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CCR3-Blocking Antibody Inhibits Allergen-Induced Eosinophil Recruitment in Human Skin Xenografts from Allergic Patients

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SUMMARY: Eosinophil, basophil, and T helper 2 (TH2) cell recruitment into tissues is a characteristic feature of allergic diseases. These cells have in common the expression of the chemokine receptor CCR3, which may represent a specific pathway for their accumulation in vivo. Although animal models of allergic reactions are available, findings cannot always be extrapolated to man. To overcome these limitations, we have developed a humanized mouse model of allergic cutaneous reaction using severe combined immunodeficiency mice engrafted with skin and autologous peripheral blood mononuclear cells from allergic donors. Intradermal injection of the relevant allergen into human skin xenografts from allergic individuals induced a significant recruitment of human $CD4^+$ T cells, basophils, and TH2-type cytokine mRNA-expressing cells, as well as murine eosinophils. Human skin xenografts, atopic status, and autologous peripheral blood mononuclear cell reconstitution were all mandatory to induce the allergic reaction. Next, we addressed the role of CCR3 in the endogenous mechanisms involved in the inflammatory cell recruitment in this experimental model of allergic cutaneous reaction. In vivo administration of an anti-human CCR3-blocking antibody selectively reduced accumulation of eosinophils but not that of $CD4^+$ cells, basophils, or cells expressing mRNA for TH2-type cytokines. These findings establish a new in vivo model of humanized allergic reaction and suggest that eosinophil migration is mediated mainly through CCR3. Finally, these results suggest that this model might be useful to test human-specific antiallergic modulators. (*Lab Invest 2002, 82:929–939*).

Allergic diseases are characterized by a tissue infiltration of eosinophils, basophils and T helper 2 (TH2) cells, which is thought to be related to the severity and chronicity of the allergic reaction. This preferential cell accumulation in tissue suggests that there are specific pathways used for their accumulation in vivo. Understanding these mechanisms could aid in developing pharmacologic therapies that would block their recruitment. This recruitment is orchestrated in part by chemokines that act through specialized surface receptors. The chemokine receptor CCR3 is of particular interest in the context of allergic reactions, because it is expressed by TH2 lymphocytes (Sallusto et al, 1997), eosinophils (Ponath et al, 1996), basophils (Uguccioni et al, 1997), and mast cells (Ochi et al, 1999; Romagnani et al, 1999), all of which are found at sites of allergen-induced late-phase response (LPR) (Frew and Kay, 1988; Gaga et al, 1991; Irani et al, 1998). The CCR3 receptor is a G-protein–coupled seven-transmembrane receptor that mediates the action of several chemokines, including eotaxin (CCL11), RANTES (CCL5), MCP-3 (CCL7), and MCP-4 (CCL13) (Ponath et al, 1996; Stellato et al, 1997). All these ligands have been shown to be overexpressed in human allergen-induced cutaneous LPR (Ying et al, 1999), in atopic dermatitis (Yawalkar et al, 1999) and in asthma (Lamkhioued et al, 1997; Ying et al, 1997), reinforcing the potential role of CCR3 as a major pathway of cell recruitment and therapeutic target in allergic reactions (Bertrand and Ponath, 2000). Hitherto, only two studies have evaluated the effect of CCR3 antagonism in vivo, one on chemokine-induced eosinophil recruitment (Sabroe et al, 1998) and the other in a parasitosis model (Grimaldi et al, 1999). In both cases, an inhibition of eosinophil recruitment was observed. However, no in vivo study has evaluated the effect of CCR3 antagonism on the other CCR3-bearing cells or in an allergy model. Moreover, CCR3 seems to be differentially regulated in the murine and in the human system (Lloyd et al, 2000). In mice, anti-CD3 stimulation, which mimics antigen effect, increases CCR3 expression on TH2 cells, whereas in humans, it down-regulates it (Sallusto et al, 1999), questioning the relevance of murine studies to human situations. For all these reasons, the develop-

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ment of a humanized model of allergic reaction was needed. We therefore used the severe combined immunodeficient (SCID) mouse and developed a model of humanized cutaneous allergic reaction. These mice have a defect in antigen receptors on the T and B lymphocytes and can be reconstituted with a functional human immune system (Mosier et al, 1988). The model of SCID mice grafted with skin alone (Christofidou-Solomidou et al, 1996, 1997b; Juhasz et al, 1993; Nickoloff et al, 1995; Yan et al, 1993) or with peripheral blood mononuclear cells (PBMC) has been previously extensively used in the field of skin diseases (Christofidou-Solomidou et al, 1997a; Gilhar et al, 1997) and to study different types of T cell–mediated cutaneous inflammation (Petzelbauer et al, 1996; Tsicopoulos et al, 1998) and graft rejection (Murray et al, 1994; Sultan et al, 1997) but never as a model of allergic reaction. In the present study, intradermal injection of allergen in SCID mice xenografted with human skin and autologous (PBMC) from the same allergic donors reproduced the features of the human allergen-induced LPR. In addition, we report in this model that the blockade of human CCR3 with a monoclonal antibody selectively decreases eosinophil infiltration.

Results

Allergen Challenge into Human Skin Xenografts from Allergic Donors Reproduces Both the Immediate and Late-Phase Allergic Reactions

In a first series of experiments, skin prick tests and intradermal injection of the house dust mite *Dermatophagoides pteronyssinus* (Dpt) into skin xenografts obtained from allergic donors induced positive cutaneous reactions within 15 minutes (a photograph is shown in Fig. 1A). No such reaction was observed in human skin xenografts from nonatopic donors or in mouse skin, which was consistent with an IgE-dependent mechanism specific to human skin xenografts from allergic donors. No delayed reaction was observed in allergic or nonatopic skin specimens. To examine the allergen-induced late-phase reaction, autologous PBMC were inoculated intraperitoneally in SCID mice bearing two human skin grafts from allergic donors and immediately followed by intradermal injection of the allergen. Twenty-four hours later, the challenged site was erythematous and swollen as compared with the contralateral diluent-injected graft. Here again, this delayed cutaneous reaction was only observed in skin xenografts from allergic patients but not in those from nonatopic donors.

