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Original article

Prolonged nasal eosinophilia in allergic patients after common cold

Background: Viral respiratory tract infections may cause both harmless common colds and severe asthma exacerbations; the differences in disease expression probably depend on the allergic status of the patient. To determine whether altered immunologic mechanisms underlie these differences, we investigated nasal inflammation during naturally acquired common cold.
Methods: In a group of 16 patients (eight allergic), nasal brush samples were taken, and nasal symptoms were recorded during common cold, 2 weeks later (convalescence), and at baseline (>4 weeks without nasal symptoms). Nasal brush cells were stained immunohistochemically for Langerhans cells, T cells, monocytes, neutrophils, B cells, macrophages, natural killer (NK) cells, mast cells, eosinophils, eotaxin, and RANTES.
Results: Four rhinovirus, four coronavirus, three RSV, one *Mycoplasma pneumoniae*, and one influenza A/enterovirus double infection were confirmed. Increased numbers of T cells, monocytes, macrophages, NK cells, eosinophils, and RANTES- and eotaxin-positive cells, but not neutrophils, were observed during common cold in allergic and nonallergic patients, and increased numbers of mast cells in allergic patients. Compared to nonallergic patients, in allergic patients eosinophil influx persisted into convalescence.
Conclusions: Prolonged nasal eosinophil influx was observed in allergic patients after common cold. What immunologic factors can induce prolonged eosinophil influx and whether this may increase the risk of subsequent allergen-induced hypersensitivity reactions must be studied further.

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Key words: adults; allergy; cell markers; chemokines; common cold; immunohistochemistry; inflammation; respiratory infection; virus infection.

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Common viral pathogens may cause both relatively harmless common cold symptoms and severe exacerbations of asthma. Differences in disease expression during common cold probably depend on the allergic status of the patient (1–3). Although adult allergic patients do not appear to have an increased annual frequency of common cold (4), increased nasal (5) and pulmonary responses have been found during and after common cold (6) in allergic rhinitis and asthmatic patients. One can therefore ask what altered immunologic mechanisms may render an allergic individual more susceptible to severe nasal and bronchial immune pathology upon viral encounter than nonallergic individuals.

Viral involvement in upper and lower airway pathology during common cold has been extensively tested and confirmed in adult volunteers. A wide variety of respiratory viruses can induce common cold symptoms (7). Several studies have used experimentally induced human infection models to study the immune pathology of common cold (1, 2). It has been shown that both mildly asthmatic and allergic rhinitis patients experimentally infected with rhinovirus type 16 have increased airway responsiveness during and after infection compared to healthy volunteers (1, 3).

Nasal and bronchial sampling studies during viral infection generally show increased numbers of lymphocytes, eosinophils, and neutrophils (8–12). Elevated levels of several cytokines and chemokines such as eotaxin, TNF-α, IL-1β, IL-5, IL-6, IL-8, and IFN-γ have also been detected during common cold in nasal and bronchial tissue (13, 14). When immunologic responses in asthmatic and nonasthmatic patients were compared during common cold, an enhanced eosinophilic response was observed in asthmatic patients during the infection, which tended to be prolonged until convalescence (9, 14, 15). With the exception of increased neutrophil and fibrinogen levels in atopics during common cold (9), no other immunologic differences, such as differences in T helper 1 and 2 cytokine balances, have been observed so far between atopic and nonatopic patients.

Only a few studies have investigated the immunologic mechanisms of naturally acquired common cold (11,

13). To clarify the immunologic mechanisms underlying virally induced immune pathology in allergic patients, we examined differences in both inflammatory cell types and eosinophil-specific chemokines in allergic and nonallergic patients in nasal brush specimens (16) during naturally acquired common cold.

