Innate Immunity of the Sinonasal Cavity

Expression of Messenger RNA for Complement Cascade Components and Toll-like Receptors

Joseph VanderMeer, MD; Quan Sha, PhD; Andrew P. Lane, MD; Robert P. Schleimer, PhD

Objective: To study the expression of important elements of the innate immune responses in human sinonasal tissue to elucidate its potential role in mucosal inflammation.

Design: We studied human sinonasal tissue from patients with chronic rhinosinusitis and an immortalized epithelial cell line to detect the expression of innate immune effectors and the responses of these cells to stimulation with compounds associated with pathogenic organisms.

Patients: Nine individuals undergoing endoscopic sinus surgery for chronic rhinosinusitis.

Main Outcome Measures: Expression of complement components and toll-like receptors.

Results: We found detectable levels of messenger RNA for all toll-like receptors in human sinonasal tissue and in the BEAS-2B epithelial cell line. Expression of several components of the alternate pathway of complement (factors B, H, and I and properdin) was constitutively present in unstimulated BEAS-2B cells and was readily detectable in human sinonasal tissue. Stimulation of BEAS-2B cells with the toll-like receptor 3 ligand double-stranded RNA resulted in increased expression of messenger RNA for factors B and H but not for properdin or factor I.

Conclusions: Toll-like receptors and the alternate pathway of complement are important components of innate immunity that are expressed in human sinonasal epithelium in vivo and in cultured airway epithelial cells in vitro. The expression of some of these components can be significantly induced by stimulation via toll-like receptors, and epithelial expression of components of innate immunity may play a role in inflammation in chronic rhinosinusitis.

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Chronic rhinosinusitis (CRS) is a prevalent health problem that affects tens of millions of Americans each year. The mechanisms that underlie inflammation in CRS remain incompletely understood, although it is becoming clear that both local and systemic factors contribute to this condition. As the first line of defense for the airway, the sinonasal cavities use innate and adaptive immune processes to combat potential pathogens as they enter the body via inspired air. Many studies have demonstrated the presence of adaptive immune elements in CRS, such as infiltrating lymphocytes and their associated proinflammatory cytokines. However, innate immune responses by the sinonasal mucosa in CRS have not been studied as extensively. In recent years, much has been learned regarding the complex pathways that regulate mucosal innate immunity in the airway and gastrointestinal tract. A variety of molecules have been identified that recognize pathogens and lead to their destruction. In particular, toll-like receptors (TLRs), a series of 10 cell surface receptors for pathogen-associated molecular patterns, have recently been suggested as pivotal elements of innate immunity. In this study, the potential role of innate immune responses in CRS is explored by examining the expression of TLRs and components of the complement cascade in human sinonasal tissue and an airway epithelial cell line.

The present study was initiated after experiments demonstrated the expression of functional TLRs by cultured human airway epithelial lines. In those experiments, stimulation of TLR3 induced ro-
bust expression of several innate immune mediators, including the gene for the complement component C3. It was therefore hypothesized that local production of complement by sinonasal epithelial cells might occur as a natural mechanism of host defense and might also have a role in the pathogenesis of CRS. The TLR family contains 10 receptors with distinct specificities for broad categories of ligands. For example, TLR3 has specificity for double-stranded RNA (dsRNA), a common product of viral replication. Similarly, TLR2 and TLR4 have specificity for peptidoglycan and lipopolysaccharide (LPS), important components of gram-negative bacterial cell walls.4 Heterodimers can be formed between TLR1, TLR2, and TLR6 that are involved in the recognition of fungal elements. Downstream from the TLRs, signaling occurs in many cases through a pathway that involves the adaptor protein MyD88, which activates interleukin 1 receptor–associated kinases. This pathway ultimately leads to activation of nuclear factor κB and subsequent induction of nuclear factor κB–dependent proinflammatory cytokine and chemokine genes.5 The activation of TLRs by pathogens can also result in the expression of a variety of other effector genes involved in host defense. Therefore, TLRs may act as early sensors for the presence of viral, bacterial, or fungal pathogens in the nasal airway and may play a sentinel role in the activation of local inflammation through the expression of innate immune mediators such as the complement cascade.

