Detection of Common Respiratory Viruses in Tonsillar Tissue of Children with Obstructive Sleep Apnea

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Summary. Background: Early life viral infection is associated with neurogenic inflammation that is present in lymphoid tissues of the upper airway in children with obstructive sleep apnea (OSA). We hypothesized that viral genomic material is present in tonsils of children with OSA. Therefore, we examined tonsils for the presence of respiratory viruses' nucleic acids in children with OSA, and in children without OSA (undergoing surgery for recurrent throat infections (RI)). Methods: Tonsillar tissue from patients with OSA and RI was subjected to multiplex quantitative real time reverse transcription PCR (mqRTPCR), analyzed for the presence of common respiratory viruses' genetic material. Results: Fifty-six patients were included, of whom 34 had OSA (age (years ± S.D), 4.22 ± 1.14) and 22 with RI (4.35 ± 1.36). Respiratory viruses nucleic acids (24 detections) were observed in 17 (50%) OSA samples. In contrast, no virus was detected in RI samples (relative frequency \(P < 0.0001\)). Viruses detected, based on frequency were Rhinovirus, Adenovirus, human metapneumovirus (hMPV), respiratory syncytial virus (RSV), and corona virus. Conclusions: Respiratory viruses are detected in OSA hypertrophic tonsils, suggestive of their role in the evolution of tonsillar inflammation and hypertrophy. Early life viral infections may contribute to the pathogenesis of pediatric OSA. Pediatr Pulmonol. 2015; 50:187–195. © 2014 Wiley Periodicals, Inc.

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BACKGROUND

Obstructive sleep apnea (OSA) is a common disorder, with a prevalence of 1–5% in the pediatric population.1 The condition is associated with harmful cardiovascular, neurocognitive, and behavioral morbidities.2,3 Although the current recommended therapy for most children with OSA is adenotonsillectomy (T&A),1 the pathogenesis of tonsillar hypertrophy in these children is poorly understood. While there are investigators who do not relate the size of the tonsil to the severity of OSA in children,4 the size of the lymphoid tissue in the upper airway of children with OSA (measured by X-ray or by MRI) seems to correlate with PSG defined OSA severity according to well-designed studies.5,6 A far as we know, in snoring children, considerable pharyngeal lymphoid tissue enlargement is present since the preschool years and persists during school years.7

Neurogenic inflammation is a name termed to describe an inflammatory process that is activated and perpetuated following a viral infection, by neurons located in proximity to airways.8 An animal model that was developed to study the association of RSV infection with childhood asthma revealed neurogenic inflammation. The investigators

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Conflict of interest: None.

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discovered that RSV infected animals had increased levels of messenger RNA (mRNA) encoding for substance P (SP) receptor (neurokinin 1 [NK1]) in peri-bronchial lymphoid tissue. In addition, nerve growth factor (NGF), a known modulator of immune and inflammatory responses and a key regulatory element of neuronal responsiveness, and its tyrosine receptor kinase (trk-A), are found in increased levels after RSV inoculation.

We have shown that in comparison to patients with RI, OSA tonsils overexpress NK1 as well as NGF, in similar to the animal model. Indeed, neurogenic inflammation is known as one of the important mechanisms linking viral infection exposure, like RSV in infancy to chronic inflammatory conditions, like early childhood asthma.

RSV is the major cause of bronchiolitis in children <1 year of age and is the most important respiratory tract pathogen during early childhood, infecting nearly all children by the age of 2 years. Interestingly, Children who had proven RSV bronchiolitis as infants had significantly higher obstructive apnea/hypopnea index compared to controls, in an overnight sleep study, suggesting a role for early life viral insult, in the pathogenesis of OSA.