Material and methods

Subjects

During the winters of 1998 and 1999, eight allergic patients (mean age 28 years) and eight nonallergic patients (mean age 31 years) with clinical symptoms of common cold participated in this study (Table 1). Within 4 days of the onset of a naturally acquired common cold, patients recorded the severity of nasal symptoms on a visual analog scale (VAS) ranging from mild or absent (0) to severe (100). The total nasal symptom score was calculated as the sum of five individual nasal symptom scores recorded (runny nose, nasal blockage, sneezing, nasal itching, and eye watering and irritation, ranging together from 0 to 500). Patients were considered to have common cold and were included in the study when the total nasal symptom score was higher than 100 and when patients presented with at least symptoms of a runny nose and nasal blockage. Allergic sensitivity was confirmed by a positive skin prick test reaction with a wheal diameter of at least 2+ (Vivodiagnost; ALK Benelux BV, Groningen, The Netherlands) or detection of specific serum IgE (Phadiatop, Pharmacia CAP System, Uppsala, Sweden) for house-dust mite, grass pollen, birch pollen, or cat and dog allergens. All sensitized patients had mild rhinitis symptoms without asthma. None of the patients had used topical and systemic corticosteroids for the previous 4 weeks. Each patient gave informed consent, and the Rotterdam University medical ethics committee approved this nasal brush study.

Study design

Nasal brush samples were taken during the acute phase of the common cold and during convalescence (2–3 weeks later). Baseline samples were taken between 1 and 12 months after the common cold and when no nasal symptoms had been present for at least 4 weeks. At each sampling moment, patients recorded the severity of several nasal symptoms reported previously and general malaise on a VAS. All samples were taken outside the pollen season.

Nasal brushes

Cells harvested from nasal brush samples were collected as previously described by Godthelp et al. (16). Cells were washed in 7 ml of RPMI 1640 medium (Life Technologies). The supernatant was collected and stored at -80°C until viral and *Mycoplasma* RNA was isolated for PCR amplification. Cells were pelleted, placed in Tissue-tek II OCT compound (Miles, Inc, Diagnostics Division, Terrytown, NY, USA), and snap-frozen in liquid nitrogen. Frozen sections (6 μm) were transferred to 10% poly-L-lysine (Sigma)-coated microscope slides and stored at -80°C until use.

Virus detection

The type of infection was confirmed in nasal brush samples within the first few days of common cold. Respiratory syncytial virus (RSV), adenovirus, influenza virus types A and B, enterovirus, and parainfluenza virus types 1 and 2 infections were detected by immunofluorescent staining of nasal brush cells with antiviral antibodies or by viral isolation from 4 ml nasal brush supernatant. Rhinovirus, coronavirus, and *Mycoplasma pneumoniae* infections were detected by amplification of viral RNA from 0.5 ml nasal brush supernatant by RT-PCR, followed by hybridization with either rhinovirus, coronavirus, or *M. pneumoniae*-specific radio-labeled probes (17).

Immunohistochemical staining procedures

Slides with frozen nasal brush cells were defrosted and fixed in acetone for 10 min, rinsed in phosphate-buffered saline (PBS, pH 7.4), and placed in a semiautomatic stainer (Sequenza, Shandon, Amsterdam, The Netherlands). To block nonspecific antibody binding, slides were incubated for 10 min at room temperature with a 10% normal goat serum (CLB) diluted in PBS supplemented with 3% bovine serum albumin (Sigma) and 0.1% azide (PBS/BSA). Subsequently, the slides were incubated for 60 min with mouse antihuman monoclonal antibodies for the cell markers and chemokines mentioned in Table 2 (diluted in PBS/BSA). The slides were rinsed in PBS, and incubated for 30 min with biotinylated goat antimouse Ig serum (1:50 in PBS/BSA plus 10% human serum), rinsed in PBS, and incubated either with streptavidin alkaline phosphatase (1:50 in PBS/BSA plus 10% human serum; Biogenex, Klinipath, Duiven, The Netherlands) for cell type staining, or with polyclonal goat antibiotin antibody (1:50 in PBS/BSA plus 10% human serum; Sigma) for chemokine staining for 30 min at room temperature. Sections were rinsed with distilled water and TRIS buffer (pH 8.5) and incubated for 30 min with New Fuchsin substrate (Chroma, Kongen, Germany). Finally, the sections were counterstained with Gill's hematoxylin and mounted in glycerin-gelatin. Control staining was performed by the substitution of primary monoclonal antibody by an isotypic control antibody.

