

The role of the C-C chemokine receptor-5 $\Delta 32$ polymorphism in asthma and in the production of regulated on activation, normal T cells expressed and secreted

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Background: There are conflicting data regarding the role of a deletion in the C-C chemokine receptor-5 gene (*CCR5* $\Delta 32$*) in the pathogenesis of asthma and whether this deletion influences the production of regulated on activation, normal T cells expressed and secreted (RANTES). RANTES is a chemokine that is known to play an important role in the pathogenesis of allergic asthma.

Objective: We sought to determine whether *CCR5* $\Delta 32$* is associated with increased RANTES production, the presence of asthma, and the severity of asthma.

Methods: A PCR assay for *CCR5* $\Delta 32$* was developed. The prevalence of *CCR5* $\Delta 32$* was determined in a group of patients with mild-to-moderate asthma, a group of subjects with severe asthma who had fatal or near-fatal asthma attacks, and a group of nonasthmatic control subjects. The level of RANTES produced by stimulated and unstimulated T cells was measured by using a commercially available immunoassay.

Results: The frequency of *CCR5* $\Delta 32$* was not significantly increased in the severe asthma group compared with in the mild-to-moderate asthma group. *CCR5* $\Delta 32$* was not increased in the asthmatic subjects versus in the control subjects. There was no significant increase in RANTES levels from T cells heterozygous for *CCR5* $\Delta 32$* compared with wild-type cells.

Conclusion: These data indicate that the *CCR5* $\Delta 32$* allele is not a genetic risk factor for the development of asthma and does not influence disease severity. The *CCR5* $\Delta 32$* allele does not influence RANTES production in the heterozygous state. (J Allergy Clin Immunol 2001;108:69-73.)

Key words: Chemokine, receptor, deletion, asthma

The pathogenesis of asthma is likely to involve the interaction of a heterogeneous genetic component with several environmental factors.^{1,2} Asthma is characterized by airway inflammation leading to structural remodeling of the airway wall and nonspecific bronchial hyperresponsiveness. In this study we investigated the contribution of C-C chemokine receptor-5 (*CCR5*) genotype to the pathogenesis of asthma.

Abbreviations used

CCR5: C-C chemokine receptor-5

MIP: Macrophage inflammatory protein

RANTES: Regulated on activation, normal T cell expressed and secreted

Chemokines are a family of low-molecular-weight cytokines that attract leukocytes to sites of inflammation. The subgroup known as C-C chemokines are primarily chemotactic for monocytes, lymphocytes, and eosinophils. The C-C chemokines are closely related proteins that include macrophage inflammatory protein (MIP) 1 α , MIP-1 β , and regulated on activation, normal T cells expressed and secreted (RANTES). CCR5 binds RANTES and MIP-1 α and is the only known receptor for MIP-1 β .^{3,4} CCR5 is expressed on T cells, monocytes, and macrophages.^{5,6} Receptors for RANTES also include CCR1 and CCR3, and for MIP-1 α , receptors include CCR1.^{7,8}

A 32-bp deletion in the *CCR5* gene (*CCR5* $\Delta 32$*) has been described and is a frame-shift mutation and results in the production of a truncated and inactive protein.^{9,10} Homozygosity for this mutation confers protection from infection by non-syncytium-inducing HIV-1 strains.^{11,12} T cells from *CCR5* $\Delta 32$* heterozygotes were shown to express 5- to 10-fold less *CCR5* than wild-type cells after stimulation.¹³ In addition, CD4⁺ T-cell clones from 2 individuals who were homozygous for this deletion produced approximately 20-fold more RANTES than wild-type clones.¹⁴

If individuals with *CCR5* $\Delta 32$* produce high levels of C-C chemokines, this would be relevant to asthma because considerable evidence suggests that C-C chemokines play an important role in allergic inflammation. The protein levels of MIP-1 α and RANTES were increased in bronchoalveolar lavage fluid from asthmatic patients.¹⁵ RANTES and, to a lesser extent, MIP-1 α are chemotactic for eosinophils.¹⁶ Eosinophils infiltrate the respiratory epithelium in asthma, and correlations have been shown between the number of infiltrating eosinophils and disease severity.^{17,18}

Two previous studies of the role of *CCR5* $\Delta 32$* in the pathogenesis of asthma produced conflicting results.^{19,20} Neither study investigated the role of *CCR5* $\Delta 32$* in influencing asthma severity. We hypothesized that the

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*CCR5*D32* allele may be a risk factor for asthma, a modifier of asthma severity, or both by virtue of an effect on RANTES production by T cells. Therefore we compared the prevalence of the *CCR5*D32* allele in a group of subjects with mild-to-moderate asthma with that in a group of nonasthmatic, nonatopic control subjects. We also genotyped a group of subjects with severe asthma for *CCR5*D32* to determine whether this allele was associated with increased severity of disease. In addition, we compared the level of RANTES secretion from wild-type T cells in vitro with the level from T cells that were heterozygous for *CCR5*D32*.

