

ORIGINAL ARTICLE**Carbon Dioxide-enriched Water Inhalation in Patients with Allergic Rhinitis and its Relationship with Nasal Fluid Cytokine/Chemokine Release**Davide Pagani,^a Emanuela Galliera,^b Giada Dogliotti,^b Mario De Bernardi di Valserra,^c Sara Torretta,^a Umberto Solimene,^{b,d} Massimiliano M. Corsi,^b and Lorenzo Pignataro^a^aDepartment of Special Surgical Sciences, Fondazione IRCCS Cà Granda-Ospedale Maggiore Policlinico, ^bDepartment of Human Morphology and Biomedical Sciences Città Studi, ^dCenter of Medical Bioclimatology, University of Milan, Milan, Italy, and ^cFonti di Rabbi SPA, Trento, Italy

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Background and Aims. Allergic rhinitis is characterized by eosinophil infiltration and accumulation in the nasal mucosa mainly due to IL-3, IL-5, and eotaxin activities. We undertook this study to investigate a possible *in vivo* effect of carbon dioxide-enriched water inhalation in patients with allergic rhinitis.

Methods. Twenty five consecutive patients inhaled carbon dioxide-enriched water at Fonti di Rabbi Spa Centre (Trento, Italy). Symptom scores for nasal obstruction, itching and sneezing were obtained before and after treatment. Nasal lavage was collected, and IL-3, IL-5, and eotaxin levels were assessed using the quantitative sandwich enzyme immunoassay technique. Cytometric analysis was performed on samples to measure total cell count, CD45+ cells, and percentages of polymorphonucleates and lymphocytes.

Results. There were statistically significant differences in chemokine levels and in cell populations between patients and healthy controls before treatment. After carbon dioxide-enriched water inhalation, we observed statistically significant improvements in symptom scores, chemokine levels, and percentages of cell populations.

Conclusions. Our results seem to confirm the role of IL-3, IL-5, and eotaxin in the pathophysiology of allergy and the beneficial effect of carbon dioxide-enriched water inhalation in patients affected by allergic rhinitis. © 2011 IMSS. Published by Elsevier Inc.

Key Words: Allergic rhinitis, Chemokine, Cytokine, Carbon dioxide-enriched water inhalation.

Introduction

Allergic rhinitis (AR) forms part of a systemic multicomplex inflammatory disorder characterized by infiltration and accumulation of eosinophils in the nasal mucosa (1–3) and can be considered an immunoglobulin E-mediated, eosinophilic and TH2-type immune disease. It is the most common atopic disease in the United States and is reaching epidemic proportions worldwide. It affects up to 25% of adults and more than 40% of children and has enormous socioeconomic costs in terms of academic performance, socialization and laboral productivity (4–7).

The main histological characteristic of AR is tissue inflammation involving the activation of T-lymphocytes and eosinophils (8), which migrate into the inflamed nasal mucosa and release pre-formed and newly generated mediators such as histamine, prostaglandin D2 and leukotriene C4, toxic granule proteins, and reactive oxygen species (ROS), thus leading to tissue damage (9–10).

The AR can be divided into early and late responses. The early response is characterized by symptoms such as sneezing, itching, rhinorrhea and congestion; it occurs within minutes of exposure to the allergen and is mainly caused by the release of a series of pre-formed and newly synthesized mast cell products such as histamine (11). The late phase is characterized by the recruitment of additional inflammatory cells (basophils, eosinophils, neutrophils, mast cells and mononuclear cells) that maintain the disease process (12).

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Eosinophils are the predominant cells in the late-phase response, and their presence correlates with disease severity and symptom progression (13–15). They release an array of pro-inflammatory mediators including cysteinyl leukotrienes, cationic proteins, eosinophil peroxidase and major basic protein and may be a major source of IL-3, IL-5, GM-CSF and IL-13 (16).

Thermal water is widely used (especially in Europe) to treat diseases such as atopic dermatitis (17) and atopic eczema (18), and can induce immunomodulation in atopic patients (19). However, although it can be considered particularly interesting, its role has been investigated in only a few published studies. The aim of the study was to verify the possible *in vivo* effects of carbon dioxide (CO₂)-enriched water inhalation in a selected group of 25 patients with AR and its relationship with nasal eotaxin, IL-3 and IL-5 release.

