

Nasal inflammatory mediators and specific IgE production after nasal challenge with grass pollen in local allergic rhinitis

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Background: Evidence exists of a new form of local allergic rhinitis (LAR) with local production of specific IgE (sIgE) and a positive response to nasal allergen provocation test (NAPT) in patients previously diagnosed with idiopathic rhinitis. However, the immunologic mechanisms involved are still poorly understood.

Objective: We explored the involvement of nasal sIgE, eosinophil, and mast cell activation in the response to NAPT with grass pollen (NAPT-grass) in a group of patients already classified with LAR.

Methods: Out-of-spring NAPT-grass was performed in 30 patients with LAR and 30 healthy controls. Nasal symptoms, acoustic rhinometry, and nasal lavage were performed at baseline and 15 minutes and 1, 6, and 24 hours post-NAPT. Tryptase, eosinophilic cationic protein (ECP), and total and sIgE to grass pollen were measured in nasal lavage by immunoassays.

Results: NAPT-grass was positive in all patients with LAR. We detected significant increases of tryptase and ECP in 40% and 43%, respectively, at 15 minutes and 1, 6, and 24 hours post-NAPT compared with baseline ($P < .05$). sIgE was increased in 30%, with significant increases at 1 and 6 hours ($P < .05$) and 24 hours ($P = .002$) post-NAPT. The maximum release of tryptase was detected 15 minutes after NAPT, whereas the maximum release of ECP and sIgE was detected 24 hours after challenge. NAPT-grass was negative in all healthy controls, with no increase in tryptase, ECP, total IgE, or sIgE.

Conclusion: These results demonstrate that patients with LAR had local production of sIgE and mast cell/eosinophil activation induced by nasal exposure to grass pollen. (*J Allergy Clin Immunol* 2009;124:1005-11.)

Key words: ECP, grass, local allergic rhinitis, nasal allergen provocation test, nasal lavage, nasal specific IgE, tryptase

Although the prevalence of idiopathic rhinitis (IR) is unknown, it appears to be common, occurring in approximately 19 million

Abbreviations used

ECP:	Eosinophilic cationic protein
IR:	Idiopathic rhinitis
LAR:	Local allergic rhinitis
NAPT:	Nasal allergen provocation test
NAPT-grass:	Nasal allergen provocation test with grass pollen
sIgE:	Specific IgE
VAS:	Visual analog scale
VOL 2-6cm:	Volume of the nasal cavity from 2 to 6cm

persons in the United States,¹ and in 23% to 71% of adults with rhinitis.^{2,3} IR is made up of a group of nasal entities of unknown etiology, usually diagnosed by exclusion.^{4,5}

There is evidence to support the existence of a new form of local allergic rhinitis (LAR) or entopy with local production of specific IgE (sIgE) and a positive response to a nasal allergen provocation test (NAPT) in patients previously diagnosed with IR.⁶⁻⁹ However, the immunologic mechanisms involved have not yet been studied in sufficient detail.

Patients with allergic rhinitis present an inflammatory IgE-mediated response¹⁰ characterized by a T_{H2} immunologic pattern,^{11,12} with mast cell and eosinophil activation and release of inflammatory mediators in response to exposure to aeroallergens.¹⁰ NAPT has proved an indispensable tool for the study of the pathophysiology of allergic rhinitis and to describe the time course of the release of several inflammatory mediators in nasal secretions.¹³⁻¹⁸

During natural exposure to aeroallergens, patients with LAR show a similar nasal inflammatory pattern to patients with allergic rhinitis, with increased levels of eosinophils, basophil-mast cells, total lymphocytes, CD3⁺ T cells, and eosinophilic cationic protein (ECP).^{8,9,18,19}

The aim of this study was to explore the involvement of nasal sIgE, and eosinophil and mast cell activation in response to NAPT with grass pollen (NAPT-grass), in patients with LAR. The clinical response, nasal patency, and nasal release of total and sIgE, tryptase, and ECP were monitored as long as 24 hours after nasal challenge in a group of patients with LAR and compared with a group of healthy controls.

Nasal mucosal exposure to grass pollen in patients with LAR induces immediate or dual responses, with local production of sIgE and mast cell/eosinophil activation with increased concentrations of tryptase and ECP.

METHODS

Study subjects

A total of 74 subjects were recruited consecutively over 12 months: 38 subjects with LAR and 36 healthy nonatopic controls. The study was performed out of the natural allergen season, when all the patients were

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This work was supported by grants from Spanish Health Ministry (FIS) (PI081572), "Consejería de Salud" Andalusia government (PI0181) and FIS network RIRAAF (RD07/0064).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication February 5, 2009; revised May 7, 2009; accepted for publication July 1, 2009.

Available online October 5, 2009.

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0091-6749/\$36.00

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doi:10.1016/j.jaci.2009.07.018

symptom-free. The local ethics committee approved the study, and informed consent was obtained.

The inclusion and exclusion criteria for patients with LAR and controls are described in this article's **Methods** section of Online Repository at www.jacionline.org.

Skin tests and serum total and sIgE

Intradermal skin test with grass pollen, skin prick test, and serum sIgE with the most prevalent aeroallergens, and serum total IgE was performed in all subjects. The detailed methods are provided in the **Methods** section of the Online Repository.

