

Effects of allergic rhinitis on the progression and recovery of acute otitis media in a mouse model

Chang Gun Cho^{a,b}, Hee-Bok Kim^a, Chul-Kyu Kim^a, Bo Hae Kim^{a,b}, Yun-Sung Lim^{a,b},
Seok-Won Park^{a,b}, Joo Hyun Park^{a,b,*}

^a Department of Otorhinolaryngology-Head and Neck Surgery, College of Medicine, Dongguk University, Ilsan Hospital, Goyang, South Korea

^b Sensory Organ Research Institute, College of Medicine, Dongguk University, Gyeongju, South Korea

ARTICLE INFO

Keywords:

Otitis media
Allergic rhinitis
Haemophilus influenza
Mice

ABSTRACT

Objectives: The objective of this study was to evaluate the effects of allergic rhinitis (AR) on the development, progression, and recovery of acute otitis media (OM) in an animal model and investigate the secondary effects of bacterial infection.

Methods: BALB/c mice were divided into four groups: AR + OM, AR, OM, and control groups. AR + OM and AR groups were sensitized with ovalbumin (OVA) and then challenged intranasally with OVA. Phosphate-buffered saline (PBS) was administered to the OM and control groups the same number of times. After AR induction, OM was induced by surgical inoculation of non-typeable *Haemophilus influenza* (NTHi) into the middle ear (ME) cavity of the mice in the AR + OM and OM groups. PBS was injected into the bulla in the AR and control groups. Each group was subdivided into sets of six mice, one for each of the four time points (0, 2, 7, and 10 days post-bacterial inoculation), at which point the mice were euthanized and ME and nasal cavity mucosa were obtained and evaluated. The occurrence of OM and the ME mucosa thickness were evaluated and compared among the four groups. Tissue expression of interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α (TNF- α) in infected ME mucosa was assessed by immunohistochemical staining. We also investigated IgE, IL-4, and IL-5 in the nasal mucosa.

Results: Most of the ears showed OM on post-inoculation day 2 in both AR + OM and OM groups. In the AR + OM group, 58.3% of ears still had OM on post-inoculation day 10, while only 16.7% of the OM group had OM. The ME mucosa of all groups increased, and the AR + OM group exhibited the thickest mucosa. The OM group showed peak thickness on post-inoculation day 2 and then decreased, whereas the ME mucosa thickness of the AR + OM group continued to increase to day 7. In the OM group, the expression of IL-1 β , IL-6, and TNF- α in the ME also increased significantly, peaking on post-inoculation day 2, and then gradually decreased. In the AR + OM group, the expression of these proteins increased until day 7 and then decreased. The IgE and Th2 response (IL-4 and IL-5) cytokines were expressed at higher levels in the AR + OM and AR groups than in the OM and control groups.

Conclusion: The inflammatory reaction to NTHi was more intense and lasted longer in the allergic group, which indicates that AR affects the progression and subsequent recovery of acute bacterial OM.

1. Introduction

Otitis media (OM) is one of the most common bacterial diseases associated with childhood and contributes a great deal to health care cost [1,2]. About 80% of children within 36 months report at least one ear infection [3]. If not treated properly, complications such as

mastoiditis, brain abscess, and meningitis can occur, and long-term hearing loss and related complications, such as developmental disorders of language, cognition, and learning ability, can produce a social burden [4–6]. Therefore, the proper diagnosis and treatment of acute OM is clinically and socially important. OM is known as a multifactorial disease. Eustachian tube dysfunction, nasal inflammation resulting from

* Corresponding author. Department of Otorhinolaryngology-Head and Neck Surgery, Dongguk University, Ilsan hospital, Siksa-dong, Ilsandong-gu, Goyang-si, Gyeonggi-do, 410-773, South Korea.

E-mail addresses: parkzzu19@naver.com, parkzzu19@dongguk.ac.kr (J.H. Park).