There are 3 main pathways of complement activation: the classic pathway, the alternate pathway, and the mannose-binding lectin (MBL) pathway.6 Each of the 3 pathways of complement consists of a cascade of serine proteases that lead to formation of C3 convertase and activation of C3 to form C3a and C3b. These 2 split products of C3, together with those of C5, act as potent chemottractants, opsonins, and inflammatory cell activators. The final downstream result of complement activation is formation of the membrane attack complex (C5b–C9), which can cause lysis of microorganisms. Components of the alternate pathway of complement activation include properdin and factors B, D, H, and I. The MBL pathway includes MBL and at least 2 separate mannose-binding lectin-associated serine proteases (MASP1 and MASP2). These proteins are functionally homologous to Clq, Cir, and Cls of the classical pathway.6

It is generally believed that the precursor forms of complement components are produced in the liver and, during times of inflammation, are carried with plasma through capillaries to the interstitial space, where they may become active. However, there is some evidence for extrahepatic production of complement proteins. Decades ago, Robertson et al7 demonstrated complement components in respiratory secretions and suggested that local production had occurred. Khirwadkar et al8 reported that several complement components are produced by an epithelial cell line. A study by Varsano et al9 showed that epithelial cells produce increased levels of C3 after stimulation with cytokines. Walters et al10 recently demonstrated that exposure to particulate matter induces airway hyperresponsiveness associated with epithelial localization of C3 in the airways. Interestingly, increased airway responsiveness was ablated, but inflam-
CELL CULTURE

The BEAS-2B cell line was isolated from human bronchial epi-
thelium transformed by an adenovirus 12-SV40 hybrid virus. The
BEAS-2B cells were grown as monolayers in Dulbecco Modified
Eagle Medium/Nutrient Mix F-12 supplemented with 5% heat-
inactivated fetal calf serum, 2mM-glutamine, penicillin (100
U/mL), streptomycin (100 µg/mL), and 5% carbon
dioxide in humidified air. Cells that had reached approximately
80% to 90% confluence were challenged with dsDNA (25 µg/
Ml), LPS (1 µg/mL), or zymosan A (100 µg/mL).

RNA EXTRACTION AND REVERSE TRANSCRIPTION

Total mRNA was isolated from the stored sinonasal tissue samples
using the TRIzol (0.8-mL) reagent (Invitrogen), and DNA was removed using a DNase treatment and removal re-
agent (DNA-free; Ambion Inc) per the manufacturer’s proto-
col. Total RNA was isolated from the BEAS-2B cells using the
RNeasy Mini Kit (QIAGEN Inc, Valencia, Calif). Isolated mRNA
was reverse transcribed with a poly(dT)15 primer (Roche Di-
agnostics Corp, Indianapolis, Ind) and streptomycin (100 µg/mL)
at 37°C with 5% carbon dioxide in humidified air. Cells that had reached approximately
80% to 90% confluence were challenged with dsDNA (25 µg/
Ml), LPS (1 µg/mL), or zymosan A (100 µg/mL).

REAL-TIME POLYMERASE CHAIN REACTION

Real-time polymerase chain reaction (PCR) was performed in
a sequence detection system thermal cycler (ABI PRISM 7700;
Applied Biosystems) to quantify target and β-actin mRNA. Prim-
ers and probes were designed using a software program (Primer
Express Version 1.5; Applied Biosystems) from sequences stored
in the GenBank sequence database. The target genes, primers,
and probes used are listed in

<table>
<thead>
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<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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</table>

Factor P

| Factor B | CCACTAAGATGAACTGATGAGT | GTCTGCAACCTCCATGGA | CTTCTGCAATGGAAC |
| Factor H | CTGGCATCTCCTGCTGCTGCTT | TCTCAATAATTATCACTGATTTGGAACATGT | CCAAAACCCCAAGAC |
| Factor I | AAAAAATGAGCAGCTGCTGCTGCTGCTT | CTTGACCACGAGGAAACAC | TCAACACTATGATACATG |
| MBL     | GGTTCGAGATGAACTGATGTCGTT | GACGGAGAGATGCTGAGTG | AGCGGAGAGATGCTGAGTG |
| MASPI   | GACCACCCGACACTGCGTT | TTTGAGACGAGATGTCGTT | AGCGGAGAGATGCTGAGTG |
| MASPI2  | ATCACCTTTGTCACAGATGATGAC | GGGAAAGAATGTCTTTCGAGATGAT | AAGATACATACAGACAGC |
| β-Actin | CTGGGGGCGGACGTGACT | GCGAGGGTGTCGACTCCTC | CACGGACGAGGCTGAGA |

Abbreviations: MASP, mannose-binding lectin-associated serine protein; MBL, mannose-binding lectin; TLR, toll-like receptor.

