

Differences in the Nasal Inflammatory Response to *Cynodon dactylon* From Rural and Urban Areas in Patients With Allergic Rhinitis

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Abstract

Background: Epidemiological and experimental studies suggest that air pollution has a negative impact on human health and modifies the environment. However, the clinical implications of changes in environmental allergens secondary to air pollution have been little studied.

Objectives: To explore if the growth conditions of the *Cynodon dactylon* (rural vs urban area) modify the inflammatory response among patients with allergic rhinitis.

Methodology: Two extracts were prepared for diagnostic test with Cyn d proteins obtained from rural and urban environment. Skin prick test (SPT), nasal challenge test (NCT), and eosinophil count in nasal mucus were performed in 3 groups: healthy subjects without rhinitis, rhinitis with (+) Cyn d, and rhinitis with (–) Cyn d.

Results: There was a 97% concordance in the positive and negative results of the SPT with the 2 extracts. However, Cyn d-urban extract generated larger wheals ($P=.03$) and a higher number of patients with rhinitis presented a positive NCT to this extract ($n = 7$ vs 14 , $P=.04$). Patients with positive NCT had a significant increase in eosinophils in mucus, but there was no difference between the extracts. The healthy controls did not react to the extracts tested in the skin or nasal test.

Conclusion: The findings suggest that the growth conditions in urban area of *Cynodon dactylon* can generate changes in the protein extract and have clinical implications in patients with allergic rhinitis.

Keywords

atopy, allergens, asthma, Cyn d, grass, pollen, pollution, rhinitis, rural, urban

Introduction

Air pollution has been recognized for many years as an important risk factor for multiple chronic diseases, especially at the respiratory level.^{1,2} Among the main pollutants are gases such as ozone, sulfur dioxide, and carbon dioxide^{3,4} and also particles of small size, which can reach the lower airways.^{5,6} In areas of high human occupation, vehicular traffic has been recognized as one of the main sources of air pollutants.^{7,8}

Patients with respiratory allergies suffer from a chronic Th2 inflammatory state that occurs due to environmental allergens that can be inhaled^{9,10} and stimulate the production of specific IgE (sIgE). Exposure to these allergens in the respiratory tract together with air pollutants⁶ promotes the Th2 pro-inflammatory state by different

mechanisms; the chronic oxidative stress appears when airway epithelial damage occurs by pollutants and favors the Th1 response.^{7,11} This Th1 inflammation in allergic patients acts facilitating the entry of allergens and in turn increase the production of the Th2

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inflammatory response. The coexposure of allergens/pollutants occurs commonly in areas with high vehicular traffic such as urban centers. In recent years, other mechanism of the allergens/pollutants interaction has been studied, and it is the protein changes that can occur in the proteins of trees, flowers, and grasses exposed to high concentrations of air pollutants during their growth.¹² These changes in flora proteins secondary to pollution are important for human health, as experimental studies suggest that it can increase the levels of allergens and its allergenicity in various pollen grains such as Cypress and Birch.^{13,14} This produces a greater lymphocyte activation and cytokine production of the Th2 cytokines and inflammatory response.^{13,14} However, most studies evaluating the relationship of air pollution and allergens come from experimental trials, and those conducted in humans have evaluated the combined exposure of allergens and pollutants,^{15,16} so it is difficult to discriminate whether changes in allergenic sources can have an effect on human health.

In this study, we intend to evaluate if the growth conditions of the *Cynodon dactylon* (rural vs urban) modify the inflammatory response among patients with allergic rhinitis, even without coexposure to air pollutants.

Methodology

Study Design

This is a cross-sectional study with 3 groups. The design was based on demonstrating or ruling out the following operative hypothesis: the exposure of the Cyn d to air pollutants during its growth can modify its immunogenicity and affect the inflammatory response in allergic subjects. To evaluate this, samples of Cyn d were collected, from 2 areas in the city of Medellín: the first one was located in the urban center less than 5 m from a main avenue (more than 100 cars for hour); the another one was located in a rural area more than 1000 m from any main avenue and with low vehicular traffic (less than 5 cars for hour). To compare the inflammatory response, we created 2 study groups: a group with rhinitis with sIgE to Cyn d (Rhinitis [+] Cyn d) and another group with rhinitis without sIgE to Cyn d (Rhinitis [–] Cyn d). To evaluate the possible irritative effect of the extracts, a third group of healthy subjects without rhinitis (Control group) was made.