We therefore hypothesized that that RSV and other common respiratory viruses' genomic material are present in tonsils of children with OSA. We speculated that its presence may underlie the aforementioned inflammatory changes and the evolution of lymphoid tissue hypertrophy in children with OSA. In this study, we investigated the presence of viral nucleic acids long after the acute infection. Therefore the objectives of our study were to detect respiratory viruses' genome in the upper airway lymphoid tissue of children operated due to tonsillar hypertrophy, and to compare the presence of viral genomic material between those with and those without OSA.

METHODS

Patients

The Soroka University Medical Center Ethics Committee approved this study. Written, informed consent was obtained from the legal caregiver of each participant. Children previously diagnosed with OSA (AHI (apnea-hypopnea index) >5/hr) based on in laboratory overnight polysomnography were recruited, as well as age, gender, and body mass index-matched control subjects with normal polysomnography findings (AHI <1/hr). The control group, included children undergoing T&A under the diagnosis of recurrent throat infections (RI), that is, >5 Strep. Group A infections in the preceding 6 months prior to surgery, that underwent a clinical PSG (with normal findings). Exclusion for all subjects included: any previous history/physician diagnosis of cardiovascular disorder, allergies, smoking of a first degree relative/daytime caregiver (kindergarten teacher, grandmother), and familial craniofacial or genetic disorders, upper airway infections 6 weeks prior to surgery and lower airway infection at least 12 months prior to surgery.

For each participant in both groups, we collected demographic data that included age and gender. We specifically looked for lower airway infections and wheezing/asthma in the past, with at least one clinical event related office visit or prescription in the 12 months prior to surgery. All the data in regard to medical history and inclusion/exclusion criteria were based on an inquiry of their medical records at the hospital and at their primary pediatrician’s office. Physical examination included height, weight, and BMI. The size of tonsils was graded from 1+ to 4+ following direct inspection of the oropharynx, at their day of surgery by the same physician. All subjects were assessed by an ear, nose, and throat surgeon and an anesthesiologist on the operation day; if acute infection was present; the patient was discharged and excluded from the study. All the children were recruited during the months of June–September.

A summary of demographic and polysomnographic data is presented in Table 1.

All patients were Caucasian. Body mass index Z-scores were calculated from (http://stokes.chop.edu/web/zscore/index.php).

Overnight Polysomnography

All participating children were studied with in laboratory polysomnography. No sleep deprivation or sedation was implemented. Children were studied in a dedicated quiet, darkened room with an ambient temperature of 24°C in the company of one of their parents.

Polysomnography was performed with a computerized, commercially available, sleep monitoring system (SensorMedics, Inc., Yorba Linda, CA). Data was streamed to an optical disk for later analysis. Polysomnography was performed as previously described. Each and every one of the studies were initially scored by a certified technician. The scores were then blindly reviewed and interpreted by two physicians experienced in pediatric polysomnography. Analysis of the polysomograms was performed with standard techniques. In brief, sleep staging was performed with the standard criteria published by the AASM in 2007, and not by the latest revision of 2012 since we recruited our last child at January 2012. The AHI was defined as the airflow with continued chest wall and abdominal movement over at least two breaths. Hypopneas were defined as a >50% decrease in nasal flow with a corresponding >3% decrease in SpO2, and/or arousal or awakening. We
determined the mean and nadir SpO2 values. Arousals were defined as recommended by the revised AASM rules.17

Tissue Collection and Preparation

Tonsillar samples were obtained in the operation room. Immediately after removal by the ENT surgeon, specimens were snap frozen in a liquid nitrogen container, and stored within 2 hr at −70°C. Nucleic acids extraction and detection was performed according to the knowledge cumulated in a previous study in the virology laboratory at the Soroka University Medical Center. That study assessed nasal and throat swabs and nasal wash for the presence of respiratory viruses’ nucleic acids.18 A 30 to 50-mg tissue portion was homogenized with 200 μl of phosphate buffered saline (PBS) using manual electricity based homogenizer (omniTH international, Zatal). Nucleic acids were extracted using AllPrep DNA/RNA mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer’s instruction. A total of 200 μl of homogenate was extracted into 50 μl of elution solution. The elution was frozen and stored at −70°C.