<https://doi.org/10.1016/j.ijporl.2020.110497>

Received 8 June 2020; Received in revised form 11 November 2020; Accepted 11 November 2020

Available online 13 November 2020

0165-5876/© 2020 Elsevier B.V. All rights reserved.

allergic rhinitis or upper respiratory infection as well as bacterial and viral infection are acknowledged to be contributing factors [7–9]. Major pathological findings of acute OM are hyperplasia and the transformation of the middle ear (ME) mucosa [10–13]. The proliferative response of ME mucosa following bacterial infection in the murine OM has been extensively reported [14–18]. Normal uninfected ME mucosa is a monolayer of simple squamous epithelium with a thickness of 15–20 μm . However, the mucosal thickness can increase to over 1000 μm in response to bacterial infection, and within a few days the mucosa can radically develop into a pseudostratified columnar epithelium containing secretory and ciliated cells [10–13,17,19]. These changes contribute significantly to the pathogenesis and symptoms of OM through the production of mucus and other bioactive secretions by various recruited inflammatory cells, as well as by ME cavity volume reduction [17].

Allergic rhinitis (AR) affects 10–30% of children and adults in the US and other industrialized countries and causes significant economic burden [20,21]. AR is strongly associated and probably causally related to Eustachian tube dysfunction, causing concomitant serous and acute OM [8]. In many epidemiologic surveys of AR and OM, especially OM with effusion, AR has been an important risk factor for OM with effusion [22,23]. There was a 33.8% prevalence of AR in a group of OM patients, which was significantly higher than the 16% prevalence in a control group of children without OM with effusion [24]. However, the mechanism by which AR causes or aggravates OM with effusion, or whether OM related to AR has a different reaction to bacterial infections with OM related other etiologic factors, has not yet been fully understood. Several possible mechanisms by which allergies could be responsible for OM with effusion were suggested. First, inflammatory swelling of eustachian tube related to inflammatory obstruction of the nose or aspiration of bacteria-laden allergic nasopharyngeal secretions into the ME cavity may result in OM with effusion [25]. The ME might function as a target organ of allergic disease [25]. The level of type 2 helper T cell (Th2) cytokines in ME effusion and mucosal biopsy was higher in patients with AR than without AR [26–28]. In a murine study using an acute AR model, the higher incidence of OM was observed in the AR group and the type 1 helper T cell (Th1) and Th2 response cytokines were expressed at higher levels in response to intratympanic lipopolysaccharide (LPS) injection than in the control group [28–30].

Thus, AR can be considered as a factor that delays or worsens the recovery of acute OM, and the process of turning to OM with effusion. Most of the studies about AR and OM were epidemiologic studies and focused on allergic casual effects on effusion. The mechanism of OM with effusion related to AR, coexistent AR, and OM was not fully elucidated. Little research about the effect of AR on development, progression, and recovery of acute OM has been reported before. There has been no complex animal model of OM and AR, especially bacterially induced OM by bacterial injection via bulla.

A complex AR and OM (AR + OM) murine model was generated successfully by using ovalbumin (OVA) and *Haemophilus influenzae*. We aimed to evaluate the effect of AR on development, progression, and recovery of acute OM, and investigated the immunologic reactions to bacterial infection using an in vivo AR + OM murine model.

2. Materials and methods

2.1. Animals

Ninety-six BALB/c mice (four-week-old male, average 15 g in weight) were purchased from KOATECH (Gimpo, Korea) and bred in a specific pathogen-free animal facility. Mice were divided into four groups, with 24 mice in each group: (a) control group, mice were sensitized, challenged, and inoculated with phosphate-buffered saline (PBS); (b) AR group, mice were sensitized with ovalbumin (OVA) plus Alum, challenged with 3% OVA, and inoculated with PBS, (c) OM group, mice were sensitized with PBS, and then inoculated with non-typeable *Haemophilus influenzae* 3655 strains (NTHi3655); (d) AR + OM group

mice were sensitized with OVA plus Alum, challenged with 3% OVA, and then inoculated with NTHi3655. Each group was subdivided into sets of six mice (12 ears), one for each of the four time points (0, 2, 7, and 10 days post-bacterial inoculation), at which point the mice were euthanized and ME and nasal cavity mucosa were obtained and evaluated. The procedures for AR and OM induction of four groups are summarized in Fig. 1.