Allergen Challenge into Human Skin Xenografts from Allergic Donors Reproduces the Cellular Infiltration Observed in Human Allergic Reactions

The human cellular infiltration in 24-hour skin xenografts obtained from the allergic SCID mice group was examined using immunohistochemistry (Fig. 2). A significant infiltration of human $CD4^+$ cells was observed at the site of allergen injection as compared with the diluent (Fig. 1, C and D), including memory $CD45RO^+$ cells. Besides these CD4 T cells, $BB1^+$ basophils (Fig. 1E) and mainly eosinophils were also recruited after allergen challenge. These human skin-infiltrated eosinophils were of murine origin ($mMBP^+$) with no human $EG2^+$ eosinophil infiltration (data not shown), which was not surprising, given the reconstitution with PBMC only. However, when SCID mice were reconstituted with both PBMC and eosinophils from allergic donors, no human eosinophil infiltration was observed in the allergen-challenged skin xenografts (not shown). When other potent eosinophil stimuli were injected into the skin, such as IL-4 plus TNF-α, or eotaxin or RANTES, again no human eosinophil infiltration was obtained (not shown), although murine eosinophils were recruited. CD4 T cell and eosinophil recruitment was not observed in SCID mice engrafted with human skin and autologous PBMC from nonatopic donors, suggesting that the cellular infiltration was strictly related to the atopic status. Allergen challenge had no effect on the number of $CD8^+$, $HLA\text{-}DR^+$, $CD68^+$ monocytes/macrophages, or $tryptase^+$ mast cells (not shown).

Allergen Challenge into Human Skin Xenografts from Allergic Donors Reproduces the Preferential TH2-Type Cytokine Profile Observed in Human Allergic Reactions

To determine the cytokine profile of the skin-infiltrated cells, TH1-type (IL-2 and IFN-γ) and TH2-type (IL-4 and IL-5) cytokine mRNA expression were evaluated by using in situ hybridization (Fig. 3). In the allergic SCID mice group, significant increases in the number of IL-4 (Fig. 1F) and IL-5 $mRNA^+$ cells were found in the allergen-injected versus the diluent-injected sites. The absence of IL-2 or IFN-γ $mRNA^+$ cells indicated a preferential recruitment of TH2-type cells. The absence of the latter cells in the nonatopic SCID mice group was consistent with a specific immunoallergic process involving human TH2 cells as observed in humans.

In Vivo Inhibitory Effect of an Anti-Human CCR3-Blocking Antibody and Functional Cross-Reactivity with Murine Eosinophils

Before assessing the in vivo effect of CCR3-blocking antibody in our model, we performed an ex vivo study to check the efficiency of CCR3 blockade (Fig. 4). Preliminary dose-response experiments showed that the optimal in vivo inhibitory concentration of the CCR3-blocking antibody was 25 μg/mouse. At this dose, sera from anti-CCR3–injected mice were able to inhibit human eotaxin-induced chemotaxis of human eosinophils (anti-CCR3 sera: -85% at 24 hours, control IgG2a sera: -19%, versus the activity of the sera before injection). Intraperitoneal coadministration of anti-CCR3 antibody and PBMC led to a residual inhibitory activity of 62% at 24 hours (versus 0% for PBMC plus IgG2a), indicating the presence of a large proportion of free anti-CCR3 antibody despite its binding on the surface of PBMC (Fig. 4A). In addition, anti-CCR3 antibody was also able to inhibit the chemotaxis of murine eosinophils (anti-CCR3 -77% versus control IgG2a -21%). These results clearly indi-

