

Evidence of Allergic Inflammation in the Middle Ear and Nasopharynx in Atopic Children with Otitis Media with Effusion

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Abstract

Background: Otitis media with effusion (OME) occurs in the setting of eustachian tube (ET) dysfunction. Previous studies have demonstrated a predominance of T helper 2 (Th2) mediators in the middle ear effusions (MEEs) of atopic children, suggesting that allergy plays a role in the pathogenesis of OME. Given that the middle ear is contiguous with the upper airway, the allergic inflammation seen in the middle ear of atopic patients with OME may also have been observed in the nasopharynx.

Objective: We hypothesize that atopic children have different cellular and cytokine profiles in MEE compared with nonatopic patients and that this allergic inflammation occurs in both the middle ear and the nasopharynx.

Methods: Forty-five patients undergoing both ventilation tube placement for OME and adenoidectomy for adenoid hypertrophy were recruited. The atopic status was determined for each patient using standard skin testing. The cellular and cytokine profiles of the MEEs and the torus tubarius and adenoid tissues were investigated using immunocytochemistry and in situ hybridization.

Results: Our results indicate that, within the atopic patient, there is a similar cellular and cytokine profile within the three regions sampled, with a predominant expression of interleukin-4 (a Th2 cytokine) and an increased infiltration of eosinophils compared with the nonatopic patient.

Conclusion: These findings confirm the association of allergy with MEE and support the hypothesis that the middle ear may be an integral part of the United Airway Concept.

Sommaire

Introduction: L'otite moyenne avec effusion (OME) est une manifestation de la dysfonction de la trompe d'Eustache. Des études ont montré la prédominance de médiateurs des *T helper* (Th2) dans l'effusion d'enfants atopiques, suggérant que l'allergie aurait un rôle dans la pathogénèse de l'OME. Puisque l'oreille moyenne est contigue aux voies respiratoires supérieures, l'inflammation allergique vue dans l'oreille moyenne des patients atopiques avec OME se retrouve peut-être aussi dans le nasopharynx.

Objectif: Nous avons émis l'hypothèse que les enfants atopiques ont un profil cellulaires et de cytokines dans leurs effusions différent de celui des enfants non-atopiques et que cette réaction allergique se retrouve autant dans l'oreille moyenne que le nasopharynx.

Méthode: Nous avons recruté 45 patients qui subissaient l'insertion de tubes de ventilation et une adénoïdectomie pour une hypertrophie des adénoïdes. Nous avons aussi déterminé le statut atopique en utilisant des kits cutanés standards. Les profil cellulaires et les cytokines ont été évalués par immunocytochimie et hybridation in situ dans les effusions, la région du torus tubarius et dans les adénoïdes.

Résultats: Nos résultats indiquent que chez les patients atopiques, il existe une similarité des profils cellulaires et des cytokines entre les trois régions. On remarque en effet une expression prédominante de l'interleukin-4 (une cytokine des Th2) et une infiltration accrue d'éosinophiles comparé aux patients non atopiques.

Conclusion: Ces trouvailles confirment l'association de l'allergie et de l'OME et supporte l'hypothèse que l'oreille moyenne fait partie intégrante du concept des voies respiratoires unies.

Key words: adenoid, allergy, atopy, interferon- γ , interleukin-4, interleukin-5, otitis media with effusion, United Airways Concept

Otitis media with effusion (OME) is a chronic inflammatory disease of the middle ear space that results in the accumulation of fluid. OME represents a major pediatric health care issue and, with an estimated prevalence of 15 to 20%,¹ is the most common indication for surgery in children. Patients with OME can have significant sequelae,² including hearing loss and delayed speech development, and are at risk of developing intratemporal and intracranial complications of chronic middle ear disease. In addition, OME has a substantial economic burden, estimated at an annual cost in the billions of dollars.³

The development of OME results from a complex interaction of numerous risk factors. Preceding bacterial and viral infections, as well as eustachian tube (ET) dysfunction, are acknowledged contributors. However, the role of allergy in the pathogenesis of OME is controversial. Most epidemiologic studies have shown that patients with OME have an increased prevalence of atopic conditions, including eczema, asthma, and allergic rhinitis,⁴⁻⁹ when compared with non-OME controls.

