

Objective monitoring of nasal airway inflammation in rhinitis

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Allergic rhinitis is an inflammatory nasal disorder in which a range of different cells participates. A variety of approaches has been used to monitor nasal inflammation objectively to investigate disease processes and to evaluate the effect of therapeutic intervention. These approaches include nasal lavage, nasal cytology, and nasal biopsy, together with the more recently established measurement of nasal nitric oxide (NO) concentration. Although all provide information about nasal mucosal inflammation, the extent of information that can be obtained by each approach, the ease of sampling, and the complexity of sample handling differ. Such considerations

influence the choice of approach when measurement of nasal inflammation is to be an objective outcome parameter in a clinical trial. In addition, the choice of approach is also determined by the questions or hypotheses that are to be addressed.

Nasal lavage is simple and rapid to perform, is well tolerated, and provides a sample that can provide information about luminal cell recruitment, cell activation, and plasma protein extravasation. Nasal cytology involves sampling and recovering mucosal surface cells. It is also easy to perform and is well tolerated in general, although some find that the procedure causes a transient unpleasant sensation. A differential cell count from the sample provides information about relative cell populations. Both nasal lavage and nasal cytology are readily applicable to clinical trials. Nasal cytology sample handling is easier, but nasal lavage offers the advantage of providing considerably greater information from the sample.

Nasal biopsy is a considerably more invasive procedure and requires expertise not only in tissue sampling but also in biopsy processing. Therefore, it is applicable only in specialist centers. However, nasal biopsy is the only sampling technique that directly informs about tissue cellular events, although these may be implied, in part from the other sampling approaches. Tissue specimens can be used to evaluate both protein and gene expression.

Measurement of nasal NO involves expensive equipment but provides an instantaneous result, unlike the other approaches, all of which require sample processing and analysis.

Recommendations for standardization of measurement have been made, and measures are considered in part to reflect allergic inflammation within the nasal mucosa. The limitations of nasal NO are that it reflects only a certain aspect of allergic mucosal inflammation, and that because a proportion of nasally measured NO is derived from the sinuses under normal circumstances, nasal NO is not specific for nasal disease. The high contribution from the sinus mucosa limits the discriminatory ability of nasal NO to reflect nasal tissue-specific alterations.

The incorporation of measures of nasal inflammation in clinical trials has distinguished anti-inflammatory therapy from symptomatic therapy and has the potential to provide information about the efficacy of novel therapies for allergic rhinitis. (*J Allergy Clin Immunol* 2005;115:S414-41.)

Key words: Rhinitis, allergic inflammation, lavage, cytology, biopsy, exhaled nitric oxide, immunohistochemistry, *in situ* hybridization, mast cells, eosinophils, epithelial cells, T-cells, plasma protein exudation

Allergic rhinitis is a nasal inflammatory disorder in which a range of different cells participates.¹ This abnormal airway inflammation involves activation and tissue recruitment of both structural cells and infiltrating

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Abbreviations used

ATS:	American Thoracic Society
ECP:	Eosinophil cationic protein
EPX:	Eosinophil peroxidase
GMA:	Glycol methacrylate
GM-CSF:	Granulocyte/macrophage colony stimulating factor
H:	Histamine
HPF:	High power field
ICAM-1:	Intercellular adhesion molecule 1
IL:	Interleukin
iNOS:	Inducible NO synthase
ISH:	In situ hybridization
LT:	Leukotriene
MIP:	Macrophage inflammatory protein
NARES:	Nonallergic rhinitis with eosinophilia syndrome
NO:	Nitric oxide
PAR:	Perennial allergic rhinitis
PG:	Prostaglandin
RANTES:	Regulated on activation normally T-cell expressed and secreted
SAR:	Seasonal allergic rhinitis
TAME:	Tosyl-arginine methyl ester

leukocytes. The tissue recruitment of eosinophils is a hallmark of untreated allergic inflammation, and mast cell activation is prominent in clinical disease expression. The tissue recruitment and retention of the eosinophil leucocyte, in contrast with the neutrophil leucocyte, is a reflection of the local release of T_H2 -orientated cytokines, in particular from activated tissue T lymphocytes and mast cells, and the consequent endothelial cell activation, with the selective upregulation of specific leucocyte endothelial adhesion molecules. This cytokine release also favors epithelial cell activation and the directed migration, under chemotactic stimulation, of cells toward the airway lumen. Mediators released from activated cells, such as mast cells, basophils, and eosinophils, can account for symptom expression because of their effects on sensory nerves, glandular components, and the nasal vasculature.¹

A range of approaches has been applied to the monitoring of nasal airway inflammation, such as nasal lavage to recover luminal mediators and cells, nasal luminal and surface epithelial cytologic examination, nasal biopsy to explore tissue cellular events, and, more recently, measurement of the nitric oxide (NO) in nasal exhaled air as an indirect marker of epithelial cell interactions in allergic inflammation. In many circumstances, different approaches are complementary, because each may provide information relating to separate aspects of the allergic inflammatory process. The monitoring of nasal airway inflammation in clinical trials provides insight into the mechanism of action of the therapeutic intervention, and the choice of approach depends on the questions to be addressed and the practical applicability of

the selected methods of sampling to the trial situation. Nasal lavage and cytology are relatively simple to perform, whereas nasal biopsy sampling is more invasive. However, only the last will directly inform about a range of mucosal tissue cellular events. Whichever approach is used, it is critical that the sampling technique and the sample processing are tightly regulated and consistent to ensure reliability of results. The different approaches to the monitoring of nasal airway inflammation and their practical applicability are assessed with these considerations in mind.

NASAL LAVAGE

The introduction of fluid into the nasal cavity and its recovery after a predetermined dwell time has been used to investigate nasal mucosal and intraluminal events in rhinitis. This process is termed *nasal lavage*. The recovered fluid can be evaluated for soluble factors to evaluate changes in cell activation, glandular secretion, and vascular permeability. Centrifugation of this recovered fluid to obtain a cell pellet enables a microscope slide to be made to evaluate the cellular content of the nasal lining fluid.

Nasal lavage has been used to investigate nasal luminal events in naturally occurring seasonal allergic rhinitis (SAR), perennial allergic rhinitis (PAR),²⁻⁹ and infective rhinitis^{10,11} to gain insight into the pathophysiologic processes underlying clinical disease expression. Because this technique is relatively noninvasive, is easy to perform, and is repeatable over relatively short periods, it has also been widely used as a research tool to gain a greater understanding of rhinitis. Through this technique, insight has been gained into the mechanisms underlying the early and delayed nasal responses to intranasal allergen challenge.¹²⁻¹⁶ Similarly, nasal lavage has been undertaken before and after nasal challenge with a range of stimuli, including histamine,¹⁷⁻¹⁹ bradykinin,¹⁹⁻²¹ capsaicin,²⁰⁻²² methacholine,^{23,24} and substance P,²⁵ to gain insight into their effects on the nasal vasculature, glandular secretion, and cell recruitment and activation. Nasal insufflation with chemokines, such as IL-8,²⁶ eotaxin,²⁷ and regulated on activation normally T-cell expressed and secreted (RANTES),²⁸ has also been used to evaluate the effect and time course of their effect on cell recruitment into the nasal lumen. Studies have also been undertaken to investigate the effects of non-IgE-related stimuli, such as hyperosmolar challenge, aspirin, and sodium metabisulfite, on mediator release within the nose,²⁹⁻³² and with or without therapeutic intervention, to gain insight into the magnitude of effect and mechanism of action of differing pharmacologic modalities on cell recruitment, cell activation, and induced vascular permeability.³³⁻⁴¹

Although these approaches have been widely used in the limited research setting, they have been less widely applied as a means of objectively monitoring nasal disease in the clinical trial setting. The use of nasal lavage to

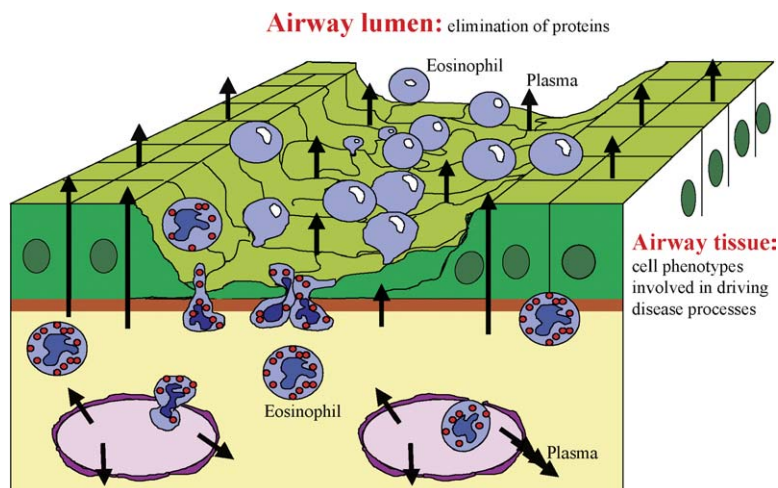


FIG 1. Airway lumen: elimination of proteins (arrows) and cells.

sample the airway provides information about nasal luminal events, but implicit in this is the assumption that these events are in part determined by tissue cellular events and that the nasal luminal findings also inform about tissue cellular processes. The airway lumen may be regarded as a major destination for proteins and cells being cleared from the airway mucosal tissue (Fig 1). Clearance of plasma proteins occurs promptly after extravasation of plasma. Luminal entry of eosinophils may be particularly pronounced at the resolution of airway inflammation.

Methods of nasal lavage

A range of techniques has been used to instill and recover fluid from the nasal cavity by using a physiologic solution, generally 0.9% NaCl, prewarmed to 37°C. The classic method is that of Naclerio et al,¹² in which subjects, in a sitting position, extend their neck gently backward to 30° from the horizontal, such that the nasal cavity is pointing upward and instilled fluid will not be lost anteriorly because of gravity. Posterior loss is limited by having subjects close their soft palate, hold their breath during the period of nasal lavage retention, and hold their mouth slightly open. The fluid is usually left within the nasal cavity for 10 seconds. The subject then leans forward and expels the fluid from the nostrils by gently exhaling into a collecting funnel that drains into a container. The recovered fluid can be measured to determine the volume retrieved. The volume instilled within each nostril is in general 2.5 mL to 5.0 mL, with approximately an 80% recovery (range, 65% to 90%). In studies in which nasal challenge induces nasal blockage, the obstruction of the nasal lumen will limit the amount of fluid that can be retained within the nasal cavity, and smaller lavage volumes need to be used under such circumstances. To overcome this, if measurement of the magnitude of nasal blockage is not an end point, intranasal α -agonist administration has been used to facilitate the undertaking of nasal lavage by decongesting the nose. This has been

shown not to influence the recovery of mediators or markers of vascular permeability within the nasal lavage fluid under challenge circumstances.⁴²

Some subjects find this technique of nasal lavage difficult to perform, because they are unable to retain the fluid within the nasal cavity and prevent it running into the oropharynx. This has led to the development of other approaches. Instead of using a syringe or pipette to introduce fluid into the nostril,^{7,9} with the neck extended, fluid has been introduced into the anterior part of the nose, with the neck flexed forward, with a seal at the anterior nares around the administering device to prevent fluid coming out anteriorly under the influence of gravity.^{21,43} This has been undertaken with either a syringe or a nasal pool device. The nasal pool device is a container that can be squeezed to expel fluid into the nostrils and then relaxed to recover the fluid after a predetermined dwell time. Radiocontrast studies have shown the distribution of nasal lavage fluid throughout the nasal cavity by this technique.

All of these techniques are acceptable and have been used satisfactorily to study nasal disease by nasal lavage. Alternative methods include the use of filter paper⁴⁴ or sponge segments for the collection of nasal secretions.

Lavage processing

The nasal lavage fluid is collected into a container on ice. After measuring the volume of recovered lavage fluid by collecting into a graded container or by weighing, having preweighed the empty container to determine the weight of the recovered lavage fluid, the nasal lavage fluid should be passed through a filter to remove mucin. The sample is then centrifuged at 4°C. Different studies have centrifuged at between 400g and 1000g for 5 to 20 minutes to sediment the cell pellet and enable the supernatant to be removed in aliquots (0.5–1.0 mL). These aliquots are put in appropriate polypropylene tubes and frozen at –20°C to –70°C until assay, depending on the end point to be measured and the time delay before the measurements are

performed. The cell pellet can be resuspended in 0.5 mL PBS containing 0.1% wt/vol human serum albumin, and 100 μ L aliquots taken to make microscope slides for differential cell counts or specific cell immunostaining. If a total cell count is required, an aliquot of the nasal lavage fluid can be taken before centrifugation and placed in a hemocytometer for this measurement to be made.

A range of mediators, cytokines, and chemokines has been measured in lavage fluid without any addition of protease inhibitors. There is no information about whether the lack of protease inhibitors modifies the absolute levels measured. In the absence of protease inhibitors, however, there are still clear disease-related differences measurable by this technique.

Nasal lavage and allergic rhinitis

Nasal lavage has been analyzed in allergic rhinitis to investigate mast cell and basophil activation by measurement of the preformed granule-associated mediators histamine and tryptase as well as the newly generated prostanoid, prostaglandin (PG) D₂. Mast cells are tissue-resident cells and are not evident in cytospin samples. Their allied metachromatic cell, the basophil, is not a normal tissue or nasal luminal constituent but can be identified in nasal cytospin samples under conditions of disease expression as a result of its airway recruitment as part of the allergic process. Similarly, eosinophils but not neutrophils are a feature of allergic nasal disease, and there are increased levels of the eosinophil activation markers, eosinophil cationic protein (ECP), and eosinophil peroxidase (EPX) in nasal lavage fluid, indicative of eosinophil activation. The cysteinyl leukotrienes (LTs), LTC₄, LTD₄, and LTE₄ may be derived from a range of cell types, including mast cells, basophils, and eosinophils. Thus, measurement of this mediator is nonspecific for cell provenance.

