

Repeatability of nasal allergen challenge results

Further validation of the allergic rhinitis clinical investigator collaborative protocols



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ARTICLE INFO

Article history:

Received for publication November 2, 2017.

Received in revised form February 5, 2018.

Accepted for publication February 6, 2018.

ABSTRACT

Background: Nasal allergen challenge (NAC) models have been used to study allergic rhinitis and new therapies. Symptoms and biological samples can be evaluated at time points after allergen exposure.

Objective: To verify protocol repeatability and adequate interval between allergen exposures.

Methods: Ten ragweed allergic participants were exposed to incrementally increasing dosages of ragweed allergen intranasally until they achieved a total nasal symptom score (TNSS) of 8 of 12 and a peak nasal inspiratory flow (PNIF) of 50% reduction or more from baseline. Three weeks later, participants were challenged with a cumulative dose equal to the sum of all the allergen doses received at screening. TNSS and PNIF were recorded at regular intervals, including a 24-hour assessment. A subsequent visit was conducted after a further 3 weeks. Nasal secretion samples were collected for cytokine and eosinophil quantification.

Results: Nine participants completed all visits. TNSS and PNIF responses followed previous patterns, with an initial peak at 30 minutes followed by a gradual decline. Most participants reported similar patterns at both NAC visits, although some did not demonstrate the same phenotype at both visits. Some experienced a secondary symptom increase 24 hours after NAC. Eosinophil and cytokine sections followed a similar pattern at both NAC visits.

Conclusion: NAC is an adequate method for modeling AR in humans, demonstrating appropriate repeatability of symptoms, nasal mucosal eosinophil, and cytokines. The 24-hour time point, previously not studied in our model, may be beneficial in evaluation of long-acting medications. This three-week interval NAC model will be beneficial for studies in which before and after treatment comparisons are desired.

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Introduction

Nasal allergen challenge (NAC) protocols are used to study allergic rhinitis (AR) pathophysiology and evaluate the efficacy and mechanism of action of novel antiallergic medications, including allergen immunotherapies. Compared with other well-established

controlled allergen challenge facilities (CACFs), which are capable of exposing a relatively large number of participants in one setting to an allergen in a controlled environment, NAC protocols allow studying AR in smaller populations at multiple locations without the need for specialized equipment.^{1,2}

A point that remains in discussion is the effect of repeated allergen exposure in this model on biological and pharmacologic outcomes. The first to describe this phenomenon was Connell³ in 1969, calling it the “priming effect” and suggesting that repeated exposure to ragweed lowers the nasal threshold of reactivity of the nasal mucosa. Priming is thought to be the result of an influx of inflammatory cells during the late phase of AR.⁴ The effects of priming can be nonspecific.^{3,5} For example, a participant who is allergic to dust mites and is exposed to the allergen at home may be considered primed and will reach the target symptom score at a CACF clinical trial much faster than a nonprimed participant.⁵ Although studies that followed found similar effects, some reported the lack of the priming phenomenon, which may be attributed to

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Disclosures: Dr Soliman reported being a current employee of Boehringer Ingelheim. no conflicts to declare. Dr Ellis reported participating in advisory boards for AstraZeneca, Johnson and Johnson, GlaxoSmithKline, and Novartis and serving as a speaker for Aralez, AstraZeneca, Boehringer Ingelheim, Mylan, Novartis, Pfizer, and Pediapharm. Her institution has received research grants from Green Cross Pharmaceuticals, Merck, Novartis, and Sanofi. No other disclosures were reported.

Funding Sources: This study was funded by the AllerGen Network Centre of Excellence, William Spear/Start Memorial Fund of Kingston General Hospital Research Institute.

the method of allergen introduction, evaluation of the response, or seasonal effects.^{6–8} The consequences of the priming effect in the NAC model are yet to be determined. Current studies are focused on comparing various NAC procedures to determine the optimal methods for reproducible responses.

In evaluating new therapies, trial design usually relies on the repeatability of AR symptoms, therefore assuming any change in the total nasal symptom score (TNSS) is the result of treatment. It is important to determine the adequate interval between allergen exposures to avoid any confounding priming effect during the second allergen challenge visit. We aimed to evaluate the Allergic Rhinitis–Clinical Investigator Collaborative (AR-CIC) protocol to determine whether a 3-week interval was sufficient for generating reliable and reproducible participant responses to allergen challenge, both with respect to symptomatic responses and objective measures.² This interval was chosen based on previous work demonstrating the effect on rhinitis symptoms of conducting a NAC with a 3-day, 1-week, 2-week, and 4-week interval.⁹ The 1- and 4-week interval resulted in a response similar to baseline challenge, whereas the 3-day interval resulted in an increased response, possibly because of priming, and the 2-week interval was suboptimal.⁹

We also aimed to study the effect of repeated challenge on various inflammatory cytokines and eosinophil recruitment to the nasal mucosa. One of the cytokines studied was interleukin (IL) 33, which is produced by epithelial cells and acts primarily on T_H2 cells, increasing IL-5 and IL-13 secretion.^{10,11} IL-33 is also a promoter of naïve T-cell differentiation into T_H2 and a chemoattractant.^{12,13} In murine models, IL-33 induces eosinophil degranulation and prolongs cell

survival and has a similar effect on mast cells and basophils.^{14–16} Mouse models of AR have demonstrated increased allergen specific CD4⁺ T cells in response to IL-33 administration, promoting airway hyperresponsiveness, eosinophilia, and increased IL-4, IL-5, and IL-13.^{17,18} Lastly, a 24-hour period was introduced to investigate the late-phase response (LPR) beyond the usual 6-hour period for biological samples and 12-hour time for symptoms and nasal airflow.

