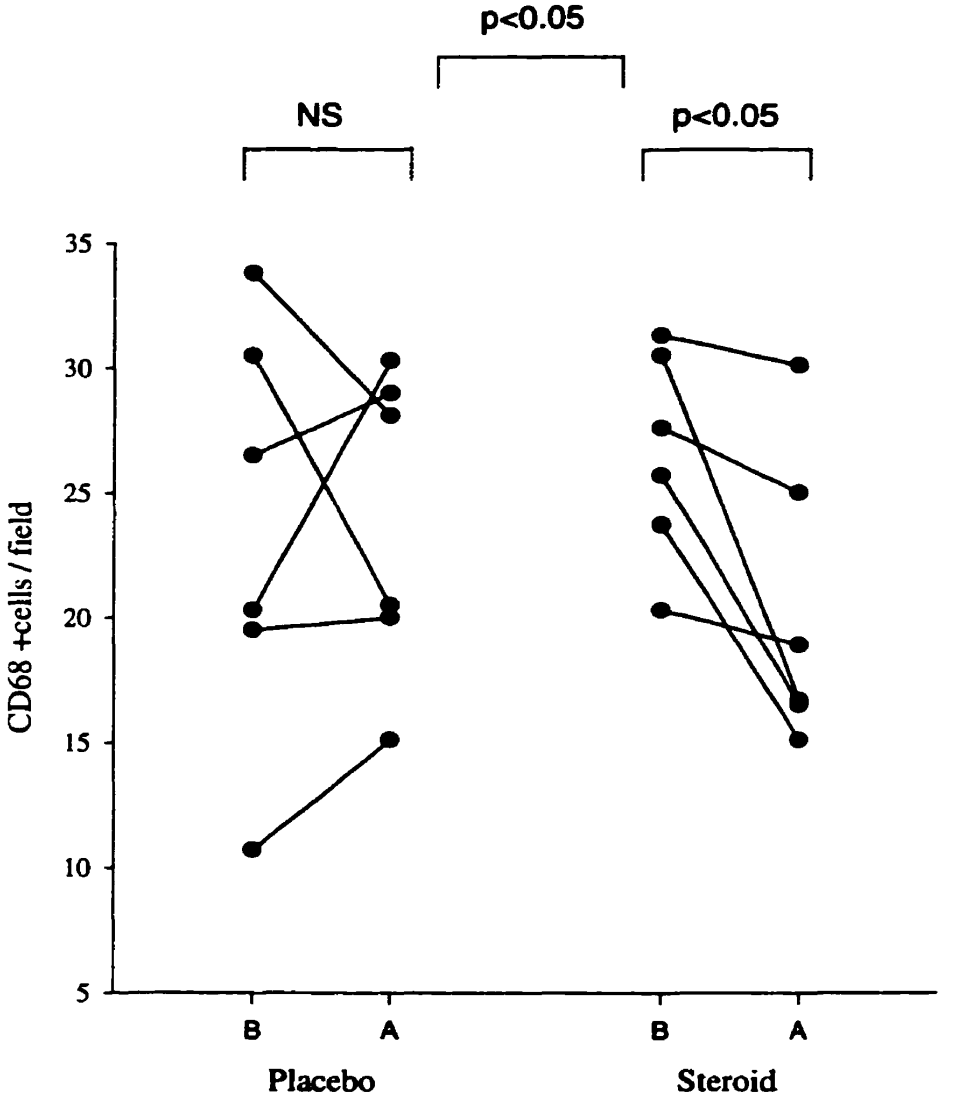


Figure 8: The number of CD68+ cells (macrophages) within the submucosa of nasal biopsy specimens obtained before (B) and 24 hours after (A) challenge in patients with allergic rhinitis either given placebo or treated with steroids. Results are expressed as the mean number of CD68+ cells per field.