Mast cells/basophils/histamine/tryptase and PGD₂

Histamine is a major preformed component of mast cell and basophil granules that is released during the immediate IgE-mediated response to allergen. It is also released by mast cells in response to stimuli that induce non-IgE-mediated degranulation, such as hypertonic challenge or adenosine exposure. Histamine is a major contributor to disease expression through its action on neural and vascular receptors within the nose, inducing nasal itch, sneeze, rhinorrhea, and nasal blockage.⁴⁵ These effects are predominantly mediated via H₁-receptors. Measures have thus been made of histamine in nasal lavage fluid, both under the controlled laboratory conditions of challenge and in naturally occurring disease.

After nasal allergen challenge, there is an immediate increase in histamine concentrations in recovered nasal lavage fluid (Fig 2). This peaks at 10 minutes after challenge and is of short duration, returning to baseline by 15 to 20 minutes postchallenge. There is a second late increase in histamine in nasal lavage, somewhere between

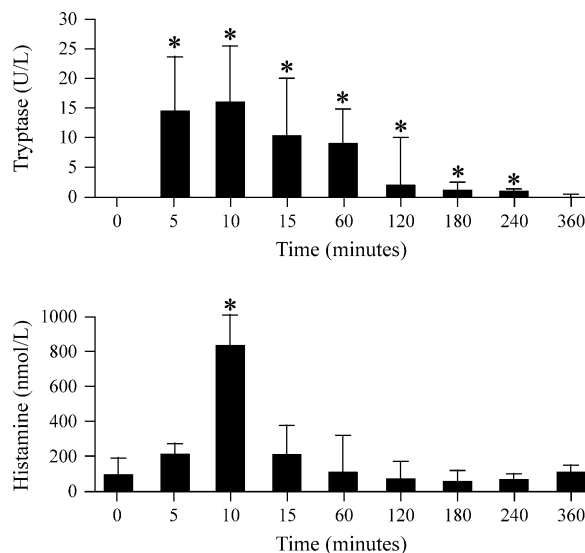


FIG 2. Changes in nasal lavage concentrations of histamine (lower panel) and tryptase (upper panel) in 15 subjects after intranasal allergen challenge. The shaded rectangles represent the mean values at each time point, with standard error bars superimposed. * $P < .05$ vs baseline prechallenge (Howarth, unpublished data, January 2005).

3 and 11 hours postchallenge. This increase, unlike the early response, is not paralleled by lavage changes in either PGD₂ or tryptase,^{46,47} suggesting that this late increase in nasal lavage histamine may relate to basophil rather than mast cell degranulation. Consistent with this, a late increase in basophil numbers has been identified in nasal lavage fluid after intranasal allergen challenge.⁴⁸ The percentage of basophils in nasal lavage fluid is small, rising from 0.2% of cells at baseline to 0.7% during the late reaction, although this increase is a reflection of an approximately 12-fold increase in total basophil numbers. Significant correlations have been reported between nasal resistance measurements and the numbers of basophils 8 hours after nasal allergen challenge⁴⁹ and between the number of basophils and the cumulative levels of histamine in nasal lavage from 3 to 11 hours postallergen challenge.⁴⁸

Baseline levels of histamine in the quiescent nose can be high, and it is standard before an allergen or other challenge in which the time course of mediator changes is being evaluated to undertake 5 baseline nasal lavages to wash out the nasal lining to obtain a low baseline value before challenge. Comparison of the histamine concentrations between the first and fifth recovered samples from 20 subjects with SAR studied outside the pollen season identifies a significant decrease with repeated lavage (Fig 3). The initial higher level in the absence of symptoms has been attributed in part to bacterial synthesis of histamine.⁵⁰ However, such levels will also be influenced by disease activity.

Measurement of nasal lavage histamine concentrations in naturally occurring disease has proved disappointing as

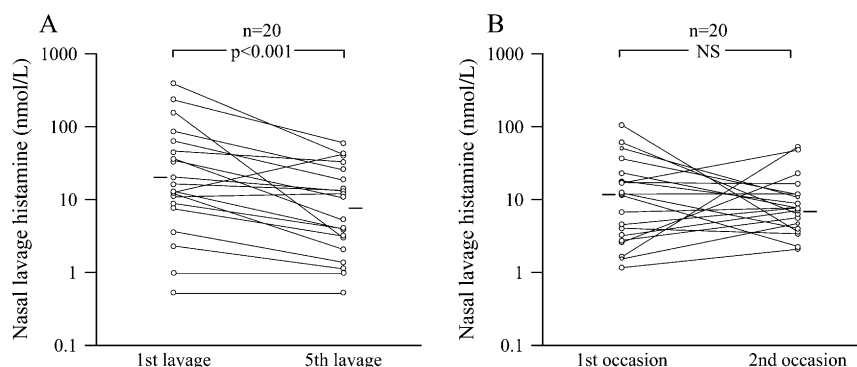


FIG 3. Measurements of histamine concentrations in nasal lavage fluid illustrating the effect of repeated nasal lavage (A) and the repeatability of the fifth lavage (B) when nasal lavage is undertaken in the same subjects 14 days apart (Howarth, unpublished data, January 2005).

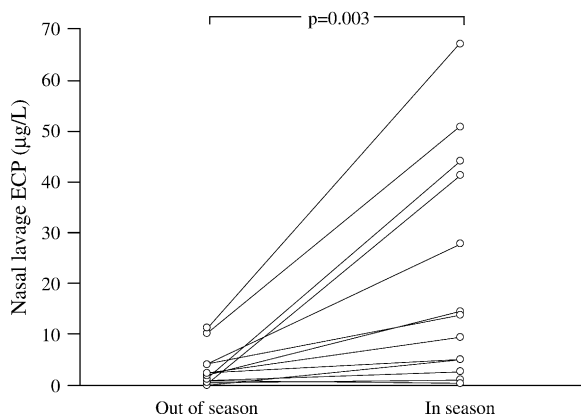


FIG 4. Seasonal changes in nasal lavage ECP concentrations. Subjects were symptomatic in season and not receiving any regular maintenance therapy for their disease (Howarth, unpublished data, January 2005).

a marker of disease activity. Several studies have failed to find elevated concentrations of histamine in nasal lavage fluid in either SAR or in PAR when a single nasal lavage sample is taken and compared with that in healthy controls.^{8,9} This may relate to either the background high baseline levels preventing the discernment of small disease-related differences or the short half-life of histamine. Histamine is rapidly metabolized by histaminases and N-methyl transferase. In this respect, other markers of mast cell degranulation, such as tryptase or PGD₂, are better biomarkers of mast cell activation. Elevated levels of both tryptase and PGD₂ are described in nasal lavage fluid recovered from subjects with SAR^{6,8,9} and PAR^{7,9} compared with healthy controls. After nasal allergen challenge, the increase in tryptase,^{14,51} another preformed granule component of mast cells, is both greater¹⁴ and more sustained than that with histamine (Fig 2). These differences are important contributors to the usefulness of this marker of mast cell activation in exploring pathophysiology in naturally occurring disease. Similarly, a previous

report has suggested that PGD₂, like tryptase, may be a more reliable *in vivo* marker of mast cell activation than histamine.⁵² A separate analysis of prostaglandin levels in nasal lavage fluid by the combination of microcolumn HPLC and a He/Cd laser-induced fluorescence system found elevated levels of PGD₂ (1.33 nmol/mL $\pm$ 0.17 nmol/mL) and PGE₂ (0.8 nmol/mL $\pm$ 0.11 nmol/mL) in allergic rhinitis compared with levels in healthy controls of 0.23 nmol/mL $\pm$ 0.16 nmol/mL and 0.29 nmol/mL $\pm$ 0.19 nmol/mL, respectively.⁶ There was no difference between the groups in measures of the nasal lavage levels of PGF₂ or 6-keto PGF_{1 α} .⁶

Eosinophils, eosinophil cationic protein, and eosinophil peroxidase

Studies are more consistent in respect to their reporting of the presence of eosinophils in nasal microscope slides or markers of eosinophil activation, such as ECP or EPX in increased concentrations in nasal lavage in active rhinitis, compared with healthy controls.^{3,4,7-9} There were clear increases in ECP levels in nasal lavage when evaluated in the same 13 subjects with grass pollen sensitization in and out of season (Fig 4). The increase in nasal lavage ECP has been reported to correlate with symptom scores ($r = 0.53$) in naturally occurring SAR,⁸ even though there is considerable intersubject variability in the nasal lavage ECP levels. Elevated nasal lavage ECP levels are also present in PAR,^{7,9} as are those for EPX.⁴ Intranasal steroid therapy has been shown to reduce the recovery of eosinophils and eosinophil activation markers in nasal lavage in SAR when used on a regular basis⁵³ or when used on an as-needed basis.⁵⁴ In both instances, differences were evident with intranasal steroid therapy in comparison with H₁-antihistamine therapy. Studies of nasal allergen challenge reveal an early increase in eosinophils between 30 and 60 minutes postchallenge in recovered nasal lavage fluid, and a second increase from 6 to 10 hours postchallenge,¹⁶ which is sustained as long as 24 hours postchallenge but not beyond.⁵⁵ This

change in eosinophils is paralleled by changes in nasal lavage EPX levels.¹⁶ The early increase in eosinophils and EPX may be a reflection of the enhanced plasma protein exudation washing resident airway cells and their free granules out into the airway lumen, because an increase in ECP in nasal lavage has also been reported after nasal histamine challenge.⁵⁶ The later increase will reflect the stimulated process of tissue cell recruitment as part of the allergic inflammatory process.

Leukotrienes

There is an early increase in LTB₄ and LTC₄ levels in nasal lavage fluid in association with histamine, tryptase, and PGD₂ after intranasal allergen challenge in sensitized subjects that occurs in association with the development of pruritus, sneezing, rhinorrhea, and nasal blockage.^{12,13} This is consistent with mast cell degranulation and the local generation and release of these arachidonic acid products. There is also a late increase in nasal lavage LTC₄ levels but not in LTB₄.^{16,46} This increase in nasal lavage LTC₄ concentration could relate to either basophil or eosinophil activation. Elevated levels of leukotrienes have also been reported in nasal lavage fluid in SAR⁵⁷ and PAR.^{4,7}

Cytokines and chemokines

Cytokines relevant to nasal eosinophil recruitment (GM-CSF, IL-3, IL-5) and the chemokines linked to this process (eotaxin, RANTES) have been measured in nasal lavage fluid both in naturally occurring disease and under circumstances of nasal allergen challenge. Although changes are described with the supraphysiologic stimulus of laboratory allergen challenge, it has been difficult to discern clear and consistent disease-related changes in naturally occurring disease.

Nasal lavage conducted in and out of the birch pollen season by using the nasal pool device in a group of subjects with severe seasonal nasal symptoms failed to find any increase in IL-1 β , IL-3, IL-5, IL-6, IL-8, or RANTES nasal lavage concentrations.⁵⁸ There was a seasonally related increase in nasal lavage GM-CSF.⁵⁸ A separate study found reduced levels of IFN- γ in nasal lavage in season in SAR and an alteration in the IL-4:IFN- γ ratio.⁵⁹ These findings in naturally occurring disease contrast with the findings in nasal allergen challenge. Under these circumstances, late increases in IL-1 β , IL-5, IL-6, IL-8, RANTES, GM-CSF, and macrophage inflammatory protein (MIP)-1 α have been described.^{60,61} Some findings are not consistent in that other workers have failed to find detectable quantities of either MIP-1 α or RANTES in nasal lavage fluid.⁶² A decrease in nasal lavage IL-8 during natural seasonal allergen exposure and a reciprocal increase in another chemokine, monocyte chemoattractant and activating factor/monocyte chemoattractant protein 1, has been reported in symptomatic seasonal allergy.⁶²

Two separate studies have reported an increase in eotaxin in nasal lavage fluid after allergen challenge.^{16,63} In one study, eotaxin was found to increase in both the

early and late phase responses to challenge.¹⁶ These changes paralleled lavage increases in both EPX and eosinophils. There was a significant correlation between nasal lavage eotaxin levels and eosinophils ($r = 0.73$) and EPX ($r = 0.67$). In the other study, daily nasal allergen challenge was undertaken for 6 days. Significant increases in eotaxin, ECP, and eosinophils were evident in nasal lavage after this period of allergen exposure.⁶³ In this study, baseline analysis revealed a significant correlation between nasal lavage eotaxin concentrations and those of ECP and α_2 -macroglobulin. These relationships were lost after the period of allergen exposure, and at the end of the study there was no relationship between nasal lavage eotaxin levels and nasal mucosal tissue eosinophil numbers.

