

Intranasal heparin reduces eosinophil recruitment after nasal allergen challenge in patients with allergic rhinitis

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Background: Recently, several studies have shown that heparin possesses various anti-inflammatory and antiallergic properties. It has been proposed that heparin might play an important role in limiting the inflammatory events associated with asthma and allergic rhinitis by neutralizing inflammatory mediators, such as eosinophil cationic protein and major basic protein, and by limiting eosinophil recruitment.

Objective: To test the hypothesis that heparin can limit the extent and magnitude of eosinophilic inflammation, we examined the effect of inhaled intranasal heparin on nasal response to allergen challenge in 10 patients with allergic rhinitis.

Methods: The capacity of heparin to reduce nasal response was studied by evaluating symptom score, eosinophil cationic protein concentration, and eosinophil counts in nasal lavage fluids 10, 60, and 360 minutes after allergen challenge.

Results: Pretreatment with intranasal heparin produced a significant reduction in symptom score 10 minutes after allergen challenge and reduced the eosinophil influx at each time point after antigen challenge, statistical significance being reached 60 and 360 minutes after allergen challenge. Similarly, the amount of eosinophil cationic protein in the nasal wash was reduced at each time point; this reached statistical significance 360 minutes after allergen challenge.

Conclusion: Heparin was shown to provide protection with respect to nasal allergen challenge. The mechanism by which heparin produces its protective activity seems to be related to the neutralization of eosinophil cationic protein as well as to the reduction of eosinophil recruitment. (*J Allergy Clin Immunol* 2001;108:703-8.)

Key words: Rhinitis, allergy, ECP, eosinophils, heparin, nasal lavages

Heparin is a highly sulfated glycosaminoglycan that is present in the mast cells of most mammals. Because of its charge density and the presence of a relatively nonspecific sequence on most of its chains, heparin is able to

Abbreviations used

BU: Biological units
EPR: Early-phase response
ECP: Eosinophil cationic protein
LPR: Late-phase response
PAF: Platelet-activating factor

bind a large number of proteins and can also interact with several cell types.¹⁻⁴ For these reasons, the role of heparin, known almost exclusively for its anticoagulant activity, has been revisited. In fact, heparin is able to interact with various growth factors^{5,6} and might modulate cellular proliferation.⁷

Several studies have shown that heparin possesses various anti-inflammatory and antiallergic properties. It modulates T-lymphocyte activities,⁴ inhibits neutrophil chemotaxis,⁸ and regulates complement activation.⁹ Other studies have shown a protective role of heparin in allergic reactions. Ahmed et al¹⁰ showed that nebulized heparin blocks the airway obstruction induced by *Ascaris* antigen in sensitized sheep. In atopic asthmatic subjects, inhaled heparin has been shown to be effective in attenuating antigen-induced cutaneous and bronchial response.^{11,12} Moreover, inhaled heparin is able to prevent exercise-induced asthma, probably by modulating mediator release.^{13,14}

It has been hypothesized that exogenously administered heparin might have a direct effect on inflammatory cells, uncoupling stimulus-secretion of mast cells by inhibiting the binding of 1, 4, 5-inositol triphosphate (which is produced during mast cell stimulation) and thus preventing the subsequent internal release of calcium (which is necessary for degranulation).¹⁵ Furthermore, we have previously observed that inhaled heparin is able to inhibit the bronchospasm induced by adenosine monophosphate in patients with asthma.¹⁶ Because inhaled adenosine provokes bronchoconstriction in asthmatic subjects by a mechanism believed to involve mast cell mediator release, it has been suggested that inhaled heparin might exert its effect in human asthma via an inhibitory modulation of mast cell functions.¹⁷

Furthermore, it has been proposed that heparin could

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Received for publication May 14, 2001; revised July 10, 2001; accepted for publication July 17, 2001.