NAPT

Symptom-free patients and healthy controls were challenged intranasally with saline and grass pollen⁹ (ALK-Abelló, Madrid, Spain). Details can be found in the **Methods** section of the Online Repository.

Statistical analysis

The data were expressed as means \pm SDs. The clinical and demographic data were compared between groups by χ^2 analysis and Mann-Whitney *U* test. The Friedman test was used to examine overall differences. If significant differences occurred, the Wilcoxon signed-ranks test was used to identify significant differences in tryptase, ECP, total IgE, and sIgE in nasal secretions, visual analog scale (VAS) symptoms, and volume of the nasal cavity from 2 to 6cm (VOL 2-6cm) in acoustic rhinometry within groups. Correlation was performed by using the Spearman rank method. All statistical analyses were carried out using the software package SPSS for Windows 15.0 (SPSS, Chicago, Ill). A *P* value $<.05$ was considered statistically significant.

RESULTS

Subjects

Of 74 subjects initially evaluated, 8 patients with LAR and 6 control subjects had positive responses to nasal saline challenge and were excluded from further analysis. All subjects with nasal hyperresponsiveness presented an immediate response to saline challenge, 64% bilateral and 36% unilateral. No differences were found between patients with LAR and controls.

Clinical and epidemiologic data are shown in **Table I**. All 30 patients with LAR complained of symptoms induced by pollen in the seasonal period, and 47% reported a history of bronchial asthma and 57% a history of conjunctivitis.

NAPT

NAPT-grass performed previously for inclusion of the subjects was repeated during the study period in all the selected subjects, with the same results: NAPT was positive in all patients with LAR and negative in all controls. All patients with LAR presented a immediate (30%) or dual response (70%); no isolated late responses were observed. The majority of patients with LAR (60%) showed a bilateral response to grass pollen with a decrease in VOL 2-6cm $>30\%$ in each nasal cavity, whereas 40% developed a unilateral response in the acoustic rhinometry, with a higher decrease in VOL 2-6cm in 1 nasal cavity (mean decrease of VOL 2-6cm, 64%) and a moderate decrease in the other cavity (mean decrease of 23%). In all cases, the total decrease in VOL 2-6cm (sum of left and right nasal cavities) was higher than 30%.

An association with asthma was similar in patients with immediate (56%) and dual responses (43%). However, the

TABLE I. Epidemiologic characteristics of patients and controls

Groups	LAR	Controls
Subjects (N)	30	30
Age (y)	39 \pm 15	37 \pm 12
Onset age (y)	31 \pm 16	NA
Disease duration (y)	7 \pm 8	NA
Sex, N (%)		
Male	11 (37)	12 (40)
Female	19 (63)	18 (60)
FHA, N (%)	13 (43)	11 (37)
Comorbidity, N (%)		
Asthma	14 (47)	NA
Conjunctivitis	17 (57)	NA
Triggering factors, N (%)		
Pollens	30 (100)	NA

FHA, Family history of atopy; NA, not applicable.

Data are expressed as means \pm SDs unless otherwise stated.

association with conjunctivitis was higher in patients with immediate (90%) than dual responses (57%) to NAPT-grass (*P* = .001).

NAPT monitoring in LAR group

VAS symptoms The patients with LAR showed a positive increase in VAS symptoms at 15 minutes and 1, 6, and 24 hours after NAPT-grass (**Fig 1**).

The patients with LAR with a clinical isolated immediate response (30%) had significant increases in VAS symptoms at 15 minutes (78%) and 1 hour (22%) after NAPT-grass (*P* $<.05$) compared with baseline. The patients with LAR with a dual response (70%) had 2 peaks of significant increases in VAS symptoms, at 15 minutes (*P* $<.001$) and 6 hours (*P* $<.05$) after challenge compared with baseline. The comparative study between immediate and dual responders showed significantly higher values of VAS symptoms in patients with a dual response at 6 hours after NAPT-grass (*P* $<.05$). Analysis of the frequency of individual nasal symptoms showed that itching and rhinorrhea (88% in both cases) were the most frequent symptoms reported by the patients with LAR during the immediate response, followed by sneezing (77%) and nasal obstruction (67%). The most frequent symptom observed in the late response of the 21 cases who had dual responses to NAPT-grass was obstruction (86%), followed by rhinorrhea (81%), itching (76%), and to a lesser extent, sneezing (38%).

Acoustic rhinometry. The clinical immediate and dual responses were accompanied by a significant reduction in nasal patency in the patients with LAR, represented by the mean value of VOL 2-6cm of the nasal cavity (**Fig 2**). The mean value of VOL 2-6cm of the nasal cavity in patients with an isolated immediate response decreased significantly from 100% at baseline to 56% \pm 4.7% (mean \pm SD) 15 minutes after NAPT (*P* $<.05$) and returned to normal values 1 hour after challenge. Of the patients with a dual response, 70% had a decrease in the mean values of VOL2-6cm ($\geq 30\%$ compared with baseline) at 15 minutes (from 100% to 63% \pm 4.9%, mean \pm SD) and 1 hour (from 100% to 67% \pm 3.2%, mean \pm SD) after challenge. Comparison between immediate and dual responders showed that the patients with LAR with a dual response had lower levels of VOL2-6cm at 1, 6, and 24 hours after nasal challenge (*P* $<.05$), and the patients