It has previously been proposed that allergic inflammation can cause OME indirectly by causing additional compromise of ET function,^{10,11} thereby leading to the development of middle ear effusions (MEEs). Other clinical studies have supported the theory of allergen-induced ET dysfunction in atopic patients. Exposure to allergen challenge consistently resulted in a dose-dependent decrease in ET patency, regardless of whether a seasonal (ragweed)¹² or a perennial (dust mite)¹³ allergen was used in the challenge.

In addition, studies have shown that the composition of the inflammatory substrate in allergic otitis media is similar to the late-phase allergic response also seen in other areas of the respiratory tract, such as in chronic sinusitis, allergic rhinitis, and asthma.¹⁴⁻¹⁶ The inflammation in allergic diseases has been characterized by an abundance of T helper 2 (Th2) cytokines (particularly interleukin [IL]-4 and -5), whereas T helper 1 (Th1) cytokines (namely, interferon [IFN]- γ) predomi-

nate in bacterial and viral infections. It has been suggested that the immunopathologic mechanisms underlying the development of OME in allergic patients are largely attributed to the effects of Th2 cytokines and activation of their receptors.

Because the middle ear space is an anatomic extension of the upper airway by way of the ET, and because the middle ear is capable of mounting an inflammatory response similar to other areas of the respiratory tract, we propose that the middle ear is part of the United Airways Concept. The United Airways Concept maintains that there is an intimate interconnection between the upper and lower airways in allergic disease, where the observed allergic inflammation is not confined to a specific target organ. Rather, the allergic disease is a disorder of the whole respiratory tract, which has a broad spectrum of clinical manifestations.

The objectives of this study were (1) to confirm findings that MEEs in atopic children with OME have cellular and cytokine profiles different from nonatopic patients and (2) to investigate the possible involvement of the middle ear space in the United Airways Concept by comparing the inflammatory profiles of the middle ear with those of the upper airway (namely, the nasopharynx).

Methods

Study Design

This prospective clinical trial was undertaken exclusively at the Montreal Children's Hospital (Department of Otolaryngology), a tertiary care pediatric hospital affiliated with McGill University. The study obtained full scientific and ethical approval from the institutional review board. Children (2-18 years of age) who required simultaneous (1) myringotomy with pressure-equalizing tube insertion and (2) adenoidectomy were eligible for the study. All subjects were enrolled in a consecutive, prospective manner. For ethical reasons, patients who were not undergoing adenoidectomy were not enrolled. In addition, children < 2 years old were not included because a diagnosis of atopy by skin testing is less reliable.

The indications for surgery were (1) OME persisting for > 3 months, producing a conductive hearing loss > 30 dB with a type B tympanogram, and (2) symptomatic nasal obstruction owing to adenoid hypertrophy. The exclusion criteria consisted of (1) Down syndrome, (2) craniofacial anomalies such as cleft palate, (3) the presence of a known immunodeficiency disorder, (4) bleeding disorders, (5) ciliary dyskinesia, (6) current middle ear infection, (7) use of antihistamine-containing medications within 1 week prior to surgery, (8) use of steroids or other immunosuppressive agents within 6 weeks prior to surgery, (9) a history of acute otitis media in the preceding 3 weeks, and

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(10) the presence of frank pus found in the nasopharynx seen at the time of surgery.

After parental consent was obtained, a history and a physical examination were performed, assessing for general medical problems and risk factors for allergy. Demographic data recorded included patient age and gender.

Diagnosis of Atopy

All patients with OME were evaluated for atopy status by skin testing by one investigator. To prevent selection bias, allergy testing was performed after patients were entered into the study.

Patients were defined as having atopy if they reacted positively to at least one allergen. Skinprick testing was performed intraoperatively for perennial and seasonal allergens using a standard panel consisting of *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, grass mix, ragweed, trees mix, cat, cockroach, dog, and the dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. Histamine (1 mg/mL) was used as a positive control and saline as a negative control. A positive skin reaction was judged as a wheal 3 mm or greater in diameter compared with the negative control. No antihistaminic or anti-inflammatory medications were given at the time of testing.

Sample Collection Methods

Middle Ear Fluid. Middle ear fluid was collected in a Juhn Tym-Taps (Xomed Treace Products, Jacksonville, FL) at the time of myringotomy and tympanostomy tube placement. Patients with pus in their effusion were not analyzed.

