

Release of both CCR4-active and CXCR3-active chemokines during human allergic pulmonary late-phase reactions

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Background: Segmental antigen bronchoprovocation has long been used as a model to study allergic pulmonary inflammatory responses. Among the characteristics of the resulting cellular infiltrate is the preferential recruitment of T_H2 lymphocytes. The mechanisms responsible for their selective recruitment remain unknown, but T_H2 cells preferentially express the chemokine receptors CCR4 and CCR8.

Objectives: We tested the hypothesis that the chemokines thymus- and activation-regulated chemokine (TARC) (CCL17) and macrophage-derived chemokine (MDC) (CCL22), whose receptor is CCR4, and I-309 (CCL1), whose receptor is CCR8, would be released at sites of segmental allergen challenge.

Methods: Segmental allergen challenge with saline or allergen was performed in 10 adult allergic subjects with asthma, who were off medications. Bronchoalveolar lavage (BAL) was performed at both the saline- and allergen-challenged sites 20 hours after challenge. BAL fluids were analyzed for total cell counts and differentials, and supernatants were assayed by ELISA for levels of TARC, MDC, and I-309. As a control, the BAL fluids were also analyzed for levels of interferon-inducible protein 10 (IP-10) (CXCL10), an IFN- γ -induced chemokine active on CXCR3, a chemokine receptor that is preferentially expressed on T_H1 lymphocytes.

Results: Allergen challenge led to an approximately 6-fold increase in total leukocytes, including lymphocytes, compared with those seen at saline-challenged sites. At antigen-challenged sites, eosinophils predominated. Chemokine levels at control, saline-challenged sites were either below the detectable limit or low, with the predominant chemokine detected being IP-10. At antigen-challenged sites, levels of MDC, TARC, and IP-10 were all significantly increased compared with saline sites, each with a median of 486 to 1130 pg/mL detected. On the basis of a comparison with serum values, BAL chemokine levels at most antigen-challenged sites could not be accounted for by transudation from plasma. In contrast, levels of I-309 were extremely low or undetectable in all BAL and serum samples tested. Finally, BAL levels of MDC significantly correlated with those for TARC, but no significant correlations were found between levels of chemokine and any cell type.

Conclusions: These data suggest that among the chemokines measured in this study, IP-10 is the predominant chemokine detected 20 hours after saline challenge, likely representing

baseline production of a chemokine that favors T_H1 cell recruitment. At antigen-challenged sites, levels of both CCR4 and CXCR3 active chemokines, but not CCR8 active chemokines, are markedly increased and are produced at levels that are likely to have biologic significance. Given the preferential accumulation of T_H2 cells at these antigen-challenged sites, the increased production of CCR4-active chemokines might contribute to this response. (*J Allergy Clin Immunol* 2003;112:930-4.)

Key words: Chemokines, pulmonary late-phase response, segmental allergen challenge, MDC, TARC, I-309, IP-10

Chronic allergic inflammation, such as seen in asthma, is a complex pathologic process associated with endothelial activation, epithelial activation and injury, and leukocyte infiltration.¹ The infiltrate is particularly enriched in eosinophils and T_H2 T lymphocytes.^{2,3} Molecules believed to contribute to this selective recruitment response include cytokines, adhesion molecules, and chemokines.⁴ Among these, chemokines provide the greatest degree of selectivity, because the 7 transmembrane receptors for individual chemokines are expressed in different patterns by subsets of leukocytes. For example, selective expression of CCR3 on eosinophils and basophils allows these cells to migrate to sites where their ligands, such as eotaxins, are produced.⁴