The animal study protocol was approved by the Institutional Animal Care and Use Committee of Dongguk University Ilsan Hospital. (IACUC#2017-04164).

2.2. Allergic rhinitis induction for mouse model

Allergic rhinitis was induced in the AR and AR + OM groups. Ovalbumin (OVA, Albumin from chicken egg white, St. Louis USA, Sigma, #A5503), and Alum (Thermo Scientific, Imject® Alum, #77161, Rockford, IL, USA; an aqueous solution of aluminum hydroxide 40 mg/ mL and magnesium hydroxide 40 mg/ mL) were used for AR induction. The mice were sensitized by an intraperitoneal (IP) injection of 25 μg OVA emulsified in 2 mg of Alum in a total volume of 200 μL phosphate-buffered saline (PBS) on day 1, followed by a 7-day rest period.

After general sensitization, mice were locally challenged with 20 μL of 3% OVA into each nasal cavity from day 14 to day 49 [29,31]. The nasal challenge was conducted for five days in the first week and three times per week for the next four weeks. Intranasal instillation was performed in the head-down position, which was maintained for 30 s after instillation to prevent pulmonary provocation and to make sure the mouse was properly fixed at the head so that the injection site was visible. After nasal infusion, bubbles form in the nasal cavity of mice. The control and OM groups were intraperitoneally injected with PBS instead of OVA on the same schedule (Fig. 1).

2.3. Confirmation of AR induction

Nasal symptom scores and mouse OVA-specific immunoglobulin E (OVA-sIgE) in nasal mucosa were evaluated to confirm successful induction of AR. After the last nasal challenge using 3% OVA, the frequencies of sneezing and nasal rubbing were observed for 10 min and recorded by blinded observer. Nasal symptom scores were counted after nasal challenge with PBS in the control and OM groups.

Nasal mucosa were scraped with a surgical spatula after dividing the nasal septum in half. Tissue Extraction Reagent I buffer (# FNN0071, ThermoFisher Scientific, Vienna, Austria) and protease inhibitor (P-2714, Sigma, St. Louis, USA) were used to prepare nasal mucosa proteins. For determination of OVA-sIgE, interleukin (IL)-4, and IL-5 level in the nasal mucosa, mouse OVA-sIgE ELISA Kit (#MBS764157, MyBioScience, MN, USA), IL-4 mouse ELISA kit (ab100710, abcam®, MA, USA), and IL-5 mouse ELISA kit (ab100711, abcam®, MA, USA) were used. Each extractive concentration was determined using

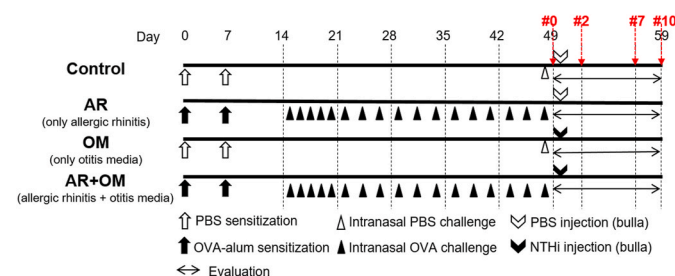


Fig. 1. Experimental protocol for generating the murine model of allergic rhinitis (AR), otitis media (OM), allergic rhinitis + otitis media (AR + OM). Ovalbumin (OVA) was used to induce AR and a non-typeable *Haemophilus influenzae* (NTHi) suspension was administered into the bullae to induce acute OM.

spectrophotometry (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA) at absorbance density 450 nm.