-Table 1-

Phenotype of MCP-3 immunoreactive cells

	% of MCP-3 immunoreactive cells		
Patient	CD3 Positive	CD68 Positive	MBP Positive
1	23	53	17.5
2	10	70	13.5
3	14.5	71.5	11.5
Mean ± SD	15.83 ± 6.6	64.8 ± 10.2	14.2 ± 3.05

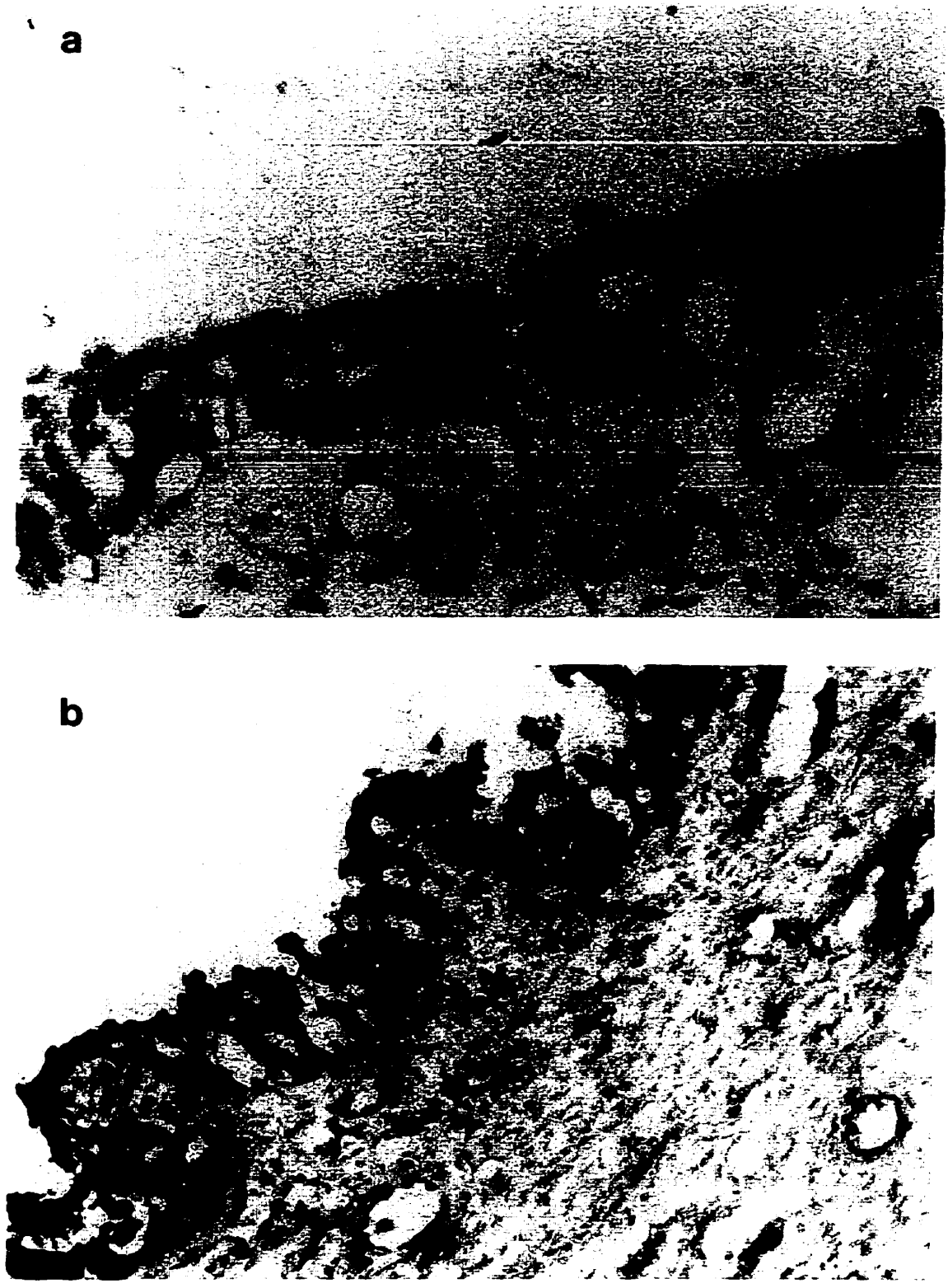
% of CD3, CD68 and MBP positive cells expressing MCP-3 immunoreactivity in nasal biopsy specimens obtained from three randomly selected placebo treated patients after allergen challenge.