Methods

The study protocol was granted ethical clearance by the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board, Kingston, Ontario. Participants were required to have a documented history of AR and a positive skin prick test response to short ragweed (*Ambrosia artemisiifolia*) (ALK-Abelló, Hørsholm, Denmark), defined as a wheal 3 mm or larger than the negative control. An initial nasal wash with 5 mL of 0.9% normal saline was used to identify and exclude participants with nonspecific hyperresponsiveness, as was a baseline challenge with the diluent control of the allergen extract. Participants reporting a TNSS greater than 2 to either were excluded. Baseline TNSS and PNIF were recorded, and an incrementally increasing allergen concentration was introduced into each nostril using the Aptar Bidose device (Aptar Pharma, Congers, New York). The screening visit, use of symptom diary cards, and PNIF training have been previously described in greater detail.²

Ten ragweed allergic participants were recruited and screened in March 2015 using the same methods used in the cumulative

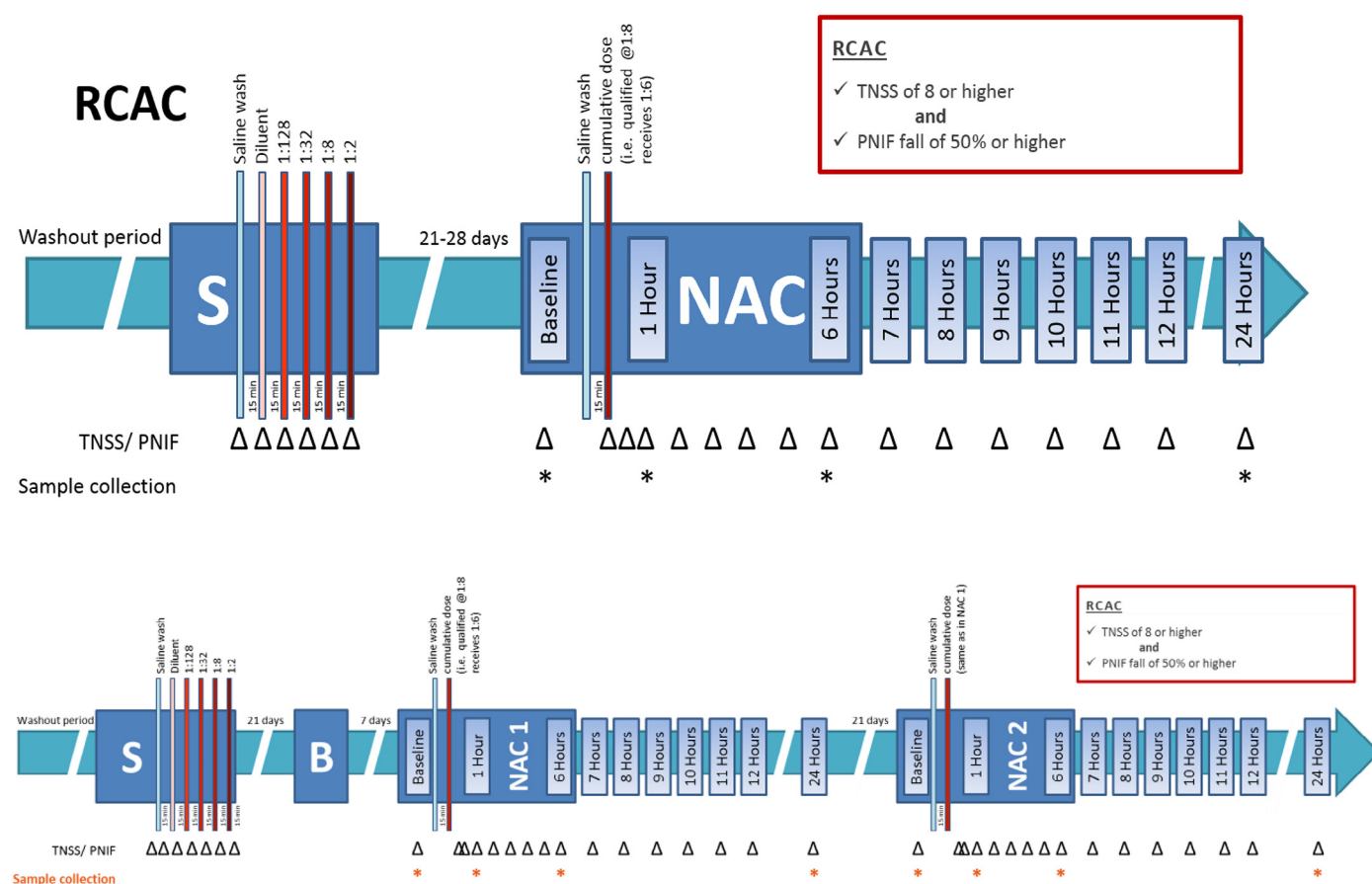


Figure 1. Repeatable cumulative allergen challenge (RCAC) participants received a cumulative allergen dose at nasal allergen challenge (NAC), an extra 24-hour sampling time point, and a 21- to 28-day interval between visits. The RCAC protocol is similar to the CAC protocol, with a 21- to 28-day interval between the screening and NAC visits. A 24-hour time point was added during both NAC visits, with nasal lavage, synthetic absorbent matrix, and nasal probe sample collection in addition to total nasal symptom score (TNSS) and peak nasal inspiratory flow (PNIF) recording.

allergen challenge (CAC) study² and similar inclusion and exclusion criteria. Participants were invited for a screening visit and 2 NAC visits with the 3-weeks interval between each of the visits (Fig 1). All visits were conducted outside the local ragweed pollen season.

Both NAC visits were conducted in the manner as previously described. Participants received their tailored CAC concentration and recorded their TNSS and PNIF at baseline, 15 minutes, 30 minutes, 1 hour, and hourly up to 12 hours. Synthetic absorbent matrix (SAM) (for nasal cytokine collection) and nasal lavage (for differential cell counts) samples were collected at baseline, 1 hour, and 6 hours, whereas nasal probe (Rhino-Probe) samples were collected at baseline and 6 hours.

After each of the NAC visits, participants were invited back the next morning, 24 hours after the allergen challenge, to record their TNSS scores and PNIF measurement and for SAM, nasal lavage, and nasal probe sampling. This extra time point was added to help assess the LPR after the NAC and provide longer-term evaluation of cytokine expression past 6 hours.