Other measures

Nasal lavage has been used to explore the influence of nasal allergen challenge on the presence and activity of the mast cell chemoattractant stem cell factor⁶⁴ on the generation and release of matrix metalloproteinase 9⁶⁵ and the protease granulocyte elastase as well as the antiproteases secretory leukocyte protease inhibitor and α_1 -antitrypsin.⁶⁶

Plasma protein exudation

Exudation may be defined as the movement of plasma, leukocytes, or both to the surface of an organ. Considerable work has been undertaken by using repeated nasal lavage to monitor changes in a range of measurements that reflect plasma protein exudation after nasal challenge with monitoring of measures such as tosyl-arginine methyl ester (TAME)-esterase activity, albumin, fibrinogen, α_2 -macroglobulin, and total protein.⁶⁷ There is increased plasma protein exudation within the nose with a range of stimuli, including allergen,^{12,13,68,69} histamine,¹⁷⁻¹⁹ kinins,¹⁹ and substance P,²⁵ that is concentration-dependent and time-dependent.¹⁸ Albumin may also be derived from glandular secretion,²³ so rather than evaluating this lower molecular weight protein (66,000 D), it is preferable to evaluate changes in a larger molecular weight protein, such as α_2 -macroglobulin (725,000 D), when evaluating the acute exudative response. Under acute challenge circumstances with histamine, however, there is a very close correlation between changes in nasal lavage albumin and α_2 -macroglobulin levels ($r = 0.98$), suggestive of a vascular source for both these proteins under such circumstances.¹⁸ An increase in nasal lavage albumin levels is evident in SAR⁹ and PAR,^{7,9} and elevated levels of α_2 -macroglobulin are also evident in SAR.⁷⁰

Measures of plasma protein exudation as a marker of tissue inflammation

Because many mediators may act on the nasal vasculature to promote plasma protein exudation, measurement of a marker of this process, such as α_2 -macroglobulin, should theoretically provide a single integrated measurement of inflammation that reflects the underlying tissue

processes. Supporting this is the identification that IgE-mediated reactions, exposure to occupational chemicals, and viral infections all evoke significant exudation of plasma proteins in nasal and bronchial airways.^{67,71} Mediators such as histamine, leukotrienes, and kinins may be considered inflammatory because they induce exudative responses in the human nose.⁶⁷ Intranasal challenge with histamine produces a dose-dependent increase in plasma exudation over a wide range of nasal mucosal surface concentrations.⁶⁷ This contrasts with histamine-induced fucose increments in nasal lavage with challenge, which reflect glandular mucinous secretion that are rapidly tachyphylactic (L. Greiff, Personal communication, May 2004). By virtue of the exceedingly profuse microcirculation of the airway mucosa, the plasma exudation response is virtually without limit.

Methacholine is a selective secretagogue in human airways. Even supramaximal secretory doses of such a muscarinic agent may produce little if any exudative effect.^{67,71} Similarly, many irritant provocations, including exposures to capsaicin, nicotine, and benzalkonium chloride, evoke secretory effects without inducing plasma exudation.^{67,72} High doses of capsaicin, with as yet unknown neural or nonneural actions, seem to be needed to produce an exudative response in the human nose, and only then in patients with underlying rhinitis. Taken together, these data indicate that the special kind of neurogenic (exudative) inflammation so readily produced in select rodent species⁶⁷ is not applicable to human airways.

The plasma exudation response in humans may thus be regarded as specific to inflammation, because secretory agents, as well as neural irritants, do not readily evoke this response. A further inference is that inhibition of airways exudation can occur without impeding the component of innate immunity that involves sensory nerve stimulation and a promptly ensuing secretory response. Conversely, antisecretory drugs such as muscarinic antagonists cannot be expected to influence plasma exudation responses. However, anti-inflammatory therapy would be expected to modify plasma protein exudation.

Influence of therapy on the exudative response

The microvascular-epithelial exudation of plasma is pharmacologically regulated at the level of the venular endothelial cells that are also key target cells for mediators and drugs. By receptor-mediated mechanisms, interendothelial gaps are transiently induced in the postcapillary venules. Through these gaps, nonsieved plasma leaves the circulation, distributes in mucosal tissues, and creates ubiquitous paraepithelial routes for its luminal entry. Histamine-induced plasma exudation in the airways is evoked by stimulation of endothelial H₁-receptors.⁷³ Hence, this is the site of action of antiexudative histamine antagonists. Similarly, the vasopermeability effect of leukotrienes, bradykinins, and other exudative autoids is selectively inhibited by the appropriate pharmacologic antagonist.

Drugs that inhibit the release of vascular permeability mediators will inhibit plasma exudation in diseased airways. This result can also be brought about, as with steroids, by reducing the number of activated inflammatory cells and thus reducing mediator availability. This indirect effect is probably the prime antiexudative mechanism of airway steroids. Supporting this view, intranasal steroid administration has only a marginal effect on histamine challenge-induced exudation. Hence, a direct stabilizing action on the venular endothelial cells appears less important in human airways than has previously been suggested in animal test systems.⁶⁷ The nasal airway microcirculation may develop increased exudative responsiveness to histamine along with development of seasonal rhinitis. Interestingly, steroids may inhibit this hyperresponsiveness.⁶⁷

Many vasopermeability agents have the additional property of being vasodilators with a capacity to increase local blood flow. In theory, this action, which would increase delivery of plasma to leaky venules, would promote exudation. Yet, presumably because of a high baseline flow in the profuse network of subepithelial microvessels, changes in blood flow may not contribute significantly to extravasation in the airway mucosa, in contrast with poorly perfused skin, in which blood flow is an important determination of extravasation. Consistent with this, decongestive doses of topical nasal vasoconstrictors do not reduce acute challenge-induced plasma exudative responses.⁶⁷

In patients without active allergic rhinitis (subjects with SAR studied out of season), antihistamines are highly effective inhibitors of allergen challenge-induced responses, completely preventing also the exudation of α_2 -macroglobulin.⁷⁴ However, the efficacy of these drugs may be reduced when the allergen challenge is administered during the active season. Interestingly, with the airway steroids, the antiexudative efficacy is the reverse. Thus, steroids may preferably inhibit allergen challenge-induced plasma exudation during active allergic disease processes,⁶⁷ and intranasal steroid therapy reduces the recovery of α_2 -macroglobulin in nasal lavage fluid in naturally occurring disease.⁷⁰ Yet steroids are not universally active as antiexudative drugs, because acute plasma exudative responses to challenge with mediators or occupational chemicals are not inhibited by single-dose steroid pretreatment. Similarly, steroids do not prevent the sustained plasma exudation that occurs with viral infections or with experimental epithelial shedding-restitution processes.⁶⁷ Thus, steroids do not seem to inhibit plasma exudation when this response potentially has important roles in innate immunity, repair, and host defense. The specificity of markers of plasma protein exudation for inflammatory rather than irritant responses and the positive effect of intervention on allergic inflammatory but not nonallergic inflammatory processes indicate that markers such as α_2 -macroglobulin, as measured in nasal lavage, provide a valid measure of allergic tissue inflammation and one that is applicable in the clinical trial setting.

Nasal lavage and naturally occurring infective rhinitis

In contrast with allergic rhinitis, nasal lavage conducted in the same way in the common cold during symptomatic upper respiratory tract infection identifies clear increases in a range of cytokines and chemokines. Elevated concentrations of IL-1 β , IL-6, IL-8, IL-10, TNF- α , IFN- γ , RANTES, and MIP-1 have been found in nasal lavage in relationship to disease expression.^{10,11} These cytokine and chemokine changes are associated with elevated levels of myeloperoxidase, ECP, and bradykinin in nasal lavage, as well as increased concentrations of albumin and soluble intercellular adhesion molecule 1 (ICAM-1). No increase in histamine has been found in nasal lavage in association with infective rhinitis.^{10,75} A further study in influenza, using nasopharyngeal sampling rather than nasal lavage, reported increased recovery of IL-6, TNF- α , and IFN- γ , as well as IFN- α , consistent with those findings from the nasal cavity.⁷⁶

Repeatability of measures in nasal lavage

The consistency of the findings in allergic and infective rhinitis for a range of different measures in nasal lavage fluid supports the concept that this method of nasal evaluation provides reliable information of relevance to disease activity. However, there is less information about the coefficient of variation of repeated measurements to provide information for power calculations to derive patient numbers in intervention studies. As indicated, repeated nasal lavage is associated with a significant reduction in histamine concentration (Fig 3). The evaluation of the fifth nasal lavage at baseline in subjects with SAR studied outside the pollen season when asymptomatic when samples were taken 2 weeks apart found no difference between the 2 time points in these measures (Fig 5). The repeatability of the nasal response to histamine has been evaluated under laboratory challenge conditions.¹⁷ These challenges, with 30-minute intervals between challenges, were undertaken in 12 subjects on one day, and the same procedure was repeated on a separate day. Repeated nasal histamine challenges were associated with a reduced sneezing response to challenge. However, nasal lavage identified that the vascular response to challenge, as monitored by change in TAME esterase and albumin concentrations in recovered lavage fluid, was reproducible both within the time frame of the repeated challenge and also the period of the 2 separate challenge days. The eosinophil response, as monitored by nasal lavage ECP levels in response to allergen challenge, is also repeatable.

However, there is considerable variability between subjects in eosinophil luminal recruitment and activation, as reflected by measurement of the activation markers ECP and EPX in grass pollen-sensitive subjects ($n = 13$) either in season (Fig 4) or after an intranasal allergen challenge that produces a comparable degree of nasal obstruction outside the pollen season, ie, when asymp-

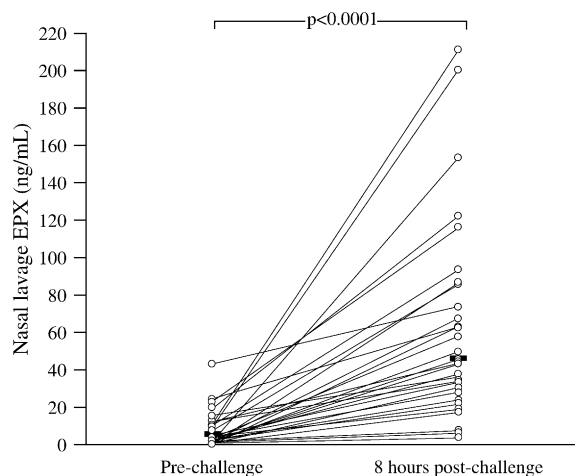


FIG 5. Nasal lavage EPX concentrations (ng/mL) in pollen-sensitive subjects, studied outside the pollen season when asymptomatic, illustrating the variability in individual response to intranasal pollen allergen challenge (Howarth, unpublished data, January 2005).

tomatic ($n = 31$; Fig 5). The biologic response to allergen exposure thus is heterogeneous in sensitized subjects, despite adjusting the allergen stimulus to account for differences in the degree of atopic sensitization in the nasal allergen challenge model.

Sources of variance

For most parameters, other than the effect of laboratory challenge, disease presence, or therapeutic intervention, there is little information on the natural variance of measures in nasal lavage. One epidemiologic study, however, has evaluated the effect of air pollution on the development of lung function in young children in Austria.⁷⁷ Repeated nasal lavage for the measurement of ECP was undertaken in 147 children over a 2-year period. Of these children, 101 underwent nasal lavage on 5 separate occasions during the first year, with an interval of a minimum of 1 week between each lavage. All children were characterized by questionnaire about their potential history for asthma, were categorized as having rhinitis (serous or purulent, depending on the nature of any nasal secretions at the time of the lavage), and were given a skin prick test (93 of the 101 children). Sixteen percent were atopic, and 3 of the 101 children had a doctor's diagnosis of perennial rhinitis. From an 8-mL total nasal lavage, there was a mean recovery of 5.7 mL in these children, whose mean age was 8.1 years. Over the 2-year period, the children had 10 to 12 separate nasal lavage samples. Over this period, 16.2% of nasal lavages were obtained when there was serous rhinitis, and 29.6% were obtained when clinically there was purulent rhinitis. Those with purulent rhinitis had the highest ECP nasal lavage concentrations (64.68 $\mu\text{g/L}$; SD, 51.26), those with serous rhinitis had intermediate ECP (46.50 $\mu\text{g/L}$; SD, 51.45), and those

without rhinitis had the lowest ECP concentrations (35.93 $\mu\text{g/L}$; SD, 33.41).

The average of the 101 individual variances across repeated ECP measurements was 2399 $\mu\text{g/L}$ (SD, 2325 $\mu\text{g/L}$).² The interclass coefficient of reliability for all measurements was 0.27 (intrasubject variance, 2274 $\mu\text{g/L}$; and intersubject variance, 841 $\mu\text{g/L}$),² suggesting that about 25% of the total variance is caused by intersubject variability. The presence of atopy did not influence the findings to a meaningful extent, nor did excluding the observations with purulent rhinitis. When purulent rhinitis samples were excluded, there was a significant correlation for individuals' mean results in year 1 compared with year 2 ($r = 0.6$; $P = .00001$).

On a bivariate level, boys had higher nasal lavage ECP levels than girls (50.95 $\mu\text{g/L}$ vs 39.13 $\mu\text{g/L}$; $P = .06$). The presence of either serous or purulent rhinitis was associated with higher nasal lavage ECP levels, as detailed, and atopic children had higher levels than nonatopic children. Passive exposure to cigarette smoke within the household did not appear to influence nasal lavage ECP levels. When atopy, rhinitis, and passive smoke exposure were excluded, normal ECP nasal lavage levels were determined to be 37.28 $\mu\text{g/L}$ for boys and 23.85 $\mu\text{g/L}$ for girls ($n = 21$). A separate, smaller study in adults ($n = 21$) evaluating the variability in cell counts in nasal lavage found an interclass coefficient of reliability of 0.88 when lavages were performed 72 hours apart.⁷⁸

Relationship to other measures

There is limited cross-reference to other indices of nasal disease—other than the absence or presence of symptoms—in studies of nasal lavage under naturally occurring conditions. One study reported a weak positive correlation between the nasal response to histamine as an index of nasal hyperresponsiveness and the percentage of eosinophils in nasal lavage microscope slide samples in PAR.⁷ This study found no relationship between any mediator measurement in the lavage fluid and nasal hyperresponsiveness to histamine. The positive relationship to histamine challenge was present when the area under the curve response for symptom reporting was evaluated, but was not evident if alternative evaluations, such as area under the curve sneeze count or nasal secretion volume, were explored.