Tissue Biopsies. At the time of adenoidectomy, a cupped forcep was used to take a 3 mm biopsy specimen of both the adenoid pad and the ET mucosa at the nasopharyngeal orifice, namely, the mucosal surface of the torus tubarius. The torus tubarius specimen was taken on the ipsilateral side of the middle ear fluid sampled.

Fluid Preparation

One millilitre of sterile phosphate-buffered saline (PBS) was added to the sample. At the laboratory, the sample was resuspended and transferred to a 15 mL Falcon tube (BD Biosciences, San Jose, CA) and centrifuged at 200 g (1500 rpm) for 5 minutes. The supernatant was stored at -80°C until assayed. One millilitre erythrocyte lysing buffer (Pharmalyse, St. Louis, MO) was added to the pellet for bloody samples. The pellet was resuspended and centrifuged, washed three times with sterile PBS, and fixed. The pellet was cytospun 300 rpm for 10 minutes onto frosted slides, and differential cell counts were made by Quick Diff (Baxter) staining and microscopic examination. Slides were also prepared for immunocytochemistry and in situ hybridization. For immunocytochemistry, the cytopins were briefly fixed

in a solution of acetone and methanol (60:40), air dried, and stored at -20°C until further use. For in situ hybridization, the cytopins were air-dried and fixed in 4% paraformaldehyde for 30 minutes, washed twice for 5 minutes with PBS, kept at 37°C overnight, and stored at -80°C until further use.

Immunocytochemistry

Immunocytochemistry was performed using the alkaline phosphatase antialkaline phosphatase method, as previously described.¹⁷ The monoclonal antibodies major basic protein (MBP) and elastase were used to detect eosinophils and neutrophils, respectively. Slides were developed using fast red substrate for alkaline phosphatase. Negative control experiments were performed by replacing the primary antibody with an isotype-matched control.

In Situ Hybridization

Digoxigenin-labelled in situ hybridization was conducted using digoxigenin-11 uridine triphosphate-labelled IL-4 and IFN messenger ribonucleic acid (mRNA) riboprobes, as previously discussed.¹⁸ Colour development in digoxigenin-labelled in situ hybridization is achieved by adding a freshly prepared substrate solution consisting of 0.175 mg X-phosphate-5-bromo-4-chloro-3-indolyl phosphate and 0.37 mg nitroblue tetrazolium salt per millilitre of equalization buffer to the slides (for 20 to 40 minutes, at room temperature). Slides were transferred to Tris Buffered Saline (TBS) washed in tap water, and counterstained with hematoxylin for 5 seconds. Positive signals were identified by a purple stain on the cells under light microscopy. Negative control experiments using sense probes and ribonuclease treatment before antisense probe application were performed to confirm probe specificity.

Quantification

For the middle ear fluid, specimens were coded and the percentage of positive cells for mRNA was counted in a blinded fashion using an Olympus microscope with an eyepiece graticule at $\times 200$ magnification. The results were expressed as the mean percentage of positive cells per cytospin $\pm$ the standard error of the mean. For the tissue specimens, the slides were analyzed for a positive signal in a blinded fashion by an investigator using an Olympus light microscope at $\times 200$ magnification with an eyepiece graticule of 0.202 mm^2 . The number of positive cells was counted and expressed as the mean per square millimetre.

Statistical Analysis

Cell counts were compared between atopic and nonatopic patients using an analysis of variance *t*-test, with $p < .01$ regarded as statistically significant.

Results

Clinical Data

Fifty-three patients with OME were enrolled in the study. Eight patients were excluded from the study. Four were excluded because they were taking antihistaminic or anti-inflammatory medications, whereas two patients had pus in the nasopharynx. The remaining two patients were diagnosed with cyclic neutropenia and Down syndrome.

Forty-five patients were therefore included in the final analysis. Eleven (24.4%) patients were found to be atopic on skinprick testing, whereas 34 (75.6%) were nonatopic and served as controls. Selected demographic and clinical characteristics for patients in each subgroup are summarized in Table 1.

Atopic patients were most commonly reactive to dust mite (64%). Forty-five percent of atopic patients were reactive to animal (cat and dog) dander, 36% to tree mix, 18% to cockroach and molds, and 9% to ragweed. No patients were reactive to grass mix.