Light microscope evaluation

For every nasal brush sample, at least 2000 cells with purple-blue-stained nuclei were counted. The number of positively stained cells was calculated as a percentage of the 2000 cells present. Positively stained cells had a bright red-stained cell membrane, red-stained cytoplasm, or both, depending on the cell type or chemokine evaluated. Cells were counted at a magnification of $\times 400$.

Statistical analysis

The statistical analysis of cell numbers was performed with SPSS. Differences in cell counts between the three sampling moments were analyzed with the nonparametric Friedman test for related samples. Differences between sampling moments were considered statistically significant when the P value was ≤ 0.05. Subsequently, differences between two sampling moments were analyzed with the Wilcoxon signed rank test for paired samples. Differences between allergic and nonallergic patients at each sampling moment were measured with the Mann-Whitney U-test. Correlations between numbers of cell types, chemokines, and clinical parameters were tested with Spearman's correlation coefficient. Differences between groups and sampling moments were considered to be statistically significant when $P \leq 0.05$.

Results

Nasal symptoms and patient characteristics

All the patients investigated had a significantly elevated total nasal symptom score (Fig. 1) and increased symptoms of general malaise during the acute phase of common cold as compared to convalescence and baseline. In 13 patients (81%), a

Table 1. Patient characteristics

	Patient	Age (years)	Type of infection	Sensitization
Nonallergic	1	32	Coronavirus OC43	–
	2	26	Coronavirus 229E	–
	3	33	Influenza A virus/enterovirus	–
	4	24	Rhinovirus	–
	5	39	Rhinovirus	–
	6	35	RSV	–
	7	29	ND	–
	8	26	ND	–
Allergic	1	31	Coronavirus OC43	HDM, grass and birch pollen
	2	23	Coronavirus OC43	HDM, grass pollen, cat
	3	26	*Mycoplasma pneumoniae*	HDM, cat
	4	37	Rhinovirus	HDM
	5	29	Rhinovirus	HDM
	6	26	RSV	Grass pollen
	7	30	RSV	Cat, dog
	8	24	ND	HDM, grass and birch pollen, cat

RSV: respiratory syncytial virus; HDM: house-dust mite; ND: not detectable.

virus or *M. pneumoniae* infection was confirmed (Table 1). During the acute and convalescent phase, no differences in systemic and local nasal symptoms were observed between allergic and nonallergic patients. At baseline, allergic patients reported slightly higher total nasal symptom scores ($P=0.05$) than nonallergic patients.

Microscopic evaluation

The number of positively stained cells was counted per 2000 nasal brush cells of which the nuclei were stained dark purple-blue. Positively stained cells had a red cell membrane (CD1a, CD3, CD8, CD19, and CD94) or cytoplasm (major basic protein [MBP], tryptase, eotaxin, and RANTES) or both (CD14, CD15, and CD68). Fig. 2 shows representative sections of nasal brush cells stained for eosinophils, and CD3-, CD68-, and eotaxin-positive cells.