-Table 2-

Phenotype of MCP-4 immunoreactive cells

	% of MCP-4 immunoreactive cells		
Patient	CD3 Positive	CD68 Positive	MBP Positive
1	20	60	11.5
2	13.5	72	9.5
3	13	70	15
Mean ± SD	15.5 ± 3.9	67.3 ± 6.4	12 ± 2.8

% of CD3, CD68 and MBP positive cells expressing MCP-4 immunoreactivity in nasal biopsy specimens obtained from three randomly selected placebo treated patients after allergen challenge.



Representative examples of immunostaining for MCP-4 in nasal biopsy specimens from placebo treated allergic rhinitic patients at baseline (a) and following allergen challenge (b). Note a constitutive expression of MCP-4 in the nasal epithelium (a) and an upregulation of MCP-4 expression following allergen challenge both in the epithelium and subepithelium of the nasal mucosa (b).

Representative examples of immunostaining for MCP-4 in nasal biopsy specimens from placebo (a) and steroid treated (b) allergic rhinitic patients following allergen challenge. Note a high expression of MCP-4 following allergen challenge in the nasal biopsy section from a subject given placebo (a). In comparison, the expression of MCP-4 in the nasal mucosa following challenge was inhibited in subjects pretreated with steroids (b).

4. Discussion

The aims of this study were to examine the expression of monocyte chemotactic proteins in allergen-induced rhinitis and to investigate their possible contribution to the late nasal response with the use of the nasal allergen challenge model. Furthermore, we assessed whether the late nasal response could be inhibited by pretreatment with topical glucocorticoid steroids. Our results demonstrate that exposure to allergen increases the expression of MCP-3 and -4 within the nasal mucosa of patients with allergic rhinitis and that these effects are abrogated by topical steroid treatment.

We observed that allergen provocation in the nasal mucosa of rhinitic patients resulted in early (0-60 min) and late (1-24 h) nasal symptoms. These responses were assessed by the number of sneezes and the degree of nasal blockage respectively, experienced by the patients during the 24 hour period following nasal ragweed challenge. These clinical observations validated the adequacy of challenge and the effectiveness of the steroid.

Budesonide was shown clinically to inhibit the late nasal response as it significantly reduced the degree of nasal obstruction as expected. On the contrary, steroid treatment didn't seem to inhibit the symptoms of the early nasal response eventhough steroids are normally clinically effective at blocking the nasal symptoms characteristic of the early nasal response. It is possible that the assessment made for the early phase response may not be very accurate given the limited number of patients

in this study. In addition, intersubject variability may also be a factor responsible for this observation.

It has previously been shown that the allergen-induced late nasal responses are accompanied by an influx of inflammatory cells particularly eosinophils and $CD4^+$ T cells and basophils (Varney *et al.*, 1992, Minshall *et al.*, 1997, Ying *et al.*, 1994). The presence of these cells and the local release of their mediators at the allergic site have been associated with the pathological features of allergic rhinitis (Varney *et al.*, 1992, Durham *et al.*, 1992). The mechanisms responsible for the recruitment of these cells to the site of inflammation, are not clearly understood, however, it has been proposed that one possibility may be the local generation of chemotactic agents that regulate this influx of leukocytes. (Baggiolini & Dahinden 1994). A number of chemotactic factors such as bacterial peptides, phospholipid metabolites, and cytokines, have been identified and found to be associated with allergic inflammation. These chemoattractants however, are weak and have little target selectivity. Chemokines, on the other hand, are potent chemoattractants, specific for a variety of leukocytes. In particular, CC chemokines have been implicated in allergic diseases, since they have been shown to act on the inflammatory cells specifically involved in allergic inflammation.