In each group, skin and challenge tests were carried out with the 2 extracts prepared from the rural (Cyn d-rural) and the urban area (Cyn d-urban). The extracts were compared between them and with a standardized commercial extract of Cyn d (Cyn d-control) (Immunotek laboratories, Madrid, Spain).

Study Population

Patients older than 18 years with persistent moderate/severe rhinitis (Allergic Rhinitis and its Impact on Asthma guidelines) were invited to participate from 2 health centers in the city of Medellín (Colombia); patients from group Rhinitis (+) Cyn d had a skin prick test (SPT) positive to Cyn d-control extract and sIgE for Cyn d measured by immunofluorescence. The group Rhinitis (–) Cyn d comprised patients with allergic rhinitis sensitized to mites but not sensitized to Cyn d or other pollen grains. Control group were healthy subjects who agreed to participate without atopy to common allergens in the region.¹⁷

Cyn d Extract Preparation

A sample of Cyn d grown in urban and rural areas was collected. The details of the procedure for extract preparation, purity of the extract, and allergenic evaluation are found in the Supplemental material.

For the skin test, an extract for the dry material with a concentration of 1 mg/mL was prepared. For nasal challenge test, a final concentration of 10 µg/mL was used, this concentration was previously selected from a titration curve (data not shown).

To analyze the purity and quantity of the proteins in the extracts, ELISA and ELISA inhibition tests were done; the 3 extracts (Cyn d-rural, Cyn d-urban, and Cyn d-control) were placed in solid phase and as inhibitors. Each extract could inhibit in more than 80% the binding of IgE to the other 2 extracts, indicating a good quality and affinity of Cyn d proteins to IgE (details in Supplemental material). The presence of the major allergen Cyn d 1 was confirmed by Immunoblotting and Immunoblotting inhibition and had similar concentration in the 3 extracts (27 µg/mL ± 8 µg).

Atopy Evaluation

The skin test was performed according to international recommendations;¹⁸ the test was read 15 min after the puncture and any reaction with a wheal diameter ≥3 mm over negative control was considered positive. The serum sIgE for Cyn d was evaluated by immunofluorescence, considering a value ≥0.35 kUI_A/mL positive.

Nasal Challenge Test

In each patient, a double-blind placebo-controlled challenge was performed with the 3 extracts, for the evaluation of the nasal challenge test (NCT) results, we use 3 assessments; change in the minimum transverse area,¹⁹ visual analogous scale (VAS), and a score of the 4 most frequent symptoms. Details of the NCT procedure are found in the Supplemental material.

Eosinophil Count

Five days before the NCT and 1 h after the test, a sample of nasal mucus was taken near to the inferior meatus, and a cytology was performed with eosin stain and methylene blue. Eosinophil count before and after the challenge test and the mean eosinophil between the groups was evaluated.

Statistical Analysis

Statistical analyses were performed with the IBM SPSS for Windows program, version 21.0. The mean was used for the descriptive analyses and as a measure of dispersion the standard deviation. The Mann–Whitney test was used for nonparametric variables. The Pearson's χ^2 test was used to evaluate the differences between the groups. The correlation analyses were performed with the Spearman method (*r*). Taking into account the operative hypothesis and the results of previous studies, a sample size of 20 patients per group is adequate to ensure a power of 90% and an alpha error of 0.05 for the main objective (comparison of the potency of 2 allergenic extracts). $P < .05$ was considered statistically significant.

For comparisons between the 3 study groups, we use the Kruskal–Wallis test, for quantitative variables; when this test showed a significant difference ($P \leq .05$), a multiple comparison test using the same analysis can be performed to compare the differences between each possible pair of groups. Because some variables were measured before and after Cyn d exposure, a generalized linear model of repeated measures was used to compare intra- and intergroup measures and the analysis of covariates.