For selective T-lymphocyte recruitment, chemokine responses vary because of the array of T-cell subtypes. For example, chemokines acting by means of CXCR3 (eg, interferon-inducible protein 10 [IP-10] or CXCL10), CCR4 (eg, macrophage-derived chemokine [MDC] or thymus- and activation-regulated chemokine [TARC], also known as CCL22 and CCL17, respectively), and CCR8 (eg, I-309, also known as CCL1) facilitate preferential migration and accumulation. With respect to T_H2 cells, however, expression of the chemokine receptors CCR3, CCR4, and CCR8 has been suggested to be useful for their identification, whereas expression of CXCR3 is considered a reliable marker of T_H1 cells.⁵⁻¹¹ Recently, however, the robustness and reliability of using CCR3 as a marker for T_H2 cells in human airways has been questioned, leaving CCR4 and perhaps CCR8 as the most consistent T_H2 cell markers.¹²⁻¹⁶

Allergen challenge has long been used as an *in vivo* human model of chronic allergic inflammation, caused in part by the strong eosinophil and T_H2 cell recruitment response that is elicited during the so-called late-phase response.^{17,18} In the lower airways, for example, bronchoscopic segmental allergen challenge results in a cel-

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Abbreviations used

BAL: Bronchoalveolar lavage
IP-10: Interferon-inducible protein 10
MDC: Macrophage-derived chemokine
TARC: Thymus- and activation-regulated chemokine

lular inflammatory response highly enriched in eosinophils and T_H2 cells, and high levels of T_H2 cytokines, such as IL-4, IL-5, and IL-13, are detected in lavage fluids.^{3,19} We therefore hypothesized that T_H2 chemokines, namely the CCR4-active chemokines MDC and TARC, along with the CCR8-active chemokine I-309,^{10,13,15} would be produced within the airways during allergic inflammatory reactions in vivo. As a control, we further hypothesized that a T_H1 chemokine, namely the CXCR3-active chemokine IP-10, would not be produced. To test these hypotheses, we performed bronchoalveolar lavage (BAL) 20 hours after segmental allergen (or saline as control) challenge of allergic subjects. Sensitive and specific ELISAs were then used to measure levels of protein for these chemokines in the samples. Levels of these chemokines were then correlated with the pattern of inflammatory cell accumulation.

METHODS

Ten subjects (5 males) with both allergic rhinitis and asthma underwent segmental challenge with saline and antigen followed by BAL. They ranged in age from 19 to 42 years (median, 28 years). The protocols were approved by the Johns Hopkins Bayview Medical Center Institutional Review Board for Human Subjects Research, and all subjects signed a consent form before entry in the study. For saline challenge, 5 mL of saline was instilled into 1 lung segment. For antigen challenge, 5 mL of low endotoxin ragweed or dust mite (*Dermatophagoides pteronyssinus*) antigen (Greer, Lenoir, NC) at a concentration of 100 PNU/mL was instilled into another segment of the opposite lung. A second bronchoscopy was then performed 20 hours later with BAL to assess the inflammatory responses to saline and allergen challenge.³ Blood was also obtained at the time of the second bronchoscopy. Cells were removed by centrifugation, and serum and BAL fluids were frozen at -80°C and analyzed at a later date. BAL cells were washed, and the number and viability (always $>90\%$) of recovered cells were determined by light microscopy by trypan blue dye exclusion. Cyto-centrifugation preparations (Cytospin 2, Shandon, Pittsburgh, Pa) were stained for differential counts with a modified Giemsa stain (Sigma Diagnostics, St Louis, Mo). Differential cell counts were expressed as a percentage of 500 total cells enumerated.

Stored BAL fluids and serum were simultaneously assayed in triplicate for levels of TARC, MDC, I-309, and IP-10 using commercial ELISAs according to the manufacturer's instructions (R&D Systems, Minneapolis, Minn). Limits of detection were 7, 62.5, 0.7 and 1.7 pg/mL, whereas maximum levels of detection were 2000, 5000, 1000 and 1000 pg/mL, respectively. If values obtained were below the level of detection, they were reported as being at the level of detection. Of note, however, is that many of the antigen-challenge samples were beyond the upper limit of detection. These were rerun at 1:10 and 1:100 dilutions, and the means of these 2 measurements were then reported after being corrected to represent what would have been detected if measured in undiluted BAL fluid.