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0091-6749/2001 \$35.00 + 0 1/81/118785

doi:10.1067/mai.2001.118785

TABLE I. Assessment of nasal response after allergen challenge

Score	Nasal blockage	Sneezing	Rhinorrhea and eye watering/irritation
0	Absent; breathing through the nose freely and easily	Absent	Absent
1	Mild; slight difficulty in breathing through the nose	1 to 5	Present, mild
2	Medium; moderate difficulty in breathing through the nose	6 to 10	Present, moderate
3	Severe; breathing through the nose is very difficult/impossible	>10	Present, severe, a persistent distraction

Nasal responses were evaluated by grading symptoms according to the reported score scale. The composite symptom score is the sum of scores for the 3 nasal symptoms. The maximum possible score is 9.

play an important role in limiting the inflammatory events associated with asthma by a mechanism that is different from mast cell inactivation.^{2,3,10} Endogenous heparin might act to reduce the severity of tissue damage induced by mediators such as eosinophil cationic protein (ECP) and major basic protein. These mediators, all of which are cationic proteins, might in fact be neutralized by binding with the highly anionic heparin.¹⁸ In addition, it has been reported that heparin binds platelet-activating factor (PAF), a potent chemotactic factor for human eosinophils¹; in several animal models, heparin was also able to limit eosinophil recruitment.^{3,19}

On the basis of these observations, it is possible to hypothesize that heparin could contribute to the self-limitation of the inflammatory process by limiting the extent of eosinophil recruitment into sites of allergic inflammation, as well as the extent of the tissue damage induced by cationic proteins.

To test this hypothesis, we examined the effect in allergic subjects of intranasal heparin on the response to nasal allergen challenge. The capacity of heparin to reduce nasal response to a specific challenge in rhinitic subjects was studied by evaluating symptom score, ECP concentration, and eosinophil counts in nasal lavages 10, 60, and 360 minutes after allergen challenge.

METHODS

Subjects

We studied 10 subjects (3 male and 7 female) aged 18 to 40 years (mean age $\pm$ SD, 31.3 $\pm$ 10.2 years), each with an obvious history of allergic rhinitis. Allergic rhinitis was defined by a positive cutaneous prick test result and a positive RAST result of class 3 or 4. Patients with allergic rhinitis had seasonal symptoms; all patients were allergic to *Parietaria judaica* (10 patients). None had nasal polyposis, chronic nasal obstruction, or other serious illness.

No subject was admitted to the study within 6 weeks of a respiratory tract infection. Each patient was studied at a time remote from his or her allergy season. All participants were required to withhold cromolyn sodium, topical and systemic corticosteroids, and antihistamines for 2 weeks.

Subjects were informed about the purpose of the investigation, and all signed forms indicating their consent to participate. The study was approved by the local ethics committee.

Study design

The study was carried out according to a sequential double-blind, placebo-controlled, randomized, crossover design. On the first study day, subjects were challenged with *P. judaica* allergen for nasal provocation testing (standardized in biological units [BU]; Neo-Abellò S.p.A, Ospiate di Bollate, Milan, Italy) to assess the develop-

ment of a nasal reaction. On the following 2 study days, which were at least 3 weeks apart, allergen challenge was preceded by administration of an aerosol of normal saline solution or heparin, the treatment being administered in a double-blinded, randomized crossover fashion. Allergen challenges were carried out 15 minutes after heparin or saline solution inhalation to limit any possibility that heparin could affect the delivery of allergen. Challenges were performed at the same time on each study day to avoid any change in the response due to diurnal rhythm. After antigen challenge, each subject was asked to stay in our ambulatory unit for 6 hours. Symptoms were monitored 10, 60, and 360 minutes after the last dose of antigen extract. Nasal lavages were performed at the same time points.