Although the expression of MCPs have previously been detected in allergic tissues isolated from patients with chronic allergic sinusitis, asthma and atopic dermatitis (Garcia-Zepeda *et al.*, 1996b, Lamkhioued *et al.*, 1997, Wright *et al.*, 1998), there is no previous evidence *in vivo,* for a role of MCPs in human allergic

rhinitis. Therefore, our findings of an increased expression of MCP-4 mRNA and MCP-3 and -4 immunoreactive cells in the nasal mucosa, as a result of allergen challenge is novel, and this increased expression may partly account for the inflammatory cell accumulation seen in allergen-induced late nasal responses.

We demonstrated a constitutive expression of MCP-1, -3 and -4 in the nasal mucosa, particularly in the epithelial cell layer. This observation suggests a role for MCPs in baseline leukocyte trafficking through epithelial tissue, as part of the natural host defenses against pathogens, as previously suggested by Garcia-Zepeda et al. While chemokines often have similar in vitro functions, their pattern of expression in a given inflammatory disease determines which chemokines may play a pathobiological role. The constitutive expression of MCP-4 has previously been detected within normal tissues, particularly with large epithelial surfaces in contact with the environment such as the intestines and the lung. This pattern of expression is similar to what has been seen for eotaxin, except that MCP-4 is expressed at higher baseline levels in the lung (Garcia-Zepeda *et al.*, 1996a, b). In addition, MCP-1 has been detected predominantly in salivary glands, and MCP-2, in the intestines and ovary (Sarafi *et al.*, 1997, Coillie *et al.*, 1997). Since MCPs have been shown to act on inflammatory cells that are not normally found in high numbers within normal tissues, the constitutive expression of MCPs and their action within these tissues needs to be further investigated (Collins *et al.*, 1995, Garcia Zepeda *et al.*, 1996, Luster *et al.*, 1997).

Although constitutively expressed at high levels within the nasal mucosa at baseline, MCP-1 immunoreactivity was not increased as a result of allergen provocation as seen with MCP-3 and –4. This finding supports those from previous studies demonstrating that MCP-1 is abundant in mononuclear cell-rich inflammatory conditions such as granulomatous inflammation, and in macrophage-rich regions of atherosclerotic lesions (Antony *et al.*, 1993, Ylä-Hertuala *et al.*, 1991). Interestingly, few eosinophils are present in these conditions unlike in allergic diseases, supporting the observations that MCP-1 may not have any chemotactic activity for eosinophils. It was recently demonstrated, that MCP-1 production is enhanced in the blood and tissue of patients with tuberculosis, suggesting that MCP-1 may contribute to the antimycobacterial inflammatory response by attracting monocytes and T lymphocytes which are characteristic of these disease (Lin *et al.*, 1998). Nevertheless, the role of MCP-1 in the pathogenesis of allergic disease can not be ruled out since MCP-1 has been detected in human allergic tissue, and is well known for its ability to chemoattract and activate basophils, which represent a major source of mediators, and an essential element of IgE-mediated hypersensitivity reactions (Schroeder *et al.*, 1995, Alam *et al.*, 1992).