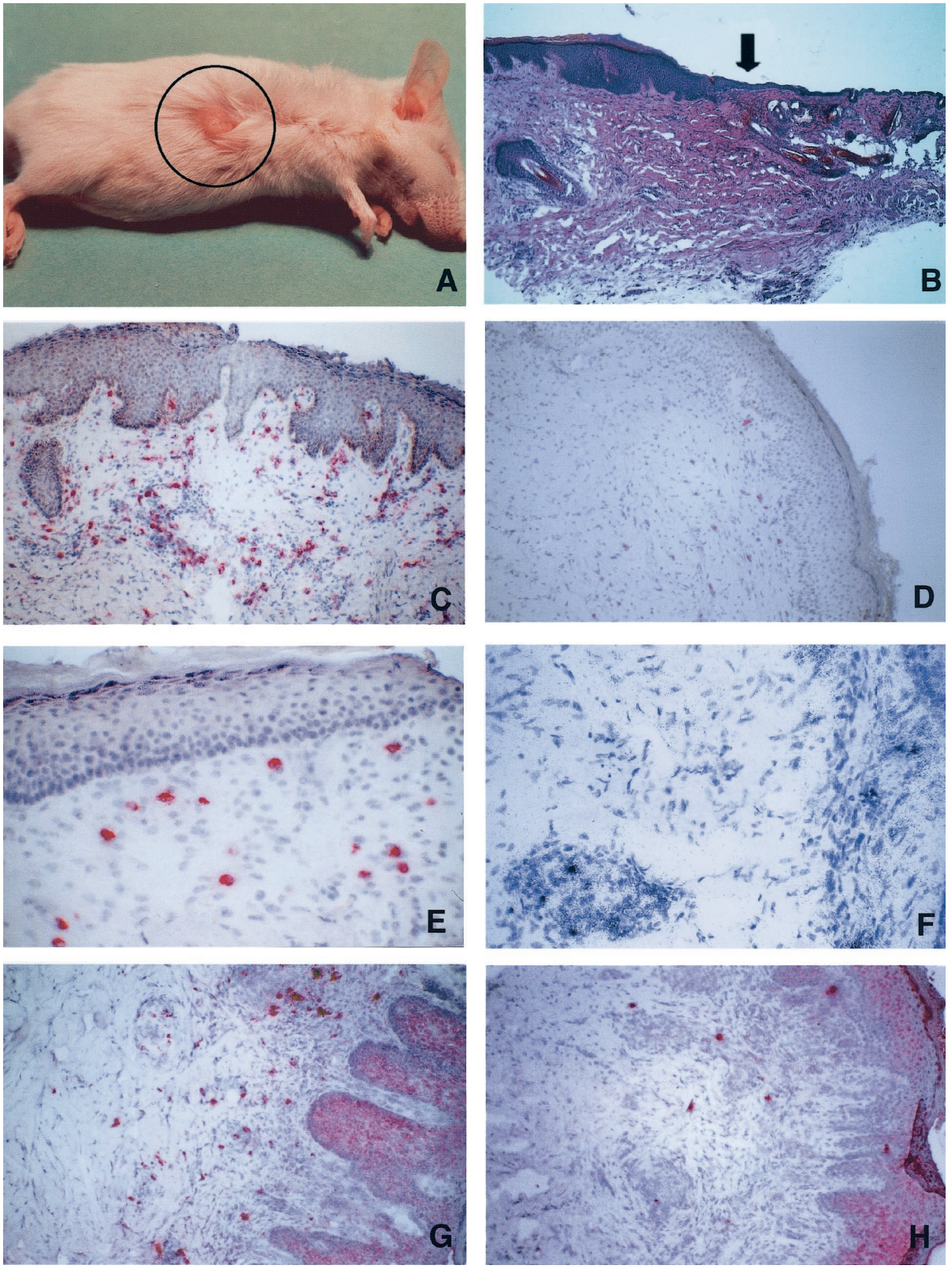

Figure 1.

A, Positive skin prick test to *Dermatophagoides pteronyssinus* (Dpt) in a severe combined immunodeficient (SCID) mouse grafted with human allergic skin. B, May-Grünwald-Giemsa–stained skin graft section showing the junction between human (on the left) and murine skin (on the right of the *arrow*) (original magnification, ×50). Cryostat sections of human allergic skin grafts 24 hours after allergen (C) or diluent (D) injections immunostained with anti-CD4 antibody (original magnification, ×100). E, Anti-BB1 staining in an allergen-injected site (original magnification, ×250). F, Autoradiograph of cryostat sections of human allergic skin graft from an allergen site hybridized with ^{35}S-labeled antisense IL-4 (original magnification, ×250). Anti-mMBP staining of allergen-injected sites in allergic SCID mice treated with IgG2a (G) or anti-CCR3 (H) antibody (original magnification, ×100). Immunostaining was performed by using the alkaline phosphatase anti-alkaline phosphatase technique.

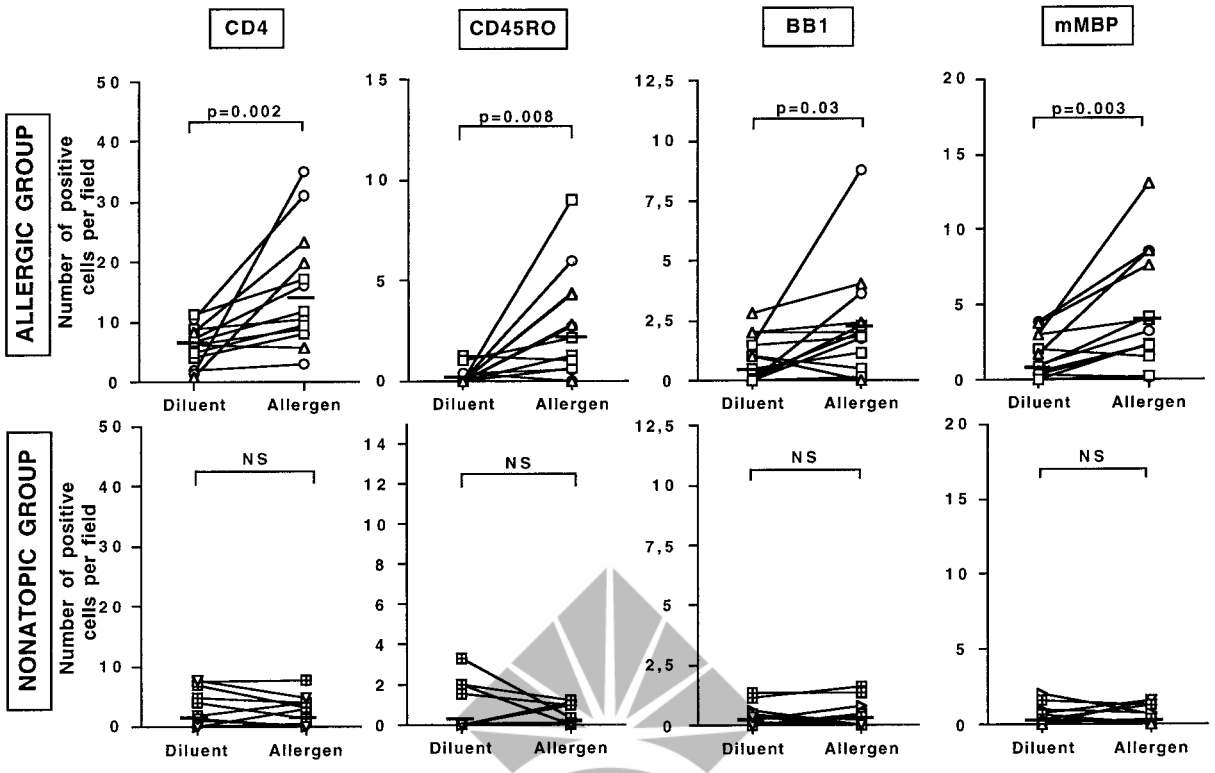

Figure 2.

Infiltration of $CD4^+$ T cells, $CD45RO^+$ memory T cells, $mMBP^+$ eosinophils, and $BB1^+$ basophils after intradermal allergen versus diluent injections in 52 skin grafts obtained from allergic or nonatopic donors (each donor is represented by a different *symbol*), 24 hours after introduction of autologous human PBMC by ip injection. Results are expressed as number of positive cells per field. Median values are represented by the *solid bars*. Within-group comparisons were performed using the ranked Wilcoxon test. NS = not significant.

cated a functional cross-reactivity between the anti-human CCR3 antibody and the murine CCR3 on eosinophils (Fig. 4B).