Ethical Considerations

The ethics review committee of the University of Antioquia and the “IPS Universitaria” institution

approved the study (Code number 2015-5383) according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all the participants.

Results

Population Characteristics

Each group consisted of 25 patients (Table 1). In the group Rhinitis (+) Cyn d, 5 patients were monosensitized to Cyn d, and 20 patients were sensitized to mites and 4 of them also to other plants. In the Rhinitis group (–) Cyn d, all were sensitized to mites, but not to plants. The control group was negative to all allergens tested.

Comparison of Skin Sensitization With Different Extracts

The 25 patients of the Rhinitis group (+) Cyn d had a positive SPT for Cyn d-control, Cyn d-rural, and Cyn d-urban. In the group Rhinitis (–) Cyn d, 2 patients were positive with Cyn d-urban extract but negative to other Cyn d extracts (Figure 1(a)). In the control group, none presented positive SPT to any of the 3 tested Cyn d extracts.

When comparing the wheal size among the 3 extracts in the group Rhinitis (+) Cyn d, we observed a significant difference between the extract Cyn d-rural and Cyn d-urban, being the average wheal and erythema major with the Cyn d-urban extract (Figure 1(b)).

NCT With Cyn d Extracts

Seven patients of the group Rhinitis (+) Cyn d were positive in the nasal test with the 3 extracts. Another 5 patients in this group were positive only with the Cyn d-urban extract (Figure 2). In the group Rhinitis (–) Cyn d, 22 patients accepted the NCT and 2 had a positive result with Cyn d-urban. These 2 patients were those

Table 1. General Characteristics.

	Rhinitis (+) Cyn d (n = 25)	Rhinitis (–) Cyn d (n = 25)	Control (n = 25)
Age	27 ± 9	30 ± 7	25 ± 11
Female	14 (56%)	13 (52%)	15 (60%)
Asthma	6 (24%)	3 (12%)	0
Atopy	25 (100%)	25 (100%)	0
slgE Cyn d (kU _A /mL)	3.14 ± 1.14	<0.35 ^a	<0.35 ^a
(+) SPT Polen	25 (100%)	0	0
(+) Cyn d	25 (100%)	0	0
(+) Mites	20 (80%)	25 (100%)	0

SPT, skin prick test.

^aThe majority of patients had an slgE result of less than 0.1 kU_A/mL, that is, the lower reading limit of ImmunoCAP. Four patients in the group with rhinitis (–) Cyn d and 2 patients in the control group had slgE between 0.1 kU_A/mL and 0.35 kU_A/mL.