Some studies have found a strong correlation between nasal lavage tryptase levels and the percentage of eosinophils in nasal smears or scrapings, and unsurprisingly, correlations also exist for ECP levels in nasal lavage and the number of nasal mucosal surface eosinophils,^{8,9} although this is more evident in SAR than in PAR.⁹

After allergen challenge, ECP levels in nasal lavage 24 hours postchallenge have been correlated with the level of NO derivatives.¹⁵ The numbers of eosinophils and the level of eosinophil activation markers postallergen challenge have also been related to the allergen-induced changes in nasal lavage eotaxin levels.¹⁶

NASAL CYTOLOGY

The usefulness of nasal cytology depends on obtaining adequate specimens, appropriately preparing and staining the samples, and knowledgeably interpreting the materials. Nasal cytology can assist in (1) distinguishing inflammatory from noninflammatory rhinopathies; (2) distinguishing between allergic, nonallergic, and infectious rhinitis; (3) distinguishing between viral and bacterial infections; (4) following the course of a disease; and (5) following the response to treatment. Measurement of nasal cellular changes may also provide information of validity to other less accessible parts of the respiratory system, because the mucosa of the upper and lower airways, as well as the sinuses, may all be involved as part of a common mucosal response in allergic disease.

Methods of measurement

Various techniques have been used for obtaining, processing, evaluating, and interpreting nasal cytology specimens.⁷⁹

Blown secretions. In this method, secretions in the nasal airways are blown onto wax paper or a plastic wrap and then placed onto a glass slide.⁸⁰ The cells are only those contained in the secretions and may thus reflect a different population from those collected from the epithelium. Another disadvantage of this technique is that many children and patients with some nasal disorders cannot produce an adequate secretion specimen.

Nasal scraping/brushing. After excess secretions are cleared, the more patent side is sampled by using a plastic curette (eg, Rhino-probe; ASI, Springville, Utah) or fine bristled brush under direct visual inspection.⁸¹ A nasal specimen of both the secretions and surface epithelium can be easily obtained by scraping the surface in the middle third of the inferior turbinate. Advantages of this method include specificity of the sampling site, minimal trauma with no need for anesthesia, ease of repetition, and adequacy of specimens at any age and in all nasal conditions. The adequacy of specimens is better with nasal scrapings than with blown secretions.⁸² The reproducibility of grading between centers is also better with scrapings compared with secretion specimens. Alternative sampling approaches to recover nasal luminal superficial cells include the use of cotton wool buds on sticks or sponges on sticks. These approaches have the disadvantage that the cells may stick to the surface of the cotton and the sponge and may not be readily transferred onto the glass slides. Furthermore, symptoms correlate better with the histologic findings from scrapings than from the cytology of secretion samples.

Nasal lavage. This specimen evaluation is performed after introducing 2.5 mL to 5 mL saline solution in each nasal cavity with the patient's head bent backward during closure of the soft palate. The expelled fluid is centrifuged and the recovered cell pellet resuspended to enable cells to be counted in a hemocytometer and the cell differential to

TABLE I. Guide for grading nasal cytograms

Quantitative analysis	Semiquantitative analysis	Grade
Epithelial cells		
NA	Normal morphology	N
NA	Abnormal morphology	A
NA	Ciliocytophthoria	CCP
Eosinophils, neutrophils		
0*	None	0
0.1-1.0*	Occasional cells	0.5+
1.1-5.0*	Few scattered cells or small clumps	1+
5.1-15.0*	Moderate number of cells and larger clumps	2+
15.1-20.0*	Larger clumps of cells that do not cover the entire field	3+
>20*	Large clumps of cells covering the entire field	4+
Basophilic cells		
0*	None	0
0.1-0.3*	Occasional cells	0.5+
0.4-1.0*	Few scattered cells	1+
1.1-3.0*	Moderate number of cells	2+
3.1-6.0*	Many cells easily seen	3+
>6.0*	Large number of cells, as many as 25 per HPF	4+
Bacteria†		
NA	None seen	0
NA	Occasional clump	1+
NA	Moderate number	2+
NA	Many easily seen	3+
NA	Large numbers covering the entire field	4+
Goblet cells‡		
0	None	0
1% to 24%	Occasional to few cells	1+
25% to 49%	Moderate number	2+
50% to 74%	Many easily seen	3+
75% to 100%	Large number, may cover the entire field	4+

*Mean of cells per 10 HPFs ($\times 1000$).

†Note presence of intracellular bacteria.

‡Ratio of goblet cells to epithelial cells, expressed as percentage.

be determined on a microscope slide.⁸³ Eosinophils are clearly identifiable in lavage microscope slides.

Biopsy. The most common site of biopsy is the lower edge of the inferior turbinate. A disadvantage of the biopsy procedure is that it is somewhat traumatic and is therefore difficult to repeat in a serial fashion. The major advantage of the technique is that it allows examination of not only the superficial epithelium but also the basement membrane and the submucosal components.

Processing methods

Fixatives for the specimen on the slide include air drying (not recommended), acetone, unscented hair sprays, Mota's basic lead acetate, buffered formalin, methyl alcohol, ether:95% ethyl alcohol (1:1), and 95% ethyl alcohol. The last preparation gives excellent results.

Histologic stains have variable advantages: Hansel's, eosinophils; Wright's, basophils; Wright-Giemsa, eosinophils, neutrophils, and basophilic cells; Papanicolaou, epithelial cells and nuclear and cytoplasmic changes; toluidine blue, basophilic cells; Leishman's, eosinophils; Alcian yellow, mast cells; Randolph's, eosinophils; Alcian blue, basophilic cells; and May-Grunwald, neutrophils.

Staining may be achieved with the Wright-Giesma dip method as follows. (1) Remove the slides from the jar containing 95% ethyl alcohol and drain the excess alcohol. Do not allow the cells to air dry. (2) Dip the slides in Wright-Giemsa stain for 10 to 15 seconds. (3) Drain the excess stain, then dip the slides in buffer for 10 to 30 seconds. (4) Drain the excess buffer, then dip the slides in hematology rinse for 5 to 10 seconds. (5) Drain the excess rinse and air-dry the specimens.

Modes of evaluation

The nasal cytogram should be viewed at high power (oil immersion, $\times 1000$). A guide for grading nasal cytograms is presented in Table I. Another method involves making a percentage calculation of specific leukocytes, eg, 10% eosinophils. The finding of 10% eosinophils in blown secretions generally corresponds to a 1+ grading seen in the nasal scraping cytogram. A guide for interpreting the nasal cytogram is presented in Table II.⁸¹

Cytologic findings in clinical conditions

Normal subjects. The normal nasal mucosal cytology of infants, children, and adults consists of numerous

TABLE II. Guide for interpreting the nasal cytogram

Cellular type	Diagnostic classification
Increased eosinophils (1-4+)	Allergy Nonallergic rhinitis with eosinophilia Aspirin sensitivity
Increased basophilic cells (1-4+)	Allergy Nonallergic rhinitis with eosinophilia Aspirin sensitivity Nonallergic rhinitis with basophilia
Increased neutrophils (2-4+)	
With intracellular bacteria	Nasopharyngitis or sinusitis
With ciliocytophthoria	Viral upper respiratory infection
With fungi	Fungal upper respiratory infection
With no bacteria	Irritant reaction, possible allergic rhinitis, sinusitis
Bacteria (2-4+), diagnostic if intracellular	Nasopharyngitis or sinusitis

epithelial cells, including ciliated columnar, nonciliated columnar, goblet, and basal cells. An adequately sampled specimen will always contain some ciliated cells and goblet cells. There are usually no eosinophils or basophilic cells ($<1+$) within the superficial layer above the basement membrane. A moderate number of neutrophils ($\leq 2+$) and a few bacteria ($\leq 1+$) can be seen, especially if the specimen is taken from the anterior portion of the inferior turbinate.^{84,85}

Allergic rhinitis. In SAR under natural conditions, as the pollen and symptoms increase, the percentage and total number of eosinophils increase. The total number of basophilic cells also significantly increases during the pollen season, but they do not start to appear until after 4 to 5 days of pollen exposure. A tendency toward an increase in the number of neutrophils is noted at the peak of the pollen season.⁸⁶

A highly significant correlation has been shown with nasal secretion eosinophilia and evidence of allergy such as nose rubbing, sneezing, runny nose, and swollen turbinates.⁸⁷ In particular, the increase in eosinophils generally correlates well with the symptom of nasal obstruction as measured by rhinomanometry.⁸⁸ The degree of nasal eosinophilia appears to correlate with the extent of allergen exposure. The presence of eosinophilia also correlates with the presence or a positive allergy skin test and, along with basophilic cells, with the serum IgE level.⁸⁹⁻⁹¹

Eosinophils are found in allergic disease at all ages. In a prospective study of infants with a strong family history of allergic disease, it has been shown that both eosinophils and basophilic cells increase in number in nasal scrapings of allergic children between birth and 4 years of age but are uncommon in children with no allergic disease.⁹²

In adults with SAR, using the Rhino-probe scraping technique, the following cytologic patterns have been reported: eosinophils in 81%, basophilic cells in 42%, neutrophils in 64%, and bacteria in 28% of those with at least a 1+ grading.⁹³ The frequency of neutrophil-positive and bacteria-positive specimens in patients with allergy is noteworthy.

In the cytology from nasal lavages of normal subjects and allergic subjects, the predominant leukocyte is the neutrophil ($73\% \pm 10\%$). Specimens from allergic subjects contain significantly more total cells, neutrophils, and eosinophils than normal subjects. Biopsy specimens from these groups of subjects reveal that the predominant leukocyte in the tissue is not the neutrophil but the mononuclear cell (most likely lymphocytes). After antigen stimulation, patients with allergy have significant increases in total cells, neutrophils, eosinophils, and mononuclear cells in the subepithelial tissue.⁹⁴

The normal metachromatic (basophilic) cell content of the nose is about 200 cells/mm³ to 400 cells/mm³ of the mucosa.⁹⁵ The great majority of these cells is located in the lamina propria. There are 3 populations of basophilic, metachromatic cells in the nose: basophil leukocytes and 2 histochemically distinct populations of mast cells.

Basophilic cells have been identified as being biologically active in allergic reactions. As the number of basophilic cells increases, the nasal symptoms become more severe, and provocative challenge responses usually become stronger.⁹⁶ However, nasal reactivity to allergen challenge can also increase without a concomitant increase in the number of surface basophilic cells. The number of basophilic cells also correlates with nasal eosinophilia in allergic rhinitis.⁹⁷ The finding of these cells and/or eosinophils increases the sensitivity of the test for confirming an allergic diagnosis to nearly 80%.⁹⁸

In a study of patients with allergic rhinitis, allergen challenge produced a 12-fold increase in the number and a 3-fold increase in the percentage of basophilic cells in nasal lavage fluid during the late-phase response.⁴⁸ At least $\frac{2}{3}$ of these cells were observed to be basophils.

Nonallergic rhinitis. Of patients undergoing evaluation for chronic perennial rhinitis, approximately 50% give a history of skin reactivity consistent with allergic rhinitis. In the remaining 50% who are nonallergic, more than $\frac{2}{3}$ are characterized by the absence of eosinophils on nasal smear. They have been classified as having nonallergic noninfective rhinitis, sometimes referred to as *vasomotor rhinitis*. In addition to the scarcity of eosino-

phils in patients with vasomotor rhinitis, there is no increase in degranulated basophilic cells or plasma cells.⁹⁹

About ¼ of patients without allergy with chronic rhinitis have eosinophilic nonallergic rhinitis or the non-allergic rhinitis with eosinophilia syndrome (NARES). In one study of patients with NARES, eosinophil percentages varied from 19% to 66% in spontaneously collected nasal secretions and from 7% to 74% in nasal wash specimens.¹⁰⁰

Another form of nonallergic rhinitis is the rhinitis of pregnancy. Unless there is an associated allergic rhinitis or bacterial rhinosinusitis, nasal cytology is normal.