Nasal challenge

The nasal challenges were performed through use of a technique similar to that described by Sim et al.²⁰ Briefly, nasal challenges were preceded by 3 lavages with normal saline solution to reduce the level of cell-free mediators. Shortly thereafter, a topical decongestant (Oxymetazoline) was sprayed into each nostril to prevent allergen-induced congestion and ensure the patency of the nasal cavity. An aerosol of heparin (Eparina vister, Parker-Davis S.p.A. Lainate Mi, Italy), 3750 U/mL (4 mL), or saline solution was then administered by mean of a Nebula nebulizer (Markos-Mefar, Monza, Italy). The patient was asked to breathe deeply, inhaling through the nose with a face mask, the mouth being closed. The specific allergen extract was insufflated into each nostril through use of nebulizer bottles containing incremental concentrations of allergen (2, 4, and 8 BU/mL); these nebulizations delivered 0.16, 0.32, and 0.64 BU of allergen, respectively. At each nebulization, the nasal reaction obtained was recorded through use of a symptom score technique. Patients were challenged with incremental doses of the allergen (0.16, 0.32, and 0.64 BU in each nostril) until a composite symptom score of 6 or more was obtained. The clinical criteria for a positive nasal provocation test result were evaluated by symptom assessment through use of a semiquantitative method similar to that used by others.^{20,21} Briefly, 3 major nasal symptoms (sneezing, rhinorrhea, and nasal obstruction) were recorded 10 minutes after nasal challenge and then every hour for the following 6 hours, according to a scale ranging from 0 (no symptoms) to 3 (severe). A total nasal symptom score was calculated by adding these scores together for each patient; the maximum possible score was 9 (Table I). Each subject had undergone preliminary challenges with allergens in a pilot study to assess the development of an early-phase response (EPR) and a late-phase response (LPR). A composite symptom score of 6 or more was the clinical criterion of a positive response to the nasal provocation test, and it was identified as a necessary prerequisite to the elicitation of a LPR (data not shown). Cumulative doses of allergen required to induce a nasal response varied from 0.48 to 1.12 BU for each nostril.

Nasal lavage

Nasal lavages were performed and lavage fluids collected immediately before nasal allergen challenge and then after 10, 60, and

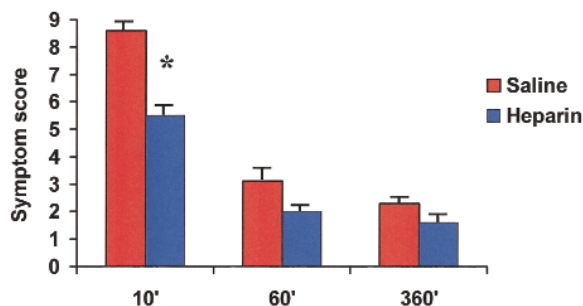


FIG 1. Symptom scores 10, 60, and 360 minutes after allergen challenge following treatment with heparin (blue bars) or saline solution (red bars). Data represent means $\pm$ SEMs ($n = 10$). * $P < .05$ between the 2 groups.

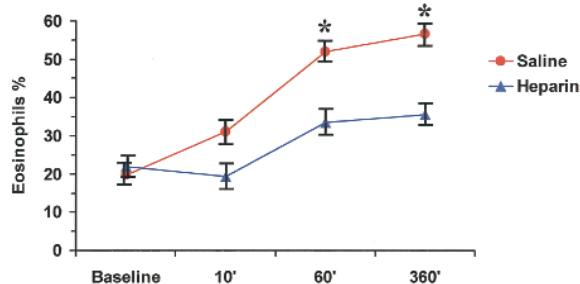


FIG 2. Percentages of eosinophils 10, 60, and 360 minutes after allergen challenge following treatment with heparin or saline solution. Data represent means $\pm$ SEMs ($n = 10$). * $P < .05$ between the 2 groups.

360 minutes. The subject, while in a sitting position, was instructed to extend the neck approximately 30 degrees from the horizontal and to refrain from breathing or swallowing. Five milliliters of normal, prewarmed (37°C) saline solution was instilled into each nostril with a pipette while the subject did not breathe or swallow. After 10 seconds, the subject flexed the neck and expelled the nasal lavage fluid into a conical polypropylene tubes (Falcon, Becton Dickinson Italia S.p.A. Milan, Italy). The amount of secretion sampled was measured by weighing the collection tube and the tube with the aliquot of saline solution before and after sampling. The wash was processed immediately.