METHODS

Subjects

The prevalence of *CCR5*D32* was determined in 3 groups of individuals: subjects with severe asthma, subjects with mild-to-moderate asthma, and nonasthmatic, nonatopic control subjects. The group of subjects with severe asthma consisted of 119 subjects who had fatal asthma attacks and 27 patients with near-fatal asthma. All of the subjects with fatal asthma were less than 50 years of age to minimize possible confusion with other diseases, such as chronic obstructive pulmonary disease. Subjects with fatal asthma had a history of asthma with recent worsening of symptoms leading up to the fatal asthmatic episode. In addition, classical asthmatic histopathology was confirmed on microscopic study of the lung by 2 pathologists. Subjects with near-fatal asthma had a history of asthma and were intubated in a hospital emergency department ($n = 25$) or had hypercapnic ($\text{PaCO}_2 > 45$ mm Hg) respiratory failure ($n = 2$) during an acute asthmatic episode.

The second study group consisted of 87 subjects with mild-to-moderate asthma. These subjects had no previous hospital admissions for asthma in the last year and were not taking oral steroids. These subjects included patients screened for inclusion in clinical trials, and all subjects fulfilled American Thoracic Society diagnostic criteria for asthma. All these subjects were atopic as defined by a positive skin test response (wheal response 2 mm larger than that produced by saline control) for at least one of 6 common aeroallergens.

In addition, we recruited a group of 148 nonasthmatic control subjects, all of whom were nonatopic. Nonasthmatic subjects had no personal history of asthma or atopy and had negative skin test responses (wheal response <1 mm larger than that produced by saline control) to common aeroallergens (ie, cat, dog, house dust mite, grass, molds, and trees).

All of the study subjects were white. Ethical approval for the study was gained from the University of British Columbia and St Paul's Hospital Ethics Review Boards.

Genotyping

DNA was extracted from either paraffin-embedded tissue blocks or peripheral blood by using previously described techniques.^{21,22} The samples were genotyped by amplification of the region containing the deletion, as determined with the PCR assay. The following primers were designed from the published sequence⁶: 5'-AGG TCT TCA TTA CAC CTG CAG C-3' and 5'-CTT CTC ATT TCG ACA CCG AAG C-3'. PCR was performed in a 20- μ L volume containing 100 ng of genomic DNA; 1 μ mol/L of each primer; 200 μ mol/L each of deoxyguanosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate, and deoxyadenosine triphosphate; 1.5 mmol/L MgCl_2 , and 0.5 units of Taq DNA polymerase. Amplification conditions were 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 second.

The PCR produced a 169-bp product from the wild-type allele and a 137-bp product from the deletion allele. The products of each

PCR reaction were resolved with electrophoresis on a 3% agarose gel stained with ethidium bromide (Fig 1). The genotyping protocol was confirmed by means of direct sequencing of PCR products from 2 homozygous wild-type individuals and 2 individuals who were homozygous for the *CCR5*D32* allele.

T-cell purification

PBMCs were purified from a venous blood sample mixed with ACD (Anticoagulant Citrate Dextrose Solution USP, Formula A) and centrifuged on a Ficoll-Hypac 1077 density gradient. The red blood cells were lysed, and T cells were isolated with Lympho-kwik T.

T-cell culture

Isolated T cells were diluted to $5 \times 10^5/\text{mL}$ with complete medium by adding RPMI-1640 containing glutamine, 10% FBS (heat inactivated), penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$). Samples were distributed in duplicate into a 24-well plate, with each well containing 10^5 cells. The cells were stimulated with either PHA-P at 5 $\mu\text{g}/\text{mL}$, IL-2 at 1 ng/mL, anti-CD3 at 100 ng/mL, or PHA-P at 5 $\mu\text{g}/\text{mL}$ plus anti-CD3 at 100 ng/mL. The cells were incubated in a humidified 37°C, 5% CO_2 incubator for 72 and 96 hours. Supernatants from the cultures were recovered and frozen at -20°C for subsequent measurement of RANTES levels by means of ELISA.