Materials and Methods

Patients

Between May and September 2009, we enrolled 25 consecutive patients (13 males and 12 females) aged 18–65 (45.3 ± 2.4) years with a diagnosis of AR who had no history of systemic diseases and had not used any intranasal steroids or systemic nonsteroidal anti-inflammatory drugs in the previous 3 months. Controls consisted of a group of 25 (12 males and 13 females) age- and gender-matched healthy subjects (46.6 ± 3.1 years).

Diagnosis of AR was based on symptoms of sneezing, watery rhinorrhea and nasal obstruction (positivity for two or three of these), and the positive results of a skin test using common aeroallergens such as house dust mites, house dust, grass mix, tree pollens, animal dander and molds (20).

Patients and Methods

All patients with AR and controls inhaled CO₂-enriched water at Fonti di Rabbi Spa Centre (Trento, Italy) once a day, 6 days a week (Monday to Saturday) for 2 consecutive weeks. In the same way, all patients also inhaled saline solution. Nasal lavage fluid was collected from patients and controls before the first inhalation and immediately after the last one. Samples were immediately divided into two parts.

In the first one, IL-3, IL-5 and eotaxin levels in the nasal fluid samples were assessed using a quantitative sandwich enzyme immunoassay. Monoclonal antibodies specific for IL-3, IL-5 and eotaxin were coated onto a microplate before standards and the samples were pipetted into the wells. Any IL-3, IL-5 or eotaxin was bound by the immobilized antibodies, and any unbound substances were immediately washed away. An enzyme-linked polyclonal antibody

specific for IL-3, IL-5 and eotaxin was then added to the wells, and any unbound substances were washed away after incubation. Finally, a substrate solution was added to the wells and its color developed in proportion to the amount of IL-3, IL-5 and eotaxin bound in the initial step. Color development was stopped by means of HCl 2N, and the intensity of the color was measured at 450 nm within 30 min.

Maximum intra- and interassay coefficients of variation (CVs) of IL-3 were, respectively, 4.1 and 7.8%, and the detection limit was 7.4 pg/mL. Maximum intra- and interassay CVs of IL-5 were, respectively, 4.2 and 7.1%, and the detection limit was 3.0 pg/mL. Maximum intra- and interassay CVs of eotaxin were, respectively, 4.2 and 10.1%, and the detection limit was 5 pg/mL. For the second one, flow cytometry measurements were obtained (total cell count, CD45+ cells, and percentage of eosinophils [E], basophils [B], neutrophils [N] and lymphocytes [L]).

Samples were centrifuged at 1500 rpm for 15 min at 4°C after dithiothreitol treatment, and several washed cells were incubated in the dark at 4°C for ~30 min with CD 45+ antibodies (a marker of leukocytes). A flow cytometer (CyFlow, PAS Partec, Görlitz, Germany) was used to analyze leukocytes and leukocytes subgroups (E, B, N, L).

Symptom scores for nasal obstruction, itching and sneezing were obtained before and after each treatment and the following scale was applied: 1 = absence of symptoms, 2 = moderate symptoms and 3 = severe symptoms.

Statistical Analysis

Data were statistically analyzed using one-way ANOVA to assess the difference in nasal fluid IL-3, IL-5, eotaxin levels and also for cytometry results between patients and healthy controls (before and after treatment); *p* values <0.05 were considered statistically significant.

Results

IL-3, IL-5 and eotaxin levels (Figure 1) in the nasal lavage fluid of patients and healthy controls before thermal water treatment were, respectively, 55.0 ± 2.2 vs. 26.4 ± 1.0 IU/mL (*p* <0.001), 36.2 ± 1.0 vs. 4.4 ± 0.3 IU/mL (*p* <0.001), and 477.0 ± 19.6 vs. 175 ± 4.7 IU/mL (*p* <0.001).

After saline solution treatment, IL-3, IL-5 and eotaxin levels in patients compared to controls were, respectively, 52.4 ± 1.6 vs. 25.5 ± 1.4 IU/mL (*p* <0.001), 33.8 ± 1.8 vs. 4.3 ± 0.9 IU/mL (*p* <0.001), and 445.0 ± 16.4 vs. 173 ± 4 IU/mL (*p* <0.001).

At the end of treatment with CO₂-enriched water, the corresponding levels were 38.7 ± 1.3 vs. 23.1 ± 0.8 IU/mL (*p* <0.01), 24.9 ± 0.8 vs. 4.1 ± 1 IU/mL (*p* <0.01), and 296.0 ± 11.3 vs. 162.1 ± 5.1 IU/mL (*p* <0.01), respectively.