Cellular examination

The lavage fluid was centrifuged at 4°C for 10 minutes at 1000 revolutions per minute; after removal and storage of the supernatants at -20°C for the following measurements, the cell pellet was resuspended in 1 mL RPMI 1640 (Gibco BRL, Life Technologies Ltd, Paisley, United Kingdom) containing 10% FCS. An equal volume of 16% N-acetylcysteine in calcium-free Hank's balanced salt solution (Gibco BRL, Life Technologies Ltd) was added to each sample and incubated for 45 minutes at 37°C to disaggregate mucus and clumps. Differential counts were performed on cytospin slides stained with DiffQuick (DADE S.p.A. Rome, Italy) and examined by means of light microscopy.

Measurement of ECP

The quantitative measurement, in nasal lavage fluids, of ECP was performed through use of a commercially available assay (Pharmacia CAP system ECP FEIA, Pharmacia & Upjohn JAB, Uppsala, Sweden), according to the manufacturer's instructions. The detection limit of the assay is 0.5 ng/mL (3 SDs from zero standard). All samples were determined in duplicate. To ensure that heparin did not interfere with the immunoassay for ECP, we performed some experiments measuring ECP in biological samples in the presence of concentrations of heparin varying from 2000 to 5000 U/mL. These experiments demonstrated that heparin does not interfere with the immunoassay measurement of ECP (data not shown).

Statistical evaluation

Statistical comparisons of the symptom scores were performed through use of a Wilcoxon nonparametric test for paired data. Statistical comparisons of number of eosinophils and levels of ECP were performed through use of a 2-way ANOVA; this was followed by Newman-Keuls testing for specific means comparisons. Results were expressed as means $\pm$ SEMs.

RESULTS

Symptom scores

As expected, nasal challenge with increasing concentration of allergen resulted in immediate symptoms in all subjects. All patients completed the study, and no severe adverse reactions during the nasal lavage and challenge procedures were noted. Results, given as mean nasal symptom scores after allergen challenge following treatment with heparin or saline solution, are presented in Fig 1. Mean symptom scores for nasal symptoms were significantly lower 10 minutes after allergen inhalation when patients received the heparin solution (5.5 ± 0.2 after heparin treatment vs 8.6 ± 0.2 after saline solution treatment; $P < .05$). A reduction in mean symptom score was also noted at 60 and 360 minutes after challenge following heparin treatment, but this did not reach statistical significance at these time points. No significant differences in cumulative allergen dose needed to elicit a positive response between the study phases of heparin and placebo were noted.

Eosinophil count and ECP levels

The percentages of eosinophils in baseline nasal lavage fluids obtained immediately before nasal allergen challenge following pretreatment with saline solution or heparin were not significantly different ($19\% \pm 2.1\%$ and $22\% \pm 1.8\%$ before saline solution and heparin pretreatment, respectively). ECP baseline levels in nasal lavage fluids were also not significantly different (9 ± 2.4 ng/mL and 7.5 ± 2.1 ng/mL in the study phases of saline solution and heparin, respectively). After allergen challenge, the percentage of eosinophils was lower after heparin treatment than after saline solution treatment at each time point considered. However, the decrease was statistically significant 60 and 360 minutes after allergen challenge ($P < .05$; Fig 2). The mean values at 60 and 360 minutes after nasal challenge after saline solution treatment were $52\% \pm 6.5\%$ and $56.7\% \pm 5.8\%$ and decreased to $33.6\% \pm 8\%$ and $35.5\% \pm 6.6\%$, respectively, when allergen challenge followed heparin treatment. We also observed a similar trend for ECP levels. In fact, after the heparin treatment