Measurement of RANTES levels

RANTES levels in the supernatants were determined by using a commercially available ELISA kit according to the manufacturer's instructions (R&D Systems, Inc). The minimum detectable concentration of RANTES with this assay was 8 pg/mL. RANTES levels were measured in T-cell cultures from 13 homozygous wild-type individuals and 5 heterozygotes. These subjects were volunteers who worked in our laboratory.

Statistical analysis

The genotype distribution in the different groups was compared by using χ^2 analysis for a 2×2 table or by using the Fisher exact test. RANTES levels in the 2 genotypic groups were compared by using an unpaired t test.

RESULTS

The characteristics of each patient population are shown in Table I. There were no significant differences between the groups in sex ratio or age.

The *CCR5*D32* genotype distribution in the study groups is shown in Table II. The genotype distributions were in Hardy-Weinberg equilibrium, both within each phenotypic group and in the population as a whole. Contrary to our hypothesis, there was no difference in genotype or allele frequencies between any of the study groups.

Preliminary studies showed that cell culture for 72 hours produced similar results to culture for 96 hours (Fig 2). Therefore in all subsequent experiments, a 72-hour culture period was used. The use of IL-2 alone did not stimulate RANTES production above baseline levels. The use of PHA-P, anti-CD3, and a combination of these agents produced similar levels of stimulation. Therefore in subsequent experiments PHA-P alone was used to stimulate RANTES production.

The production of RANTES from T cells homozygous for the wild-type *CCR5* allele was compared with the production from cells heterozygous for the *CCR5*D32* allele

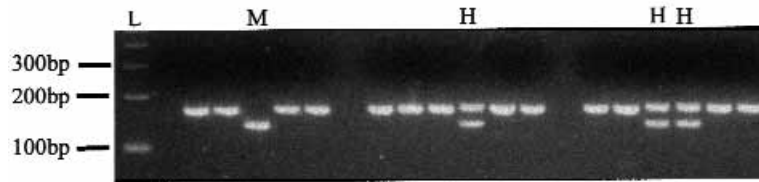


FIG 1. Genotyping of *CCR5* alleles by means of PCR amplification. Lane L, 100-bp ladder; Lane H, heterozygotes; Lane M, homozygous mutant; other lanes, homozygous wild-type.

TABLE I. Characteristics of the study subjects

Phenotype	Nonasthmatic subjects	Subjects with mild-to-moderate asthma	Subjects with fatal or near-fatal asthma
No.	148	87	146
Age, y (mean $\pm$ SE)	37.7 $\pm$ 1.2	35.0 $\pm$ 1.6	34.4 $\pm$ 1.3
Male sex (%)	54.7	44.0	44.8

There were no significant differences among the groups.

TABLE II. Frequency of *CCR5**D32 genotypes in the study groups

Phenotype	Homozygous wild-type	Heterozygous	Homozygous <i>CCR5</i> *D32
No asthma (%)	115 (78)	30 (20)	3 (2)
Mild-to-moderate asthma (%)	65 (75)	21 (24)	1 (1)
Fatal or near-fatal asthma (%)	106 (73)	35 (24)	5 (3)

There were no significant differences among the groups.

(Table III). Contrary to our hypothesis, there was no significant difference in RANTES level between the genotypic groups under basal conditions or with PHA-P stimulation.

DISCUSSION

In this study we examined the potential role of the *CCR5**D32 deletion in the pathogenesis of asthma. The results of a previous study suggested that the *CCR5**D32 allele may lead to upregulation of RANTES and MIP-1 α . Several studies have shown the importance of RANTES and MIP-1 α levels in asthma. Sputum levels of RANTES and MIP-1 α increase in asthmatic patients before the late-phase response of an asthma attack.²³ Furthermore, RANTES levels are increased in skin biopsy specimens from atopic subjects after allergen challenge, and this increase is correlated with infiltration by T cells and macrophages.²⁴ The level of MIP-1 α is increased in bronchoalveolar lavage fluid after antigen challenge in asthmatic subjects.²⁵ Antibodies to RANTES or MIP-1 α reduced the intensity of eosinophil recruitment to the lung and airway in a mouse model of allergic airway disease.²⁶ These data suggested that the *CCR5* gene was a good candidate gene for asthma.