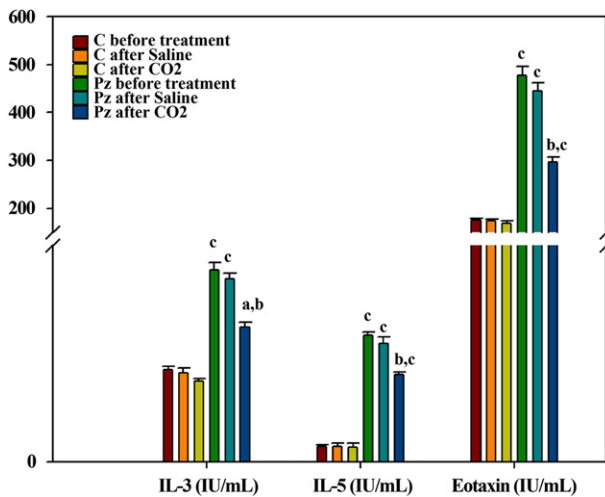


Figure 1. Graphic representation of IL-3, IL-5 and eotaxin levels in nasal lavage in patients (pz) and controls (c) before and after saline and CO₂-enriched water inhalation. $p < 0.01$ (a) vs. C, (b) vs. Pz before treatment; $p < 0.001$ (c) vs. C, (d) vs. Pz before treatment. (A color figure can be found in the online version of this article.)

In the nasal lavage fluid of patients, IL-3, IL-5 and eotaxin levels before and after saline solution treatment were not statistically significant ($p > 0.05$), whereas statistically significant levels after thermal water treatment were observed, compared with those before and after saline treatment ($p < 0.001$ and $p < 0.01$ for all molecules, respectively). No differences were observed in healthy controls.

Total cell count and leukocyte subgroup populations are shown in Table 1. Leukocytes were $>50\%$ of total cell count in all samples. Percentage of leukocytes subgroups were not statistically significant except for eosinophil percentage which was, in patients and controls, respectively, before treatment, 50 ± 13 vs. 18 ± 10 ($p < 0.001$), after saline treatment 48 ± 9 vs. 19 ± 7 ($p < 0.001$), and after CO₂-enriched water 30 ± 8 vs. 15 ± 6 ($p < 0.001$).

Moreover, in patients, a statistically significant improvement in eosinophil percentage count before and after CO₂-enriched water inhalation was observed ($p < 0.001$).

Comparison of symptoms demonstrated a statistically significant difference between patients and healthy controls before and after each treatment (Table 2). In patients, a statistically significant variation was observed after

CO₂-enriched water inhalation but not after saline solution treatment.

Discussion

We analyzed IL-3, IL-5 and eotaxin levels in the nasal fluid of a select group of patients with AR who underwent CO₂-enriched water inhalation treatment. These chemokine and chemotactic agents seem to play pivotal roles in the pathophysiology of allergic diseases including IgE production, mast cell and eosinophil recruitment and activation, mucus hypersecretion, subepithelial fibrosis and tissue remodelling (21,22) and also attract eosinophils with different degrees of selectivity (23–25).

Interleukin-3 is a 28-kD glycoprotein that has many colony-stimulating factor activities. It stimulates the proliferation, differentiation and activation of macrophages, neutrophils, mast cells, eosinophils, and other cells (26,27), increases macrophage cytotoxicity and adhesion, and promotes the function of eosinophils, basophils and mast cells (28). It modifies allergic reactions by priming basophil secretion and regulating other leukocytes (29).

IL-5 is a disulfide-linked dimer that is mainly produced by activated Th2 cells but also produced by mast cells and eosinophils (30). It participates in the terminal differentiation, priming, activation, chemotaxis, migration and survival of eosinophils during the allergic reaction (31,32) and seems to be related to symptomatic episodes and the severity of AR and asthma (33–35).

Eotaxin is a CC chemokine that is derived from antigen-stimulated T lymphocytes and airway epithelium cells and whose expression is increased in the airways of allergic and asthmatic subjects (36,37). It contributes to selective eosinophil chemotaxis and transendothelial migration by the eotaxin receptor CCR-3 (38): immediately after binding, CCR-3 is internalized, partially degraded and finally re-expressed, which suggests that, in addition to stimulating eosinophil migration, eotaxin may be involved in stopping leukocyte movement (39).