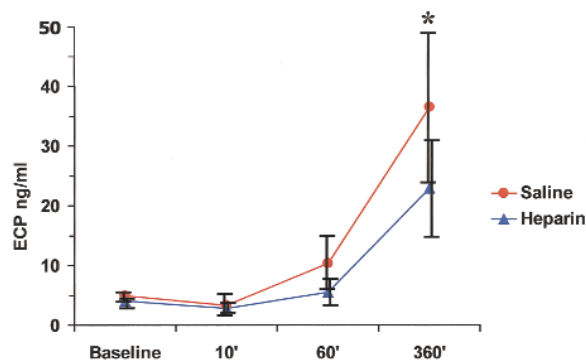


FIG 3. ECP levels 10, 60, and 360 minutes after allergen challenge following treatment with heparin or saline solution. Data represent means $\pm$ SEMs ($n = 10$). * $P < .05$ between the 2 groups.

there was a decrease in the amount of ECP at each time point; this reached statistical significance 360 minutes after allergen challenge ($P < .05$; Fig 3). The mean ECP value was 36.6 ± 12 ng/mL at 360 minutes after saline solution treatment; this decreased to 23 ± 8 ng/mL when allergen challenge was preceded by heparin inhalation.

DISCUSSION

In patients with allergic rhinitis, nasal antigen challenge has been successfully used to monitor allergic inflammatory responses during both the EPR and the LPR.^{22,23} Mast cells are crucial for the development of EPR; in contrast, eosinophils are considered the most prominent effector cells in the LPR, and their role in the pathogenesis of allergic diseases is believed to be critical. Eosinophils are in fact able to produce a wide array of proinflammatory cytokines and have the capacity to release cytotoxic proteins, including major basic protein, eosinophil-derived neurotoxin, and ECP, potentially harmful for the integrity of the nasal and bronchial mucosa. Accordingly, a full comprehension of those mechanisms capable of modulating the recruitment and inflammatory activities of this important cell appears to be crucial.

In 1991, Fredens et al²⁴ described a possible interaction between ECP and heparin, suggesting that heparin could neutralize the deleterious effects of ECP. More recently, Page²⁵ and Tyrell et al²⁶ revised and widened this concept, hypothesizing for mast cell–derived heparin an important role in limiting the tissue damage that characterizes allergic diseases, through the inhibition of cationic proteins and reduction of the eosinophil recruitment into sites of allergic inflammation.

Interestingly, in a study performed in patients with atopic asthma, Diamant et al¹² showed that multiple doses of inhaled heparin reduced the early and late asthmatic response to inhaled house dust mite extract, expressed as corresponding areas under time-response curves. In animal models of asthma, heparin was demonstrated to be effective in reducing eosinophil infiltration,^{3,19} though a recent study performed in neonatally immunized rabbits

showed that heparin failed to inhibit the increase in inflammatory cell number after antigen challenge.²⁷

As a consequence of these observations, the hypothesis that heparin can reduce the influx of inflammatory cells, especially eosinophils, in the airway mucosa of allergic subjects is still debated. To address this question, we investigated the protective effect of inhaled heparin on the inflammatory response after nasal challenge in allergic subjects and its capacity to limit eosinophil influx into nasal mucosa.

We note first that though this study was performed outside the *P judaica* pollen season in symptom-free patients, the percent of eosinophils in baseline nasal lavage fluids showed a relatively high number of eosinophils. This is in agreement with studies that found a minimal persistent inflammation in patients with seasonal allergic rhinitis.^{28,29} In fact, in these patients, markers of allergic inflammation are always detectable, even when symptoms are not present and during the pollen-free season. Moreover, in our region, a low level of *P judaica* pollen is present most of the year (beyond the seasonal peak); pollen counts registered by our pollen spore trap at the time of the study documented this (data not shown).