The Mann-Whitney *U* test was used to compare levels of cells and chemokines at saline-challenged and antigen-challenged sites.

The Spearman rank test was used to determine correlations between various chemokine levels and BAL cell levels.

RESULTS

As expected,³ allergen challenge induced a vigorous inflammatory cellular response (Table I). Total cell numbers were approximately 6-fold greater at allergen-challenged sites than saline sites. Most cells found at the saline sites were macrophages and neutrophils. At allergen sites, eosinophils were more prevalent than any other cell type. Although the percentages of lymphocytes and other cells did not significantly increase (data not shown), the absolute numbers of most cell types, including lymphocytes, were significantly increased ($P = .001$).

Shown in Fig 1 are the ELISA results for TARC, MDC, and IP-10 using BAL fluids and serum obtained 20 hours after segmental challenge. BAL and serum measurements for I-309 are not included, because they were all undetectable (<0.7 pg/mL) or <4 pg/mL (data not shown). TARC and MDC levels were below the limit of detection in most of the samples from saline sites but were detected in most BAL fluids from antigen sites, albeit over a wide range. The same pattern was seen for IP-10, except that levels were detectable in BAL fluids at every saline site (median, 66 pg/mL). In comparing levels at antigen-challenged sites, TARC, MDC, and IP-10 were significantly increased compared with those at saline sites, with median levels of 1130, 1033, and 486 pg/mL, respectively. Comparing the range of values detected, levels of TARC were among the highest observed (ie, 3 values >10 ng/mL). Given the simultaneous median serum values obtained for TARC, MDC, and IP-10 (308, 549, and 107 pg/mL, respectively, which are well within the range of those reported in the ELISA package inserts), the levels detected in antigen-challenged BAL fluids cannot be accounted for by plasma leakage alone. This is consistent with a lack of any significant correlation between chemokine levels in serum versus BAL (data not shown).

Finally, to explore whether levels of chemokines might explain the BAL cytologic findings, a series of correlations was determined by comparing the chemokine ELISA results in BAL with that of BAL cytologic findings, using data from antigen-challenged sites ($n = 10$). Among chemokine levels in BAL fluids, only levels of MDC and TARC were correlated with each other ($P < .005$). No significant correlations were seen between levels of any chemokine and either the percentage or absolute numbers of any specific cell type, including lymphocytes and eosinophils, although MDC levels in BAL showed a strong trend with eosinophil percentage in BAL ($P = .06$).

DISCUSSION

To our knowledge, this article is the first to report levels of MDC, TARC, I-309 (essentially undetectable), and IP-10 in late-phase BAL fluids after segmental allergen