TNSS recorded per time point in each NAC was compared to the corresponding score, both at a per participant basis and by comparing the means. In a similar manner, the cytokine levels were analyzed to establish the protocol's repeatability on a biological level and to understand the allergic response past the 6-hour time point.

Cytokine Analysis

Nasal secretions were collected using 2 SAM strips at baseline, 1 hour, 6 hours, and 24 hours after allergen challenge. The SAMs were cut to a size of 7 × 30 mm and placed back to back, for 2 minutes, on the lateral surface of the inferior turbinate of the right nasal cavity using blunt-ended forceps. The strips were then removed and placed directly into a microtube that contained 300 µL of Dulbecco phosphate-buffered saline with 0.5% bovine serum albumen. Each microtube that contained the SAM strips was then placed on ice and later spun, aliquoted, and stored at –80°C.

Cytokine levels were measured from the SAM samples using Bio-Plex Pro Human Cytokine 17-plex Assay (IL-1 β , -2, -4, -5, -6, -7, -8, -10, -12, -13, and -17, monocyte chemoattractant protein 1 [MCP-1], macrophage inflammatory protein 1 β [MIP-1 β], tumor necrosis factor α , granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor, interferon γ) (Bio-Rad Laboratories, Hercules, California) on a Bio-Plex 200 system (Luminex xMAP multiplex technology), according to the manufacturer's protocol. CAC samples and samples from 4 nonallergic participants were also analyzed using a custom 2-plex assay (IL-25, IL-33) (Bio-Rad Laboratories).

Nasal Lavage Samples and Eosinophil Analysis

Nasal lavage samples were collected at baseline, 1 hour, 6 hours, and 24 hours after allergen challenge, using a modified nasal pool method. Participants were instructed to sit in a forward-flexed neck position (60° from the upright position) to prevent fluid from reaching the nasopharynx. A nasal olive attached to a 10-mL syringe that contained 5 mL of sterile 0.9% saline at room temperature was placed into the opposite nostril to the side used for SAM sampling to ensure a tight seal. The nasal cavity was gently flushed with saline 20 times over a 1- to 2-minute period, then the sample was transferred into a 15-mL polypropylene tube and placed on ice.

Total cell counts using trypan blue were determined before the preparation of cytospin slides for each lavage sample, which were subsequently fixed and stained using Diff-Quik to obtain differential cell counts. Each slide was counted by 2 team members (M.S. and D.A.) and the average recorded.