Real Time PCR

Each sample was examined for RSV, Influenza B, Influenza A, human metapneumovirus (hMPV), Adenovirus, parainfluenza 3 virus, Rhinovirus, and Coronavirus NL63 as described previously,18 using real time reverse transcription polymerase chain reaction (mqRTPCR) and hydrolysis multiplex quantitative probes. In addition, each sample was tested for human endogenous retrovirus ERV3 as an internal control (IC). Each sample was tested by two sets of mqRTPCR (Table 1 and Table 2). qRTPCR for beta-actin and RNAseP housekeeping genes was done in all samples, as well. Amplification of panel 1 was carried out in final volume of 25 μl in a Real Time PCR System, Thermocycler 7500 (Applied Biosystems, Foster City, CA) with 5 μl of nucleic acids elution. Amplification of panel 2 was carried out in final volume of 20 μl on Real Time PCR System LC480 (Rhoche, Mannheim, Germany) with 4 μl of nucleic acids elution.

Data Analysis

Results are presented as means ± SD, unless otherwise stated. All analyses were conducted using statistical software SPPS version 17.0. All numeric data were subjected to statistical analyses with either t-tests or two-way analysis of variance procedures for repeated measures, as described by Neuman–Keuls. Post-hoc tests were performed as appropriate. A two-tailed P < 0.05 was considered statistically significant.

RESULTS

Tonsillar tissues were obtained consecutively from 56 children (34 OSA and 22 with RI). Demographic and medical history as well as polysomnographic key data of the participants is shown in Table 3. Three children from the OSA group and two children of the RI group underwent adenoidectomy as well.

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Table 1—Primers and Probes used in Panel 1 RTGPCR Assay

| Viral set          | Primers and probe sequence | Concentration (nM) | Target gene | Reference or source |
|--------------------|----------------------------|--------------------|-------------|---------------------|
| Influenza B        | AAATACGGTGGAATTAATAAAAGCAA  | 600                | HA          | 15                  |
|                    | CCAGCATAAGCTTGCAAGGAA       |                    |             |                     |
|                    | VIC-CACCATATGCTGGCAATTTCCTATGTCAG-TANRA | 600                |             |                     |
|                    | GACCATCCTGTCACCTCTGAC        | 300                | NS          | Retrieved from 16   |
|                    | AGGGCATTYTGAGCAAAKCTCTCTTA  | 300                |             |                     |
|                    | FAM-TGAGTCGCTTCACCTGTGGCGACG-BHQ | 200                |             |                     |
|                    | CATATAAGCATGCTATATTAAAAG    | 200                | Nucleocapsid protein | 17 |
|                    | CCTATTCTGAGCATATTTTGATCACG | 250                |             |                     |
|                    | VIC-CACCATATGCTGGCAATTTCCTATGTCAG-TANRA | 80                 |             |                     |
| RSV                | GCCAAAATTTTITCCCAATA         | 250                | L           | 14                  |
|                    | TCTTCATCACCATACCTTTTCTGTAA  |                    |             |                     |
|                    | IC                          | CATGGGAAGCAAGGGAACTAATG | 232 | Human ERV3 | 14 |
|                    | CACCGCGACATACAGAAATTT       | 232                |             |                     |
|                    | CCCAGCGAGCAATACAGAAATTT     |                    |             |                     |
|                    | C5-TCTTTCCCTCGAGCCATCACCATCBBQ | 232                |             |                     |

1Symbols and abbreviations: HA, hemagglutinine; NS, nonstructural protein; L, L protein; FAM, 6-carboxyfluorescein; BHQ, black hole quencher; MGB, minor groove binder; ROX, carboxy-X-rhodamine; BBQ, blackberry quencher; IC, internal control.

2Boldface values indicate hydrolysis probes.
Previous (more than 12 months) LRTI was documented in all (34) children in the OSA group. In two patients of the RI group a past lower respiratory tract infection record was not found in their medical files, although their legal guardians indicated they had one. Therefore only in 20 out of 22 controls there is verified information about past infection.