Inflammatory cell influx

During common cold, significantly increased numbers of cells positive for CD3, CD14, CD68, and CD94 were observed as compared to convalescence and baseline samples (Fig. 3). During baseline and convalescence generally, fewer than 5% monocytes, macrophages, and natural killer cells (NK cells) were detected. This figure increased sharply during common cold to levels of up to 20% of cells present in the nasal brush samples. Baseline levels of CD3-positive T cells varied considerably between 0 and 6%. This increased to a maximum of 13% of the cells present during common cold. Fewer CD8-positive T cells than CD3-positive T cells were detected in nasal brush samples (range 0–6.6%), but there was a slight trend toward increased numbers during common cold ($P=0.1$; Friedman test) (Fig. 3). High numbers of neutrophils (CD15-positive cells) were observed (range 0.7–62.7%), but no significant differences were observed between the three sampling moments (Fig. 3). Langerhans cells (CD1a positive) (median; range: 0; 0–0.3% positive) and B cells (CD19 positive) (median; range: 0; 0–0.4% positive) were detected in only a few nasal brush samples. No

Table 2. Monoclonal antibodies used for immunohistochemical staining

Antibody	Specificity	Cell type/chemokine	Titer	Source
OKT6	CD1a	Langerhans cells	1:25	Dept. of Immunology, EMCR, Netherlands
T3-4B5	CD3	Total T-cell pool	1:100	DAKO, Denmark
Leu2a	CD8	Cytotoxic T cells	1:100	B & D, Dorset, UK
Mon/1	CD14	Monocytes	1:600	CLB, Amsterdam, Netherlands
80H5	CD15	Neutrophils	1:25	Immunotech, Marseille, France
IOB4a	CD19	B cells	1:200	Immunotech, Marseille, France
EBM11	CD68	Macrophages	1:300	DAKO, Denmark
HP-3B1	CD94	Natural killer (NK) cells	1:50	Coulter, Netherlands
G3	tryptase	Mast cells	1:100	Chemicon, UK
BMK-13	MBP	Eosinophils	1:100	Sanbio, Uden, Netherlands
αEotaxin	Eotaxin	Chemokine	1:15	R & D systems, UK
αRANTES	RANTES	Chemokine	1:50	Chemicon, UK

MBP: major basic protein.

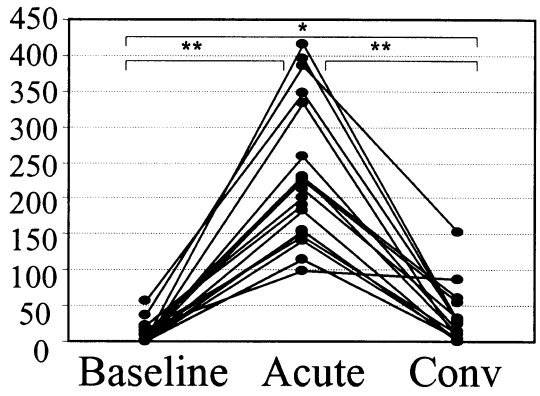

Figure 1. Total nasal symptom score during baseline, acute phase (acute), and convalescent phase (conv) of common cold (*$P<0.05$, **$P<0.01$).

differences in the cell types mentioned were observed between allergic and nonallergic patients during the acute and convalescent phases of common cold. At baseline, we observed significantly more macrophages in nonallergic than in allergic patients ($P=0.02$).

Eosinophils, mast cells, and eotaxin- and RANTES-positive cells

During the acute phase of common cold in allergic and nonallergic patients, significantly increased numbers of eosinophils (MBP-positive cells) were detected as compared to baseline. No differences between allergic and nonallergic patients were observed during common cold and at baseline. However, during convalescence, allergic patients had significantly higher eosinophil levels than nonallergic patients ($P=0.03$) (Fig. 4a).

In allergic patients during the acute phase of common cold, increased numbers of mast cells (tryptase-positive cells) were found as compared to convalescence and baseline (Fig. 4b), while no differences were found in nonallergic patients between the three sampling moments. The numbers of mast cells during common cold were significantly higher in allergic than nonallergic patients ($P=0.04$).