Anti-CCR3 Antibody Treatment Inhibits In Vivo Eosinophil Recruitment Induced by Allergen Challenge into Human Skin Xenografts from Allergic Donors

After intraperitoneal coadministration of anti-CCR3 antibody and PBMC and intradermal allergen challenge, skin biopsies performed 24 hours later revealed a significant reduction of recruited murine eosinophils, which was not observed with the use of the control IgG2a antibody (Fig. 1, G and H, and Fig. 5). Surprisingly, anti-CCR3 antibody treatment had no significant effect on the numbers of recruited CD4 and $BB1^+$ cells, which were similar to the control IgG2a-treated group. Also, there was no increase in numbers of $CD8^+$, HLA-DR^+, $CD68^+$, and $tryptase^+$ cells after allergen challenge, whatever the group (not shown). Treatment with anti-CCR3 versus control IgG2a antibodies did not affect the number of recruited IL-4 and IL-5 mRNA-expressing cells. The same TH2-type cytokine profile was observed in the IgG2a-treated group and in the allergic group of the pilot study. There was no expression of TH1-type cytokines in any groups. These results strongly suggest that anti-CCR3 Ab affects specifically the recruitment of eosinophils in the context of the cutaneous allergic challenge.

Discussion

There is considerable evidence to support an important role for chemokines and their receptors in the pathophysiology of allergic diseases such as asthma and atopic dermatitis (Lukacs and Tekkanat, 2000; Schroder and Mochizuki, 1999). A detailed understanding of the molecular mechanisms that govern the inflammatory cell recruitment into tissues during inflammation is essential if chemokine-based therapies are to be developed for treatment of allergic diseases. Although animal models of allergic reactions are available, findings cannot always be extrapolated to man. Therefore we took advantage of the SCID mouse model to set up an in vivo model of humanized allergic reaction, using SCID mice engrafted with skin and PBMC from allergic donors.

Human cutaneous allergic reaction is usually divided into two phases: a first immediate reaction occurring within 15 minutes and a delayed or late-phase reaction consisting of a leukocyte infiltration at the site of allergen administration (Frew and Kay, 1988). In the humanized SCID mouse model of allergy, we are able to reproduce both the immediate and late cutaneous allergic reactions. The immediate allergic reaction was only observed in skin xenografts from allergic donors but not in skin from nonatopic donors and not in mouse skin. No PBMC reconstitution was needed, suggesting that the human skin xenografts

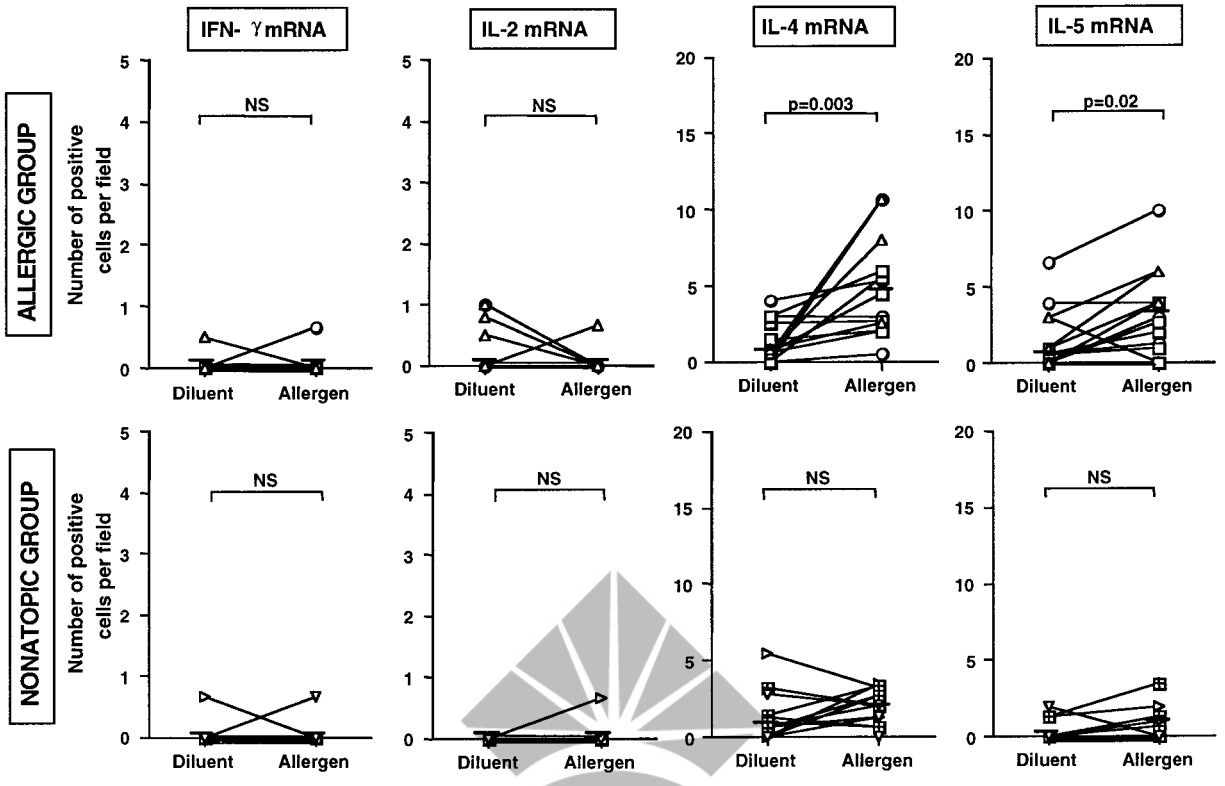

Figure 3.
Cytokine mRNA expression profile after intradermal allergen versus diluent injections in 52 skin grafts obtained from allergic or nonatopic donors (each donor is represented by a different *symbol*), 24 hours after introduction of autologous human PBMC by intraperitoneal injection. Analyses are as in Figure 2.

retained all the components required for the immediate allergic reaction. In our model, the late allergic reaction occurred only with autologous PBMC reconstitution and allergenic challenge of skin xenografts. Human skin and PBMC from allergic donors were both necessary and sufficient for the development of allergic LPR. No human cell migration was observed when allergen was injected into mouse skin in the reconstituted SCID mouse model, and without PBMC, no LPR occurred in allergen-injected human allergic skin of grafted SCID mice. Taken together, the allergen challenge of skin xenografts from allergic donors in reconstituted SCID mice resulted in the development of an immediate reaction and a delayed reaction exactly as observed in skin from allergic patients.