The total number of patients in whom viral nucleic acids were detected is 17 (50% of 34 OSA patients). Respiratory viral nucleic acids were not detected in any of the 22 participants of the RI group (relative frequency OSA vs. RI, \( P \leq 0.001 \)) (Fig. 1).

Overall there were 24 respiratory viral nucleic acids detections in 17 patients with OSA (50%). Four patients had detections of two different viruses; one patient had quadruple (four different viral nucleic acids) detections (Fig. 2).

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**TABLE 3—Demographic, Medical History, and Polysomnographic Key Findings**

|                         | OSA (n = 34) | RI (n = 22) | \( P \) value |
|-------------------------|--------------|-------------|---------------|
| Age (y)                 | 4.22 ± 1.14  | 4.35 ± 1.36 | NS            |
| Sex (M:F)               | 15:19        | 9:13        | NS            |
| BMI (Z score)           | 0.61 ± 1.08  | 0.57 ± 1.23 | NS            |
| Tonsils size            | 3.21 ± 1.01  | 3.11 ± 1.12 | NS            |
| Medical history         |              |             |               |
| History of LRTI\(^1\)   | 34/34        | 20/22       | NS            |
| History of recurrent wheezing or asthma | 7/34        | 3/22        | (0.42)        |
| Polysomnography         |              |             |               |
| AHI (events/hour)       | 12.1 ± 4.08  | 0.61 ± 0.22 | \(<0.001\)    |
| Oxygen min. Sat\(^1\)   | 84.2 ± 5.36  | 94.2 ± 2.14 | \(<0.001\)    |
| Arousal index (arousal/hr) | 19.6 ± 10.3 | 18.3 ± 8.5 | NS            |
| Sleep efficiency (%)    | 85.3 ± 7.5   | 87.6 ± 6.3  | NS            |

\(^1\) LRTI, lower respiratory tract infection; Sat, oxygen saturation.
Rhinovirus was the most common respiratory virus (33.3%), followed by adenovirus (29.2%), hMPV (25.0%) and RSV (8.3%), and corona virus (4.2%) (Fig. 3). Statistical analysis revealed that the differences were significant only for rhinovirus, adenovirus and hMPV. Table 4 summarizes the frequencies of virus detection in the study group compared to controls.

**DISCUSSION**

Our study revealed the presence of viral genome in the upper airway lymphoid tissue of children with OSA. Our findings demonstrate an evidence for persistent infection of the tonsillar tissue at least a year following any demonstrated LRTI.

Previous studies have raised the suspicion of a possible association between LRTI during infancy to the emergence of respiratory manifestation a few years later. In our cohort 50% of children with OSA who experienced lower respiratory tract infection more than a year prior to surgery, present nucleic acids of respiratory pathogens in their tonsils. Since we couldn’t identify any presence of respiratory virus nucleic acid in any of the RI samples, we now suggest that respiratory viruses may be involved in the evolution of tonsillar hypertrophy and OSA.

Our theory is, that a respiratory viral acute infection triggers a cascade which amplifies the proliferation of lymphoid tissue in susceptible young children’s airways, resulting with OSA.

There is growing evidence for the manifestation of local inflammation in the upper airway of both adults and pediatrics OSA patients, as reflected by higher levels of leukotrienes and enhanced expression of their receptors, measurable elevation of inflammatory markers in upper airway secretions as well as substantial increase in tissue inflammatory cell infiltration. Several investigators also found an evidence of systemic inflammation, reflected by elevated C reactive protein (CRP) levels in both adults and children with OSA.

Furthermore, T&A is associated with a decrease in systemic inflammatory markers, and treatment of children with non-severe OSA with an anti-inflammatory medication, improve PSG findings, imaging and children’s symptoms.