Irritant rhinitis. Nasal cytology has been studied after exposure to various intranasal stimuli. Some observations include the following: saline, no change in number or types of cells in nasal lavage fluid; distilled water, no change in number or types of cells in nasal lavage fluid¹⁰¹; formaldehyde, occasional epithelial metaplasia and dysplasia¹⁰²; cold air, no change in number or types of cells in nasal lavage fluid¹⁰³; excessive α -adrenergic agents resulting in rhinitis medicamentosa, no change in number or types of cells in nasal lavage fluid; and nonatopic or atopic individuals receiving irrelevant allergen challenges, increased neutrophils possibly induced by repeated scraped nasal sampling.¹⁰⁴ Only environmental tobacco smoke causes an allergic-like cell infiltrate in the nasal mucosa of nonatopic children, with the findings of increased eosinophils and basophils.^{87,105}

Infectious rhinitis

Large numbers of bacteria, especially intracellular bacteria, support the diagnosis of infection. Fewer ciliated cells can be a finding of recurrent infections. Two studies have shown nasal cytology to be suggestive of the diagnosis of sinusitis. Using a wax paper blow for specimen collection, one study of 300 children and adults found that sinus radiographs were significantly more likely to be abnormal when there were >5 neutrophils/high power field (HPF; $\geq 2+$).¹⁰⁶ The presence of >5 neutrophils/HPF was 86% sensitive and 40% specific in predicting radiographic pathology. In the second study, when the Rhino-probe scraping technique was used, the results in 20 children showed that ≥ 5 neutrophils/HPF correlated with radiographic sinusitis to a greater degree. The sensitivity and specificity were 100% and 53%, respectively. Counts of other nasal cells, such as eosinophils, epithelial cells, and bacteria, did not yield significant correlations with radiographic sinusitis. The high sensitivity of the test suggests that if no neutrophils are found in cytology, the chance of the patient having occult chronic sinusitis is very low. Because of the low specificity of the Rhino-probe in diagnosing chronic sinusitis, confirmation via radiographs is still necessary if any neutrophils are present on the Rhino-probe cytology.¹⁰⁶

Increased eosinophilia has been seen in surgical sinus mucosal specimens from patients with nonallergic, chronic sinusitis. This may represent an extension of the NARES process into the adjoining sinuses. Basophilic

cells have not been shown to increase with infectious rhinosinusitis.¹⁰⁷

In viral nasal infections, ciliated epithelial cells undergo destructive changes termed *ciliocytophthoria*. The features of the cytopathic effects of the viruses include clumping of the nuclear chromatin material, margination of the pyknotic chromatin mass attached to inclusion material within the nucleus, halo formation around the nucleus, increased granulation of the cytoplasm, constriction of the ciliated cell, and finally separation of the nucleus-containing basal portion from the ciliated apical portion.¹⁰⁸

Nasal polyps. Nasal polyps have been studied histologically. In comparison with healthy nasal and sinus mucosa and mucosa of patients with chronic sinusitis, polyps show a statistically significant increase in the number of eosinophils. In patients with nasal polyps, basophilic cells are also more numerous, found in 65% of these patients, compared with 5% of normal controls, 14% of patients with chronic sinusitis, and 91% of patients with nasal allergy.¹⁰⁹

Atrophic rhinitis. Atrophic rhinitis on cytologic examination reveals an epithelial squamous metaplasia and a chronic inflammatory cell infiltrate.¹¹⁰⁻¹¹⁴ Table III summarizes the current data on nasal cytology findings in various clinical conditions.

Influence of therapeutic intervention on nasal cytology

Antihistamines. Oral antihistamines such as terfenadine and cetirizine given before an allergen challenge have no significant effect on the increase in the number and percentage of eosinophils noted after allergen challenge.¹¹¹ Some have reported that antihistamines given orally or topically for therapy produced little change in the presence of eosinophils, basophilic cells, or neutrophils in patients with allergic rhinitis.¹¹²⁻¹¹⁴ However, one recent report indicated an effect of levocetirizine on the nasal lavage eosinophil and neutrophil counts in SAR,¹¹⁵ whereas another found that the combination of cetirizine and montelukast reduced nasal lavage eosinophils in SAR.¹¹⁶

Decongestants. No data are available on the oral and intermittent topical use of decongestants. Rhinitis medicamentosa as a result of intranasal α -adrenergic agents appears to cause no significant change in nasal cytology.

Anticholinergic agents. No significant changes in the percentages of eosinophils, basophilic cells, neutrophils, or bacteria have been found in nasal scrapings after ipratropium therapy.¹¹⁷

Sodium cromoglycate. In one clinical study in which patients were treated with intranasal sodium cromoglycate 4% 4 times a day for 4 weeks, there was a statistically significant decrease in eosinophils, but not in basophilic cells or neutrophils.¹¹²

Intranasal corticosteroids. With intranasal allergen challenge, the immediate nasal response in the number of eosinophils and neutrophils in nasal secretions is not

TABLE III. Nasal cytology in various clinical conditions

	Eosinophils	Metachromatic (basophilic) cells	Neutrophils	Bacteria	Ciliated cells	Comments
Normal	0	0	0-1+	0		
Allergic rhinitis	1-4+	1-4+	1-4+	0		
Vasomotor rhinitis	0	0	0-1+	0		
NARES	1-4+	1-4+	?	0		
Pregnancy	0	0	0-1+	0		
Rhinitis medicamentosa	0	0	0-1+	0		
Irritants	0	0	1-4+	0		May see dysplasia, metaplasia
Environmental tobacco smoke	1-2+	1-2+	1-4+	0		
Bacterial rhinosinusitis	0	0	1-4+	1-4+		Bacteria may be intracellular; may not see bacteria
Viral rhinitis	0	0	1-4+	0	Decreased	Ciliocytophthoria
Polyps	1-4+	0-4+	?	?	Decreased	
Atrophic rhinitis	0	0	1-4+	0	Decreased	Metaplasia

affected by pretreatment with the topical corticosteroid budesonide.¹¹⁸ However, if treatment has been sustained for a period before the challenge, such as in a study in which the intranasal steroid flunisolide was given for 1 week before allergen challenge, such treatment significantly prevents the influx of eosinophils, neutrophils, and basophilic cells (68% identified as basophils) in the late-phase response examined in nasal washings.⁴⁸ This effect with more prolonged treatment can be accounted for by the steroid-induced reduction in surface inflammatory cells, in particular mast cells.

In clinically symptomatic patients with allergic rhinitis treated with intranasal fluticasone propionate, examination of nasal scraping specimens revealed a significant decrease in the scores for eosinophils and basophilic cells in multiple reports. In one study in which symptomatic patients received 1 of 3 doses of fluticasone propionate (25 µg, 100 µg, or 400 µg twice daily) or placebo, more than 80% had nasal eosinophilia before treatment. The graded eosinophil score decreased in 50% to 70% of patients treated with fluticasone propionate for 2 weeks and in only 20% of placebo-treated patients. Forty-two percent of the patients had basophilia before treatment. After treatment with fluticasone propionate, basophil numbers decreased in a significantly greater number of patients (~35% to 45%) compared with after placebo (~15%).⁹⁵ Fewer patients had neutrophils after treatment with fluticasone propionate, but the difference from placebo in most studies was not statistically significant. Approximately 30% of the studied patients had bacteria noted in their nasal mucosal specimens at baseline. There was no significant change in this percentage after any treatment regimen. All patients had similar numbers of goblet cells before and after treatment.¹¹⁹ Treatment with intranasal budesonide has also been shown to result in a reduced eosinophil and basophilic cell luminal infiltrate. The reduction in these inflammatory cells occurred at doses of 32 µg/d, 64 µg/d, 128 µg/d, and 256 µg/d.¹²⁰

Oral corticosteroids. Oral prednisone has been studied for its effect on the late nasal response to allergen challenge. Pretreatment for 2 days with prednisone will block influx of eosinophils but not neutrophils or mononuclear cells.³³

Immunotherapy. Immunotherapy can decrease eosinophil influx.¹²¹ After 3 and 6 months of immunotherapy, the number of basophilic cells was also significantly reduced.¹²²

NASAL BIOPSY

Nasal biopsies have been used extensively over the period of the past 15 years both in immunohistochemical and in situ hybridization studies. They have provided information on the normal structure and cell populations of the nasal mucosa.¹²³⁻¹²⁵ In addition, they have been used to look at the effect of disease states such as perennial and seasonal rhinitis on the normal architecture.¹²³⁻¹²⁸ Nasal biopsies have also provided useful data on the effect of treatment on the nasal mucosa.¹²⁹⁻¹³²

Schneider first described the histology of the nasal mucosa in 1660.¹³³ It consists of epithelium normally coated with mucus and separated from the underlying lamina propria by the basement membrane. Stratified squamous epithelium lines the vestibulum and first few millimeters of the nose, whereas the rest of the nasal cavity is lined with ciliated columnar epithelium (with or without goblet cells) and/or partially stratified cuboidal epithelium. Several migratory cells have been demonstrated in the epithelium: T lymphocytes, eosinophils, neutrophils, mast cells,¹²³⁻¹²⁵ macrophages,¹³⁴ B cells,¹³⁵ basophils,¹³⁶ and dendritic cells.¹³⁷ The lamina propria is usually composed of a relatively cell-rich, collagen-poor, subepithelial layer with most of the mucous glands and a deeper collagen-rich, cell-poor layer lying on the supporting skeleton. All of the inflammatory cells found in the epithelium have also been demonstrated in the lamina propria.

Examination of the nasal cytokine profile in nasal biopsies by in situ hybridization (ISH) identifies an imbalance in T-cell cytokine production in favor of a T_H2-type response with increased IL-5, GM-CSF, IL-4,¹³⁸ and IL-13 mRNA¹³⁹ in patients with both SAR and PAR. Such cytokines are also evident stored preformed in mast cell granules within nasal biopsy tissue using immunohistochemical staining.¹²⁵⁻¹³⁰

Although nasal lavage, mucosal scraping, mucosal imprints, blown secretions, nasal smears, and nasal brushings all allow evaluation of nasal inflammatory processes, it is only nasal biopsy that permits the study of cells in all layers of the nasal mucosa¹²³ and of the activation status of structural airway cells such as the vascular endothelium.¹²⁴ It is an invasive but well tolerated technique.¹⁴⁰ Before selecting any specific sampling technique, it is important that the purpose of the study has been clarified and the questions to be addressed formulated to determine whether nasal biopsy or an alternative approach is the optimum method to obtain the information required.

Nasal biopsy procedure

Methodology for sampling must be standardized. A constant location of biopsy site is important because of possible regional differences in the histology of the nasal mucosa.¹⁴¹ The most common site of biopsy is the undersurface of the inferior turbinate, 1 cm to 2 cm posterior to its anterior insertion to the lateral wall of the nose. The reason for this is mainly practical, because it is the most accessible part of the nasal cavity, with a comparably lower risk of perioperative and postoperative bleeding. In addition, it is the part of the nose that is most exposed to the influence of inspired air.¹⁴² Local anesthesia is achieved by applying a small cotton wool plug soaked in 10% cocaine or 4% lignocaine immediately below the inferior turbinate for 10 minutes. The addition of 0.025% adrenaline to the anesthetic induces localized vasoconstriction. This is helpful because the paler nasal mucosa, where the anesthetic is also localized, is clearly discernible, and the vasoconstriction limits bleeding during and after the procedure. High quality 2.5-mm biopsy specimens can be taken under direct vision with nasal biopsy forceps, such as Gerritsma forceps,¹⁴⁰ which do not visibly damage the epithelium of the sample and provide sufficient depth of lamina propria.

Nasal biopsy processing

The handling of small biopsies presents some specific issues. This has been addressed in a recent review considering aspects of tissue fixation, embedding, and analysis.¹⁴³ Intact biopsies can be processed for electron microscopy to evaluate ultrastructure, for immunohistochemistry to detect protein expression, and for ISH to detect gene expression. A range of methods has been used for tissue fixation and processing, depending on the assessment to be undertaken. Whichever approach is taken, there needs to be a standard operating protocol

that is adhered to throughout the study to ensure consistency of results. This is particularly important when comparing samples taken before and after challenge or therapeutic intervention. It is important for immunohistochemistry and ISH that the protocol followed has a balance concerning the fixation time. This needs to be long enough to preserve tissue morphology but not so long as to mask antigens, reduce accessibility, or destroy mRNA/DNA. Typically, fixation times vary between 4 and 24 hours, and for ISH, fixatives need to be free of enzymes that degrade DNA and RNA. The following are methods used in tissue processing relevant to immunohistochemistry and ISH.

Snap-freezing tissue. The immediate snap-freezing of freshly obtained samples in melting isopentane (ie, previously cooled in liquid nitrogen) and cryostat sectioning has the advantage of a rapid processing method that preserves antigenicity well. The disadvantages of snap-frozen samples are that they may have poorly preserved morphology and, potentially, ice crystal artifact. In the absence of previous fixation, there may be unwanted diffusion of antigens and proteins, and there is a need for low-temperature storage (−80°C in a freezer or storage in liquid nitrogen). In cut and stored sections, some antigens are lost quite rapidly. Tissue sections are usually cut at a thickness of 5 μm to 7 μm. Because of the thickness of these sections, less information can be obtained from each biopsy sample compared with paraffin and glycol methacrylate (GMA), both of which use thinner sections.

Paraffin wax embedding. Paraffin wax embedding is the processing of samples into paraffin wax, usually after fixation in 10% neutral-buffered formalin for immunohistochemistry or 4% paraformaldehyde or 10% neutral-buffered formalin for ISH. This method offers some advantages over snap-freezing. Tissue architecture is better preserved, there is little or no diffusion of antigens, specialized storage facilities are not required, and facilities for processing are universally available. However, antigen preservation may not be so good, and antigen retrieval techniques may be required because of the use of cross-linking fixatives that may mask selected antigens from detection by immunohistochemistry.

Plastic resin. A hydrophilic resin, GMA has recently been used for immunohistochemistry¹⁴⁴ and has been well validated in studies. When coupled with acetone fixation (with protease inhibitors), GMA offers some advantages over both frozen and paraffin preparations. Acetone is a noncross-linking fixative that, together with processing at a low temperature, ensures both good antigen preservation and good morphologic preservation. The resin allows sections of only 1 μm to 2 μm thick to be cut and immunostained. More information can thus be obtained from 1 biopsy, and because of their thinness, adjacent sections can show more than 1 cut through a single cell, enabling the detection and colocalization of more than 1 epitope to a cell. As with paraffin embedding, GMA-embedded samples can be stored safely for long periods. However, GMA embedding is more time-consuming and

less widely used than either frozen or paraffin preparations and is not yet suitable for studies of RNA/DNA and their visualization by ISH. This method is thus specific for immunohistochemistry.