In humans, allergen-induced cutaneous LPR is associated with an infiltration of $CD4^+$ T cells, eosinophils, activated $CD25^+$ cells (Barata et al, 1998; Tsicopoulos et al, 1994), and basophils (Irani et al, 1998; Ying et al, 1999). A preferential TH2-type cytokine mRNA expression with almost no TH1 expression is found at the sites of such cutaneous allergic reactions (Kay et al, 1991; Tsicopoulos et al, 1994). In the humanized SCID mouse model of allergy, allergen-injected skin xenografts from nonatopic individuals did not exhibit cell infiltration, whereas those from atopic subjects reproduced all the main findings in human systems. This cellular infiltration was not a result of MHC incompatibility, based first on the use of autologous mononuclear cells and skin specimens and second on the difference in cell recruitment observed between the diluent- and allergen-injected sites. The cellular infiltration after allergen challenge was not observed in all mice. This was not related to variability among donors, as can be observed from the individual data presented for each donor. Rather, these variations probably reflect the complexity of the model, which requires a good chimerism between the human and the murine systems to be functional. One interesting difference is that the recruited eosinophils were exclusively of murine origin. The accumulation of murine eosinophils in the human skin suggests that some human mediators may attract murine eosinophils. Such cross-reactivity has been demonstrated for human IL-5 and eotaxin (Fattah et al, 1990; Teixeira et al, 1997). There was no infiltration of human eosinophils, in agreement with the mode of reconstitution of SCID mice that involved only mononuclear cells. In a separate set of experiments, we tried to attract human eosinophils to the site of the cutaneous allergic reaction by reconstituting SCID mice with both PBMC and eosinophils from the same donors. No human eosinophil infiltration was observed (not shown). The same results were obtained when cytokines or chemokines known to attract eosinophils were injected into the skin (not shown). Taken together, these results suggest either a lack of eosinophil survival mediators in the vicinity of the peritoneal cavity or an absence of cross-reactivity between human eosinophil adhesion molecules and murine vessels counter receptors, leading to an absence of migration to the skin.

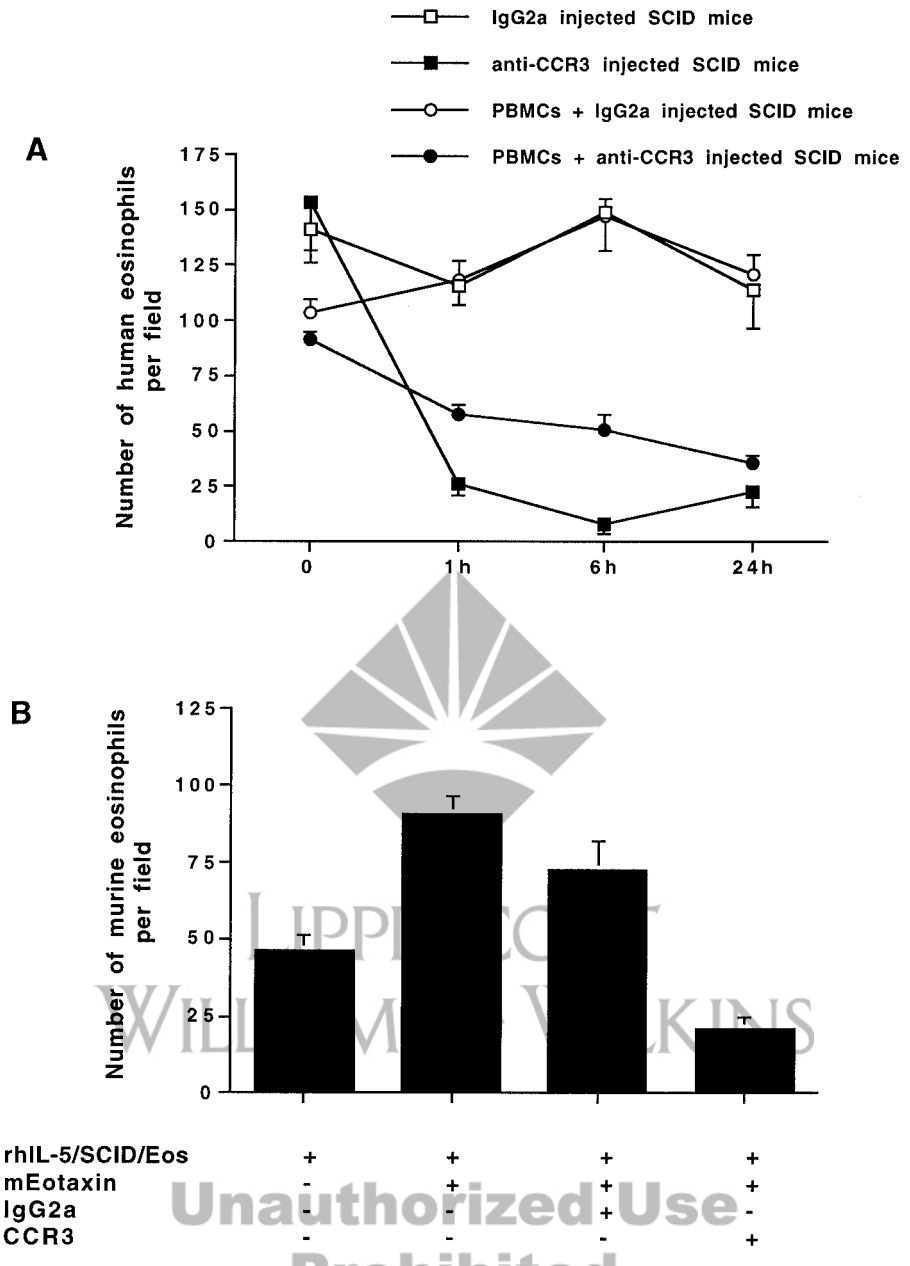

Figure 4.