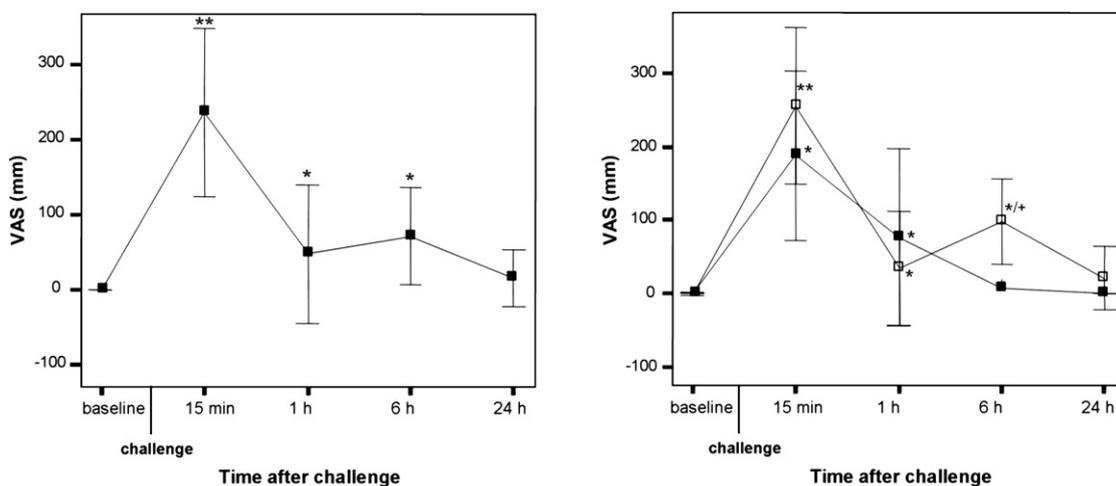


FIG 1. Changes in total VAS symptoms in all patients with LAR (*left*), and between immediate (■) and dual (□) responders (*right*) after NAPT-grass. Significant differences between baseline and 15 minutes and 1, 6, and 24 hours after challenge are indicated by * $P < .05$ and ** $P < .001$. Significant differences between immediate and dual responders are indicated by + $P < .05$. Data are expressed as means and SDs.

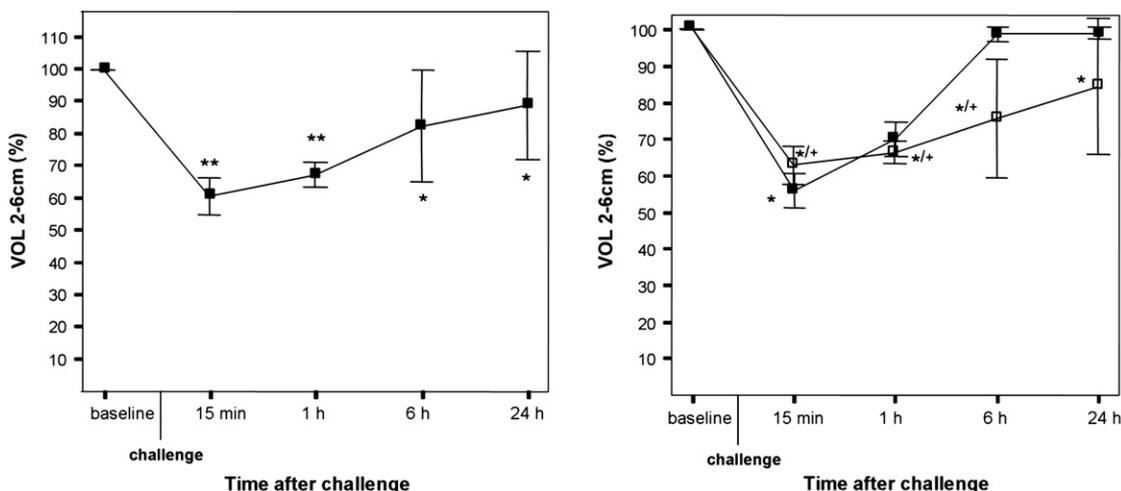


FIG 2. Changes in VOL 2-6cm in all patients with LAR (*left*), and between immediate (■) and dual (□) responders (*right*) after NAPT-grass. Significant differences between baseline and 15 minutes and 1, 6, and 24 hours after challenge are indicated by * $P < .05$ and ** $P < .001$. Significant differences between immediate and dual responders are indicated by + $P < .05$. Data are expressed as means and SDs.

with LAR with an isolated immediate response had lower levels 15 minutes after NAPT-grass ($P < .05$).

Tryptase. Tryptase was detected in 40% of the patients (42% isolated immediate response and 58% dual; Fig 3). The patients with LAR had an increases of tryptase into nasal secretions at 15 minutes ($P < .01$), 1 hour ($P < .01$), 6 hours ($P < .05$), and 24 hours ($P < .01$) postchallenge compared with baseline. The maximum level was detected 15 minutes after challenge, decreasing over time and normalizing at 24 hours. When the subjects were studied according to their pattern of clinical response, we observed that the patients with LAR with an isolated immediate response presented significantly increased levels of tryptase at 15 minutes and 1 hour postchallenge ($P < .05$) compared with baseline values and a higher release of tryptase into nasal secretions 15 minutes after NAPT-grass than those patients with a dual response ($P < .05$).