Immunocytochemistry

The percentage of MBP-positive cells (indicating eosinophils) was significantly higher in the MEE of atopic patients ($7.2 \pm \text{SEM } 0.4$) compared with nonatopic patients ($1.2 \pm \text{SEM } 0.2$) ($p < .01$). In addition, eosinophil levels were significantly higher in both the torus tubarius tissue and the adenoid tissue of atopic patients ($6.1 \pm \text{SEM } 0.6$; $6.4 \pm \text{SEM } 0.9$) compared with nonatopic patients ($3.1 \pm \text{SEM } 0.2$; $2.5 \pm \text{SEM } 0.3$) ($p < .01$) (Figure 1).

On the other hand, a significantly lower percentage of elastase-positive cells (indicating neutrophils) is found in the MEE of atopic patients ($18.4 \pm \text{SEM } 0.9$) compared with nonatopic patients ($44.4 \pm \text{SEM } 1.3$) ($p < .01$). In addition, neutrophil levels were significantly lower in both the torus tubarius tissue and the adenoid tissue of atopic patients ($26.6 \pm \text{SEM } 1.9$; $22.9 \pm \text{SEM } 1.2$) compared with nonatopic patients ($37.2 \pm \text{SEM } 1.2$; $34.1 \pm \text{SEM } 1.0$) ($p < .01$) (Figure 2).

In Situ Hybridization

The percentage of IL-4 mRNA-positive cells is significantly higher in the MEE of atopic patients ($8.4 \pm \text{SEM } 0.7$) compared with nonatopic patients

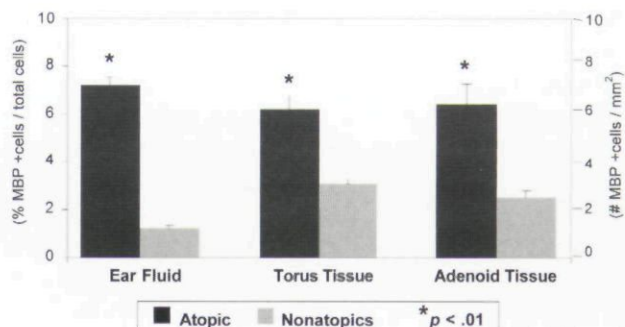


Figure 1 Eosinophils. (Middle ear fluid levels measured in percentage of total cells. Torus tubarius and adenoid tissue levels measured in number of cells per mm^2).

($1.7 \pm \text{SEM } 0.3$) ($p < .01$). In addition, IL-4 levels were significantly higher in both the torus tubarius tissue and the adenoid tissue of atopic patients ($8.0 \pm \text{SEM } 1.1$; $10.3 \pm \text{SEM } 0.9$) compared with nonatopic patients ($1.3 \pm \text{SEM } 0.2$; $1.5 \pm \text{SEM } 0.2$) ($p < .01$) (Figure 3).

On the other hand, levels of IFN- γ -positive cells were found to be significantly lower in the MEE of atopic patients ($5.3 \pm \text{SEM } 1.1$) compared with nonatopic patients ($9.3 \pm \text{SEM } 0.7$) ($p < .01$). In addition, IFN- γ -positive cell levels were significantly lower in the adenoid tissue of atopic patients ($3.3 \pm \text{SEM } 0.6$) compared with nonatopic patients ($6.7 \pm \text{SEM } 0.5$) ($p < .01$). However, the levels of IFN- γ -positive cells in the torus tubarius tissue were not significantly lower in atopic patients than in nonatopic patients, although there was a trend ($p = .09$) (Figure 4).

Discussion

In our study, the incidence of atopy was 24.4% among all patients with OME. We demonstrated that atopic patients have significantly higher levels of eosinophils and IL-4 mRNA-positive cells in MEE and torus

Table 1 Demographic and Clinical Characteristics

	Atopic Group	Nonatopic Group (Controls)	Excluded Group
n	11	34	8
Mean age, yr (SEM)	6.5 (0.86)	5.3 (0.45)	3.25 (0.39)
Gender (male:female)	2.7:1	1:1	1.7:1

SEM = standard error of the mean.