While the expression of several C-C chemokines such as eotaxin and RANTES have previously been shown to be upregulated in the nasal mucosa in allergen-induced rhinitis (Bazan *et al.*, 1997, Minshall *et al.*, 1997), and to be associated with the infiltrating inflammatory cells, the factors responsible for the infiltration of leukocytes in disease states have not been clearly defined. To determine whether the local

upregulation of MCP-3 and –4, which we observed in the nasal mucosa after allergen challenge, is associated with the infiltrating cells, we examined the numbers and phenotype of cells within the nasal mucosa after allergen challenge. Our data demonstrated a significant eosinophil, and $CD4^+$ T cell infiltration following allergen challenge, consistent with previous findings (Varney *et al.*, 1992, Ying *et al.*, 1993, Minshall *et al.*, 1997). This increase may be associated with the upregulation of MCP-3 and-4 within the nasal mucosa. The presence of these cells and the release of their mediators such as cytotoxic proteins or Th2 type ctyokines, has been associated with the pathological features of allergic rhinitis (Varney *et al.*, 1992, Durham *et al.*, 1992). In contrast, no change in $CD68^+$ cells were observed in the nasal mucosa following challenge when compared to baseline biopsies, consistent with our previous findings (Minshall *et al.*, 1997, Rajakulasingam *et al.*, 1997). Monocytes generally infiltrate Th1 type granulomatous diseases (Lin *et al.*, 1998). The fact that MCP-1 immunoreactivity was not increased in the nasal mucosa after challenge may also account for this finding, given that macrophages are one of the main sources of MCP-1, and MCP-1 levels seem to be enhanced particularly in macrophage rich conditions (Ylä-Hertuala *et al.*, 1991).

Furthermore, to determine if the expression of MCP-3 and -4 in the epithelial and subepithelial cell layers was quantitatively associated with the infiltration of inflammatory cells, we performed correlation analyses. The fact that there were no significant correlations between the infiltration of inflammatory cells and MCPs expression may suggest that other chemokines, such as eotaxin and RANTES, play a

substantial role in the acute inflammatory cell influx following allergen provocation. In addition, the CC chemokines MIP-1α, MIP-1β, have also been shown to be chemotactic for monocytes, differentially chemotactic for lymphocyte subsets, and chemotactic for eosinophils in vitro and presumably in allergic responses (Taub *et al.*, 1993, Schall *et al.*, 1993, Rot *et al.*, 1992). The lack of correlation observed may also be due to the time course of chemokine production and its influence on the kinetics of leukocyte recruitment. A disadvantage of the nasal biopsy technique is that it is not amenable to the performance of time-course studies for ethical reasons. We chose the 24 hour time point based on previous studies showing identifiable changes in inflammatory cells particularly eosinophils and T lymphocytes at this particular time point (Varney *et al.*, 1992).

In our tissue specimens, we observed MCP-3 and –4 expression within the nasal epithelium and resident inflammatory cells in the nasal submucosa. It is well known that chemokines are often secreted from activated leukocytes presumably as part of amplification and desensitization mechanisms. Phenotyping the cells expressing MCP-3 and MCP-4, demonstrated that macrophages, eosinophils and T cells were significant cellular sources of these chemokines, with macrophages being a prominent source within the submucosa. However, the expression of MCP-3 and –4 was most strongly localized to the ciliated pseudostratified epithelium in the nasal mucosa in our clinical specimens. This is consistent with previous findings showing that MCPs are strongly expressed in bronchial epithelial cell lines after cytokine stimulation (Stellato *et al.*, 1997, Garcia-Zepeda *et al.*, 1996). This however, is not only the case for

MCPs, but for other CC chemokines as well, such as eotaxin and RANTES (Minshall *et al.*, 1997, Ponath *et al.*, 1996, Rajakulasingam *et al.*, 1997)