The patients with LAR with a dual response had a significant increase in tryptase at 15 minutes and 1 and 6 hours compared with baseline values ($P < .05$). Although higher levels of tryptase at 6 and 24 hours after challenge were observed in dual than in immediate responders, these differences were significant only at 6 hours after challenge ($P < .05$; Fig 3). Only 1 patient with a dual response had a detectable concentration of tryptase in nasal secretions at 24 hours after NAPT-grass (1.16 ng/mL).

ECP. Nasal release of ECP was detected after NAPT in 43% of the patients with LAR (38% isolated immediate response and 62% dual; Fig 4). A significantly increased release of ECP into nasal secretions was detected at 15 minutes ($P < .01$) and 1, 6, and 24 hours ($P = .008$) after NAPT compared with baseline, rising to reach maximum levels at 24 hours. There were no significant differences in nasal release of ECP between patients with LAR with an immediate or a dual response (Fig 4).

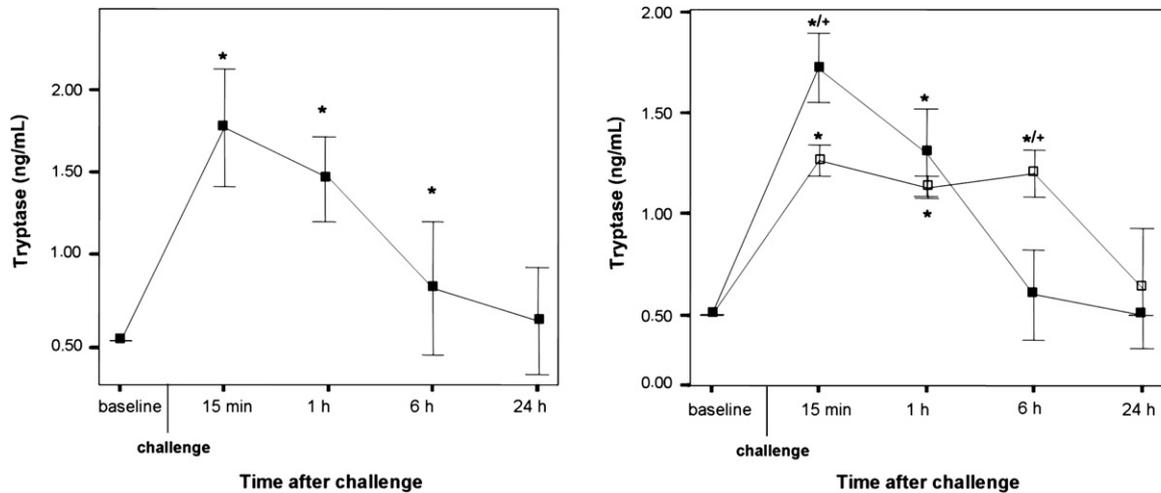


FIG 3. Nasal levels of tryptase in all patients with LAR (*left*), and between immediate (■) and dual (□) responders (*right*) after NAPT-grass. Significant differences between baseline and 15 minutes and 1, 6, and 24 hours after challenge are indicated by * $P < .05$. Significant differences between immediate and dual responders are indicated by + $P < .05$. Data are expressed as means and SDs.

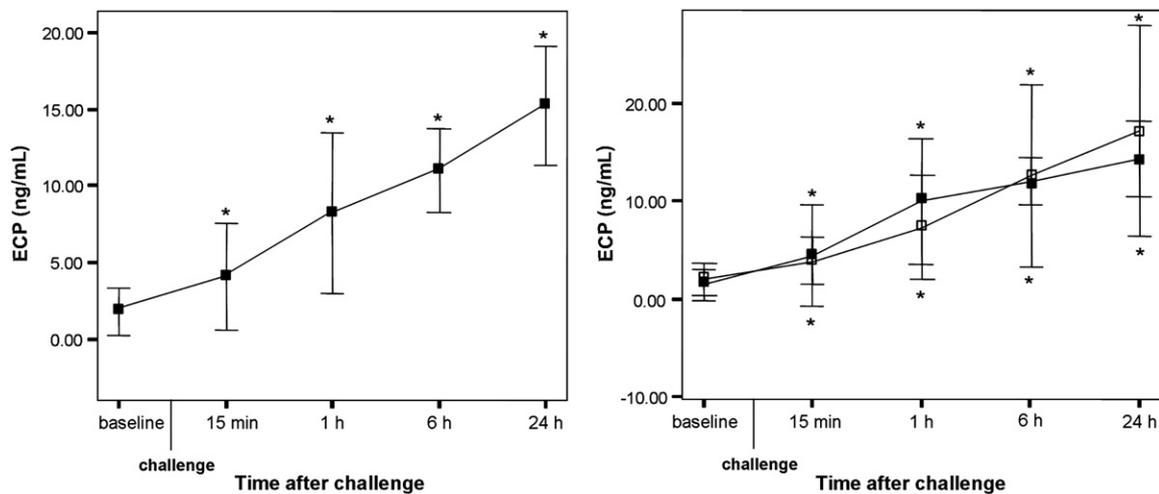


FIG 4. Nasal levels of ECP in all patients with LAR (*left*) and between immediate (■) and dual (□) responders (*right*) after NAPT-grass. Significant differences between baseline and 15 minutes and 1, 6, and 24 hours after challenge are indicated by * $P < .05$. No significant differences between immediate and dual responders were found. Data are expressed as means and SDs.