A, Kinetics of in vivo inhibitory effect of CCR3-blocking antibody on eosinophil chemotaxis. Human eosinophils were incubated with sera from SCID mice collected before (0) and at 1, 6, and 24 hours after ip injection of 25 μg of anti-CCR3 or IgG2a antibodies with or without PBMC, and with human eotaxin as chemoattractant. B, Functional chemotactic cross-reactivity between anti-human CCR3 and murine eosinophils. Eosinophils from recombinant human IL-5-SCID mice were incubated with 20 μg/ml of anti-CCR3 or control IgG2a antibodies and chemoattracted using murine eotaxin. Results are expressed as mean $\pm$ SEM number of recruited eosinophils per field ($n = 3$ for A and B).

One important chemoattractant receptor expressed on infiltrating cells during allergic reactions is represented by CCR3. To investigate its role in allergic reactions, we evaluated the effect of a CCR3-blocking antibody in our humanized model of allergen-induced LPR. A selective inhibition of mouse eosinophil recruitment was observed after skin allergen challenge in the anti-CCR3–treated group. This suggests that in response to allergen, the human skin xenograft produced chemoattractant mediators, such as eotaxin, as already shown in human allergen-induced cutaneous LPR (Ying et al, 1999), able to trigger murine eosinophils through CCR3. The high level of CCR3 expression on both human and murine eosinophils (Ponath et al, 1996) and their similar sensitivity to human eotaxin are consistent with this hypothesis. Moreover, the high amino-acid sequence homology (80%) between murine and human CCR3 explains the cross-reactive inhibitory effect of the anti-human CCR3 antibody on murine eosinophil chemotaxis. Thus, in this humanized SCID mouse model of allergy, we can conclude that the recruitment of eosinophils into human skin xenografts challenged with allergen is mainly mediated by CCR3 and its ligands.

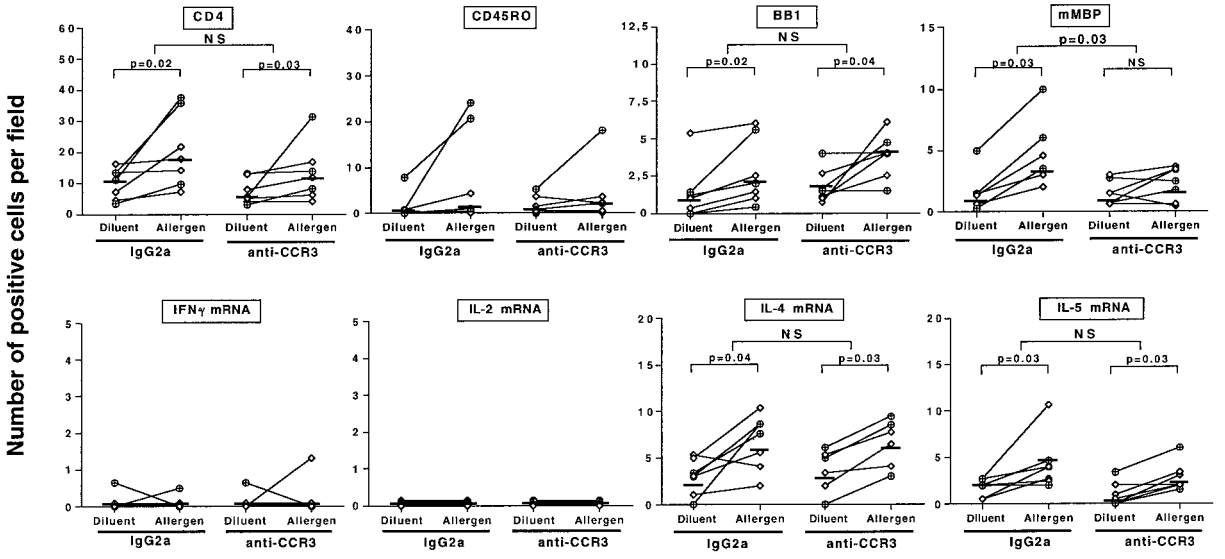

Figure 5.

Infiltration of $CD4^+$ T cells, $CD45RO^+$ memory T cells, $mMBP^+$ eosinophils, $BB1^+$ basophils and cytokine $mRNA^+$ cells after allergen versus diluent challenges in 28 skin grafts obtained from allergic donors (each donor is represented by a different *symbol*), 24 hours after ip introduction of IgG2a- or anti-CCR3-treated autologous human PBMC. Results are expressed as number of positive cells per field. Median values are represented by the *solid bars*. Differences within the four groups were first evaluated by using the Kruskal-Wallis test and were found statistically significant for CD4, BB1, mMBP, IL-4, and IL-5 mRNA but not for CD45RO, IFN-γ, and IL-2 mRNA. When significant, comparisons within allergen and diluent values were performed by using the Wilcoxon test. Comparisons between IgG2a and anti-CCR3-treated groups were performed by using the Mann-Whitney *U* test after subtraction of the diluent values from the allergen values. *p* values are indicated and were considered significant when less than 0.05.

The role of CCR3 in eosinophil recruitment has been studied in other animal models. In a guinea pig model of eosinophil cutaneous recruitment, pretreatment of labeled eosinophils in vitro with a blocking CCR3 antibody inhibited their accumulation in response to eotaxin in vivo (Sabroe et al, 1998). In a model of *Nippostrongylus brasiliensis*-infected mice, in vivo depletion of CCR3-expressing cells led to a reduction in lung eosinophilia (Grimaldi et al, 1999). In the present study, we show for the first time that in a humanized mouse model of cutaneous allergy, allergen-induced recruitment of eosinophils is also dependent upon CCR3. Hitherto, the effect of CCR3 antagonists has only been evaluated on eosinophils, even though CCR3 is also highly expressed on basophils (Uguccioni et al, 1997). In the present study, there was no inhibitory effect of anti-CCR3 treatment on basophil migration, suggesting that these cells may be attracted to the site of the reaction through additional pathways. It is of note that in human allergen-induced cutaneous LPR, it has been recently shown that the expression of CCR3-binding chemokines correlated with the presence of eosinophils but not basophils (Ying et al, 1999). Basophils are known to express CCR4 in particular (Power et al, 1995), which might be another potential target involved in allergic inflammation (Lloyd et al, 2000).