Results

Nine participants completed both NAC visits; one person did not complete the second NAC because of a scheduling conflict. TNSS reached a peak at 15 minutes in both visits (mean, 8.0 for NAC1 and 7.33 for NAC2) followed by a gradual decline, similar to results of previous studies (Fig 2). Mean PNIF decreased to a minimum at 30 minutes (mean, 61 L/min for NAC1 and 74 L/min for NAC2), followed by a gradual return to near baseline values. Participants reported similar results at both NAC visits, as depicted by both curves being superimposed at several time points (Fig 2). There was no significant difference between the visits when comparing mean TNSS at each time point, using 2-way repeated-measures analysis of

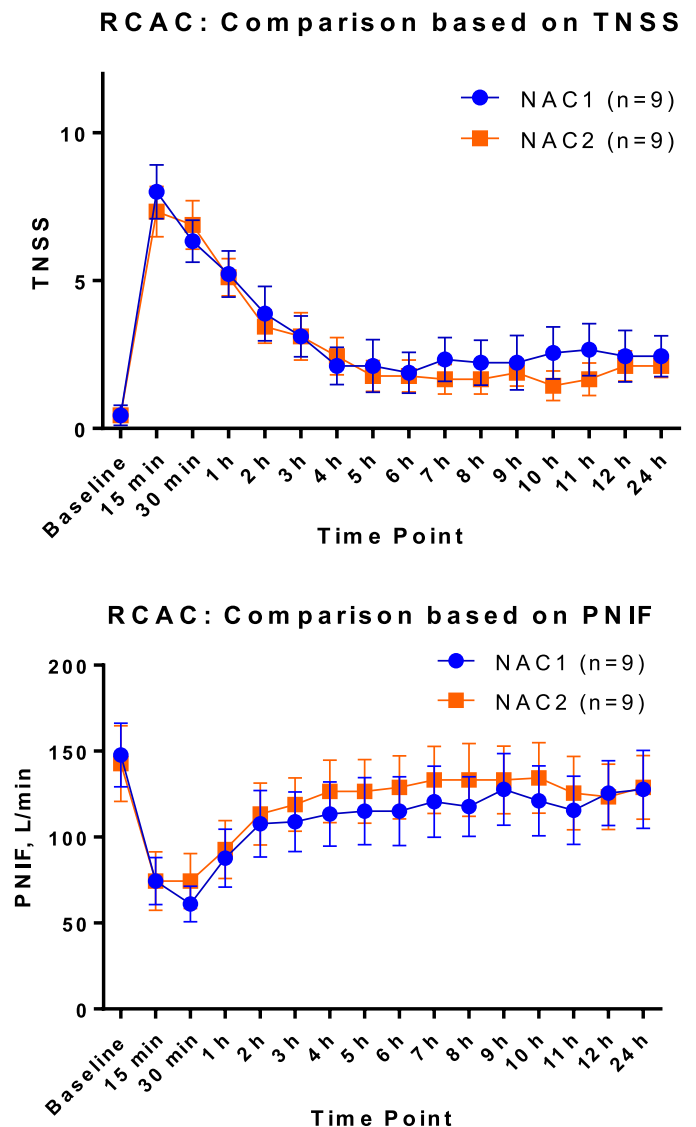


Figure 2. Repeatable cumulative allergen challenge (RCAC) participants achieved similar total nasal symptom score (TNSS) and peak nasal inspiratory flow (PNIF) results during both nasal allergen challenge (NAC) visits (NAC1 and NAC2), suggesting that the protocol is repeatable. Participants experienced an initial peak in TNSS at 15 minutes followed by a gradual decline. PNIF measurements mirrored the TNSS findings, initially decreasing to the lowest flow at 30 minutes after the allergen challenge, followed by a gradual return to airflow measurements close to baseline. Participants experienced the same trend as observed in the previous CAC protocol. Both NAC visits had similar results with the graphs superimposed on multiple time points. There was no statistical difference between the 2 visits when comparing them based on TNSS or PNIF.