Regarding the protective effect of heparin, our results show that aerosolized intranasal heparin significantly attenuates the nasal response to allergen challenge in atopic rhinitic subjects. Pretreatment with inhaled heparin in fact produces a significant reduction in symptom score 10 minutes after allergen challenge and reduces the eosinophil influx at each time point after antigen challenge, reaching statistical significance at 60 and 360 minutes after the challenge. Similarly, the amount of ECP found in the nasal wash is reduced at each time point, though this reached statistical significance at 360 minutes. The reduction of the amount of ECP is probably strictly correlated with the inhibition of eosinophil influx, even if heparin, a highly anionic molecule, could neutralize ECP merely as a matter of charge, determining a reduction of the amount of detectable ECP in the nasal washes.

As far as we know, this is the first study showing that heparin is effective in reducing eosinophil influx after allergen-specific challenge in the human airway mucosa, and this suggests a complex mechanism of action of this molecule. The ability of heparin to prevent the release from mast cells of mediators such as histamine might in fact explain the significant reduction in symptom score observed 10 minutes after allergen challenge; on the other hand, its protective effect on eosinophil influx and activation, still exerted at 60 and 360 minutes after allergen challenge, requires some speculation.

Migration of eosinophils from the peripheral blood through the endothelial surface into nasal mucosa is a complex process. It requires a sequence of events that is initiated by eosinophils rolling along the endothelium, mediated by multiple low-affinity interactions between P- and E-selectin on endothelium and selectin receptors on the eosinophil surface; this is followed by firm adhesion and diapedesis, largely dependent on the expression

of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 on endothelial cells. It is well known that mast cells are potent regulators of adhesion molecule expression on endothelial cells. In fact, mast cells, through their capacity to bind IgE-antigen complexes, can release both preformed and newly generated chemical mediators (histamine and leukotrienes) and cytokines (IL-4 and TNF- α) that are able to induce an upregulation of P-selectin, E-selectin, intercellular adhesion molecule 1 expression, and vascular cell adhesion molecule 1 expression.³⁰⁻³¹

Thus heparin, through its capacity to prevent mast cell mediator release, could indirectly produce a downregulation of the expression of a wide range of adhesion molecules and therefore limit eosinophil migration into the nasal mucosa. Interestingly, heparin can also directly regulate cellular diapedesis through the endothelium by reducing the adherence of leukocytes to endothelial cells, probably by increasing the negative charge of the endothelial cell surface.³⁴

Moreover, other mechanisms have been thought to explain how heparin can modulate eosinophil recruitment and activation. Heparin can modulate the activity of soluble mediators crucial to the promotion of eosinophil migration. It has been reported that heparin might inactivate PAF,¹ a cationic protein with a potent chemotactic activity for human eosinophils. In this case, the ability of heparin to bind PAF would result in a reduced cell influx into the tissue. Another study showed that heparin binds to IL-5, the major cytokine regulating eosinophil migration and activation.³⁵

In our study, the capacity of heparin to limit eosinophil migration into the airway mucosa, observed at 60 and 360 minutes after challenge, produced a reduction in symptom score at the same time points, though this did not reach statistical significance. This is in agreement with other studies^{22,36} that have shown that neither the magnitude of the inflammatory cell influx nor ECP concentration correlates significantly with the symptoms present during the acute response or the late response to antigen challenge, suggesting that other factors might also contribute to symptoms.

In conclusion, heparin has been shown in the present study to provide protection with respect to nasal allergic challenge. This confirms that the mast cell products, heparin and related proteoglycans, could act as natural anti-inflammatory molecules, playing a critical role in limiting inflammatory response in allergic diseases, and it suggests the hypothesis that an altered heparin release from mast cells in atopic subjects could play a major role in the pathogenesis of allergic diseases. The mechanism by which heparin produces its protective activity is more complex than has been thought; considerations include mast cell stabilization as well as ECP neutralization and reduction of eosinophil recruitment. The reduced adhesion and subsequent diapedesis of the eosinophils from the blood into the tissue could be one of the mechanisms by which this molecule produces its articulated anti-inflammatory activity. A better understanding of the mole-

cular mechanism by which heparin produces this complex anti-inflammatory activity is required for the use of this interesting molecule in allergic diseases to be optimized.

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