There was no difference in the frequency of the *CCR5**D32 allele in asthmatic subjects compared with that in control subjects in this study. There was also no difference in *CCR5**D32 allele frequency in subjects with severe asthma compared with those with mild-to-moderate asthma. This suggests that *CCR5**D32 does not confer increased risk for the development of asthma. These data support the results of Mitchell et al,²⁰ who used the transmission disequilibrium test in 313 families, each

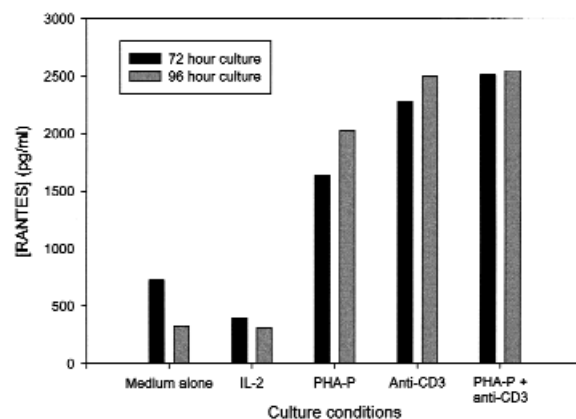


FIG 2. Levels of secreted RANTES under different cell-culture conditions.

TABLE III. Production of RANTES in T cells homozygous for the wild-type *CCR5* allele versus cells heterozygous for the *CCR5**D32 allele

	RANTES production, pg/mL (mean $\pm$ SD)	
	Homozygous wild-type	Heterozygous
No.	13	5
Basal conditions	244 $\pm$ 177	165 $\pm$ 42
With PHA (5 μ g/mL)	1377 $\pm$ 1205	1306 $\pm$ 888

Cells were cultured for 72 hours before measurement of RANTES levels. There were no significant differences in RANTES levels between the genotypic groups.

with at least 2 affected individuals. The *CCR5***D32* allele was not associated with atopy or asthma in this study. In contrast Hall et al¹⁹ showed that *CCR5***D32* was protective against the development of asthma. The odds ratio for *CCR5***D32* in this study of 98 asthmatic children and 317 control subjects was 0.36. The authors proposed that *CCR5***D32* was protective against asthma because individuals would be less responsive to RANTES, MIP-1 α , and MIP-1 β as a result of reduced levels of CCR5. However, this may not be the case because both RANTES and MIP-1 α can mediate their effect through other C-C chemokine receptors.

The reasons for the conflicting data from these genetic studies are not known. Although all of the studies of *CCR5***D32* enrolled white individuals, each study recruited participants from different countries. Therefore there may be differences in the study populations, either in genetic background or exposure to environmental factors. Such differences could result in an association in one population that does not occur in others. Alternatively, the association reported by Hall et al¹⁹ could be spurious because of undetected population stratification.

In this study we have used fatal or near-fatal asthma as a marker of severity. The definition of asthma severity is difficult because factors such as poor medical care and patient noncompliance can increase the severity of symptoms and the risk for a life-threatening attack. Nevertheless, fatal and near-fatal asthma attacks are clearly defined clinical end points, and it is important to identify factors that predispose an individual to disease of this severity. However, there was no significant increase in the frequency of *CCR5***D32* in the subjects with severe asthma compared with that in the group with mild-to-moderate disease. This suggests that *CCR5***D32* does not influence the degree of asthma severity.

In a previous study T cells derived from individuals homozygous for *CCR5***D32* were shown to secrete approximately 20-fold more RANTES and approximately 10-fold more MIP-1 α than wild-type cells in vitro.¹⁴ The reason for this elevated chemokine production is not known, but it could reflect the lack of negative feedback on chemokine production caused by the absence of the functional *CCR5* gene. In contrast, a recent study showed no difference in RANTES or MIP-1 α production in whole blood samples from individuals heterozygous for *CCR5***D32* compared with samples from homozygous wild-type individuals.²⁷ The differences between these studies may be because *CCR5***D32* is a recessive allele or may be because of the different experimental approaches (ie, use of isolated T cells vs whole blood). In this study of isolated T cells, we also were unable to show any difference in RANTES production between heterozygotes and wild-type homozygotes. This is an interesting finding because heterozygotes are known to have lower levels of CCR5 compared with wild-type individuals.¹³ It is possible that the lack of difference between heterozygotes and wild-type homozygotes is due to the considerable redundancy in the chemokine system. In heterozygotes the reduction of CCR5 may be

compensated for by another chemokine receptor. However, cells may not be able to compensate for the complete absence of CCR5 in *CCR5***D32* homozygotes, resulting in excessive chemokine production.

In summary, we found that *CCR5***D32* was not associated with asthma or disease severity. In addition, heterozygosity for *CCR5***D32* did not result in upregulation of RANTES. We conclude that *CCR5***D32* is unlikely to play a major role in the susceptibility for asthma.