Total and sIgE in nasal lavage. Nasal sIgE to grass pollen was detected in 30% of patients with LAR after NAPT-grass (NAPT⁺/sIgE⁺) and remained undetectable in the others (NAPT⁺/sIgE⁻). No increases in total IgE were detected.

Patients with NAPT⁺/sIgE⁺ had significant increases in nasal concentrations of sIgE at 1 and 6 hours ($P < .05$) and 24 hours ($P = .01$) compared with baseline; 4 of these patients had detectable baseline levels of sIgE to grass pollen out of spring. The nasal concentration of sIgE to grass pollen showed a progressive increase, reaching a maximum 24 hours after challenge. These data are shown in Fig 5.

Of the 9 patients with LAR with NAPT⁺/sIgE⁺, 4 presented an isolated immediate response to NAPT-grass and 5 a dual response. There were no significant differences in nasal levels of sIgE to grass pollen between patients with LAR with an immediate or a dual response (Fig 5).

An overall comparison between NAPT⁺/sIgE⁺ and NAPT⁺/IgE⁻ patients showed a similar pattern of NAPT response, with a majority of dual and bilateral responders. However, significant differences were detected in nasal levels of tryptase and ECP, with significant increases after nasal challenge in 100% of NAPT⁺/sIgE⁺ patients but in only 14% (tryptase) and 19% (ECP) of NAPT⁺/IgE⁻ patients ($P = .001$).

Correlations between nasal symptoms and nasal production of tryptase, ECP, and sIgE. A significant association was observed between the increases in the nasal production of tryptase and the increase in the intensity of itching ($r = 0.788$; $P = .001$), sneezing ($r = 0.763$; $P = .001$), rhinorrhea ($r = 0.505$; $P = .001$), and nasal obstruction ($r = 0.425$; $P = .003$) after NAPT-grass (Fig 6). A similar association was observed between the increase in nasal ECP production and nasal obstruction ($r = 0.559$; $P = .001$) after allergen provocation (Fig 6). No

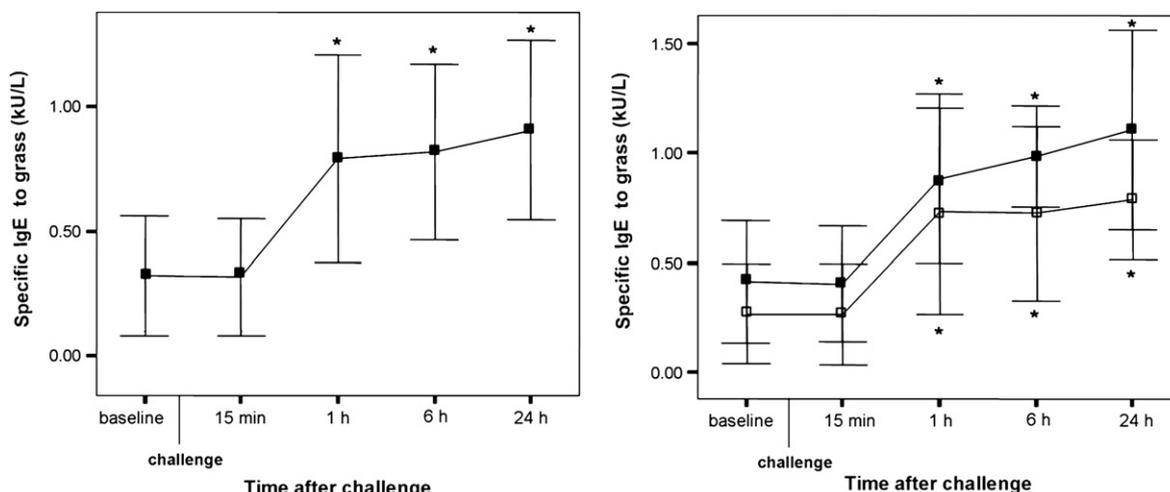


FIG 5. Nasal levels of sIgE in all patients with LAR (*left*), and between immediate (■) and dual (□) responders (*right*) after NAPT-grass. Significant differences between baseline and 15 minutes and 1, 6, and 24 hours after challenge are indicated by * $P < .05$. No significant differences between immediate and dual responders were found. Data are expressed as means and SDs.

significant association was detected between nasal levels of sIgE and the increase in intensity of nasal symptoms.

NAPT monitoring in the control group. Nasal challenge was negative in all healthy controls. In these subjects, no significant increases were detected in total VAS symptoms, VOL 2-6cm, inflammatory mediators, or IgE.

DISCUSSION

Although NAPT is a useful tool to investigate the pathophysiology of allergic rhinitis,¹³⁻¹⁸ it has been used less in IR. Several studies have shown an inflammatory response in nasal mucosa of patients with allergic rhinitis with mast cell and eosinophil activation and release of inflammatory mediators that can be detected in nasal secretions after NAPT.¹⁴⁻¹⁷ This approach enables us not only to quantify the inflammatory mediators involved but also to estimate the presence and production of nasal IgE antibodies in patients with LAR. We therefore decided to explore the involvement of nasal sIgE, and eosinophil and mast cell activation, in the inflammatory response to NAPT-grass in a well defined group of patients with LAR.