In cooperation with IL-5, eotaxin stimulates the migration of eosinophils from bone marrow into inflamed tissue (40). It has been observed *in vitro* and *in vivo* that IL-5 participates in eosinophil mobilization from bone marrow,

Table 1. Cytometric features (expressed as mean $\pm$ SD) in patients and controls before and after saline and CO₂-enriched water inhalation

	Patients before treatment	Patients after saline treatment	Patients after CO ₂ treatment	Controls before treatment	Controls after saline treatment	Controls after CO ₂ treatment
Total of cells	9250 $\pm$ 1923	8996 $\pm$ 2012	9185 $\pm$ 1945	9135 $\pm$ 1734	9234 $\pm$ 2201	9256 $\pm$ 2167
CD45+	68 $\pm$ 19	67 $\pm$ 22	62 $\pm$ 20	58 $\pm$ 18	56 $\pm$ 21	55 $\pm$ 22
Neutrophils %	11 $\pm$ 6	10 $\pm$ 8	15 $\pm$ 6	17 $\pm$ 10	16 $\pm$ 13	17 $\pm$ 15
Eosinophils %	50 $\pm$ 13 ^a	48 $\pm$ 9 ^a	30 $\pm$ 8 ^{a,b}	18 $\pm$ 10	19 $\pm$ 7	15 $\pm$ 6
Basophils %	1.05 $\pm$ 1.23	0.98 $\pm$ 1.11	0.87 $\pm$ 1.09	0.85 $\pm$ 0.94	0.84 $\pm$ 1.08	0.80 $\pm$ 0.99
Lymphocytes %	0.89 $\pm$ 1	0.86 $\pm$ 0.87	0.77 $\pm$ 0.65	0.43 $\pm$ 0.5	0.42 $\pm$ 0.52	0.33 $\pm$ 0.29

$p < 0.001$ ^avs. controls ^bvs. patients before treatment.

Table 2. Symptom scores (expressed as mean $\pm$ SD) in patients and controls before and after saline and CO₂-enriched water inhalation

	Patients before treatment	Patients after saline treatment	Patients after CO ₂ treatment	Controls before treatment	Controls after saline treatment	Controls after CO ₂ treatment
Nasal obstruction	2.53 $\pm$ 0.62 ^c	2.11 $\pm$ 0.6 ^c	1.70 $\pm$ 0.58 ^{c,d}	1.3 $\pm$ 0.47	1.18 $\pm$ 0.39	1.11 $\pm$ 0.48
Itching	2.59 $\pm$ 0.61 ^c	2.47 $\pm$ 0.62 ^c	1.68 $\pm$ 0.46 ^{a,d}	1.41 $\pm$ 0.5	1.23 $\pm$ 0.43	1.17 $\pm$ 0.39
Sneezing	2.41 $\pm$ 0.71 ^c	2.34 $\pm$ 0.7 ^c	1.89 $\pm$ 0.3 ^{b,c}	1.35 $\pm$ 0.49	1.29 $\pm$ 0.47	1.05 $\pm$ 0.24

$p < 0.01$ ^avs. controls; ^bvs. patients before treatment; $p < 0.001$ ^cvs. controls ^dvs. patients before treatment.

and eotaxin induces their trafficking to tissues and thus amplifies their response (41,42).

In line with previously published data (43), we found that the levels of IL-3, IL-5 and eotaxin are increased in the nasal fluid of patients affected by AR, thus confirming their role in the pathophysiology of the disease. We also found that their levels significantly decrease after inhalation of CO₂-enriched water, although they remain higher than those observed in healthy controls. Our findings seem to indicate that the beneficial effects of inhaled CO₂-enriched water may be due to its ability to reduce the concentration of IL-3, IL-5 and eotaxin in nasal fluid. This was also attested by statistically significant improvement in symptom scores after treatment.

Flow cytometry permitted the identification of leukocytes and their subgroup populations in nasal lavage. There are many disorders such as asthma and AR in which eosinophils are increased, either in peripheral blood or in different tissue samples, as the expression of a systemic inflammatory status (44,45).

Our study underlined that CO₂-enriched water inhalation not only ameliorates inflammatory status but also reduces also the most common symptoms of AR such as nasal obstruction, itching and sneezing.

These data seem to confirm the therapeutic role of inhaled thermal water in AR, which has traditionally and empirically been attributed to its composition and mineral concentration as well as its temperature (46).

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