Biopsy evaluation

Immunohistology. Immunohistology, ie, the use of antibodies to selectively label cells or unique antigens on the surface of individual cells, can be performed by using a variety of techniques. Two commonly used methods that amplify the signal, enabling easy recognition of the antibody binding to the tissue antigen, are the alkaline phosphatase antialkaline phosphatase method and streptavidin-biotin techniques. Positively stained cells can then be enumerated. Discrete, individually stained cells such as lymphocytes, mast cells, and eosinophils can be counted by using a hand-counter or computer-assisted graphics tablet. The standard practice is to count the number of stained cells in a tissue section by using light microscopy and to normalize these counts to submucosal area, excluding glands and large blood vessels, or to the length of the epithelial reticular basement membrane, depending on whether a cell count in the lamina propria or epithelium is required. Results are thus presented as cells per square millimeter or cells per millimeter of epithelium, respectively. Counting epithelial cells can be undertaken reliably only in areas of intact epithelium. A criticism of this approach is that the probability of a stained cell being counted in a 2-dimensional section is proportional to its size, density, and orientation. This method thus introduces a bias in favor of higher cell counts for larger cells. However, assuming that nuclear size varies less than overall cell size, opting to count only those cells with a visible nucleus in the plane of the tissue section theoretically reduces this bias and is thus usually recommended. Some workers count only defined areas within a biopsy rather than the whole biopsy, such as 1 grid depth (0.45 mm) beneath the basement membrane along the whole length of the membrane or cells within a set number of random HPF. The latter approach is open to bias in the selection of the areas to be counted. Cell counts are still expressed per square millimeter of submucosa. The parameters for counting need to be appropriate to the type of staining so that intraobserver coefficients of variation for repeat counts are within acceptable limits, 5% to 8%.¹⁴⁰

Counting can also be automated by use of digital image analysis systems provided that positively stained cells are readily distinguishable from any background staining.¹⁴⁵ For staining that is less easy to define in snap-frozen tissue, a rating scale has been used, such as for the endothelial staining for ICAM-1,¹⁴⁶ although with resin embedding and thin sections cutting, adjacent section staining with an endothelial marker and the well preserved morphology allows enumeration of the percentage of vessels that express each different leukocyte endothelial adhesion molecule.¹²⁴

ISH. ISH with antisense, isotopic, or nonisotopic labeled riboprobes can be used to detect specific mRNA within tissue.¹⁴⁷ With the use of riboprobes, there is an absolute requirement to minimize RNase contamination. To preserve mRNA, tissue must be fixed immediately in 4% paraformaldehyde for 2 hours (depending on tissue size), then washed in 2 changes of 15% sucrose/PBS, left overnight, blocked in mounting medium, and stored at -80°C . Different types of probes are available to visualize mRNA, including DNA oligonucleotide probes and complementary RNA probes. RNA probes are more commonly used because they offer the advantage of being more sensitive and specific for the detection of a particular mRNA. The probes can be labeled with radioisotope or with biotin, digoxigenin, or fluorescein isothiocyanate for detection. Radiolabeled probes provide great sensitivity and should be used to detect mRNA encoding of actively synthesized proteins that are rapidly turned over and therefore have a relatively small amount of intracellular mRNA. However, radioactively labeled probes also have disadvantages in that they are difficult and can on occasion be dangerous to handle. Furthermore, they have a short half-life and require skill in tissue section autoradiography to visualize the tissue localization of the radioactively labeled probes. Nonradiolabeled probes are ideal for genes that are expressed at a high level (ie, present in high copy number). They are safe to handle and can be stored for relatively long periods (ie, 6 months). However, they are considered to be less sensitive than radiolabeled probes, and the required immunodetection procedure can result in nonspecific labeling, particularly of epithelia. The major advantage of immunolabeled mRNA hybrids is that they can be visualized by bright field microscopy without the need for autoradiography. Also, unlike the autoradiography of radiolabeled probes, which takes a week or more to develop, nonradiolabeled probes can be viewed immediately after the immunohistochemistry detection. However, there are many pitfalls in the performance of ISH, and this work needs to be undertaken in an experienced laboratory.

Repeatability of measurements

A potential disadvantage of nasal biopsy is that the samples obtained are small (2.5 mm), and the data generated often come from a limited number of sections of the biopsy. A recent study on bronchial biopsies demonstrated that inflammatory cells are not scattered randomly through the subepithelial tissue but may be clustered within airway tissue.¹⁴⁸ As a consequence, it is difficult to predict the area of tissue that should be inspected to obtain a mean with an acceptable confidence limit. Descriptive analysis led to the suggestion that at least 6 sections or a zone beneath 5 mm to 10 mm of reticular basement membrane should be included per cell type. In the nose, the minimum number of sections required to give a sufficient number of fields to assure acceptable accuracy (5%) was determined to be 2¹⁴⁹ on the basis of a summation average graph.¹⁵⁰ The nose differs from the lower airways in having a much smaller surface area and in not branching and thereby not offering inhaled

particles, such as allergens, a variety of routes. This may explain the lesser variability evident within nasal biopsies.

To gain further information concerning this issue to provide data for the rhinitis workshop, Jacobson and Durham examined 5 biopsies from subjects with grass pollen allergy during the hay fever season, ie, when symptomatic (data first published in this article). The variability in eosinophil cell counts was evaluated in 12 different 6- μ m tissue section slices cut at 72- μ m intervals by using an antibody against eosinophil cationic protein (EG2). The biopsies had been fixed immediately in 4% paraformaldehyde for 2 hours and transferred to 15% sucrose in PBS for 1 hour, then overnight in fresh sucrose at 4°C, followed by embedding in optimal cutting temperature medium and snap-freezing in isopentane precooled in liquid nitrogen. There was variation in cell counts both between patients and within patients (Fig 6). The number of sections that should be analyzed from each individual biopsy to yield reliable data was determined by studying the reliability of the subject-means.¹⁵¹ Although the data set consisted of 5 patients with at least 12 observations per patient, the samples were used to assess what the reliability of a subject-mean would be when the number of observations per subject was equal to 12, 10, 8, 6, 4, and 2. This involved taking 5 random samples of these sizes for each individual. The reliability coefficients for each of the random samples generated for each sample size are displayed graphically (Fig 7) and give a picture of the variability of the reliability coefficient.

These results show that, for this example, the reliability of EG2 counts begins to drop and becomes more variable when the number of observations per person is <8. It should be borne in mind, however, that this study was performed by using biopsies from only 5 subjects. If it were performed with more subjects, the number of observations required to give a satisfactory reliability coefficient would likely decrease.

Indeed, despite variation in cell numbers, blinded studies involving 2 to 4 sections per biopsy have consistently detected significant differences in cell numbers whether the comparison was between preseason and peak season,^{152,153} normal subjects versus patients with allergy,¹⁵⁴ or placebo treatment versus an active treatment.¹⁵⁵

Sources of variation

When seeking to compare data from biopsy studies from different centers or before and after treatment, it is important to bear in mind the possible sources of variation. These include biologic factors such as differences in seasonal pollen counts and exposure to other antigens. Patient-related factors are also important. Patients differ in their degree of sensitization and in their level of exposure within the same region, because some may work outdoors and some in air-conditioned offices. Additional factors, such as intercurrent viral infections, concomitant drug therapy, and smoking habit may all be other variables of relevance. Other factors such as age, sex, diurnal variation, and within-biopsy variation need to be considered. Data

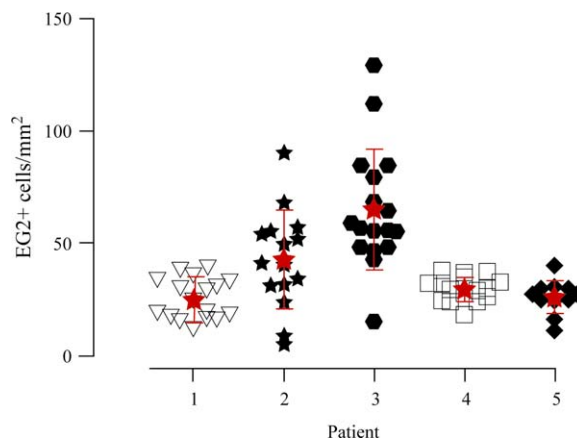


FIG 6. EG2⁺ cells/mm² nasal submucosa from multiple sections taken from 5 subjects with seasonal allergic rhinitis. Each point represents the cell count from a single section. The red stars and bars represent the mean $\pm$ SD (Jacobson and Durham, unpublished data, January 2005).

can also differ between studies as a result of methodologic differences, whether in biopsy technique, method of fixation, thickness of sections, immunostaining, or ISH technique. In addition, the reagents used, the counting parameters, and the intraobserver and between-observer counting variability all need to be borne in mind before comparisons can be made between studies.

Comparison between nasal biopsy and nasal brushing

Nasal biopsy and nasal brush techniques have been compared in a group of 46 patients with grass pollen SAR undergoing a double-blind, placebo-controlled trial of fluticasone propionate.¹⁵⁵ Patients receiving fluticasone propionate showed a significant increase in symptom-free days for sneezing, nasal discharge, blockage, and itching ($P < .001$). Tryptase-positive mast cell and BMK13-positive eosinophil counts within cytopsins prepared from the nasal brushings showed a highly significant inhibition of seasonal increases in the fluticasone propionate group ($P < .00001$; Figs 8 and 9). This inhibition was mirrored by mast cell and eosinophil counts made in the nasal epithelium of biopsies (Figs 8 and 9). Both the eosinophil and mast cell counts in the microscope slides correlated with those in the epithelium of biopsies.

This study demonstrated clearly the comparability of these 2 techniques for looking at the nasal epithelium. The sampling method selected for a given study would thus depend on the number of samples required per patient and whether epithelial data were sufficient. Nasal brushing is a simple technique that does not require anesthetic and has the advantage of being more economical than nasal biopsy both in terms of time and money. Nasal brushing would be particularly well suited to studies looking at the effect of treatment on inflammatory cells within the epithelium or on epithelial cells themselves, especially in studies involving children, large study groups, or multiple measure-

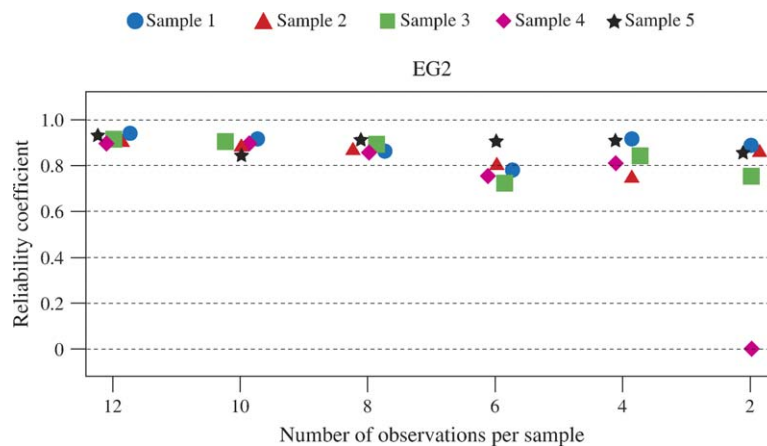


FIG 7. Reliability of the subject means for EG2⁺ cells based on the number of nasal biopsies observed per subject when selected randomly from the complete set of biopsies (Jacobson and Durham, unpublished data, January 2005).

ments. Nasal biopsy enables studies of both the nasal epithelium and submucosa and thus provides a broader picture of the nasal architecture.

Relevance of allergen challenge studies versus natural exposure

Nasal biopsies may be taken either at baseline before relevant exposure or after allergen exposure. Allergen exposure may occur naturally—for example, during the grass pollen season—or may be provoked by using allergen challenge. Within minutes of nasal allergen challenge, immediate symptoms of sneezing and itching occur, followed within 5 to 15 minutes by increased rhinorrhea and nasal blockage.¹⁵⁶ Depending on the allergen dose and the susceptibility of the individual, a proportion of subjects go on to develop a late-phase response that is maximal at 6 to 24 hours and resolves within 1 to 3 days. Local allergen provocation may be performed by application of allergen solution on the surface of small filter discs to the nasal mucosa.¹⁵⁶⁻¹⁵⁸ Alternatively, nasal allergen challenge may be performed by using an atomizing nasal spray of defined output.

In contrast with nasal allergen provocation, which involves high-dose exposure over minutes, natural exposure occurs to particulate allergen over a prolonged period. It is therefore not surprising that the nasal inflammatory cell profile differs depending on the method of exposure. Tissue eosinophilia is a reproducible feature of allergic rhinitis, whereas a local increase in neutrophils is observed only after allergen provocation.^{123,156} Conversely, during the pollen season, mast cells migrate into the epithelium^{123,125,130,155} but are completely absent after local allergen challenge.^{132,152} CD4⁺ T lymphocytes and CD25⁺ (anti-IL-2 receptor) cells increase during allergen challenge but not during natural seasonal exposure. At the mRNA level, there is increased expression of IL-3, IL-4, IL-5, and GM-CSF after local allergen provocation.¹³⁸

This is consistent with activation and proliferation of T_H2 lymphocytes. By contrast, the T_H1-type cytokines IL-2 and IFN- γ are not increased. An increase in the local nasal expression of IL-4, C ϵ , and I ϵ RNA^{156,157} has been reported after both nasal allergen provocation and seasonal exposure, which might suggest that IgE synthesis occurs within the nasal mucosa. Biopsies taken during the late phase after local allergen provocation thus provide some findings qualitatively comparable with those during natural exposure. Ex-ceptions include increases in epithelial counts for mast cells, eosinophils, and CD1a dendritic cells during the pollen season, features not observed after a simple allergen challenge.