2.4. Otitis media induction in the AR mouse model

Acute bacterial OM was induced in the OM and AR + OM groups. Non-typeable *Haemophilus influenzae* (NTHi) strains 3655 were colonized with chocolate II Agar (#221169, GC II Agar with Hemoglobin And IsoVitalax, USA) and inoculated into the middle ear cavity after overnight growth in BHI culture (#B9500, Brain heart infusion broth, Teknova, USA). OM was surgically induced by inoculation with a suspension of NTHi 3655 into the ME cavities of the mice through the tympanic bulla using a ventral surgical approach via an anterior neck incision [18,32]. All surgeries were performed under anesthesia, and all efforts were made to minimize the animals' suffering. The bullae of the mice were bilaterally injected with 10 μ l PBS containing 10⁵ cells/ml NTHi 3655. In the control and AR groups, PBS was inoculated into bulla instead of NTHi on the same schedule. Before and after NTHi or PBS inoculation, the tympanic membrane was visually confirmed to be intact using a digital otomicroscope (#AM4113, Dino-Lite Digital Microscope, ANMO Electronics Corporation, USA) and a 2.8 mm 30° endoscope. The tympanic membranes of each group were observed on post-inoculation day 0, 2, 7, and 10 to confirm the occurrence and progress of OM.

2.5. Evaluation of the middle ear mucosa thickness

The bullae were fixed in 4% neutral buffered formalin for 24 h, decalcified overnight using a decalcifying solution (D0818, Sigma-Aldrich, St. Louis, MO, USA). The tissue was embedded in paraffin and sliced to a thickness of 4 μ m on a microtome (RM2235, Leica®, Nussloch, Germany). For the analysis of mucosal thickness, hematoxylin (HES32, Sigma-Aldrich) and eosin Y (E6003, Sigma-Aldrich; H&E) stains were used on the prepared slides. After H&E staining, 200-fold images were stored and analyzed using an optical microscope. ME mucosa thickness analysis was performed by measuring three widths in a photograph and then collecting the measurement widths for each group to obtain an overall average.

2.6. Expression of cytokines and allergic factors in the middle ear mucosa

To determine the expression of proinflammatory cytokines and allergic factors in ME mucosa, after all slides were deparaffinized, 3% fetal bovine serum was applied. Antibodies for with interleukin (IL)-1 β (ORB228386, Biorbyt Ltd., CB4 0WY, UK), IL-6 (ORB303667, Biorbyt Ltd., CB4 0WY, UK), tumor necrosis factor (TNF)- α (ab6671, abcam®, MA, US), IL-4 (ORB10908, Biorbyt Ltd., CB4 0WY, UK), and IL-5 (16-7052-81, Thermo, MN, USA) were diluted at 1:100, reacted at 4 °C, and then coupled with 3, 3'-diaminobenzidine (DAB; Code K3468, Dako North America Inc., California, USA) for color development. We obtained microscopic images and photographs of each slide at X400 magnification. All the detected antibodies were collected using Qwin V3 (Software Version6, Leica Microsystems Imaging Solutions Ltd, Cambridge, UK) digital image processing and analysis software.

2.7. Statistical analysis

All data are presented as mean $\pm$ standard error of the means (SEM). The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) for Windows, Version 18.0. The criterion for statistical significance was set at $p < 0.05$. Fisher's exact test was used to compare the occurrence of OM at each time point among groups. ME mucosa thickness was analyzed by using one-way ANOVA with post-hoc comparison among groups. Kruskal-Wallis tests were used to compare differences in each of the proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) and Th2 cytokine (IL-4 and IL-5) expression among groups, and Mann-Whitney U tests were used to test for differences between two

groups. A Mann-Whitney U test was used to evaluate nasal symptom scores and IgE levels between the AR induction group (AR and AR + OM groups) and the other groups (control and OM groups).

3. Results

3.1. Allergic rhinitis symptom scores and OVA-specific IgE expression

The number of nasal rubbings and sneezing was significantly higher in the AR induction group (AR and AR + OM groups) than in the non-AR groups (Control and OM groups; Fig. 2A; $p < 0.001$).

AR induction groups showed higher OVA-sIgE levels than non-AR groups on day 0, 2, 7, and 10 (Fig. 2B; $p < 0.001$). The OVA-sIgE expression was not different among the post-inoculation day 0, 2, and day 7. However, the expression of OVA-sIgE on post-inoculation day 10 (98.2 ± 25.1 ng/ml) showed significantly lower level than those of day 0 (148.5 ± 27.2 ng/ml), day 2 (177.4 ± 23.9 ng/ml) and day 7 (171.6 ± 35.3 ng/ml), although this still significantly higher than non-AR group (Fig. 3B).