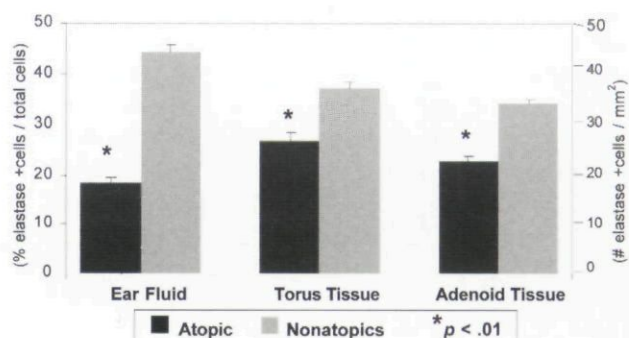


Figure 2 Neutrophils. (Middle ear fluid levels measured in percentage of total cells. Torus tubarius and adenoid tissue levels measured in number of cells per mm^2).

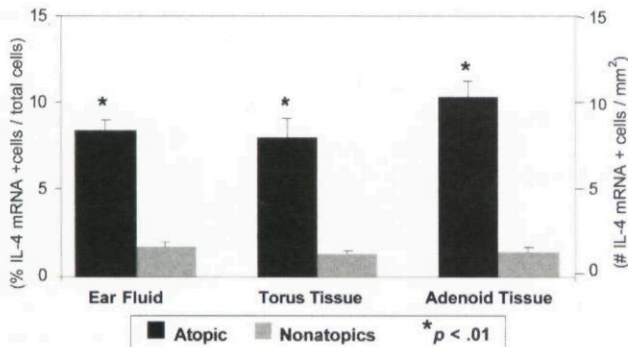


Figure 3 Interleukin-4 (IL-4) messenger ribonucleic acid (mRNA). (Middle ear fluid levels measured in percentage of total cells. Torus tubarius and adenoid tissue levels measured in number of cells per mm².)

tubarius and adenoidal tissue when compared with nonatopic patients.

Previous studies have attempted to examine the inflammation seen in the ear in atopic patients with OME. Hurst and Venge reported increased levels of eosinophilic cationic protein in the supernatant of atopic OME patients compared with nonatopic controls.¹⁹ Wright and colleagues demonstrated increased expression of IL-5 and MBP (eosinophils) in the middle ear mucosa of patients with OME compared with normal controls.²⁰ Sobol and colleagues recently found significantly higher numbers of T lymphocytes and eosinophils, as well as IL-4 and IL-5 mRNA-positive cells, in atopic compared with nonatopic MEE.²¹ Our findings concur with these studies and confirm that allergy may play a role in OME.

In addition, our study, for the first time, correlates cellular and cytokine profiles found in the MEE with those found in ET mucosa at the nasopharyngeal orifice and in adenoidal tissue. We have shown that in atopic patients, there is uniform allergic inflammation in all three sites and that these changes resemble the pathologic changes found in the lower airways of the lungs

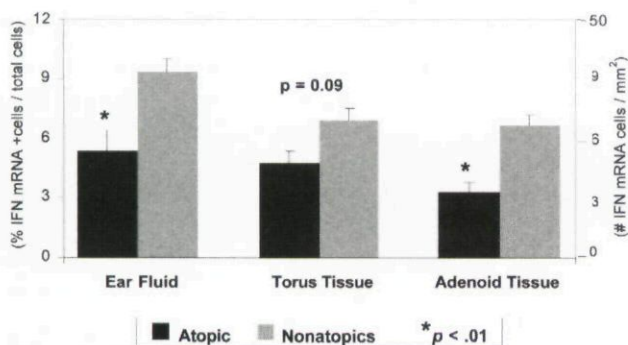


Figure 4 Interferon-γ (IFN-γ) messenger ribonucleic acid (mRNA). (Middle ear fluid levels measured in percentage of total cells. Torus tubarius and adenoid tissue levels measured in number of cells per mm².)

with the late-phase allergic response. This response is also seen in other areas of the respiratory tract and is implicated in the pathogenesis of asthma, allergic rhinitis, and chronic sinusitis.¹⁴⁻¹⁶ Our data support the concept that the middle ear may be part of the United Airways Concept and may behave in a fashion similar to that of the lungs under allergic inflammatory insults.