RESULTS

One microliter of each complementary DNA preparation, corresponding to 25 µg of mRNA, was diluted in a ratio of 1:10,
and 5 µL of this preparation was placed in a total volume of 25 µL
with the following components: 1× TaqMan PCR buffer,
5.3mM magnesium chloride, 0.25mM deoxynucleotide triphos-
 phosphates (deoxyadenosine 5'-triphosphate, deoxyctyidine 5'-triphosphate, deoxyuridine 5'-triphosphate, and deoxyguano-
sine 5'-triphosphate). 0.25 U of AmpErase uracil N-glycosylase,
0.75 U of Ampli Taq Gold, 0.4µM each primer, and 0.2µM Taq-
Man probe (Applied Biosystems). The cycle parameters used were
50°C for 2 minutes to activate uracil N-glycosylase and 95°C for 10
minutes to activate Taq polymerase, followed by 40 cycles at
95°C for 15 seconds and 60°C for 1 minute. The fold change of
target mRNA was expressed as 2^ΔΔCt = the change in threshold
cycles for target and β-actin; ΔΔCt = the difference in
Differences were considered statistically significant at P<.05.

STATISTICAL ANALYSIS

Raw data from real-time PCR were entered into a spreadsheet
(Excel; Microsoft Corp, Redmond, Wash), and statistical analy-
sis was performed using a software program (StatView; SAS In-
institute Inc, Cary, NC). Data are expressed as mean±SEM. Sta-
tistical significance of differences was determined using the
t test. Differences were considered statistically significant at P<.05.

Figure 1: A demonstrates that sinonasal tissue samples con-
tained detectable levels of all 10 TLRs on screening with
standard reverse transcription PCR. To begin to assess the
relative levels of TLRs, we developed TaqMan probes and
primer sets for human TLRs (Table 1). Figure 1B shows the
threshold cycle values for levels of mRNA for all 10 hu-
man TLRs quantified in 5 patients with sinusitis. Values were relatively consistent among the 5 subjects, and the SEM for each TLR was 0.67 or fewer PCR cycles (Figure 1B). Among the TLRs, relatively high values were detected for TLR1, TLR2, TLR3, and TLR5; intermediate values were detected for TLR6, TLR7, TLR8, TLR9, and TLR10; and low values were detected for TLR4 (Figure 1A).

In recent studies using microarrays, Sha et al observed that activation of airway epithelial cells with the TLR3 ligand dsRNA leads to induction of the third component of complement (C3). We decided to determine whether human sinonasal tissue contains mRNA for the genes of the complement pathway. To validate the TaqMan probe/primer pairs, we used human liver mRNA as a positive control. **Figure 2** shows a standard reverse transcription PCR demonstrating expression of complement component mRNA from normal human liver as well as a sinonasal mucosal specimen. By quantitative real-time PCR, the RNA from human liver contained substantial levels of mRNA for the alternative and MBL pathways (Figure 3A). Nine sinonasal samples from patients with CRS were tested for mRNA for complement factors using TaqMan real-time PCR (Figure 3B). The sinonasal tissue samples had uniformly detectable levels of factor B, factor H, and properdin; somewhat lower levels of factor I; low but detectable levels of MBL; and essentially undetectable levels of MASP1 and MASP2. Although levels of mRNA for complement proteins were generally lower in the sinonasal samples than in the liver, the substantial quantities of mRNA for the alternate pathway components and the relative paucity of mRNA for the proteases of the MBL pathway were notable.