CCR3 was the first chemokine receptor reported to be preferentially expressed on human TH2 cells (Sallusto et al, 1997), in particular cells producing IL-4 (Sallusto et al, 1998). No previous studies have directly addressed the role of CCR3 in vivo on TH2 cell recruitment. Indirect arguments have been provided by assessing the effect of anti-eotaxin antibody on TH2 cell recruitment in a murine model of allergic airway disease. In this study, the involvement of the eotaxin/CCR3 pathway was confined to early stages of the TH2 response in vivo (Lloyd et al, 2000). It is of note that CCR3 expression is differentially regulated in the human and the mouse system. In mice, anti-CD3 stimulation increases CCR3 expression on TH2 cells (Lloyd et al, 2000), whereas it down-regulates it in humans (Sallusto et al, 1999). These species differences underline the interest of the human xenograft model to assess effects of molecules devoted to human usage. In our human skin xenograft model of allergy, anti-CCR3 treatment had no effect on CD4 T-cell recruitment, which is in line with the very small proportion of peripheral blood T cells expressing CCR3 (Sallusto et al, 1997), but also had little or no effect on TH2-type cytokine mRNA-expressing cells. It is probable that, as for basophils, TH2-type $mRNA^+$ cells may be recruited in vivo through additional chemokine receptors, such as CCR4 and CCR8, expressed on the latter cells (Bonecchi et al, 1998). In this context, it has been reported that in human allergic cutaneous reactions, there is an overexpression of thymus activation regulated chemokine (CCL17) (Kakinuma et al, 2001; Vestergaard et al, 2000) and macrophage-derived chemokine (CCL22) (Galli et al, 2000), which bind CCR4 (Imai et al, 1997, 1998). The in vivo relevance of the CCR4 pathway on TH2 cell recruitment in allergic inflammation has been assessed indirectly using antibody against macrophage-derived chemokine in a murine model, where it was mainly effective on the chronic recruitment of TH2 cells (Lloyd et al, 2000). The CCR8 receptor has also been recently shown to be implicated in TH2 func-

tional responses in vivo. Interestingly, CCR8-deficient mice display impaired TH2 cytokine production and reduction in eosinophils in a model of ovalbumin-induced allergic airway inflammation (Chensue et al, 2001). Taken together, these data suggest that chemokine receptors may play a redundant role in basophil and TH2 cell recruitment in allergic reactions.

In conclusion, the present study establishes an efficient humanized model of allergic cutaneous reaction by using human skin and PBMC xenografts in SCID mice. In addition, the study demonstrates that CCR3 is a major mechanism of eosinophil recruitment in allergic cutaneous LPR, although additional pathways are likely to be involved in the recruitment of the noneosinophilic cells. Finally, these data open new insights in the evaluation of potential human-specific antiallergic molecules.

Materials and Methods

Reagents

Anti-CD4 monoclonal antibody was purchased from Becton Dickinson (Pont de Claix, France). EG2 was obtained from Pharmacia (St. Quentin en Yvelines, France). Anti-CD8, anti-CD45RO (T-cell memory phenotype), anti-CD68 (macrophages), anti-HLA-DR, rabbit anti-mouse Ig, monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP), and control IgG antibodies were from Dako (Trappes, France). Anti-tryptase antibody was from Chemicon (Temecula, California). BB1, a human basophil-specific monoclonal antibody, and rabbit antiserum specific for murine eosinophil major basic protein (mMBP) were produced as previously described (Filley et al, 1981; McEuen et al, 1999). Biotin-labeled goat anti-rabbit antibody, extravidin-alkaline phosphatase, and Fast Red TR/naphthol ASMX tablets were from Sigma (St. Quentin Fallavier, France). Dpt extract was obtained from Stallergènes (Antony, France). The endotoxin concentration was less than 0.125 EU/ml for the dose of Dpt used as assessed by the Limulus amebocyte lysate test (BioWhittaker, Walkersville, Maryland). Anti-human CCR3-blocking monoclonal antibody (7B11), from Leukosite Inc., was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Heath et al, 1997; Ponath et al, 1996). A control monoclonal antibody of the same isotype (IgG2a) was purchased from Diaclone (Besançon, France).

Human Donors

The human donors were separated into two groups according to their atopic status. Allergic donors ($n = 5$) had a history of perennial rhinitis or asthma and exhibited positive skin prick tests to Dpt extracts and positive radio allergo sorbent tests (RASTs). The total IgE level was 632 ± 284 KU/ml (mean ± SEM). Nonatopic donors ($n = 3$) served as a control group, and their mean total IgE level was 125 ± 54 KU/ml. Skin from human donors was obtained from plastic surgery or from truncal operations where skin was discarded and blood was collected on heparin 6 weeks later. The protocol was approved by the Centre Hospitalier Regional et Universitaire Ethical Committee (no. 96–102). All donors signed an informed consent form.

Animals and Skin Grafting

Inbred mice with severe combined immunodeficiency (CB-17 SCID mice) were maintained at the Institut Pasteur de Lille in sterilized isolators. Leaky mice (displaying spontaneous IgG production) were discarded. The mice were housed under pathogen-free conditions. Animals were handled according to the ethical principles of animal experimentation established by the European Center of Tufts University. Skin grafting was performed as previously described (Tsicopoulos et al, 1998; Yan et al, 1993). Two full-thickness human skin grafts of the same size were placed onto the wound beds on each mouse. The transplants were held in place with 6-0 silk suture material and covered with an adhesive wound dressing and then with an adhesive bandage. The dressing material and sutures were removed 10 days after transplantation.