Numerous publications have discussed the differences between the two tonsillar conditions that usually necessitate their removal; that is, OSA and RI. Leukotrienes and their receptors for example, are more abundant in pediatric tonsillar tissue in the OSA group. Interestingly, leukotriene modifier therapy improves both upper airway patency and severity of sleep disordered breathing. In this respect, leukotrienes mediate neurogenic inflammation in lungs of young rats, following their infection with respiratory syncytial virus (RSV). Other studies showed an over expression of steroid alpha receptors (as opposed to beta receptors) This biological feature is associated with an increased
responsiveness to steroids, a pediatric common anti-inflammatory agent. It is also known, that a number of viruses may cause persistent tonsillar infection. Considering the aforementioned data, we decided to expand our search to the most common respiratory viruses that trigger early infancy and early childhood upper and lower respiratory infections. The additional viruses included RSV, Adenovirus, as well as the hPMV whose importance is being recognized among pediatricians in light of the clinical spectrum of disease it causes, and the clinical similarity to RSV.

Another newly included virus was Rhinovirus. Several publications by leading research centers unfold that the pathogen known particularly as the commonest cause of common cold, is the most significant predictor of the subsequent development of asthma at age 6 years compared to other respiratory viruses.

Recently, investigators in Brazil reported on viral nucleic acid detections in children with adenotonsillar disease. They examined tonsils that were removed due to several indications. Apparently, in only 11 (9%) of them the diagnosis of OSA was confirmed (no details regarding PSG formal diagnosis were mentioned). The lowest rate of virus detection was found in tonsils (68%, similar to our 50%), followed by nasopharyngeal secretions and adenoids. Furthermore, in 20.7%, viral co-infections were detected in palatine tonsils, similar to our findings (29%). In similar to our findings they also found adenovirus, rhino and hMPV to be the most prevalent viruses in tonsils, with the exception of enterovirus (that wasn’t on our RTPCR panel). Although they detected viral nucleic acids in many of their patients, it is hard to compare their findings to ours, mainly because of the uncertainty of OSA diagnosis.

The RTPCR results indicate clear tendency of respiratory viruses to remain for an extended period of time in the lymphoid tissue of patients with OSA, but not at the age and sex matching group without OSA, whose lymphoid tissue proliferation mechanisms are conceivably the result of other mechanisms.

Histological studies of enlarged tonsils, revealed follicular enlargement, suggesting hyperplasia with a substantial increase in lymphoid cells within germinal centers. It is also conceivable that some respiratory viruses may directly affect tonsill cell survival, by countering apoptosis. Reduction in lymphoid cell apoptosis is a well characterized mechanism in the pathogenesis of tonsillar hypertrophy, which may be initiated by viruses. Another mechanism is activated by the human coronavirus that inhibits apoptosis by the mitochondrial antiviral signaling adaptor (MAVS).

The presence of prolonged viral infection in tonsils with OSA can also be explained by virus products that may function as pathogen-associated molecular patterns (PAMPS) that would preserve and amplify inflammatory pathways by the stimulation of the innate immunity, with injury and repair mechanism, instigating hyperplasia and hypertrophy of tonsils, whose enlarged sizes will promote obstructive breathing during sleep.

Further studies should determine if viral proteins persist, in addition to viral genomes in OSA, and perhaps enhance the inflammatory process.

Upper airway lymphoid tissue is recognized as a site of persistent infection of certain viruses like Epstein–Barr virus, human herpes virus 6, HIV, measles virus, and enterovirus. However, studies of the most common respiratory viruses in lymphoid tissues are practically missing, emphasizing the novelty of our reported data.

In fact, adenovirus was first identified and its name was coined based on its close association with adenoid tissues, and our findings confirm the strong tropism of this agent for lymphoid tissues. The molecular details of adenovirus persistency are not completely understood, but the prolonged presence of this pathogen in nasopharyngeal tonsils allows for periodic and asymptomatic virus shedding that enables successful transmission of the virus.