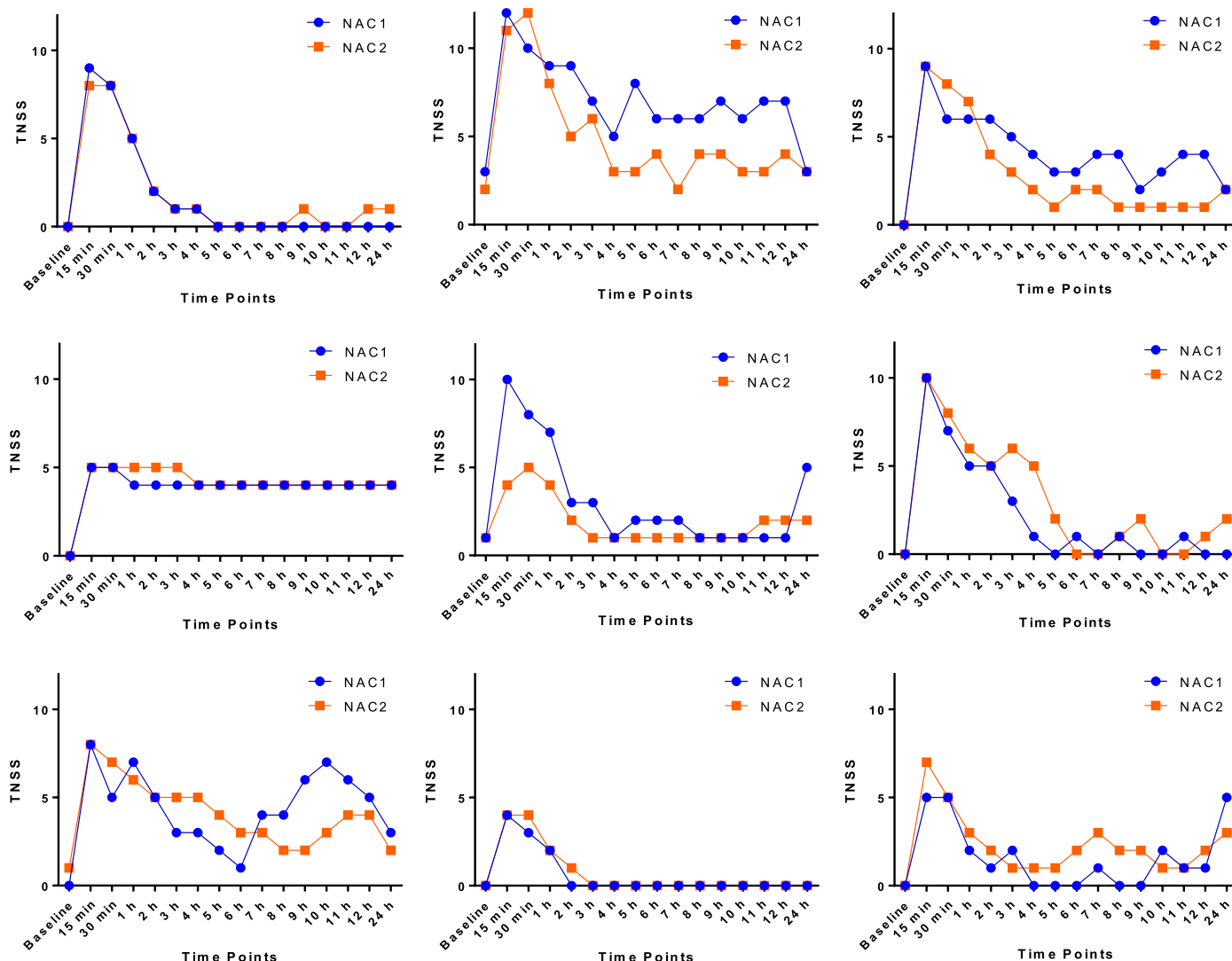


Figure 3. Separate repeatable cumulative allergen challenge (RCAC) evaluations of total nasal symptom score (TNSS) for each participant showed similar TNSS trends for both nasal allergen challenge (NAC) visits (NAC1 and NAC2) for most participants. TNSSs for each NAC visit are compared for each participant separately. All participants except for 1 reported similar peak TNSSs at 15 minutes. There was no significant difference between both visits when comparing each participant's results separately (paired, 2-tailed, *t* test). One participant experienced a dual-phase response and one experienced a protracted early-phase response (EPR) during NAC1, while reporting an EPR during NAC2. All other participants reported an EPR in both visits. Although the second half of the visits were more variable, each participant's TNSS plots followed a similar trend in both NACs.

variance with Bonferroni corrections and a paired 2-tailed *t* test at each time point.

Each participant's TNSS recorded throughout both NAC visits was plotted separately to assess individual variability (Fig 3). Most participants reached similar TNSS at both visits, with one exception reporting a markedly reduced peak TNSS during the second NAC.

Phenotyping definitions used in previous studies were applied to the repeatable cumulative allergen challenge (RCAC) data for consistency.^{2,19} Seven participants experienced an early-phase response (EPR) at both visits, whereas one participant experienced a dual-phase response (DPR) at NAC1 and another experienced a protracted EPR but reported values consistent with EPR at the NAC2 (Fig 3). Although these participants did not retain their designated reaction during NAC2, they followed the same trend in both visits, although with a less pronounced second increase in TNSS after the sixth hour.

Two participants reported a late increase in their TNSS at 24 hours after the allergen challenge of at least 2 TNSS point increases. Other participants reported worsening of symptoms overnight, the

severity of which was not captured at the 24-hour time point, by which time symptoms had partially subsided.

Eosinophil Cell Counts During a 24-Hour Period During Subsequent NAC Visits

Eosinophils as a percentage of white blood cells in nasal lavage increased at 1 hour (mean, 12.79% for NAC1 and for 4.48% NAC2) from baseline (mean, 2.54% for NAC1 and 1.70% for NAC2) after allergen challenge (Fig 4). This was followed by a decline at 6 hours for NAC1 (mean, 7.97%) but was sustained during NAC2 (mean, 4.67%). A further increase was observed in both visits at 24 hours (mean, 16.48% for NAC1 and 12.96% for NAC2). There was no statistical difference between both NAC visits at each of the 4 time points, although NAC1 had a slightly greater mean percentage of eosinophils at all 3 time points after allergen challenge. There was no statistical difference between the time points because of the variability in the numbers among the samples.