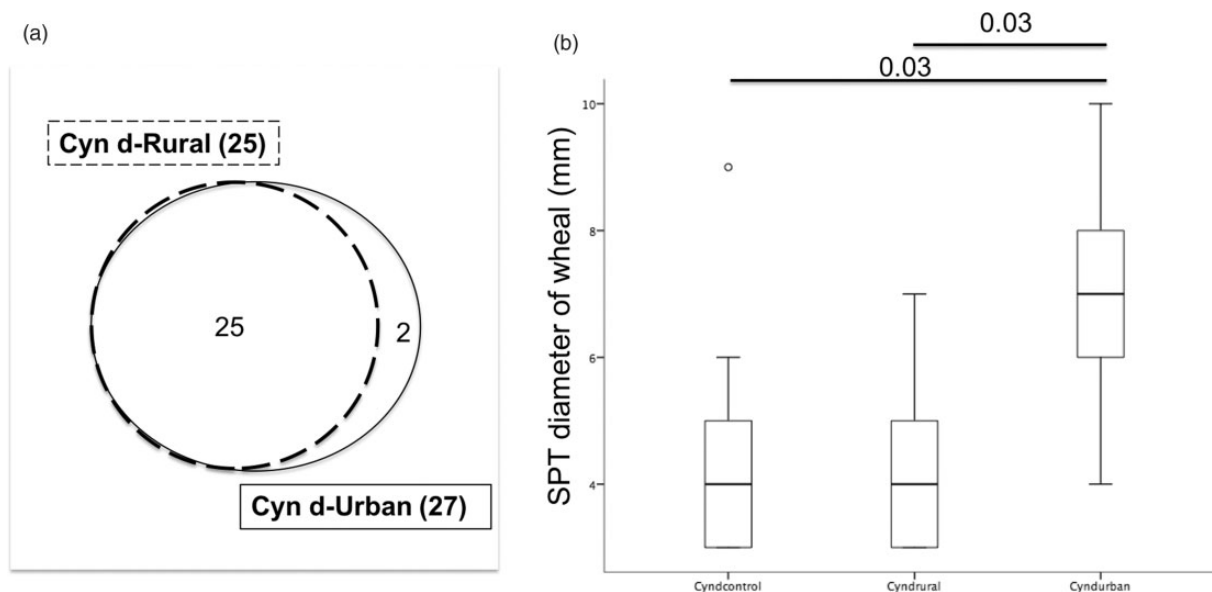


Figure 1. Skin test with Cyn d. (a) Patients with positive skin prick test. All patients (n = 25) of Rhinitis group (+) Cyn d were positive to Cyn d-rural and Cyn d-urban. Two patients of the Rhinitis group (-) Cyn d were positive to Cyn d-urban. (b) The wheal diameter of the skin test with the extract Cyn d-control, Cyn d-rural, Cyn d-urban among patients with positive skin test. $P < .05$ was considered statistically significant.