Outcome measures and power

In most clinical trials, the outcome measure of major interest will be a clinical variable (such as a reduction in medication requirements or an increase in symptom-free days) selected in advance of statistical analysis. The number of patients recruited into the trial should be based on a sample size calculation to have a high chance of detecting, statistically significantly, a worthwhile effect if it exists, and thus to be reasonably sure that no such benefit exists if it is not found in the trial.¹⁵⁸ In such studies, changes in inflammatory cells within nasal biopsies will be secondary outcome measures and as such should be interpreted cautiously, perhaps as ideas for further research. If biopsy data are to provide the major outcome variable, then power calculations should be performed on that basis. Data from previous trials can be used in these calculations. For example, by using previous data from EG2 cell counts in nasal biopsies before pollen season and peak pollen season,¹⁵⁹ it has been possible to calculate the number of patients required for a future study to ensure detection of a 50% reduction in cell numbers in the treatment group compared with the placebo. This was done by calculating the change that each individual experienced during the pollen season and then using

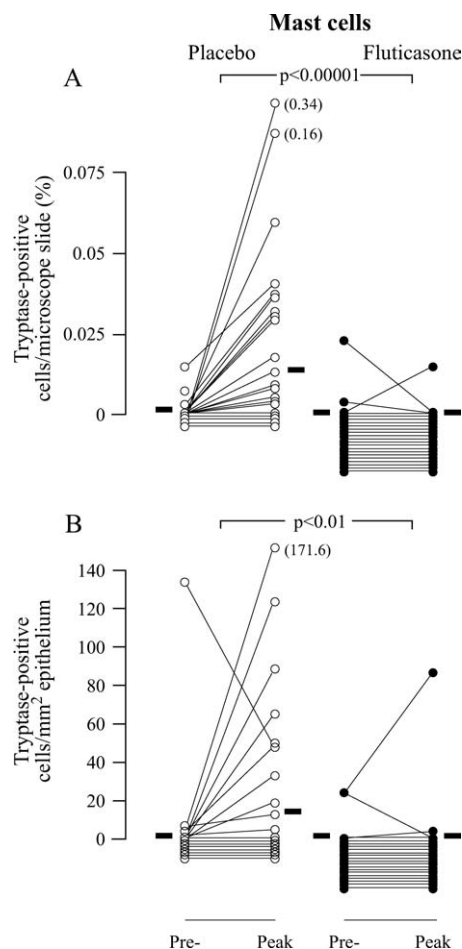


FIG 8. Tryptase⁺ mast cells in nasal cytospins (A) and nasal biopsies (B) from grass pollen-sensitive individuals preseason and during season showing the superficial accumulation of mast cells in natural disease and the inhibitory effect of 6 weeks of therapy with nasal fluticasone propionate. Reproduced with permission from Blackwell Publishing.¹⁵⁵

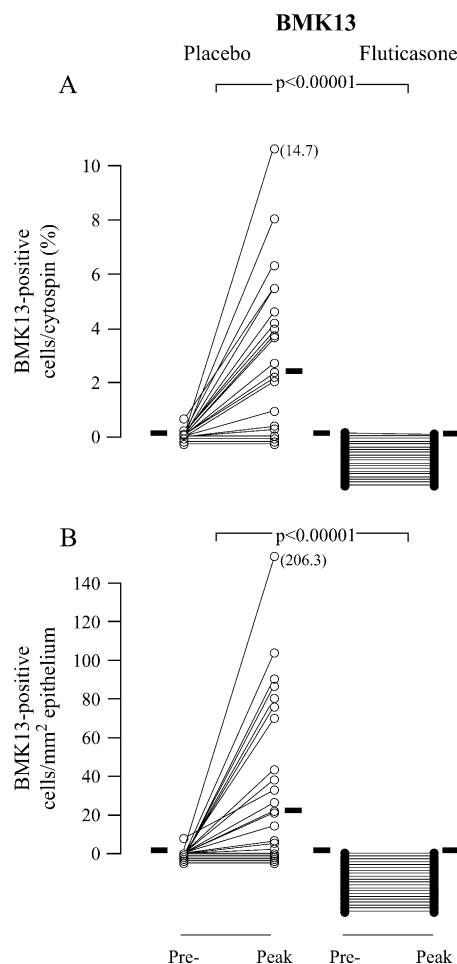


FIG 9. BMK13⁺ eosinophils in nasal cytospins (A) and nasal biopsies (B) from grass pollen-sensitive individuals preseason and during season showing the superficial accumulation of mast cells in natural disease and the inhibitory effect of 6 weeks of therapy with nasal fluticasone propionate. Reproduced with permission from Blackwell Publishing.¹⁵⁵

standard sample-size calculation techniques in STATA (version 6) to estimate the power to reject the null hypothesis if the difference between the 2 groups was as small as 2.3 (2-sample 1-sided test; $\alpha = 0.05$). A difference of 2.3 was chosen because it represented a 50% drop from the mean change in the placebo group. Such considerations found that the number of individuals in each group required to get 80% power was 52, although one might add 10% to the sample size to account for the fact that the data were skewed and nonparametric tests would need to be used in future analyses. This theoretical figure contrasts with real life, in which it has been consistently possible to detect significant changes in eosinophil numbers, for example, with intervention in studies with 15 to 25 patients per group.

In conclusion, nasal biopsies provide a valuable source of tissue for studies of upper airway disease. Good quality biopsies can be taken with little discomfort to the patient.

NASAL NO

Nitric oxide is an endogenous ubiquitous mediator involved in both physiologic and pathologic responses.^{160,161} The supravascular upper airway generates NO concentrations in the parts per million range, several-fold greater than the lower respiratory tract.^{162,163} The NO concentration is particularly high in the paranasal sinuses.^{164,165} The upper airway is a complex system of communicating cavities, including the 2 nasal cavities, paranasal sinuses, middle ear, and nasopharynx. Each of these areas probably contributes to nasal NO output.

Both low-output constitutive NO synthases (cNOSs), which are calcium-calmodulin dependent and steroid-insensitive, and high-output inducible NO synthase (iNOS), classically calcium-calmodulin independent and steroid-sensitive, are found in normal upper airway epithelium.^{166,167} There is evidence, however, that normal

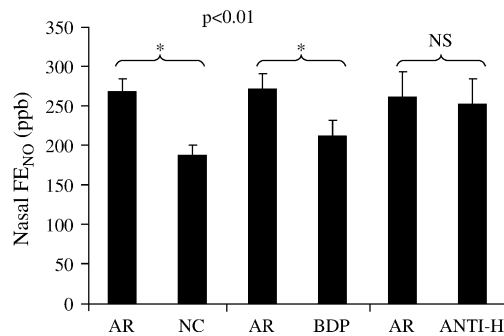


FIG 10. Nasal NO in allergic rhinitis (AR) compared with normal controls (NC) and the effect of nasal beclomethasone dipropionate (BDP) and nasal antihistamine (ANTI-H) on nasal FE_{NO} in AR. Adapted from Baraldi et al.¹⁸²

sinus epithelium constitutively expresses a unique type of iNOS that is calcium-dependent and steroid-insensitive.¹⁶⁸ High NO output from this iNOS expression might explain the high levels of nasal NO found even in normal subjects. The epithelial expression of iNOS has been confirmed with RT-PCR analysis of gene expression in resected tissue, with expression found to be increased in allergic rhinitis, with a RAST score of 5 or 6.¹⁶⁹ In these subjects, there was a reciprocal reduction in constitutive NO synthase mRNA.¹⁶⁹ The use of 4,5-diamino fluorescein diacetate (DAF-2DA) imaging in this study also reported increased NO production by nasal epithelial cells in allergic rhinitis compared with healthy controls.

The physiologic functions of nasal NO are unknown. However, the high concentration found in the paranasal sinuses is sufficient for an important antibacterial and antiviral effect,¹⁷⁰ perhaps ensuring sinus sterility in health. NO also has been reported to modulate ciliary function.¹⁷¹ Inhalation via the nose, which conditions the inspire with endogenous NO, has been reported to have discernible effects on ventilation-perfusion matching in the lung.¹⁷² This may represent an important physiologic mechanism controlling matching of ventilation to pulmonary blood flow. This supports grandma's advice to breathe through your nose! Studies using the NO synthase inhibitor NG-nitro-L-arginine-methyl ester-HCL have shown a reduction in nasal NO in allergic rhinitis, although this reduction has no effect on either resting nasal airway resistance measures or on the nasal obstructive response to nasal allergen challenge, suggesting that NO is not a primary determinant of nasal patency.^{173,174}

Physiologic roles aside, the measurement of nasal inflammation in allergic rhinosinusitis would rely on detecting increased levels of nasal NO resulting from increased expression of iNOS, similar to the much-reported increase of lung exhaled NO in asthma.¹⁷⁵

Nasal NO in allergic rhinitis

Low output constitutive NOS isoforms are found in nasal epithelium in healthy and allergic subjects. However, high-output iNOS is increasingly expressed in the respiratory epithelium in allergic rhinitis in a manner

similar to that seen in asthma.¹⁷⁶ The relatively large amounts of NO and pro-oxidant derivatives of NO, such as peroxynitrite, produced by iNOS in the nasal cavity can promote nasal inflammation through tissue damage but also through the recruitment of eosinophils,²⁷ promotion of vascular congestion,¹⁷⁷ and increase in nasal secretion.¹⁷⁸ Thus, nasal NO is a mediator of the inflammatory response.

More recently, nasal NO measurement (nasal NO) has been proposed as a noninvasive marker of nasal inflammation, similarly to exhaled NO in asthma.¹⁷⁵ Clear changes are evident in NO concentrations in nasal exhaled air in active allergic rhinitis. A report by Martin et al¹⁷⁹ identified higher levels in nasal exhaled air than orally exhaled air in both healthy individuals and in those with SAR, consistent with constitutively greater generation within the upper airways than lower airways. Higher levels were seen in subjects with rhinitis than in subjects without rhinitis, consistent with enhanced generation in untreated active allergic inflammation. These measurements were shown to be flow-dependent, indicative of the ongoing generation and release of this gas, and the importance of nasal airflow as a measurement variable.

In one study, Kharitonov et al¹⁸⁰ measured nasal NO in subjects with symptomatic allergic rhinitis during the pollen season and compared this with normal subjects and patients on nasal corticosteroids. Nasal NO was significantly ($P < .001$) elevated in patients with untreated rhinitis ($1527 \text{ ppb} \pm 87 \text{ ppb}$; $n = 12$) compared with normal individuals ($996 \text{ ppb} \pm 39 \text{ ppb}$; $n = 46$) or subjects treated with nasal steroids ($681 \text{ ppb} \pm 34 \text{ ppb}$; $n = 10$). Arnal et al¹⁸¹ also reported that nasal NO was substantially higher in patients with allergic rhinitis ($382 \text{ ppb} \pm 20 \text{ ppb}$) versus controls ($235 \text{ ppb} \pm 11 \text{ ppb}$; $P < .0001$). They remarked that even patients without symptoms on the day of the test had a clear-cut increase in nasal NO.

The effect of an intranasal steroid (beclomethasone dipropionate $400 \mu\text{g/d}$) and a nasal antihistamine (levocabastine $200 \mu\text{g/d}$) on nasal NO was elegantly studied by Baraldi et al¹⁸² (Fig 10) in 21 children (age 5-17 years) with perennial allergic rhinitis; nasal NO was also measured in 21 healthy children. At baseline nasal NO in allergic rhinitis, mean $\pm$ SEM, was $267 \text{ ppb} \pm 18 \text{ ppb}$, compared with $186 \text{ ppb} \pm 15 \text{ ppb}$ in the control group ($P < .01$). Nasal NO in children with allergic rhinitis ($n = 13$) treated with beclomethasone for 10 days demonstrated a significant ($P < .05$) decrease ($271 \text{ ppb} \pm 21 \text{ ppb}$ to $212 \text{ ppb} \pm 20 \text{ ppb}$), whereas in the group treated with levocabastine, nasal NO was not significantly changed ($261 \text{ ppb} \pm 33 \text{ ppb}$ to $252 \text{ ppb} \pm 31 \text{ ppb}$). Other studies have also confirmed a clear increase in nasal NO in allergic rhinitis with a fall after treatment with intranasal steroid.¹⁸³ Increased expression of iNOS in nasal and sinus epithelium in allergic rhinitis compared with healthy controls, as shown by Kawamoto et al,¹⁶⁷ suggests that this enzyme is the source of the increases in nasal NO in allergic rhinitis. The fall in nasal NO after steroid is entirely consistent with the known steroid sensitivity of iNOS.

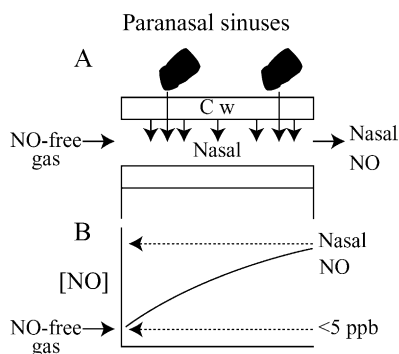


FIG 11. Conditioning of transnasal airflow with NO diffusing caused by a gradient between the nasal wall NO concentration (Cw) and lumen, with passage of NO from the paranasal sinuses. NO concentration rises progressively as gas traverses the nasal cavity.

In general, the relative increase of nasal FE_{NO} in allergic rhinitis is less than that seen in lung FE_{NO} in asthma, possibly related to dwarfing of the allergic rhinitis-related increase in nasal NO by high background levels. Thus, even in normal subjects, nasal NO is in the low hundreds of parts per billion, whereas exhaled NO is in the 10 ppb to 20 ppb range when measured at the same flow rate (50 mL/s).

Nasal NO measurement

Compared with other nasal outcomes, upper airway NO level is perhaps the newest tenant on the block. The development of standardized and reproducible methods for measurement of nasal NO has come on the heels of methods developed for measurements of exhaled NO. Nasal NO measurement techniques have been addressed by a European taskforce,¹⁸⁴ and more recently by an official statement of the American Thoracic Society (ATS),¹⁸⁵ reflecting the growing interest in these measurements.