We next evaluated cultured epithelial cells for inducible expression of complement components. **Figure 4A** shows the threshold cycle values for basal expression of mRNA for factor B, factor H, factor I, and properdin in BEAS-2B cells. Based on the threshold cycle values, the relative expression of these factors was factor B > factor H = properdin > factor I. Evaluation of BEAS-2B cells for expression of MBL, MASP1, and MASP2 revealed levels that were in most cases undetectable (data not shown). To determine whether stimulation of TLRs might activate expression of complement genes, we tested mRNA from BEAS-2B after activation with dsRNA, LPS, and zymosan A. Figure 4B shows that dsRNA stimulated a 158-fold induction of mRNA for factor B (P = .04) and a 53-fold induction for factor H (P < .01). No appreciable induction of factor I or properdin was detected. Throughout the experiments performed on BEAS-2B cells, mRNA for the MBL pathway components was consistently below the detectable limits in all samples. No statistically significant induction of complement proteins was detected in cells stimulated with either LPS or zymosan A.

**COMMENT**

The mucosal surface of the nasal airways is a site of substantial exposure to airborne particulates and potentially pathogenic microorganisms. Despite the well-established finding that exacerbations of CRS may be associated with upper respiratory tract viral infections and with bacterial or fungal colonization of the sinonasal cavities, relatively little is known about innate immune responses of the nose and sinuses to such pathogens. We demonstrate in the present study that human sinonasal tissue from individuals with CRS contains mRNA for TLRs and mRNA for proteins of the alternative pathway of complement. We also present evidence from in vitro studies using cultured epithelial cells that dsRNA, a ligand for TLR3, induced mRNA for the alternative pathway of complement.

The complement cascade is a well-characterized innate immune pathway that is believed to be essential for effective mucosal immunity and the prevention of microbial infections. To our knowledge, this study is the first demonstration of the expression of mRNA for proteins of the alternate pathway of complement in upper airways constitutively and in response to pathogen stimulation. Findings from screening for the presence of mRNA for complement factors in sinonasal tissues suggest that C3 and the entire alternate pathway are locally ex-
pressed. Expression of the MBL pathway seems to be low and not readily inducible. We speculate that the promoters of the alternate pathway genes and the MBL pathway genes are regulated differently in epithelial cells and other mucosal cells. Mannose-binding lectin is a member of the collectin family, which also includes SpA and SpD; these genes all cluster on chromosome 10 in the region 10q21-24. Airway epithelial expression of SpA and SpD may be obviating the need for local expression of MBL. Regardless of the molecular mechanisms of expression, these findings suggest that locally produced complement proteins may be concentrated in sites exposed to triggers of epithelial activation and may contribute to inflammation and host defense.

Components of the complement pathway are primarily produced in the liver and circulate as proenzymes in the plasma. At times of inflammation, increased vascular permeability allows leakage of these components from the vasculature, resulting in activation in situ. During the past decade, several studies have found expression of complement component mRNA in extrahepatic tissues. Although our study represents the first description of complement expression in sinonasal tissue, it supports the concept that complement expression occurs in extrahepatic sites. Our findings also suggest that despite the significant homology between the 3 distinct pathways of complement, there is differential expression within tissues. In contrast to the classical and MBL pathways, which are surface-bound only, the alternate pathway is initiated in the soluble state before becoming membrane associated under the influence of factor P. In addition, formation of the chemoattractant split products C3a and C5a in the sinonasal cavity may help explain the abundance of inflammatory cell infiltrates in CRS.

Expression and function of TLR is important in innate and adaptive immunity. Some evidence exists that the process of aging decreases the function and host defense properties of TLRs and that individuals with polymorphisms of TLR4 have reduced release of inflammatory cytokines and increased susceptibility to bacterial infection. Studies more closely related to nasal inflammation have linked airway hyperresponsiveness to particular matter with the production of C3. A study by Diamond et al detected expression of TLR2 and TLR4 in airway epithelial cells and demonstrated production of β-defensins in response to TLR ligand stimulation. Recently, Claey S et al described similar levels of expression for TLR2 and TLR4 in the nasal epithelium and tonsils but low expression of defensins in the nose compared with tonsil or adenoid tissue. In another study, Shuto et al found elevated TLR2 expression in the airway epithelium in the setting of concurrent otitis media and demonstrated further TLR2 induction in cultured cells exposed to nontypable Hemophilus influenzae.