Numbers of RANTES- and eotaxin-positive cells increased during common cold as compared to baseline in allergic and nonallergic patients (Fig. 5). Although the numbers of eotaxin-positive cells decreased after common cold, still higher numbers were detected during convalescence than baseline. The

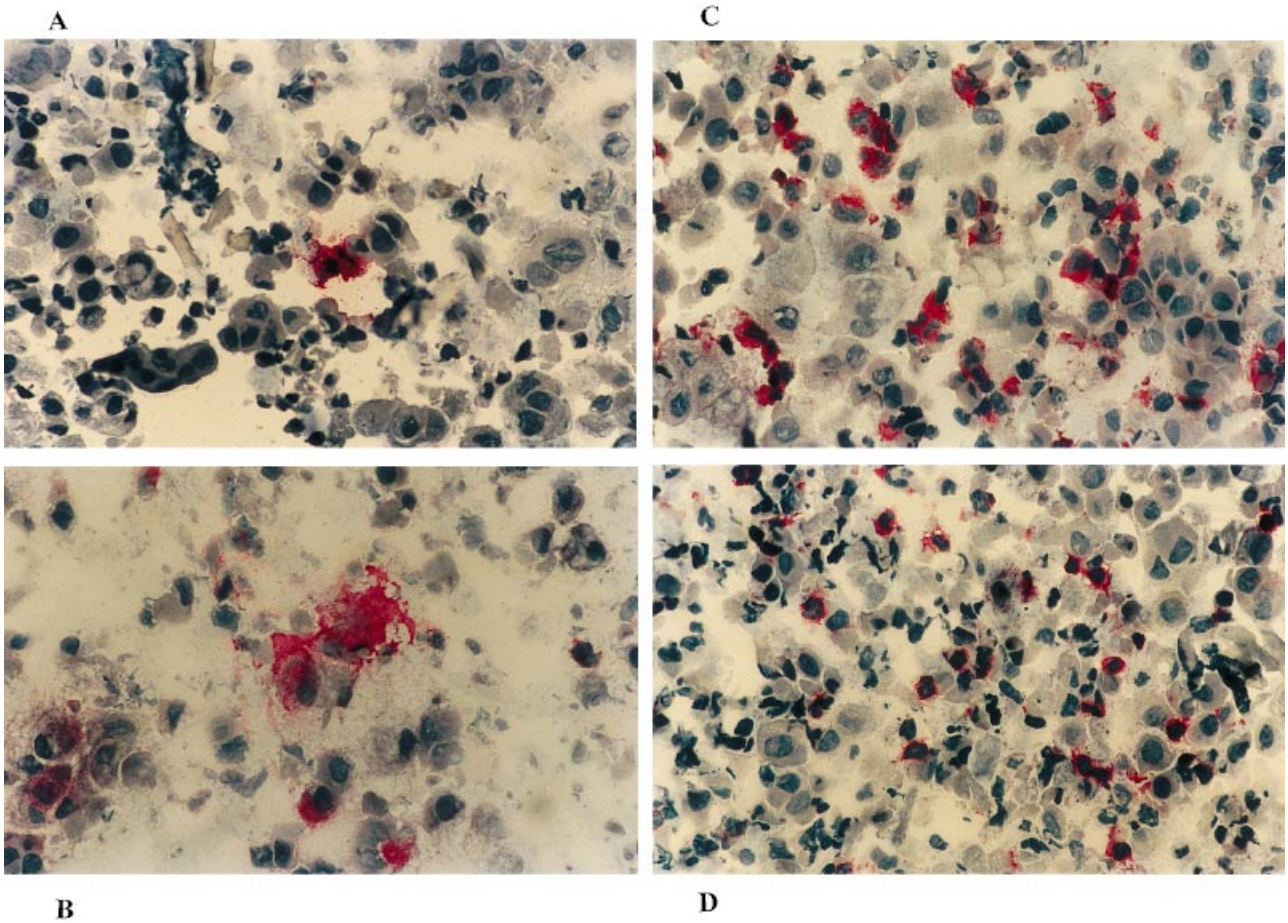

Figure 2. Nasal brush samples stained for A) MBP (eosinophils), B) eotaxin, C) CD68 (macrophages), and D) CD3 (T cells).