3.2. Occurrence of otitis media

Middle ear effusion was observed in 10% of ears of the AR and AR + OM groups after AR induction, just before the NTHi inoculation. All mice in the other groups showed normal eardrums. After NTHi or PBS inoculation into the bullae, the tympanic membrane was evaluated at each time point. On post-inoculation day 2, most of the ears showed OM in both AR + OM (100%) and OM groups (91.7%). Several ears in the AR group (12.5%) and the control group (8.3%) also showed OM on day 2. On post-inoculation day 7, OM was observed in 93.8% of the AR + OM group and 43.8% of the OM group. On post-inoculation day 10, 56.3% of AR + OM group still had OM, while only 18.8% of OM group had OM. The occurrence of OM was significantly different among the four groups on post-inoculation day 2, 7, and 10 (Fig. 3; $p < 0.001$, $p < 0.001$, and $p = 0.001$, respectively). The AR + OM group showed higher OM occurrence than the OM group on the post-inoculation day 7 ($p = 0.001$) and day 10 ($p = 0.033$) statistically (Fig. 3).

3.3. Middle ear mucosa thickness

The ME mucosa thickness before inoculation was 29.2 ± 2.8 μ m in the AR group and 32.3 ± 2.7 μ m in the AR + OM group, and both were thicker than those of the control group (14.7 ± 1.4 μ m) and OM group (17.6 ± 1.6 μ m). The ME mucosa of all groups increased after NTHi or PBS inoculation, and the AR + OM group exhibited the thickest mucosa. The mucosa thickness of the AR + OM group was significantly thicker than the ME mucosa of the OM group on post-inoculation day 2 (219.2 ± 10.7 μ m vs. 126.0 ± 2.8 μ m). The AR + OM group showed 281.1 ± 16.9 μ m, the thickest ME mucosa on post-inoculation day 7. The thickness of the ME mucosa was 100.3 ± 7.0 μ m and 112.6 ± 4.4 μ m in the AR and OM groups. The ME mucosa of the AR + OM group grew more than that of the OM group at post-inoculation day 2, 7, and 10 ($p < 0.001$). The OM group showed peak thickness on post-inoculation day 2 and decreased gradually, whereas the ME mucosa thickness of the AR + OM group increased until day 7 (Fig. 4).

3.4. Immunohistochemical staining analysis for IL-1 β , IL-6, and TNF- α

Immunohistochemical staining showed that the proinflammatory cytokine IL-1 β detection area increased on day 7 ($8.03 \pm 0.47\%$) compared with day 2 ($5.43 \pm 0.4\%$) in the AR + OM group, while it decreased on day 7 ($2.5 \pm 0.18\%$) compared with the day 2 ($4.42 \pm 0.2\%$) in the OM group (Fig. 5A). This difference in the detection area was statistically significant on days 7 ($p < 0.001$) and day 10 ($p < 0.001$). The AR + OM group exhibited a statistically significant increase in expression compared to the other groups on post-inoculation day 7 and