Study Design: Pilot, CCR3, and Eosinophil Studies

Six weeks after human skin transplantation, anti-asialo GM1 (50 μl; Wako, Osaka, Japan) was injected ip to neutralize murine natural killer activity. Twenty-four hours later, SCID mice were reconstituted ip with 15 to 20×10^6 PBMC (hu-PBMC-SCID mice), purified from the donor's blood using a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient after platelet depletion and resuspended in PBS at a concentration of 40×10^6 cells/ml. One IR unit of Dpt (index reactivity unit) in 20 μl of endotoxin-free saline was then injected intradermally immediately, with 5% Evans blue dye (Sigma) to mark the site of injection. The contralateral graft of each mouse was injected with endotoxin-free saline diluent containing 5% Evans blue dye to serve as internal control. In the CCR3 study, the same protocol was followed, except that PBMC were incubated with either the 7B11 anti-CCR3 antibody or with an irrelevant isotype-matched IgG2a antibody at the predetermined optimal dose (see chemotaxis assays) of 25 μg/mouse for 30 minutes before ip reconstitution.

In a different set of experiments, human eosinophils, obtained from allergic donors and purified as described below, were injected together with autologous PBMC at a concentration of 10^6 cells. One graft was injected with the diluent, and the second one with allergen, as described above, or the cytokines IL-4 (2000 U) or IFN-γ plus TNF-α (2000 U each), or chemokines (eotaxin or RANTES at 4 μg). In all experiments, mice were killed 24 hours after the injections, and the skin grafts were cut into two halves through the center of the injection site marked by the Evans blue dye. One half was immediately embedded in OCT compound (Labonord, Villeneuve d'ascq, France), snap-frozen in isopentane precooled in liquid nitrogen, and stored at −80° C. Cryostat

sections (6 μm) were cut, air-dried, fixed in a mixture of 60% acetone and 40% methanol, dried, wrapped in foil, and stored at −20° C until used for immunohistochemistry. The other half was fixed in 4% paraformaldehyde (for in situ hybridization studies) and washed in 15% phosphate-buffered saline/sucrose. Cryostat sections (10 μm) were cut onto poly-L-lysine–coated RNase-free slides and stored with silica gel at −80° C until use.

Mice with spontaneously activated grafts, as evidenced by leukocyte infiltration in the diluent-injected site, were discarded. The pilot study included three allergic donors and three nonatopic donors as a control group, with a total of 13 mice in each group representing 52 grafts. In the CCR3 study, two additional allergic donors were included, with a total of 14 mice representing 28 grafts.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Tsicopoulos et al, 1994) using a modified APAAP method for anti-human monoclonal antibody staining. Briefly, sections were incubated with the primary antibody for 30 minutes, washed in tris-buffered saline, and successively incubated for 30 minutes with rabbit anti-mouse and then APAAP diluted in 20% normal human serum. For anti-mMBP staining, the second reagent was a biotinylated goat anti-rabbit antibody, and the third reagent was extravidin-alkaline phosphatase. The color was developed using Fast Red. Sections were hematoxylin counterstained. Substitution of the primary antibody with an irrelevant antibody of the same species was used as negative control. No cross-reactivity was observed with murine cells.

In Situ Hybridization

In situ hybridization was performed as previously described (Hamid et al, 1987; Tsicopoulos et al, 1998). Briefly, sections were hybridized with ^{35}S-labeled riboprobes (2×10^6 cpm per section) in hybridization buffer containing 50 mM dithiothreitol (Promega, Madison, Wisconsin). Sections were hybridized overnight at 45° C and washed in high-stringency conditions (45° C, 0.1× SSC). Human peripheral blood-derived polarized TH1 or TH2 cells that expressed mRNA for IL-2 and IFN-γ or IL-4 and IL-5 were used as cytokine expression positive controls. For negative controls, skin sections were hybridized using sense probes for the relevant cytokines. Additional sections were treated with RNase-A before the prehybridization step with antisense probes. Specific hybridization was recognized as clear dense deposits of silver grains in the emulsion overlying cells in the tissue preparations. Cells were identified as dense, discrete, well-circumscribed areas of silver grains. When hybridized cells were in close proximity, their numbers were determined by visualizing the number of individual nuclei, using dark field illumination.

Human and Murine Eosinophil Chemotaxis Assay

Highly purified human eosinophils (95.4 ± 1.6%), obtained from peripheral blood of hypereosinophilic patients, were produced as previously described (Fahy et al, 2000) using magnetic beads coated with anti-CD16 (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany) and resuspended in RPMI at a concentration of 10^6 cells/ml. Human eosinophils were incubated with sera from SCID mice diluted 1:5 with RPMI, which had been collected before and at 1, 6, and 24 hours after ip injection of either anti-CCR3 or IgG2a control antibody (10 or 25 μg/mouse) with or without PBMC. The chemotaxis protocol was performed with a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, Maryland) using 5-μm pore polycarbonate filters (Nucleopore Corporation, Pleasanton, California) during 1 hour at 37° C in 5% CO_2. Chemotaxis was induced using human eotaxin at 10^{-7} M (Tebu, Le Perray en Yvelines, France).

Murine eosinophils were obtained from recombinant human IL-5-SCID mice as previously described (Fattah et al, 1990). Briefly, SCID mice were treated using 25 μg of recombinant human IL-5 (R&D, Minneapolis, Minnesota) delivered during 7 days through subcutaneous osmotic pumps (Charles River, St. Aubin, France). After death, spleen eosinophils were resuspended at a concentration of 10^6 cells/ml and incubated with medium, control IgG2a, or anti-CCR3 antibody at a final concentration of 20 μg/ml. Chemotaxis was induced using murine eotaxin at 10^{-7} M (Tebu).

Quantification and Statistical Studies

Slides were encoded and counted in a blinded fashion at ×250 magnification using an eyepiece graticule. The upper edge of the grid was placed at the epidermal junction. For each specimen, at least three sections were evaluated, from which three to nine fields were counted for both immunohistochemistry and in situ hybridization studies. The absolute number of positive cells was counted per field (0.4 mm^2). Statistical analysis was performed using Statview 5 software. Comparisons within allergen- and diluent-injected sites were performed by using the Wilcoxon matched-pairs signed rank test. Differences within anti-CCR3- and IgG2a-treated groups were first evaluated for the four subgroups using the Kruskal-Wallis test. When statistical significance was observed, differences were subsequently analyzed by using the Wilcoxon test for diluent/allergen pairs and the unpaired Mann-Whitney *U* test for anti-CCR3- and IgG2a-treated groups after subtraction of the diluent value from the allergen value. Only *p* values less than 0.05 were considered significant.

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