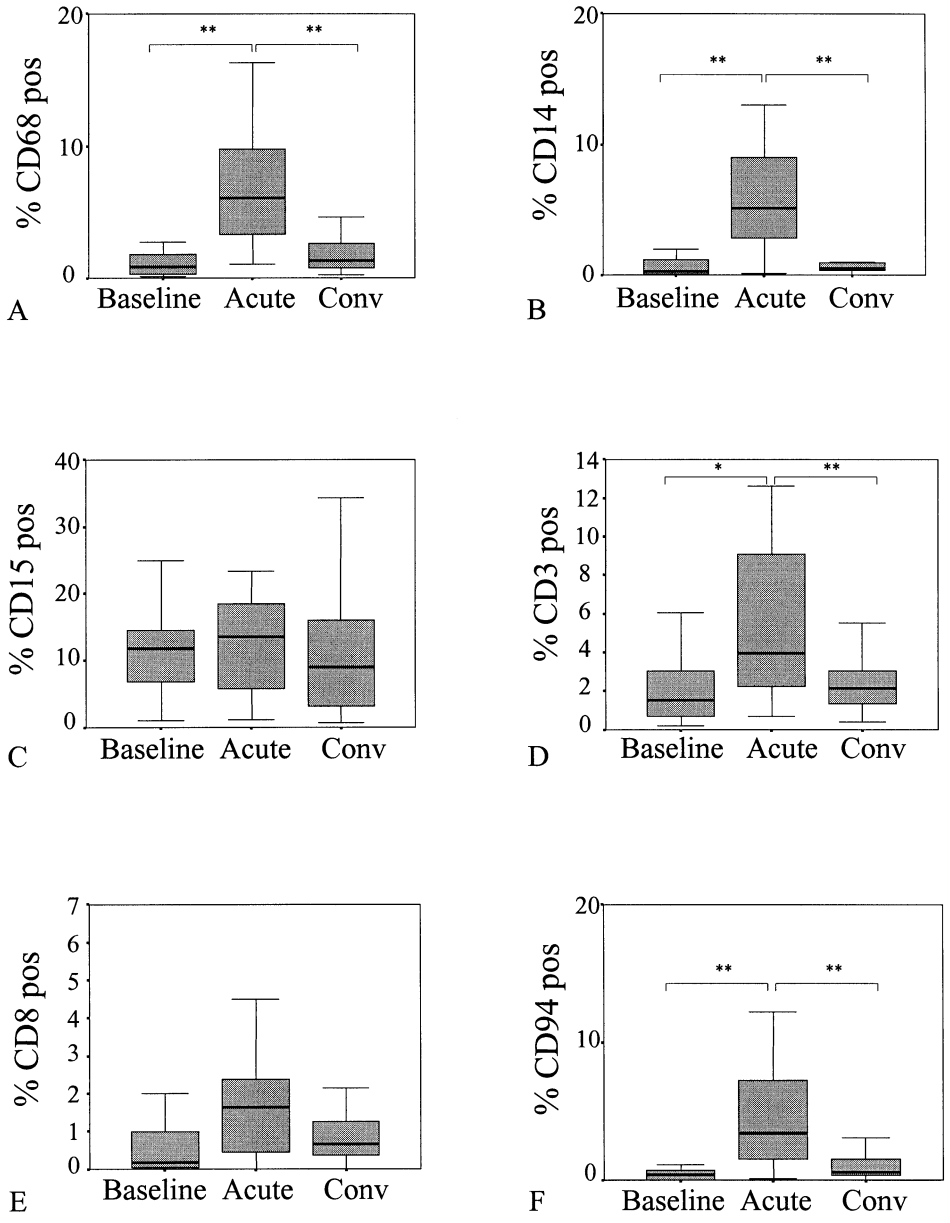

Figure 3. Percentage of A) macrophages (CD68), B) monocytes (CD14), C) neutrophils (CD15), D) CD3-positive T cells, E) CD8-positive T cells, and F) natural killer cells (CD94) during baseline, acute phase (acute), and convalescent phase (conv) of common cold (*$P<0.05$, **$P<0.01$).

numbers of RANTES-positive cells also remained elevated after common cold. No differences between allergic and nonallergic patients in numbers of eotaxin- and RANTES-positive cells were observed at all three sampling moments.