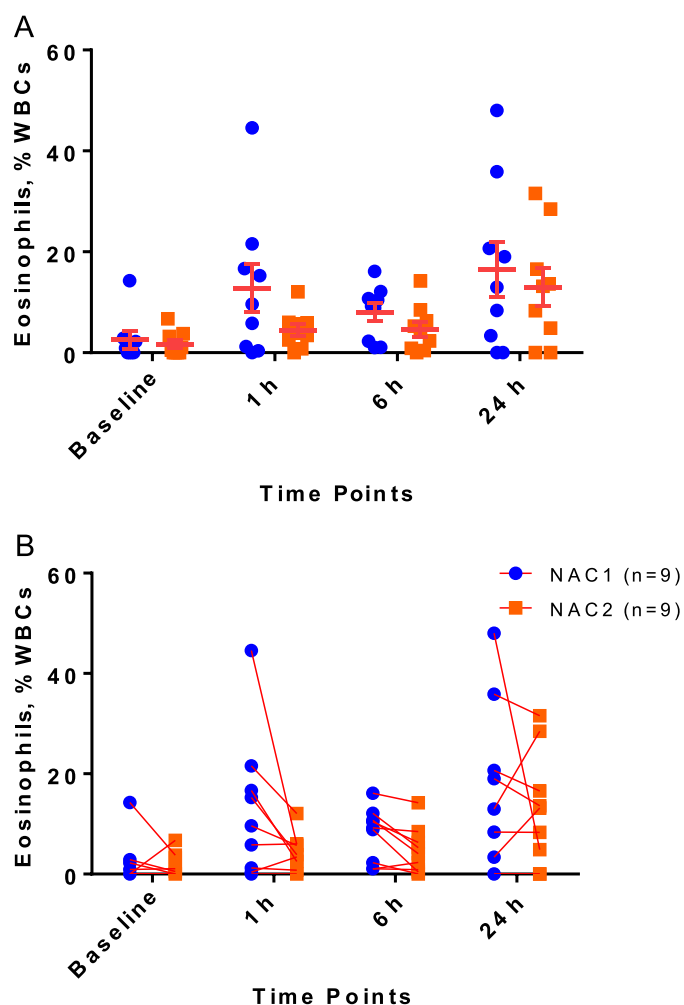


Figure 4. The overall repeatable cumulative allergen challenge (RCAC) percentage of eosinophils in nasal lavage samples were repeatable, no significant difference between both nasal allergen challenge NAC visits (NAC1 and NAC2). The percentage of white blood cells (WBCs) that are eosinophils in nasal lavage is shown for each participant at each time point for each NAC. A: Mean percentage of eosinophils and SEM at each time point of each NAC. There was no significant difference between the 2 NAC visits at any of the time points, although NAC1 had a slightly higher percentage. Eosinophils as a percentage of WBCs initially increased at 1 hour followed by a slight decrease at 6 hours and a second increase 24 hours after allergen challenge. B: Difference between NAC1 and NAC2 for each participant. One participant had a lower percentage of eosinophils at baseline and 1 hour at NAC2 compared with NAC1.

Further analysis of the RCAC eosinophil data looked at the differences between the NAC visits for each participant (Fig 4). Baseline eosinophil percentages were comparable for both studies, whereas the 1-hour time point showed a decrease in the percentage during the NAC2 visit for 4 participants. At the 6-hour time point, there was less visible change in the percentages between the 2 visits (Fig 4). The 24-hour time point provided greater variability in the results, with almost all participants' samples showing an increase or decrease at NAC2 compared with NAC1. The variability is seen in both visits, similar to the changes observed with TNSS (Fig 4).

Repeatability of Cytokine Response

Nasal cytokines collected at baseline, 1 hour, and 6 hours followed similar trends as previously observed in CAC samples. Samples collected from each participant at the 2 separate NAC visits were not significantly different for most cytokines at most time points.

MCP-1 was significantly increased during NAC1 vs NAC2 at 6 hours ($P < .05$; Mann-Whitney U test) (Fig 5), whereas MIP-1 β was significantly higher in NAC1 at 1 hour ($P < .05$). IL-6 was statistically elevated in NAC1 at baseline and 6 hours ($P < .05$). G-CSF was the only cytokine to be significant higher during NAC1 at baseline, 1 hour, and 6 hours after the allergen challenge ($P < .05$). This variability was mostly observed at the 6-hour time point. At 24 hours after the allergen challenge, there was a second increase in MCP-1 and MIP-1 β cytokines at both NAC visits. IL-5 and IL-13 concentrations were markedly lower than CAC protocol findings. IL-33 was measured in NAC1 samples and was initially increased at 1 hour, remained increased at 6 hours, unlike in the CAC samples, and decreased at 24 hours below the concentration observed at baseline (Fig 6).

Discussion

TNSS and PNIF results from both NAC visits indicate that the CAC protocol is repeatable. Having a 3-week interval between each visit but not an increase in allergen concentration was key to achieve repeatability. A retrospective look at the interval between screening and NAC visits during the previous ragweed challenge studies revealed that most participants had only a 2-week interval between visits, which could explain the lack of adequate repeatability. Although the spacing of allergen challenges into 2 administrations during the multiple CAC study helped to compensate for the short interval, cytokine and chemokine measurements were compromised.²⁰ During the NAC visit, participants in the multiple CAC study received a cumulative dose of allergen concentrations administered during screening, excluding the final qualifying dose. The final allergen concentration at which the participant qualified was then administered 15 minutes later. Thus, the RCAC protocol provides the best balance between the appropriate allergen concentration delivered to participants and the period between studies.