In this work, the group of patients with LAR studied had a history of rhinitis of more than 7 years of evolution, with the presence of concomitant conjunctivitis (57%) and bronchial asthma (47%), and a positive response to nasal challenge with grass pollen in the previous 12 months. NAPT-grass was positive in all patients with LAR, and negative in all control subjects, indicating the consistency and reproducibility of these observations when the response to NAPT is monitored using 2 parameters: nasal symptom scores and objective data of nasal obstruction by acoustic rhinometry. Other authors have observed that the use of the nasal symptom score as a sole evaluation parameter in NAPT may lead to frequent false-positive responses.²⁰

Sixty percent of the patients developed a bilateral positive response after nasal challenge; similar data were observed by Carney et al²¹ in a group of patients with IR with positive NAPT with house dust mite, pollens, and cat/dog epithelia. The physiological nasal cycle may explain why some patients with LAR developed a positive response to NAPT with a

predominant unilateral nasal decrease in VOL 2-6cm in acoustic rhinometry.^{22,23}

In our study, all the patients with LAR had a positive immediate response, and 70% also had a late response, but no isolated late response was observed. These data are in agreement with those observed by our group in patients with LAR with positive NAPT to *Dermatophagoides pteronyssinus*,⁸ grass, and/or olive pollen.⁹ The existence of a late response with recurrence of symptoms and release of inflammatory mediators after NAPT has been demonstrated by several authors in patients with allergic rhinitis with and without asthma.^{14,17,24-27}

A late bronchial response after allergen inhalation in allergic asthma has also been reported in approximately 60% of asthmatic allergic subjects.²⁸ This response has been considered a more important sign of airway inflammation than the immediate allergic airway response. Whether this occurs in LAR needs further evaluation.

In our study, an important group of patients with LAR presented a significant increase in nasal release of tryptase, ECP, and sIgE to grass pollen after nasal allergen challenge, with a different time course of nasal release. No increases were detected in healthy controls. Tryptase and ECP were chosen as specific markers of mast cell and eosinophil activation, respectively. The maximum level of tryptase was detected at 15 minutes, decreasing over time and normalizing at 6 hours in immediate responders and at 24 hours after challenge in dual responders. We found a significant correlation between the increase in nasal concentration of tryptase and ECP and the intensity of nasal symptoms. These correlations were more intense between tryptase and itching and rhinorrhea, and also between ECP and nasal obstruction. We also found significant differences between immediate and dual LAR responders. Patients with an isolated immediate response had a greater association with conjunctivitis, and a stronger nasal response to nasal challenge, with significantly higher decrease in nasal patency at 15 minutes after NAPT-grass and a higher peak of maximum nasal concentration of tryptase 15 minutes after challenge than dual responders, with the nasal levels of tryptase decreasing over time and returning to baseline levels at 24 hours. Patients with LAR with a dual response to NAPT-grass, however, had sustained high nasal levels