Correlation between cells, cytokines, and nasal symptom severity

During the acute phase of common cold, several statistically significant positive correlations were found between different cell markers, chemokines, and clinical parameters. The numbers of macrophages, monocytes, T cells, and NK cells all correlated well with each other (data not shown). The number of eotaxin-positive cells correlated well with T cells and NK cells ($r=0.7$, $P=0.003$ and $r=0.8$, $P=0.001$, respectively). There were also positive correlations between the numbers of eotaxin-positive cells and both upper respiratory symptoms and the feeling of general malaise ($r=0.6$, $P=0.015$, and $r=0.6$, $P=0.02$, respectively).

Discussion

To clarify the immunologic mechanisms underlying immune pathology during common cold in allergic patients, we examined differences in inflammatory cell types and eosinophil-specific chemokines between

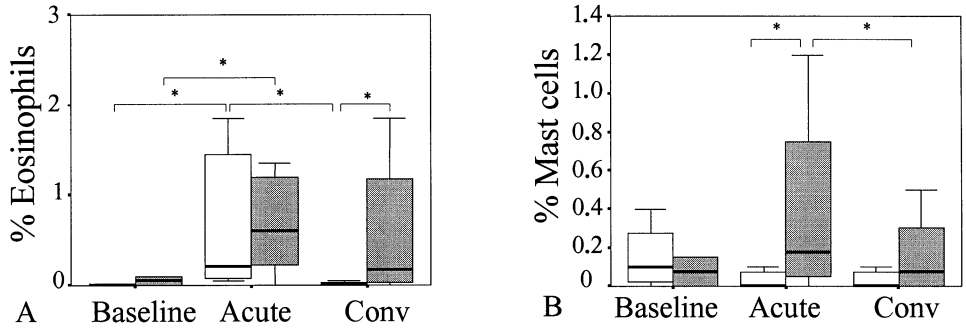

Figure 4. Percentage of A) eosinophils (MBP) and B) mast cells (tryptase) during baseline, acute phase (acute), and convalescent phase (conv) of common cold in allergic (gray bars) and nonallergic patients (white bars) ($*P < 0.05$).

allergic and nonallergic patients in nasal brush specimens during naturally acquired common cold. Both allergic and nonallergic patients showed an initial response of macrophages, monocytes, T cells, NK cells, and eosinophils in the nasal epithelium. However, a mast-cell response was observed during the acute phase only in allergic patients. In addition, the increase in numbers of eosinophils during the acute phase persisted only into convalescence in allergic patients. These different immunologic responses may underlie different disease expression during common cold in allergic and nonallergic patients.

A wide variety of respiratory viruses can induce common cold symptoms (7, 18) and can induce airway hyperresponsiveness in allergic patients (1, 3). However, until now, almost all common cold studies used experimentally induced infection models with rhinovirus type 16 in man (2, 19). Allergen provocation studies (20) have shown that immunologic data derived from experimental studies can be very different from data acquired in naturally occurring disease, stressing the importance of studying natural disease. Little is known about the role of viruses other than rhinovirus in inducing inflammatory responses related to common cold.