The newly added 24-hour time point revealed the possibility of experiencing a DPR beyond the 12-hour period that was used in the initial studies. Participants' phenotype designation may be changed as a result of this addition. The lasting effect of a single NAC can further the application of the protocol. Samples collected at the 24th hour after allergen challenge may allow identifying one or more biomarkers associated with developing a DPR and would help in assessing the long-term effect of novel therapies on suppression of AR symptoms. Future direction should include the assessment of the phenotyping definitions in light of this finding. Some participants also reported severe overnight symptoms that partially subsided by the 24th hour. An overall assessment of symptoms, between the 12th and 24th hours, should be included to capture a general idea of the missed data points. Such findings might help estimate the effect a medication has on improving the quality of life of participants by improving sleep patterns secondary to symptom reduction.

Mild asthma studies using the inhalation challenge model have found that 50% of participants experience a LPR based on a decrease in forced expiratory volume in 1 second of 20% or more.²¹ In contrast, 10% to 25% of the AR-CIC NAC study participants experienced an LPR. Most NAC studies conducted so far recruited 10 participants at a time; a more comparable LPR percentage might be achieved as more participants are enrolled in NAC-type studies. A further assessment of this finding is required in the future.

The cytokine response was, for most targets, not significantly different between the 2 NAC visits. The small variability in IL-6, MCP-1, and G-CSF cytokine concentrations that arose at the 6-hour time point could be explained by the change in phenotype of some of the participants during the second NAC. Increase in G-CSF during NAC1 compared with NAC2 is the outlier because it was the only cytokine to be significantly higher at multiple time points during