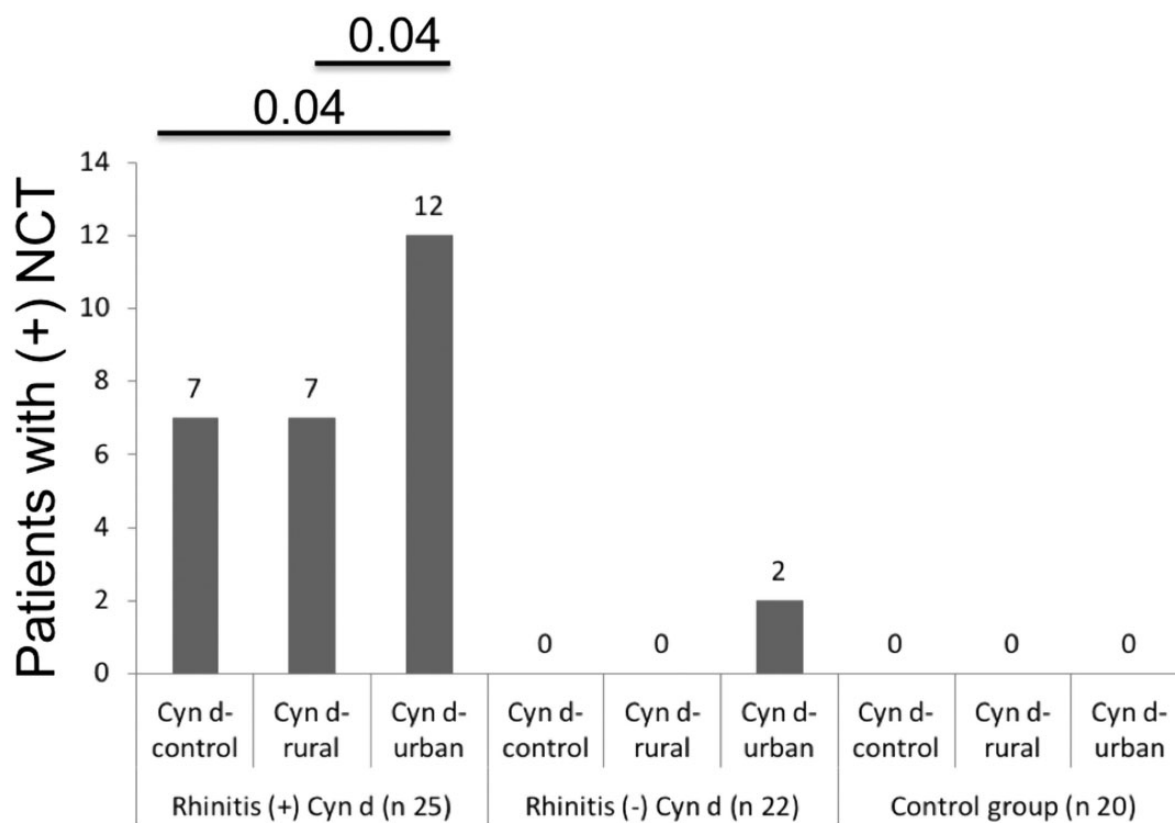


Figure 2. Patients with (+) nasal challenge test. Seven patients from the Rhinitis group (+) Cyn d were positive in the NCT with Cyn d-control, Cyn d-rural, and Cyn d-urban. Five additional patients in this group were positive for PPT with Cyn d-urban and 2 of the Rhinitis group (-) Cyn d. $P < .05$ was considered statistically significant.

who had a positive SPT to Cyn d-urban extract. The levels of sIgE to Cyn d in these 2 patients were 0.17 kUI_A/mL and 0.23 kUI_A/mL, respectively. Twenty subjects of the control group accepted the performance NCT being negative in all cases (Figure 2).

Among the subjects with positive NCT, the intensity of the symptoms was evaluated according to the rhinometry, the VAS scale, and the symptom score. In all 3 scales, the intensity of the symptoms was greater with the Cyn d-urban extract, in comparison with the Cyn d-rural or Cyn d-control extract (Table 2). We did not observe differences between patients with positive or negative challenge according to age, sex, or levels of sIgE to Cyn d.

Eosinophil Count and Nasal Challenge

Among patients with a positive test, there was a statistically significant increase in the number of eosinophils after the challenge (Rhinitis [+] Cyn d $4.9\% \pm 5\%$ vs $13.9\% \pm 8\%$, $P = .05$ and Rhinitis [–] Cyn d $4.6\% \pm 6\%$ vs $15.2\% \pm 8\%$, $P = .04$). The increase in the number of eosinophils was slightly higher with the Cyn d-urban extract, but it was not statistically significant ($P = .08$). Among subjects with negative NCT, there was no significant change in the number of eosinophils.

Discussion

Multiple studies show the impact of air pollution on the health of human beings. Its spectrum of action is not only respiratory diseases, but they can also aggravate cardiovascular and skin diseases.^{20,21} Fujieda and Diaz et al.^{15,16,22,23} conducted different controlled studies in which a group of patients with allergic rhinitis underwent nasal exposure to an allergen together with diesel and demonstrated that this coexposure leads to greater expression of total IgE and nasal inflammation than exposure alone to diesel or allergens. Liu et al. in a similar experiment observed changes in the methylation of inflammatory genes according if the exposure of the allergen occurs with air pollutants or not.²⁴ Although the coexposure allergen/pollutants potentiate the

inflammatory response, little has been studied of how the pollution can modify the allergens and whether these changes affect their allergenicity properties. Experimental studies indicate that modifications in proteins due to air pollution can persist in the plant even after the pollutant disappears;^{12,25} the modifications of these proteins increase their pro-inflammatory and allergenic capacity, favoring lymphocyte activation due to their greater structural stability. Zhao et al.²⁶ observed changes in foliage density and coloration when comparing samples of shrubs from city to urban area; these changes in their morphology were also associated with changes in protein expression. Although these changes suggest that plants after exposure to pollutants may acquire properties that would make them more inflammatory for allergic patients even without coexposure with contaminants, studies carried out so far are limited to experimental evaluations. We wonder if these previous experimental results could have a clinical impact on the allergic population, so we evaluated whether changes in Cyn d due to the place of growth (rural vs urban) could affect the inflammatory response in patients with allergic rhinitis. We chose Cyn d because it is one of the main pollens causing IgE sensitization in several regions of the world,^{17,27} and we found that patients sensitized to Cyn d presented a more intense inflammatory reaction in the cutaneous test and in nasal challenge when were exposed to Cyn d extract from a zone of high vehicular traffic. We also observed that none of the subjects of the control group had a positive skin or nasal reaction with Cyn d extracts, indicating that in rhinitis patients the nasal and skin reaction is due to the Th2 response and not due to irritative effect on the nasal mucosa.