We demonstrated that human sinonasal tissue contains mRNA for all 10 TLRs and that some were highly expressed (TLR1, TLR2, TLR3, and TLR5), some were moderately expressed (TLR6, TLR7, TLR8, TLR9, and TLR10), and 1 was only weakly expressed (TLR4). Further studies need to be performed to confirm expression of these receptors at the protein level and to assess what cell types are expressing them in the sinonasal mucosa. There is an interesting parallel between the airway epithelium and gastrointestinal epithelium, where TLR4 is also either weakly expressed or undetectable. In the gut, expression of TLR4 is found primarily on the basal surface of the epithelial cells, and it has been proposed that the lack of inflammation induced by gut-colonizing

**Figure 2.** Agarose gel electrophoresis of polymerase chain reaction amplification products encoding complement components in human liver (A) and human sinonasal tissue (B). Top columns: ladder; complement factors B, H, and I; and properdin. Bottom columns: ladder, mannose-binding lectin-associated serine protease 1 (MASP1), MASP2, mannose-binding lectin, C3, and β-actin. Representative of 9 separate samples (sinonasal) or a single sample (liver).
bacteria may relate to this fact. Because the lungs are sterile in normal conditions, in contrast to the gastrointestinal tract, it would not be surprising if expression patterns for TLRs differ in the airways compared with the gut. Although TLR expression in the airway has not yet been linked to a disease state, a dysregulation of TLR4 in the gastrointestinal tract has been associated with inflammatory bowel disease.

The role of mucosal TLRs and complement expression in sinonasal immunity or inflammatory diseases is unknown. Expression of TLRs in sinonasal mucosa is likely to be important in triggering innate immune responses in the nose and sinuses. Exposure to TLR ligands can initiate the production of chemokines, cytokines, and growth factors that trigger the influx and activation of numerous cell types involved in the immune response, including neutrophils, monocytes, lymphocytes, and dendritic cells. The response of the epithelium and the subsequent immune and inflammatory response may depend on the TLR involved. For example, some TLRs (eg, TLR2, TLR5, and TLR7) activate only the MyD88 adaptor protein and lead to a limited array of cytokines and chemokines. Notably absent from the products produced by ligands for these receptors are interferons and interferon-induced genes. Other TLRs, such as TLR3, TLR4, and TLR10, activate the interferon-dependent pathways via adaptor molecules distinct from MyD88. Thus, exposure of the sinus or nasal mucosa to single pathogens is likely to lead to distinct response patterns depending on the TLRs activated by those particular agents.

The idea that epithelial or mucosal TLRs may be involved in the triggering of exacerbations of sinonasal disease represents an appealing model. For example, acute viral upper respiratory tract infection is the number one cause of illness for which patients seek medical care in the United States, and acute bacterial infection is thought to occur in 0.5% to 2% of patients with an initial diagnosis of viral upper respiratory tract infection. Stimulation of epithelial cells via TLR3 by virus-derived dsRNA can cause a substantial increase in the expression of inflammatory mediators and innate immune effectors, including the alternate complement pathway. If epithelial expression of these proteins is important in innate immune responses of the sinuses and upper airways, insufficiencies of these responses could lead to disease by increasing susceptibility to local infections. On the other hand, it is also possible that overexpression of comple-

Figure 3. Mean cycle threshold of reverse transcription polymerase chain reaction amplification products of complement components in normal human liver (A) and nasal tissue of patients with chronic rhinosinusitis (B). MBL indicates mannose-binding lectin; MASP1, mannose-binding lectin-associated serine protease 1. Error bars represent SEM.

Figure 4. Real-time polymerase chain reaction analysis of the inducibility of alternate pathway complement components in BEAS-2B airway epithelial cells. A, Mean cycle threshold for messenger RNA of alternate pathway complement components in unstimulated BEAS-2B cells. B, Mean relative expression of alternate pathway complement components in BEAS-2B cells stimulated with various pathogen components compared with medium control. dsRNA indicates double-stranded RNA; LPS, lipopolysaccharide. Error bars represent SEM.
ment proteins by the sinonasal epithelium could lead to episodic or chronic inflammation due to the known inflammatory properties of activated complement. We have not yet compared the level of expression of either complement proteins or TLRs in sinonasal tissue of patients who have CRS with levels in normal sinonasal tissue. Such a comparison, which is the subject of ongoing investigations, should be informative regarding the potential role of TLRs and local complement production in CRS.

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Correspondence: Andrew P. Lane, MD, Department of Otolaryngology–Head and Neck Surgery, Johns Hopkins Outpatient Center, Sixth Floor, 601 N Caroline St, Baltimore, MD 21287.

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REFERENCES