Among the immunoregulatory cells, eosinophils play a particularly important role in the late-phase allergic response through the release of various mediators. These effector cells are rapidly recruited and activated after allergen stimulation.^{22,23} The increase in eosinophils in atopic patients (compared with nonatopic patients) is much greater in the MEE than that seen in the torus tubarius or adenoid tissue. These results suggest that, in addition to participating in a generalized allergic inflammation of the airway, the middle ear is capable of a more intense local inflammation. These may be due to superimposed local factors in the middle ear, such as chemokines, which can be produced by structural cells.

There were significantly higher numbers of IL-4 mRNA-positive cells of allergic patients compared with nonatopic control subjects in all three sites. The increase in IL-4 means that the middle ear compartment and adenoidal tissue are capable of promoting local immunoglobulin E production, influencing activated naive T cells to express predominantly Th2 cytokines, and facilitating eosinophil infiltration by enhancing endothelial expression of vascular cellular adhesion molecule 1 (an important adhesion molecule). These findings confirm that these sites are capable of a sustained late-phase allergic response and of participating in allergen-specific inflammation.

Nonatopic patients reveal different cellular and cytokine profiles in their MEE and in their torus tubarius and adenoid tissue. Neutrophils were the predominant cell type, constituting a significantly higher percentage of the total number of cells when compared with their atopic counterparts. On the other hand, the presence of neutrophils in MEE of atopic patients, albeit at low levels, may represent the residual effects of a previous infectious process, despite the fact that patients with purulent effusions or a history of a recent ear infection were excluded.

IFN-γ-positive cells were present in significantly higher percentages in nonatopic children in our study. In contrast to allergic OME, Th1 cytokines seem to orchestrate the inflammatory response in nonallergic OME, reflecting the possible role of bacterial and/or viral products in the stimulation of MEE production. As well, within the atopic patients, another possible explanation for the low levels of IFN-γ seen is that there is suppression of the basal levels of IFN-γ by the increased levels of IL-4, thereby maintaining a balance of Th1 and Th2 cytokines.

The mean age difference between nonatopic (5.3 years) and atopic (6.5 years) groups is 1.2 years. Although statistically different, there is unlikely to be an observed clinical or immunologic difference. T-cell priming to inhalant allergens and the development of Th2 responses are believed to occur principally in early childhood. Yabuhara and colleagues showed that compartmentalization of inhalant allergen-specific T-cell immunity into adult-equivalent Th1 patterns is likely to be established by the age of 5 years.²⁴ In addition, Musiatowicz and colleagues did not find any statistically significant differences among Th and T-suppressor cell (T_S) subpopulations in the adenoids of children ages 0 to 5, 5 to 10, and 10+ years.²⁵ Other studies examining the cellular infiltrates of tonsillar tissue have found differences only after the first decade of life.²⁶

There are limitations to the study owing to ethical reasons. First, mucosal biopsies within the ET mucosa were not taken. Therefore, we sampled the torus tubarius, which we felt was the most easily accessible and representative tissue. Second, patients with adenoidal hypertrophy were enrolled in the study. Ideally, tissues would be sampled from patients undergoing only pressure-equalizing tube insertion, regardless of their adenoid size. That being said, Bernstein and colleagues examined the cytokine profiles in adenoids and found no difference between patients who underwent adenoidectomy for otitis media or for nasal obstruction.²⁷

Conclusion

In conclusion, our study demonstrates that MEEs and the torus tubarius and adenoidal tissue of atopic children with OME were characterized by higher levels of eosinophils and IL-4 mRNA-positive cells when compared with nonatopic controls. Our study supports the hypothesis that allergic inflammation may contribute to the pathogenesis of OME in atopic children. In addition, we have shown for the first time that in atopic patients, there is uniform allergic inflammation in the middle ear and nasopharynx. This suggests that the middle ear may be part of the United Airways Concept.

Patients with OME do not all respond consistently to conventional management. By identifying the subgroup of atopic patients in whom allergic inflammation may be a contributing factor in the etiology of OME, treatment options may include anti-inflammatory medications and immunotherapy. If the middle ear proves to play a role in the United Airways Concept, then management strategies should take into consideration the common underlying systemic inflammation in this chronic allergic respiratory syndrome.

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