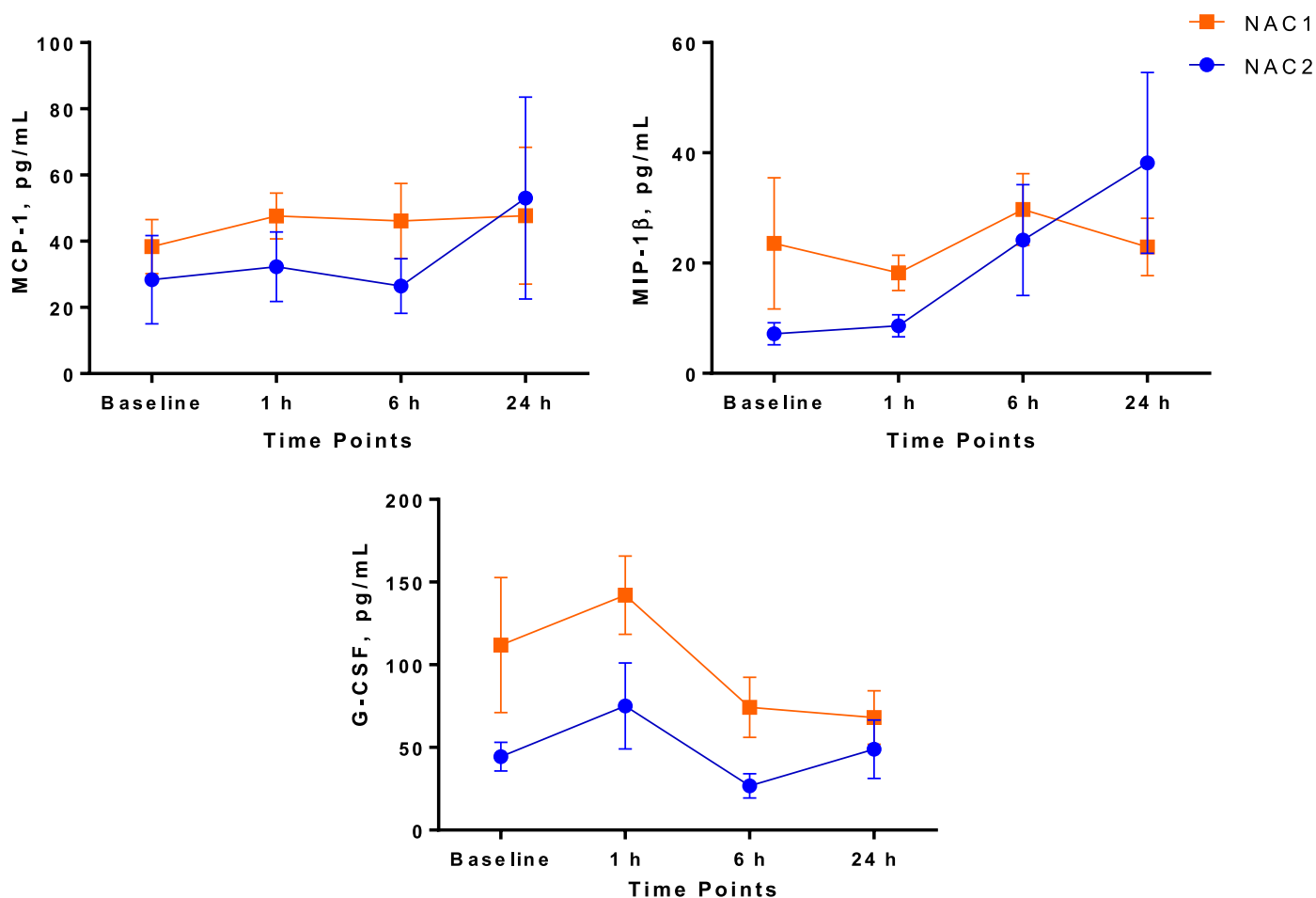


Figure 5. Similar trends in cytokines for both nasal allergen challenge (NAC) visits (NAC1 and NAC2). NAC2 had lower cytokine levels but were not significant for most cytokines. Sample cytokines are shown above. Cytokine secretion followed a similar trend to that observed in previous NAC studies. There was no significant difference between NAC1 and NAC2 for most of the cytokine levels at most of the time points. Small significance was achieved at 6 hours for monocyte chemoattractant protein 1 (MCP-1), at 1 hour for macrophage inflammatory protein 1 β (MIP-1 β), and at baseline, 1 hour and 6 hours for granulocyte colony-stimulating factor (G-CSF) ($P < .05$). MCP-1 and MIP-1 β continued to increase at the 24-hour time point.

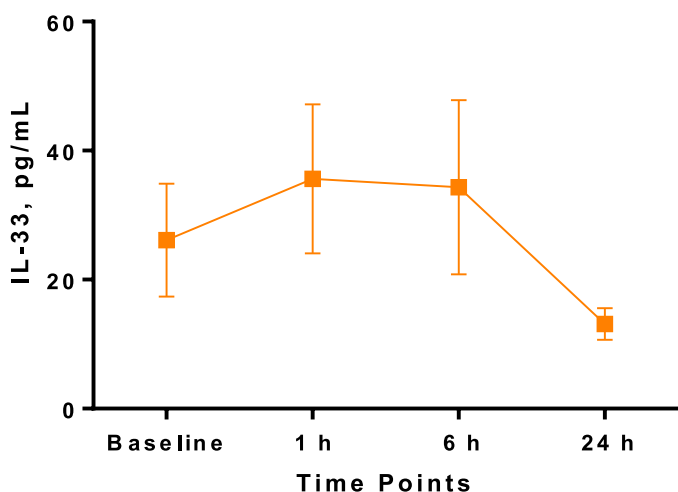


Figure 6. Interleukin (IL) 33 increased at 1 hour from the baseline low, was maintained at 6 hours counter to cumulative allergen challenge results, and decreased below baseline at 24 hours after nasal allergen challenge (NAC). IL-33 initially increase at 1 hour and was maintained at 6 hours after the allergen challenge. This was followed by a decrease below baseline levels during NAC1 ($n = 10$). There was increased variability at 1 and 6 hours, but all samples had decreased concentrations with less variability at the 24th hour. The decrease at 24 hours might be an indication of inflammation resolution.

NAC1. With the enrollment of more participants, such variability might be potentially eliminated. Most participants experienced an EPR during NAC2, which is mirrored by the decrease in cytokine levels at the sixth hour. IL-33 was the first cytokine, from the panel under investigation, to increase at 1 hour after the NAC, confirming previous findings, although the high levels were maintained at 6 hours. Correlating these results with the results of gene expression analysis at baseline, 6 hours, and 24 hours will help explain this new finding. The 24-hour time point may help explain the second increase in TNSS experienced by some participants, as seen in the increase in MCP-1 and MIP-1 β 24 hours after the allergen challenge. The addition of this time point will aid in examining the pathophysiology of the LPR and better assessing novel medications.

Participants experienced reliable symptoms of AR, and both dual responders and early phase responders were noted, an observation that resembles symptoms experienced by the general population.²² This observation can be monitored in a study that evaluated the efficacy of a novel medication during the full length of its duration of action.^{23,24} Likewise, NAC can be used to study how symptoms and cytokines are affected before and after immunotherapy and in drug safety studies.^{25,26} Using the combined criteria of TNSS and PNIF decrease is a more robust method of determining a positive challenge result to nasal allergen administration.

The NAC protocol provides a valuable tool for researchers to study AR pathophysiology in humans. IL-33 is a prominent recently discovered cytokine and possibly a target for novel therapies for difficult cases of AR and possibly asthma.¹² IL33 gene expression and the IL-33 cytokine can be studied and further analyzed in relation to clinical symptoms and other cytokines to better study the effect of future novel therapies that target the IL-33 cytokine.

The CAC protocol is the optimal protocol from the variations tested and can be used in evaluating novel therapy with a 3- to 4-week interval between screening and NAC1. The first NAC would establish a baseline symptom progression after the allergen challenge. This will then be followed by the treatment under investigation and a second NAC to observe the effect of this medication on symptoms and biomarkers. NAC2 should take place at least 3 to 4 weeks after NAC1.

The AR-CIC's optimized CAC protocol is capable of adequately and efficiently modeling AR in humans. The NAC protocol reliably reproduces clinical and biological results, ensuring that any change would be purely due to medication under investigation in a clinical trial setting.

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