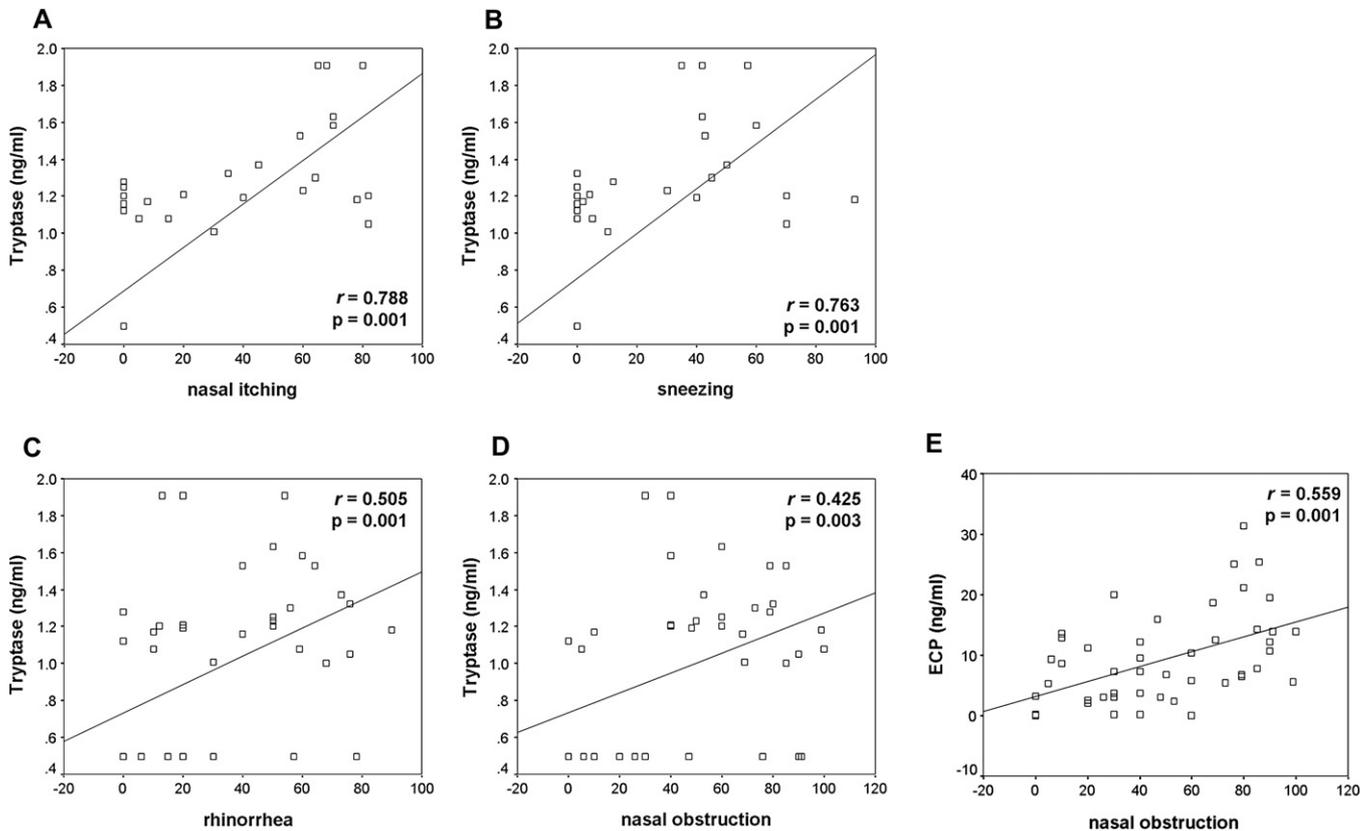


FIG 6. A-D, Correlation of nasal levels of tryptase (y -axis) with intensity of nasal symptoms (x -axis) after NAPT-grass in patients with LAR: nasal itching (A, Spearman $r = 0.788$, $P = .001$), sneezing (B, Spearman $r = 0.763$, $P = .001$), rhinorrhea (C, Spearman $r = 0.505$, $P = .001$), and nasal obstruction (D, Spearman $r = 0.425$, $P = .003$). E, Correlation of nasal levels of ECP with intensity of nasal obstruction after NAPT-grass in patients with LAR (Spearman $r = 0.559$, $P = .001$).

of tryptase during the first 6 hours after challenge. The reasons for these differences are unknown. Graaf-in't Veld et al²⁹ reported short peaks of tryptase during the late-phase response to NAPT with *D pteronyssinus* in patients with allergic rhinitis, but high sustained release of tryptase because of persistent mast cell activation during the late phase of allergic reactions has not been reported.²⁵ Whether there was a limited degradation of tryptase in our cases was not determined.

Eosinophilic cationic protein is stored in the granules of eosinophils and secreted on cell activation. It has been used in previous studies as a specific marker for eosinophil activation in late responses to NAPT in subjects with allergic rhinitis, showing a significant increase 4 to 9 hours after challenge.^{29,30} In our group of patients with LAR, the release of ECP was detected 15 minutes after the NAPT, and then increased progressively, reaching the maximum nasal concentration at 24 hours after provocation. There were no differences between an immediate and a dual response. These results reflect eosinophil activation during the immediate and late response to nasal challenge in patients with LAR, not just during the late response, as has been reported in atopic patients.^{27,29-31}

An important finding of this study was the detection of significantly increased nasal levels of sIgE to grass pollen at 1, 6, and 24 hours after NAPT in 30% of the patients with LAR. This rapid nasal release of sIgE after nasal challenge, with the detection in some patients of baseline levels of sIgE to grass

pollen out of spring, supports the existence of a persistent local synthesis of sIgE in nasal mucosa of patients with LAR that rapidly enhances after nasal exposure to aeroallergens.

T lymphocytes are one of the principal cells that regulate and coordinate immune responses in allergic diseases. In atopy, T_H2 cells regulate IgE synthesis and cell recruitment at the sites of inflammation.⁴ Indeed, the mucosal inflammation in allergic rhinitis is characterized by tissue infiltration of T lymphocytes ($CD4^+$ T cells and $CD25^+$ [activated] T cells) in both the submucosa and the epithelium.^{32,33} A significant correlation has been reported between the increase in $CD4^+$ T cells during the late-phase allergic reaction after an allergen challenge and the number of infiltrating eosinophils in the mucosa.³² In previous studies, our group observed an increase in T lymphocytes during the symptomatic period in nasal secretions from patients with LAR.^{8,9} These cells may contribute to the recruitment of eosinophils and to the production of the IgE antibodies in patients with LAR. In fact, we are currently trying to identify lymphocyte subpopulations in the nasal secretions of patients with LAR during NAPT.

In the current study, patients with LAR showed their maximum nasal levels of ECP and sIgE to grass pollen at 24 hours after nasal challenge, the final time point of the evaluation period in this study. Whether nasal production of ECP and/or sIgE increases further beyond this time point is a question for further analysis.

Previous data from our group have shown an elevation of ECP and sIgE to both house dust mite⁸ and grass pollen⁹ during natural

exposure to aeroallergens, although tryptase levels were not measured. In this first kinetic study to be performed in patients with LAR, we provide sequential data and demonstrate a significant correlation between intensity of nasal symptoms and nasal production of tryptase and ECP after nasal allergen provocation with grass pollen. No further determinations were made after 24 hours, although studies of the release kinetics of inflammatory mediators over a longer period are currently being undertaken by our group.

In this study, increased nasal levels of tryptase, ECP, and/or sIgE to grass pollen were not detected in all the patients. This may be explained by the limited sensitivity of the method, or perhaps to another mechanism. Accordingly, this issue needs to be addressed in further studies.

In summary, we showed that NAPT is a good approach to confirm the immunologic mechanism involved in LAR and to detect the presence of sIgE antibodies. The increase in the local production of sIgE to grass pollen observed in this study requires further investigation to assess the kinetics of the production of sIgE antibodies and the lymphocyte subpopulations and cytokines involved.