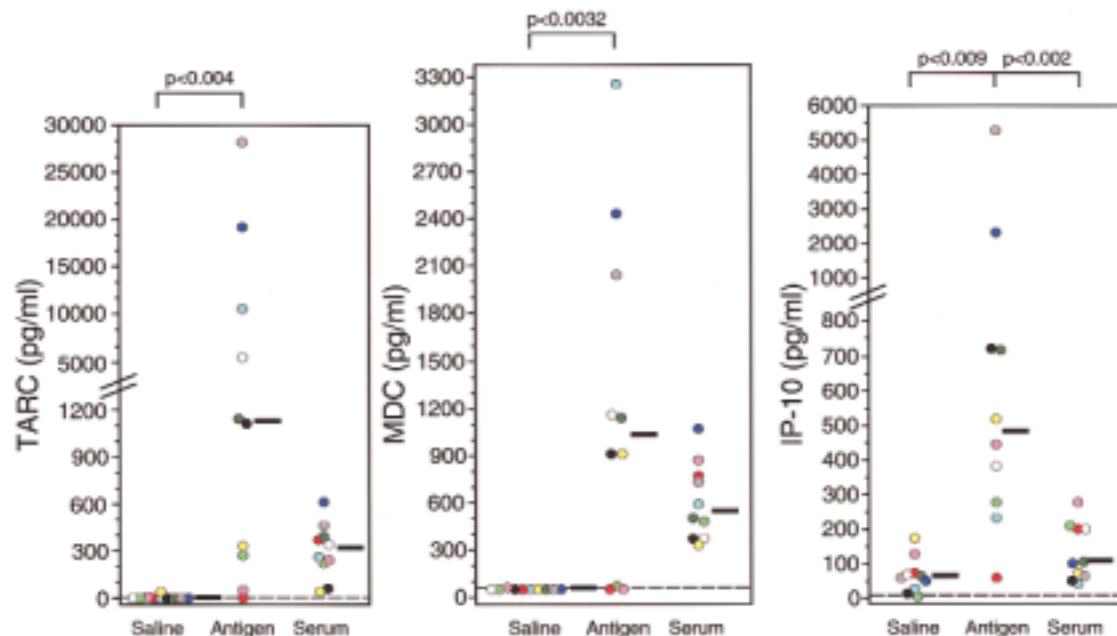


FIG 1. Levels of TARC, MDC, and IP-10 measured by commercial ELISAs in serum and in BAL fluids obtained 20 hours after segmental allergen challenge of atopic subjects with saline or allergen ($n = 10$; each colored dot represents a different subject). Short solid lines represent median values. Limits of detection are represented by dashed horizontal lines. P values are from the Mann-Whitney U test.

TABLE I. Cytology of BAL fluids obtained 20 hours after segmental challenge with saline or allergen

	Saline	Allergen
Total cells ($\times 10^6$)	19 (8-54)*	150 (18-240)†
Macrophages ($\times 10^6$)	10.1 (4.8-23.4)	17.5 (5.4-39.8)
Lymphocytes ($\times 10^6$)	1.7 (0.7-6.2)	15.7 (1.1-29)†
Neutrophils ($\times 10^6$)	2.2 (0.1-40.7)	11.4 (3.3-100.1)†
Eosinophils ($\times 10^6$)	0.2 (0-2.4)	63.6 (2.6-192)†
Epithelial cells ($\times 10^6$)	0 (0-0.6)	0 (0-0.4)

*Values are median (range) number of cells; $n = 10$.

† $P < .001$ compared with saline.

challenge. Increased levels of MDC and TARC are consistent with data reported by others in asthma and after allergen challenge using less quantitative measures such as in situ hybridization and immunohistochemistry.^{13, 15} An advantage of this study is that it confirms release of chemokines into the airway lumen and permits their quantitation to determine whether biologically relevant levels are produced. On the basis of the ELISA data, among the 4 chemokines measured at saline-challenged sites in allergic subjects, the predominant chemokine present at 20 hours was IP-10, albeit at low levels (median levels of 66 pg/mL). This is likely to represent a level of IP-10 at baseline without challenge, although a non-specific response to bronchoscopy or saline has not formally been excluded. Regarding levels of chemokines in BAL fluids 20 hours after allergen challenge, levels of MDC, TARC, and IP-10 were easily detectable, with median levels in the low nanogram per milliliter range.

These levels are within the range of those shown to have biologic effects, such as chemotaxis, in vitro,⁸ and are 2 to 3 logs higher than those reported by others for other chemokines, such as eotaxin, in similar late-phase BAL fluids.²⁰ The levels of TARC reported herein are about 10-fold higher than those reported in BAL fluids from patients with eosinophilic pneumonia.²¹ Indeed, on the basis of the estimated 50- to 100-fold dilutions caused by the BAL procedure,^{22,23} it seems that in many cases, the BAL levels for chemokines might be as much as 10- to 100-fold greater than serum levels. Given the variability of the BAL dilution effect, however, it is difficult to compare these values directly. Nevertheless, the levels detected suggest that a gradient from blood to lung for these chemokines would exist in vivo as a result of the allergen challenge procedure.