In this study we also examined the effects of nasal topical corticosteroids on the expression of MCPs and on the cellular infiltrate in allergen-induced rhinitis. Allergic rhinitis has been demonstrated to be amenable to treatment with topical nasal corticosteroids (PipKorn *et al.*, 1987). Numerous studies have demonstrated the efficacy of steroids in the suppression the clinical symptoms in this disease. Studies have demonstrated that prolonged treatment with topical corticosteroids inhibits both early, and late nasal responses and the associated tissue eosinophilia following allergen provocation, by inhibiting the expression of Th2 cells and the release of their cytokines (Rak *et al.*, 1994, Masuyama *et al.*, 1994). We demonstrated that pretreatment with budesonide abrogates the increase in MCP-1, -3 and -4 protein expression both in the epithelium and subepithelium as a result of allergen provocation in the placebo treated subjects. These data are consistent with previous studies showing that topical corticosteroid treatment leads to the inhibition of the LNR (Rak *et al.*, 1994) and with previous *in vitro* studies showing that budesonide may be an effective inhibitor of MCP-4 mRNA in cultured BEAS-2B epithelial cells activated with cytokines (Stellato *et al.*, 1992). We postulate that the suppression of cellular infiltrate after allergen challenge in subjects undergoing glucocorticoid therapy occurs partly through the inhibition of MCPs, effectively attenuating leukocyte transendothelial migration. Epithelial cells are the first cell type to come in contact with inhaled glucocorticoids and since we and others have shown that epithelial cells are the main source of

chemokines (Stellato *et al.*, 1992), this cell type may be an important target of the anti-inflammatory effects of inhaled glucocorticoids. Furthermore since application of topical corticosteroids represent in many cases the drug of first choice for the treatment of seasonal allergic rhinitis, these findings must be considered of relevance to the therapeutic management of rhinitis.

In summary, we have demonstrated a constitutive expression of MCP-1, -3 and -4 in the nasal mucosa of allergic patients with a substantial increase in MCP-3 and -4 immunoreactivity aswell as MCP-4 mRNA expression following allergen challenge. The increases in these chemokines were accompanied by an upregulation of inflammatory cells, particularly eosinophils, within the nasal submucosa. Furthermore, prolonged treatment with topical corticosteroids was associated with a significant attenuation of allergen-induced increases in MCP-1, -3 and -4 immunoreactivity and in MBP, CD68, and CD4 positive cells. These data suggest that the increased expression of MCP-3 and -4 may be relevant to the pathophysiology of latephase reactions in allergen-induced rhinitis through their ability to recruit leukocytes into the nasal mucosa in response to antigen.

We believe that CC chemokines, in particular MCPs are likely to play a critical role in regulating the inflammation characteristic of allergic diseases and inhibition of their production may thus serve as a favorable approach for the treatment of allergic diseases. Chemokine antagonists are of therapeutical interest as an approach for anti-allergic therapy. It has been shown that antagonists are effective, and some low-molecular-weight compounds that block chemokine receptors are already at hand

(Baggiolini & Moser 1997). However, whether strategies directed against MCP-3 and -4 , such as synthesis inhibitors or receptor antagonists might modify the response to allergen challenge and the course of allergic rhinitis, remains to be investigated.

5. Future Studies

In order to further investigate the role of MCPs in the pathogenesis of allergic rhinitis, additional studies must be conducted which can help to answer many of the questions raised in this study. *In vitro* chemotaxis assays, which examine chemotactic activity more precisely, will allow us to assess the actual potency of MCPs as chemoattractants. Since the processes governing the recruitment of inflammatory cells are likely to be the result of a combination of chemokines, with the use of neutralizing antibodies we may be able to identify the chemoattractive contribution and potency of each individual chemokine. Furthermore, with the use of an *in vitro* assay we can examine whether the chemoattractant effect of MCPs is enhanced by the addition of other mediators or cytokines. To determine whether there is a local upregulation of MCPs in the nasal mucosa after challenge, *ex vivo* studies isolating the nasal mucosa from systemic infiltration would provide us with such insight. Given the limitations of the *in vivo* allergen challenge model, the nasal explant model can also serve as a means of performing time course studies to examine the kinetics of MCPs expression. At the same time, the regulation of MCPs expression can be investigated more precisely by incubating the explanted tissue with various mediators, in particular Th2-type cytokines.

These *in vitro* studies may thus provide valuable insight in determining more accurately the regulation and function of monocyte chemotactic proteins in allergic inflammation.

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