We recently reported how children with OSA carry increased risk for lower respiratory tract infections. It is conceivable that the infections are preceded by viral shedding from the tonsils, which decreases tolerance to infections in these children. The circumstances under which acute infection turn into a persistent one, in children with OSA is yet to be discovered, and so are the mechanisms that determine the evolution of neurogenic inflammation, leading to tonsillar hypertrophy. In the last few years, premature babies are receiving the RSV immunoglobulin during the winter months in their first year of life. Indeed, it would be interesting to assess the occurrence of OSA in such a cohort.

It was recently shown, how respiratory virus like RSV can persist in a latent state in extrapulmonary reservoirs: specifically, the bone marrow stroma (BMS). Piedimonte and coworkers were able to demonstrate how PCR-amplifiable RNA sequences with virtually complete homology to the RSV genome, are detected in naive primary bone marrow stromal cells (BMSCs) from adults and pediatric BMS tested. Since no homologous sequence was identified in the human genome database, supporting the message identified in BMSCs was indeed of viral origin.

Numerous lines of confirmation can explain how RSV infection ends in a non-pulmonary target. RSV can enter the pulmonary microcirculation and then spread through the bloodstream to extra-pulmonary sites. The virus has been detected in a variety of human tissues, including the central nervous system, heart, and liver. Interestingly, a previous study using nested PCR detected RSV RNA in the blood of 63% of neonates and 20% of infants whose nasal washes tested positive for RSV antigen.
suggested that RSV viremia may descend during acute infection to other organs, conceivably the tonsils.

There is no strong evidence to suggest that the acquisition of the viruses is different between the two groups with similar respiratory infection rates.

Therefore, it may be the immune response to the virus that allows persistence of the virus to occur only in the OSA group. Tan et al.\textsuperscript{53} described recently how pediatric OSA is associated with reduced T regulatory lymphocytes population and altered Th1:Th2 balance toward Th1 predominance. This condition is not only pro-inflammatory but also accompanied by an altered T cell response to certain stimuli.

Ye\textsuperscript{54} found that in adults with OSA, the condition may be associated with peripheral Th17/Treg imbalance that serves as an alternative explanation for the substantial activation of immune cells in OSA. Indeed, it was recently shown that Th17 cells may also play a role in regulating inflammation in the RSV-infected lung. This CD4+ T cell subset is enriched at mucosal sites and characterized by production of IL-6 as well as IL-17, a cytokine that induces chemokine production by respiratory epithelial cells causing leukocyte infiltration.\textsuperscript{55}

These similar reports in children and adults with OSA suggest that their immune system responds different then non-OSA subjects. Overall, these finding may explain why we see this persistence of viral genomic material in OSA. An understanding of the unique nature of the mucosal immune system of respiratory organs (like the tonsils), is extremely important for future prevention and treatment of OSA.

A major study limitation was our inability to retrospectively reveal viral identification during the acute viral infection experienced earlier in life. A prospective study with viral identity during the acute respiratory infection will be needed in order to relate the events and strengthen our data. Furthermore, only RSV is currently associated with neurogenic inflammation, and although it is plausible that many viruses can trigger this phenomenon, it is yet to be proven. Another limitation is the sample size, and although the statistical differences are significant we believe that studies with larger number of subjects will reassure the field that this is not a “by chance” finding, but a real biological phenomenon that is important in the pathophysiology of OSA in childhood. Since we did not include assessment of the adenoids in this study, it is also a limitation since children may suffer from OSA due to adenoid hypertrophy. However, we couldn’t find a difference between our two groups in terms of adenoidectomy rates. Therefore, we think that in these cohorts, this limitation does not bias the results.

In summary, we present data that indicates persistent infection of the upper airway lymphoid tissue by respiratory viruses in children with OSA. If our findings will be corroborated, it is plausible that new measures will need to be considered in order to decrease the prevalence of tonsillar hypertrophy during childhood and eventually OSA.

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