Although it has been proposed that sIgE values less than <0.35 kUI_A/mL are usually not associated with symptoms, Hamizan et al.²⁸ observed in a systematic review that up to 26.5% of patients with nonallergic rhinitis were positive in NCT. A local but not systemic production of sIgE to an allergen have been proposed to explain this rhinitis phenotype, but it has also been proposed that factors such as environmental conditions, allergen concentrations, and exposure time may influence the NCT result.^{28–30} We observed that 2 patients

Table 2. Severity of Symptoms During the Nasal Challenge Test.

	Cyn d-control (n = 7)	Cyn d-rural (n = 7)	Cyn d-urban (n = 14)	P
Rhinometry	37 ± 8	35 ± 10	46 ± 16	.04/.03
VAS (0–10 points) ^a	6 ± 5	6 ± 4	8 ± 3	.05/.05
Symptoms score (0 to 12 points)	7 ± 4	7 ± 5	10 ± 3	.04/.04

^aHere, the variation between the baseline and after the provocation test is observed. Seven patients were positive in the nasal challenge test with Cyn d-control, Cyn d-rural, and Cyn d-urban. Seven additional subjects were positive only with Cyn d-urban; 5 come from rhinitis patients and 2 from control group. The P value represents the comparison of Cyn d-urban versus Cyn d-control/Cyn d-urban versus Cyn d-rural. VAS, visual analog scale.

with low sIgE to Cyn d but negative SPT and NCT to Cyn d-control and Cyn d-rural showed positive results for both tests with Cyn d-urban. These results indicate that allergens sourced from materials grown in urban areas induce a stronger inflammatory response in patients with rhinitis when compared to equivalent allergen source material cultivated in rural areas (Cyn d-rural). Therefore, air pollution not only causes a direct effect at the patient level but also by changing the molecular characteristics of the substances in their environment. Alternative explanation would be an irritative effect of the tested extract; however, it seems unlikely for 2 reasons; first, as detailed in the Supplemental material, the extracts (Cyn d-rural and Cyn d-urban) were compared with the control extract showing similarities in protein bands according immunoblotting; also Cyn d-control produce an inhibition greater than 80% with Cyn d-rural and Cyn d-urban in the ELISA. Second, the negative results of the skin and nasal tests in the control group with Cyn d-urban suggest that these extracts do not produce an irritative effect. The results of this study also have implications in clinical practice; if the extract used for the SPT or for the nasal challenge is obtained from an area with low air pollution, it is possible that the sensitivity of the allergy tests will be affected, with the consequent error in the medical diagnosis. In addition, extracts used for treatments such as immunotherapy with allergens, usually come from cultivation areas with low exposure to pollutants present in urban areas; it is difficult to predict if the clinical response could be favored or not by using extracts that are closer to what the patient living in the urban area is exposed to.

Our study has some limitations: most patients in rhinitis groups were sensitized to other sources, especially mites; however, considering the origin of the extract, it is unlikely that the extracts had contamination of mite proteins. Also, the cosensitization of patients to other plants was low, so it is unlikely that positive results in the tests were due to cross-reactivity. Nevertheless, even if that were the case, the positivity in the NCT indicates a clinical relevance to Cyn d. Because the samples were collected in a wild environment, it is possible that there is some contamination in the extracts with irritating substances, but as we explained earlier, we performed several procedures to minimize this probability, and the results found in the control group suggest that this is unlikely. In addition, the results observed in the ELISA and immunoblotting (see Supplemental material) show a high concordance between the skin tests using the 2 prepared extracts and the control extract.

In conclusion, these findings suggest that the growth conditions of the Cyn d modify its allergenicity; when it occurs in urban centers the severity of the clinical response in the allergic patient worsens. Although it

must be proven, it is logical to think that these changes also occur in other sources of allergens.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

This study was approved by our institutional review board.

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Statement of Human and Animal Rights

All procedures were performed after the approval by the ethical committee.

Statement of Informed Consent

All patients signed informed consent.

Supplemental Material

Supplementary material for this article is available online.

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