Principles underlying nasal NO measurement

Measurement of nasal NO output requires generation of airflow through the nasal cavity (transnasal airflow). As the gas traverses the nasal cavities, it is conditioned with NO diffusing from the sinuses and the nasal epithelium (Fig 11). Flow through the nasal cavities in series can be achieved by aspirating or insufflating air via one naris while the vellum is closed, so that air circulates from one naris to the other around the nasopharynx. Transnasal flow with the nasal cavities in parallel, mimicking natural breathing, is achieved in several ways¹⁸⁶: (1) exhaling via one or both nasal cavities, (2) aspirating via the mouth with air entrained into both nares, or (3) aspirating from one or both nares with air entrained via the mouth. With parallel exhalation via nasal cavities, however, NO originating from the lower respiratory tract contaminates the sample. In subjects with severe unilateral or bilateral nasal obstruction, generation of transnasal airflow may be problematic, making measurement of nasal FE_{NO} impossible.

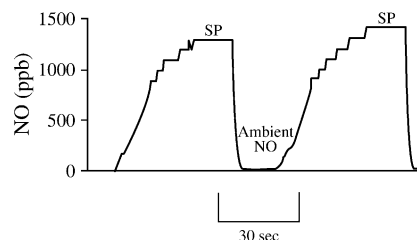


FIG 12. Nasal NO concentration versus time during 2 separate determinations showing a rise to a steady nasal NO plateau (SP), which is highly reproducible. Ambient NO is recorded in the portion of the tracing between the measurements. Adapted from Kharitonov.¹⁸⁵

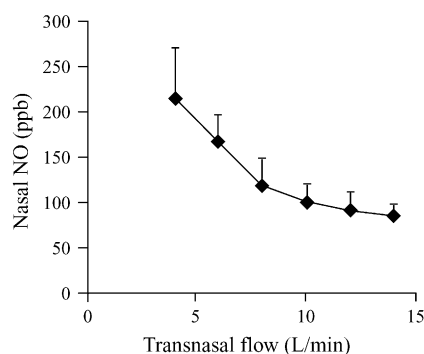


FIG 13. Flow dependence of nasal NO. Adapted from Am J Respir Crit Care Med.¹⁸⁵

With all methods, a constant transnasal flow rate produces a washout phase followed by the establishment of a steady NO plateau seen in the profile of NO versus time, analogous to that seen in the lower respiratory tract (Fig 12). Importantly, nasal NO concentration is inversely related to the transnasal airflow rate¹⁸⁶⁻¹⁸⁸ (Fig 13). In addition, different flow rates could result in changes in the physics of airflow (eg, laminar versus turbulent flow) and different pathways of flow through the nasal cavities,¹⁸⁹ thereby affecting nasal NO.^{190,191} For all of these reasons, standardized methods should precisely control transnasal airflow rate.

Velum closure in nasal NO measurement

With transnasal airflow in series, velum closure is essential to prevent leakage of nasal NO into the oropharynx, or conversely, dilution of the sample with gas from the lower respiratory tract. Velum closure can be achieved by orally exhaling against a resistance,¹⁹² pursed lips breathing via the mouth,¹⁹³ breath holding with velum,¹⁹⁴ or voluntary elevation of the soft palate.¹⁹⁵ During the test, measurement of nasal CO_2 , which should remain low, can verify velum closure.

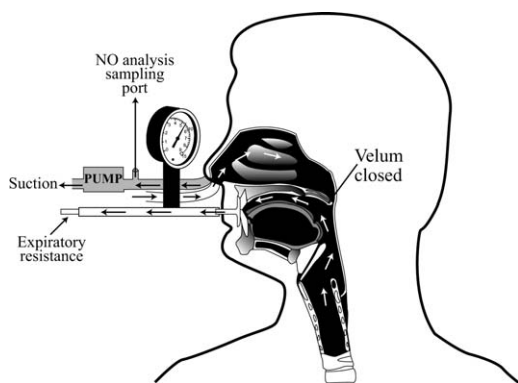


FIG 14. One configuration of the apparatus required to measure nasal NO according to the method recommended by ATS.¹⁸⁵

ATS recommendation for nasal NO measurement

Of the several methods that have been described for nasal NO measurement, an official statement by the ATS has recommended using the method of constant transnasal flow in series.¹⁸⁵ This method is currently the most prevalent and best-validated one.^{180-182,184,186,190,195-198} Importantly, nasal NO is sampled in isolation from the lower respiratory tract. Velum closure, which prevents leak of nasal NO via the posterior velopharyngeal aperture, is reliably achieved by slow oral exhalation against a resistance of at least 10 cm H₂O.^{188,192} Biofeedback display of airway pressure to the subject facilitates maintenance of a steady exhalation pressure within the desired range. Notwithstanding, any method that reliably closes the velum is acceptable to the ATS.¹⁸⁵

Description of method

One configuration of the NO measuring apparatus is shown in Fig 14. Two soft nontraumatizing nosepieces with a central lumen are positioned to occlude the nares; a suction pump aspirates gas via one naris, and air is entrained via the other. A side port just distal to the aspirating nosepiece samples gas for NO analysis. The seated subject inserts a mouthpiece, inhales to total lung capacity, and exhales through an expiratory resistance while targeting a mouth pressure of 10 cm H₂O to close the velum. An acceptable alternative to aspiration of air via a suction pump is insufflation of air into one nostril and sampling of nasal NO as air exits the other nostril.¹⁸⁶ This insufflation method may be desirable when nasal cavity obstruction leads to dynamic alar collapse during the aspiration technique.

Transnasal airflow rate

The flow dependence of nasal NO requires a constant transnasal flow. The ATS has recommended sampling at a flow of 3 L/min (50 mL/s), identical to that recom-

mended for exhaled NO measurement.¹⁸⁵ This flow provides a steady plateau in the nasal NO versus time profile within 20 seconds in most subjects. Higher flow rates may create excessively negative intranasal pressures with resultant alar collapse, whereas very low flows leads to an excessively long time until nasal NO concentration plateaus.

Factors influencing nasal NO values

Several factors may affect values of nasal NO. The most important ones are briefly discussed.

Ambient NO. Ambient NO may reach several hundred parts per billion in urban areas. If ambient air is passively entrained into the nosepiece during the test, this will affect the gradient for NO diffusion in the upper airway and hence make it impossible to ascertain the endogenous portion of the effluent NO measured. This extraneous NO could also influence nasal physiology. In an extreme situation, if ambient NO concentrations were greater than nasal mucosal wall concentrations, no net excretion of nasal NO would occur. For these reasons, an air source containing low concentrations of NO (<5 ppb) is recommended.¹⁸⁵ Ambient NO should be recorded at the time of each test.

Exercise. Nasal NO concentration falls during heavy physical exercise.^{187,199,200} It is therefore prudent to refrain from exercise for 1 hour before the measurements.

Nasal volume/sinus access. Changes in nasal cavity volume can affect nasal NO by altering NO uptake into nasal blood and modulating the nasal epithelial surface area. Also, the communication of the nasal cavity with the communicating sinuses, which produce NO, could be altered. Evidence concerning the influence of nasal volume on nasal NO is contradictory currently,²⁰¹ although free flow between the sinuses and the nasal cavity is an important determinant of nasal NO because, paradoxically, there are low levels of nasal NO in patients with extensive nasal polyposis, and these levels rise after successful surgery.²⁰²

Humming. Consistent with the importance of a patent orifice between the sinuses and the nasal cavity, it has been shown that humming, which increases sinus ventilation, increases nasal NO 15-fold,²⁰³ and that repeated humming, in comparison with a period of silence, decreases nasal NO.²⁰⁴ This suggests that the oscillating sound waves generated by repeated humming wash out the sinuses and deplete the reservoir of high NO that contributes to measurements of nasal NO. These effects of humming are not evident in nasal polyposis because of the ostiomeatal obstruction, and it has been proposed that measures of nasal NO prehumming and posthumming may represent a clinical test of ostiomeatal patency.

Nasal aerodynamics. The physics of airflow through the nasal cavity could alter the area of nasal mucosa contributing to the uptake of NO in the transnasal airflow. At low flows, laminar flow may predominate, and the pressure fluxes in the nasal cavity will be less than at high flows, reducing the efflux of gas from the paranasal

sinuses. Variations in nasal aerodynamics may explain some of the flow dependency of nasal NO output.¹⁹⁰

Medications and nasal NO. Medications have been shown to affect NO and should be recorded. Those reported to have an effect on nasal NO include nasal decongestants, which decrease nasal NO output by about 15%.²⁰¹ Nasal steroids were reported to have no effect in normal subjects in one study,²⁰⁵ but to reduce nasal NO output after 2 weeks in normal subjects²⁰⁶ and patients with asthma²⁰⁷ in other reports. Antibiotic therapy had no effect on nasal NO in one study in normal subjects,²⁰⁶ but nasal NO rose after treatment of sinusitis in another.¹⁹⁷ Vasodilators (eg, papaverine) increase nasal NO output.²⁰⁸

Smoking. A small decrease in nasal NO has been observed in smokers.²⁰⁹

Reproducibility of measurements

Several methods of nasal NO measurement, including that recommended by the ATS, have excellent reproducibility at the same session. Silkoff et al¹⁸⁶ reported that the intraclass correlation coefficient for triplicate measurements for 5 nasal NO methods at the same session were in the 0.95 to 0.99 range. Bartley et al²¹⁰ examined same-day and between-day reproducibility of the ATS-recommended method and reported coefficients of variation of 13.4% and 11.8%, respectively. This suggests that a clinically relevant change would be in the 20% to 25% range. Studies of reproducibility over weeks have not been reported. However, in allergic rhinitis, in which nasal inflammation may fluctuate and nasal congestion could affect NO diffusion from paranasal sinuses, reproducibility might be poor. Indeed, the change over time in relation to changes in disease activity is the very reason for developing new surrogate markers. Careful attention to accurate control of transnasal flow, well-fitting nosepieces, and reliable velum closure help achieve reproducibility. The use of nasal decongestants to facilitate nasal NO measurement requires study because there are reports that nasal NO may fall after decongestion.

Comparison with other nasal outcomes

Nasal NO is a relatively new marker, and there are few studies comparing this with other more standard outcomes. Olin et al²⁰⁹ reported no significant relationship between nasal symptoms and nasal NO and symptoms. Chatkin et al²⁰¹ reported no correlation between nasal NO measured with the ATS method and nasal volume assessed by acoustic rhinometry. In general, nasal NO, similar to exhaled NO in asthma, does not correlate with indices of nasal obstruction; however, exhaled NO has shown significant correlation to bronchial reactivity in steroid-naïve subjects.²¹¹ We await studies examining how nasal NO relates to nasal reactivity and the presence and degree of nasal inflammation in lavage and biopsies.

SUMMARY

A range of approaches has been developed to evaluate inflammatory changes within the nose in relationship to disease expression, to provide insight into pathophysiologic mechanisms, and to enable investigation of the effects and mechanisms of action of therapeutic intervention. These include nasal lavage, nasal cytologic sampling, and nasal biopsy. These techniques allow characterization of the cells and mediators participating in the allergic response. These techniques vary in their ease and level of invasiveness. Common to all is the need to process the material obtained. More recently, evaluation has been made of measurement of the concentration of nasal NO as an indirect marker of nasal inflammation and a measurement that is instantaneous.

Nasal lavage is a simple way of sampling the nasal lining fluid to investigate disease-related changes in cellular and solute composition. Despite its widespread use under challenge conditions, in which a range of stimuli have been found to lead to substantial changes in nasal lavage fluid composition and have provided insight into pathophysiologic processes, nasal lavage has been less used under clinical or epidemiologic circumstances. However, its use under such circumstances has been valuable in providing information on cellular recruitment and activation underlying clinical disease expression and the tissue response, such as that relating to plasma protein extravasation. The sources of variability and the repeatability of the findings are poorly substantiated under field conditions, however, and further information is required under such circumstances to enable the use of nasal lavage endpoints in appropriately powered therapeutic intervention studies. Nasal lavage is simple to undertake and easily applicable to such studies.

Nasal cytologic studies, accomplished by the proper obtaining and staining of the specimen and interpretation by an experienced microscopist, can provide valuable information on the cells in the nasal mucosa. These data can be used to understand better the mechanisms of the symptoms, the pathophysiology of the specific diseases, and the responses to different therapeutic interventions by pharmacologic and immunologic agents. Nasal cytologic sampling is also simple to perform and applicable to clinical trials. Information obtained pertains to the superficial cellular changes within the nasal mucosa and does not provide information about deeper tissue cellular events.

Nasal biopsy is required to evaluate such tissue cellular changes. It is a routine clinical procedure that is well tolerated under local anesthesia, in experienced hands, and representative biopsies can be obtained from the inferior turbinate. Such biopsies provide tissue for evaluation by immunohistochemistry, ISH, and PCR or can be used for *ex vivo* explant studies. Although small, nasal biopsies give a representative picture of the region of the nose from which they have been taken and provide information about tissue pathologic processes and the effect of therapeutic intervention. Such an approach is restricted to specialist centers.

Nasal NO measurement is reproducible, simple, and inexpensive to perform, and is therefore suitable for repeated application. However, the initial outlay for equipment is still expensive. From a pragmatic standpoint, the result is immediate. Nasal NO is high in allergic rhinitis, compared with normal controls, and falls after nasal steroid therapy but not with H₁-antihistamine treatment. Further studies are required to examine how nasal NO correlates with other objective measures of nasal inflammation.

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