We thank Ian Johnstone for help with the final English language version of this article.

Clinical implications: The results confirm the existence of a new form of LAR with local production of sIgE, tryptase, and ECP induced by aeroallergens.

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METHODS

Study subjects

The inclusion and exclusion criteria for patients with LAR and controls were as follows:

1. Inclusion criteria for LAR: age 18 to 70 years, a history of persistent rhinitis for at least 2 years, a positive response to NAPT and/or nasal sIgE to grass pollen, but a negative skin prick test (SPT) and serum sIgE to common environmental aeroallergens, and a negative intradermal skin test to grass pollen.
2. Exclusion criteria for LAR: another immunologic disease, chronic rhinosinusitis and/or nasal polyposis (computed tomography scan), vasomotor rhinitis (clear rhinorrhea and response to ipratropium bromide) or respiratory infection for 4 weeks. Treatment with systemic or nasal corticosteroids (4 weeks), or systemic antihistamines or nasal vasoconstrictors (2 weeks). Pregnant or breast-feeding patients.
3. Inclusion criteria for controls: age 18 to 70 years, healthy, no allergic or nasal diseases, no pregnancy or lactation, and negative SPT, serum sIgE to aeroallergens, intradermal skin test and NAPT to grass pollen.

SPT

The SPT was performed by using the most prevalent inhalant allergens, including house dust mite (*D pteronyssinus*, *Dermatophagoides farinae*, *Lepidoglyphus destructor*, *Blomia tropicalis*), grass pollens (*Poa*, *Phleum*, *Lolium*), casuarina, eucalyptus, cypress (*Cupressus*), plane tree (*Platanus*), olive tree (*Olea europaea*), sunflower (*Helianthus*), lamb's quarter (*Chenopodium album*), English plantain (*Plantago lanceolata*), mugwort (*Artemisia*), wall pellitory (*Parietaria judaica*), Russian thistle (*Salsola kali*), red sorrel (*Rumex*), castor oil plant (*Ricinus*), molds (*Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*), and dog, cat, and hamster epithelia (ALK-Abelló, Madrid, Spain).

Intradermal skin test

Intradermal skin testing was performed with freshly reconstituted freeze-dried allergen solutions of grass pollen mixture (0.01 and 0.1 $\mu\text{g}/\text{mL}$; ALK-Abelló), as described.^{E1}

Total and sIgE in serum

Total IgE and sIgE were determined by immunoassay (UniCAP; Phadia, Uppsala, Sweden). For serum sIgE, we used the same allergens as for the SPT. The cutoff value for sIgE was 0.35 kU/L.

NAPT

Symptom-free patients and healthy controls were challenged intranasally with saline to exclude nasal hyperactivity. If negative, NAPT was performed 1 week later with freshly reconstituted grass pollen (0.1 $\mu\text{g}/\text{mL}$) extract (ALK-Abelló). The allergen dose (2 puffs, 100 μL) was administered in each nasal passage. A positive NAPT was considered to be an increase $\geq 30\%$ in the total nasal symptoms and a decrease $\geq 30\%$ in the total volume of the nasal cavities from 2 to 6 cm (VOL 2-6cm) in acoustic rhinometry compared with the baseline test. A positive response was categorized as immediate (15 minutes to 1 hour after challenge), late (6-24 hours after challenge), or dual (both immediate and late).

NAPT monitoring

Nasal allergen provocation test with grass pollen was monitored before and 15 minutes and 1, 6, and 24 hours after the challenge by nasal symptoms, acoustic rhinometry, and measurement of inflammatory mediators and total and sIgE to grass pollen in nasal secretions.^{E2,E3} The subjects remained in the laboratory throughout the first hour and for 30 minutes before the 6-hour and 24-hour time points to limit their potential exposure to changes in temperature or irritant odorants.

Nasal symptoms

The subjects used a VAS of 100 mm, with a total range of 0 to 400 mm, to record nasal symptoms of obstruction, rhinorrhea, itching, and sneezing before and during the NAPT.

Acoustic rhinometry

Nasal patency before and during the challenge was assessed by acoustic rhinometry with the use of a SRE 2000 rhinometer (Rhinometrics, Lyngø, Denmark) following the guidelines of the Standardization Committee on Acoustic Rhinometry.^{E4} The parameter used (VOL 2-6cm), the volume of the nasal cavity corresponding to the lower turbinate, was analyzed in each nostril. The changes in VAS and acoustic rhinometry were previously validated in symptomatic patients with persistent allergic rhinitis and using mediator release (histamine).

Nasal inflammatory mediators, total IgE and sIgE measurements

A bilateral nasal lavage by the Naclerio method^{E5} was performed with 8 mL physiologic saline with the subjects seated with their necks extended approximately 30° from the vertical. After 10 seconds, the subjects flexed their necks and expelled the sample of mucus and saline into a container. The procedure was then repeated in the other nostril.

To increase cell viability, sample processing was kept at 4°C. Samples were centrifuged at 2000 rpm (1069g) for 7 minutes at 4°C, and the supernatant was stored at -20°C for measurement of tryptase, ECP, total IgE, and sIgE to grass pollen by immunoassay (UniCAP). The cutoff value of the immunoassays was 1 ng/mL for tryptase, 2 ng/mL for ECP, and 0.35 kU/L for sIgE.

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