REFERENCES

- Sandford A, Weir T, Paré P. The genetics of asthma. *Am J Respir Crit Care Med* 1996;153:1749-65.
- Platts-Mills TA. How environment affects patients with allergic disease: indoor allergens and asthma. *Ann Allergy* 1994;72:381-4.
- Samson M, Labbe O, Mollereau C, Vassart G, Parmentier M. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemistry* 1996;35:3362-7.
- Combadiere C, Ahuja SK, Tiffany HL, Murphy PM. Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1 α , MIP-1 β , and RANTES. *J Leukoc Biol* 1996;60:147-52.
- Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhardt M, et al. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996;381:661-6.
- Raport CJ, Gosling J, Schweickart VL, Gray PW, Charo IF. Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1 β , and MIP-1 α . *J Biol Chem* 1996;271:17161-6.
- Neote K, DiGregorio D, Mak JY, Horuk R, Schall TJ. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell* 1993;72:415-25.
- Ponath PD, Qin S, Post TW, Wang J, Wu L, Gerard NP, et al. Molecular cloning and characterization of a human eosinophil receptor expressed selectively on eosinophils. *J Exp Med* 1996;183:2437-48.
- Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 1996;86:367-77.
- Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, et al. Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 1996;382:722-5.
- Huang Y, Paxton WA, Wolinsky SM, Neumann AU, Zhang L, He T, et al. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat Med* 1996;2:1240-3.
- Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* 1996;273:1856-62.
- Wu L, Paxton WA, Kassam N, Ruffing N, Rottman JB, Sullivan N, et al. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. *J Exp Med* 1997;185:1681-91.
- Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, et al. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 1996;381:667-73.
- Alam R, York J, Boyars M, Stafford S, Grant JA, Lee J, et al. Increased MCP-1, RANTES, and MIP-1 α in bronchoalveolar lavage fluid of allergic asthmatic patients. *Am J Respir Crit Care Med* 1996;153:1398-404.
- Rot A, Krieger M, Brunner T, Bischoff SC, Schall TJ, Dahinden CA. RANTES and macrophage inflammatory protein 1 α induce the migration and activation of normal human eosinophil granulocytes. *J Exp Med* 1992;176:1489-95.
- Carroll N, Cooke C, James A. The distribution of eosinophils and lymphocytes in the large and small airways of asthmatics. *Eur Respir J* 1997;10:292-300.
- Bousquet J, Chané P, Lacoste JY, Barneon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033-9.
- Hall IP, Wheatley A, Christie G, McDougall C, Hubbard R, Helms PJ.

- Association of CCR5 Δ 32 with reduced risk of asthma. *Lancet* 1999;354:1264-5.
20. Mitchell TJ, Walley AJ, Pease JE, Venables PJW, Wiltshire S, Williams TJ, et al. Delta 32 deletion of CCR5 gene and association with asthma or atopy. *Lancet* 2000;356:1491-2.
21. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Press; 1989.
22. Cooper CS, Stratton MR. Extraction and enzymatic amplification of DNA from paraffin-embedded specimens. In: Matthew C, editor. *Methods in molecular biology*, vol 9: protocols in human molecular genetics. Clifton (NJ): The Humana Press Inc; 1991. p. 133-40.
23. Kurashima K, Mukaide N, Fujimura M, Schroder JM, Matsuda T, Matsushima K. Increase of chemokine levels in sputum precedes exacerbation of acute asthma attacks. *J Leukoc Biol* 1996;59:313-6.
24. Ying S, Taborda-Barata L, Meng Q, Humbert M, Kay AB. The kinetics of allergen-induced transcription of messenger RNA for monocyte chemoattractant protein-3 and RANTES in the skin of human atopic subjects: relationship to eosinophil, T cell, and macrophage recruitment. *J Exp Med* 1995;181:2153-9.
25. Cruikshank WW, Long A, Tarpy RE, Kornfeld H, Carroll MP, Teran L, et al. Early identification of interleukin-16 (lymphocyte chemoattractant factor) and macrophage inflammatory protein 1 alpha (MIP1 alpha) in bronchoalveolar lavage fluid of antigen-challenged asthmatics. *Am J Respir Cell Mol Biol* 1995;13:738-47.
26. Lukacs NW, Strieter RM, Warmington K, Lincoln P, Chensue SW, Kunkel SL. Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in allergic airway inflammation. *J Immunol* 1997;158:4398-404.
27. Yang JY, Togni M, Widmer U. Heterozygous defect in HIV-1 coreceptor CCR5 and chemokine production. *Cytokine* 1999;11:1-7.