The influx of inflammatory cells such as T cells, monocytes, macrophages, and NK cells found in this study is comparable to what has been found in common cold studies in allergic patients and in controls (10–12). In contrast to these studies, we did not observe a nasal influx of neutrophils (8, 9). Since high numbers of neutrophils are found in the nose during all sampling moments but no differences were observed between allergic and nonallergic patients, it would not seem very likely that neutrophils play a role in differentiating the two patient groups. However, functional differences, such as in cytokine production, may explain differences in airway hyperreactivity.

The mast-cell response in this study, and studies showing increased levels of histamine in lavage samples during common cold in allergic subjects (21) indicate the role of mast cells in airway hyperresponsiveness. In addition, mast cells after allergen challenge have been shown to produce mediators and cytokines which attract and activate eosinophils, leading to priming of subjects and induction of airway hyperresponsiveness. However, mediators such as histamine production alone do not result in priming phenomena. Therefore, this is not a mast-cell product which is very likely to explain airway hyperresponsiveness after common cold in allergic disease.

The most likely candidates for inducing immune pathology seem to be the eosinophils. Although increased numbers of eosinophils were detected in the nose of allergic and nonallergic patients during common cold, it was only in allergic patients that this eosinophilia persisted into convalescence. In asthmatic patients also, prolonged eosinophilia has been found

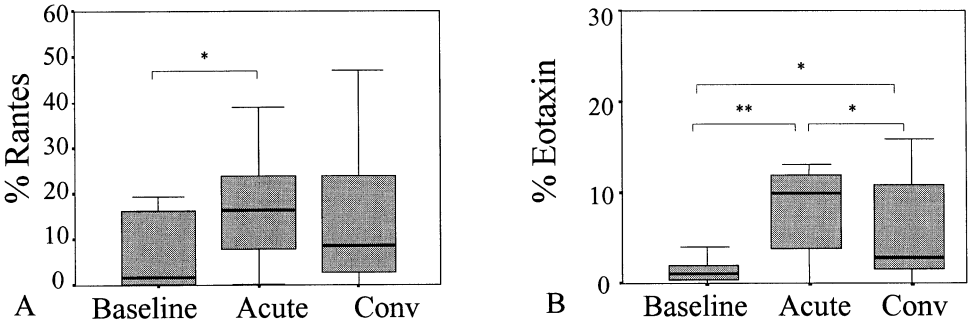

Figure 5. Percentage of A) RANTES- and B) eotaxin-positive cells present in nasal brushes during baseline, acute phase (acute), and convalescent phase (conv) of common cold ($*P < 0.05$, $**P < 0.01$).

after induced common cold in bronchial (9, 15) and nasal samples (14). To our knowledge, these observations are the first which show persistent nasal eosinophilic responses in relatively mildly allergic subjects after naturally acquired common cold. There have been reports of increased nasal and bronchial hyperreactivity in subjects with allergic rhinitis (1). Influx of mast cells, release of mast-cell products, and persistence of eosinophils in the airway mucosa may cause an increase in airway hyperreactivity in allergic patients compared to nonallergic controls.

Contrary to what we expected on the basis of the findings above, no differences were found between allergic and nonallergic patients in eosinophil-specific chemokine positive cells. An increase in RANTES- and eotaxin-positive cells was observed during and after common cold in both groups. However, eosinophils may display enhanced susceptibility in allergic individuals for the chemokines mentioned; for example, through altered chemokine receptor expression (22, 23). Other mechanisms, such as increased expression of ICAM-1 in inflammatory and epithelial cells (11, 24–26), or decreased apoptosis of eosinophils, may account for the prolonged eosinophilia in the nose after common cold (27). The next step will be to measure the actual quantities of chemokines produced by nasal brush cells in both allergic and nonallergic patients.

In conclusion, increased numbers of mast cells during infection and enhanced nasal eosinophilia after common cold in allergic patients may explain nasal and bronchial hyperreactivity and asthma exacerbations after viral infection. Further studies are needed to clarify why eosinophils tend to persist longer in the noses of allergic patients than in those of nonallergic patients after common cold.

Acknowledgments

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