Unlike levels of the other chemokines measured, concentrations of the CCR8 agonist I-309 were extremely low or undetectable. As additional controls when doing the ELISA, we spiked aliquots of saline and allergen-challenged BAL fluids with recombinant I-309 (100 pg/mL) immediately before assay or subjected spiked samples to 1 freeze-thaw cycle before assay. Recovery ranged between 52% and 80%, so at most, levels might represent $\approx 50\%$ of actual levels, which would still be exceedingly low. Although this addresses some aspects of the assay, namely nonspecific interference or unreliability, it leaves open the possibility that I-309 released into the airway lumen might be degraded or metabolized, which unfortunately cannot be addressed directly. It is also possible that I-309 is produced at a different time point during the late-phase response.

Unexpectedly, levels of the T_H1 chemokine IP-10 were quite similar to those of the T_H2 chemokines MDC and TARC. In fact, the levels of IP-10 detected here are very similar to those measured in acute lung rejection and in bronchiolitis obliterans syndrome associated with lung transplantation.²⁴ The cellular target for IP-10 is CXCR3, found on T_H1 cells but also endothelium, monocytes, and natural killer cells but not neutrophils,²⁵ so recruitment of the latter cell type cannot be explained by IP-10. Because we did not determine which cells in BAL expressed these receptors, it is not possible to determine which portion of the cellular infiltrate might have been due to effects of IP-10. Although expression of IP-10 is more typically seen in the airways in T_H1 -related diseases including sarcoidosis, a small but significant increase in IP-10 positive cells was reported in BAL cells from subjects with asthma compared with normals.²⁶ One shortcoming of our study is that we are unable to determine whether the pattern of chemokine production during the late-phase response was unique to patients who have allergic asthma, because patients with allergic rhinitis were not studied. Another shortcoming is that ELISAs do not determine the source of the detected chemokines, although presumably BAL cells as well as the respiratory epithelium represent the major sources.^{13,15,26,27}

When chemokine levels were correlated with each other and with the pattern of cellular infiltration at 20 hours, the only correlation seen was between levels of TARC and MDC. The finding that cytokines such as $TNF-\alpha$ and IL-4 induce epithelial production of TARC and MDC in vitro^{8,15,28,29} and the detection of these cytokines in the segmental allergen challenge model^{3,30} is consistent with the possibility that the allergen-driven T_H2 cell recruitment response by means of these chemokines could, at least in part, be due to these cytokines acting on the respiratory epithelium. Surprisingly, no correlations were seen between the percentage or absolute numbers of lymphocytes and chemokine levels. Even if eosinophils are used as a marker of the intensity of the late-phase allergen challenge response, no significant correlations were seen, although a correlation ($P < .06$) between MDC and eosinophils was seen. This is of potential interest, because MDC has been reported to have modest chemotactic activity for eosinophils.³¹ One possible explanation for the relatively few correlations seen might be the choice of the 20-hour time point for measurement of cytologic findings and chemokines. Indeed, it is difficult without kinetic analyses to determine whether this is an optimal time point at which to measure chemokine levels, or whether earlier time points would have been more useful. Also, we did not specifically determine levels of T_H1 versus T_H2 cells in the lavage fluids, although the cytokine profile in this model supports the predominance of T_H2 lymphocytes.^{3,19}

In summary, measurements of chemokines in BAL fluids obtained 20 hours after segmental allergen challenge revealed biologically relevant levels of both T_H1 -associated (IP-10) and T_H2 -associated (MDC and TARC but not I-309) chemokines. In contrast, only the CXCR3

chemokine IP-10 was consistently detectable at saline-challenged sites. Whether CCR4 would be useful as a therapeutic target in allergic diseases must await testing with specific antagonists in human trials.

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