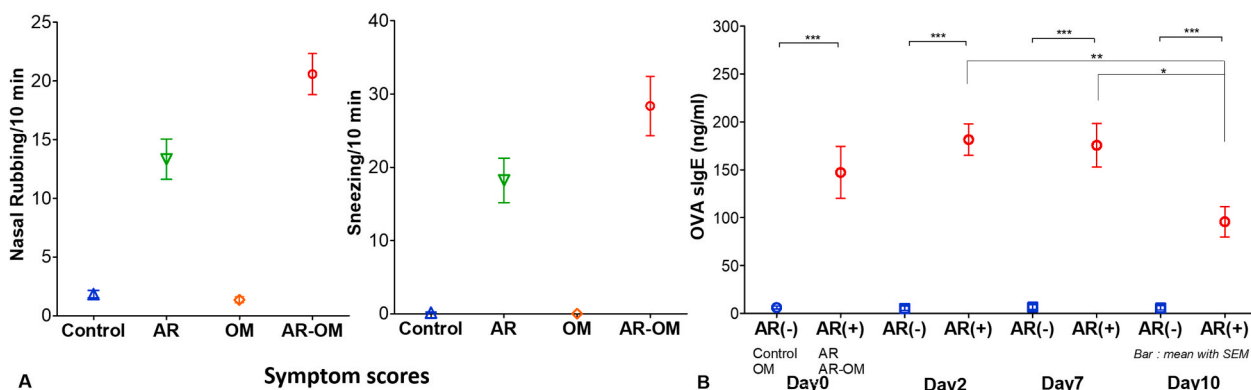


Fig. 2. Symptom scores and expression of OVA-specific IgE in nasal mucosa. The number of nasal rubbings and sneezing (A) and OVA-specific IgE expression (B) were all significantly higher in the AR induction groups (AR and AR + OM groups) than in the non-AR groups (control and OM groups). The mean $\pm$ 1 SEM is shown; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

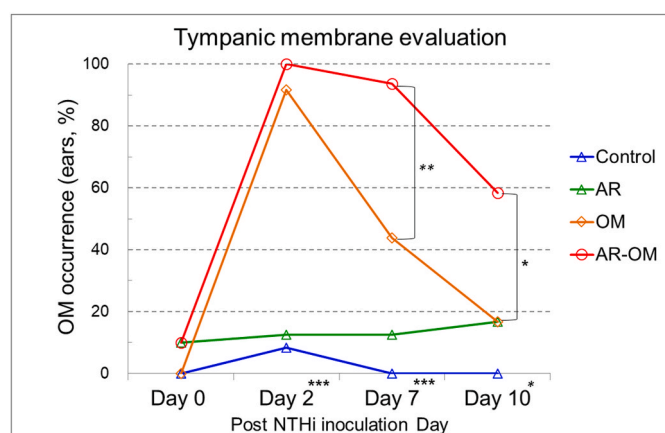


Fig. 3. Occurrence of OM. Most of the ears with bacterial administration show OM on post-inoculation day 2. The AR + OM group showed higher OM occurrence than the OM group on post-inoculation days 7 and 10. The mean $\pm$ 1 SEM is shown; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

10. The IL-6 expression area in the AR + OM group was significantly higher compared to the other groups on post-inoculation days 7 ($4.25 \pm 0.27\%$) and 10 ($2.60 \pm 0.34\%$). The detection area of IL-6 in the AR + OM group was significantly higher compared to the OM group on the post-inoculation day 7 and 10 ($p < 0.001$; Fig. 5B). The TNF- α expression area was $4.26 \pm 0.36\%$ in the AR + OM group and $3.52 \pm 0.39\%$ in

the OM group on post-inoculation day 2. TNF- α expression decreased after day 2 in the OM group ($2.19 \pm 0.12\%$), but increased more in the AR + OM group ($5.14 \pm 0.34\%$) on post-inoculation day 7; difference was significant ($p < 0.001$; Fig. 5C). Expression of IL-1 β , IL-6, and TNF- α exhibited a similar pattern with changes in mucosa thickness in four groups.

3.5. Expression of IL-4 and IL-5 in the nasal mucosa

IL-4 expression levels were higher in the AR induction group (AR and AR + OM groups) than in the non-AR groups (control and OM group) on day 0 ($p < 0.001$) and on post-inoculation days 2 ($p = 0.005$), 7 ($p = 0.004$), and 10 ($p = 0.027$; Fig. 6A). On post-inoculation day 2, IL-4 expression in the nasal mucosa tended to increase in all groups, although the change was not significant. Higher IL-5 expression was also observed in the AR induction groups than the non-AR groups on day 0 (after AR induction, $p = 0.001$) and on post-inoculation days 2 ($p = 0.003$), 7 ($p = 0.002$), and 10 ($p = 0.008$; Fig. 6B).

4. Discussion

In this study, we evaluated the effects of AR on acute OM, focusing on the period needed for OM recovery after bacterial infection and the proliferative response of ME mucosa, which is the major pathology in OM. Most of the ears showed OM in both AR + OM and OM groups. Seven days after NTHi infection, nearly half of the OM group showed normal TM, while AR + OM mouse mostly still exhibited OM on tympanic membrane evaluation. More than half (58.3%) of the AR + OM

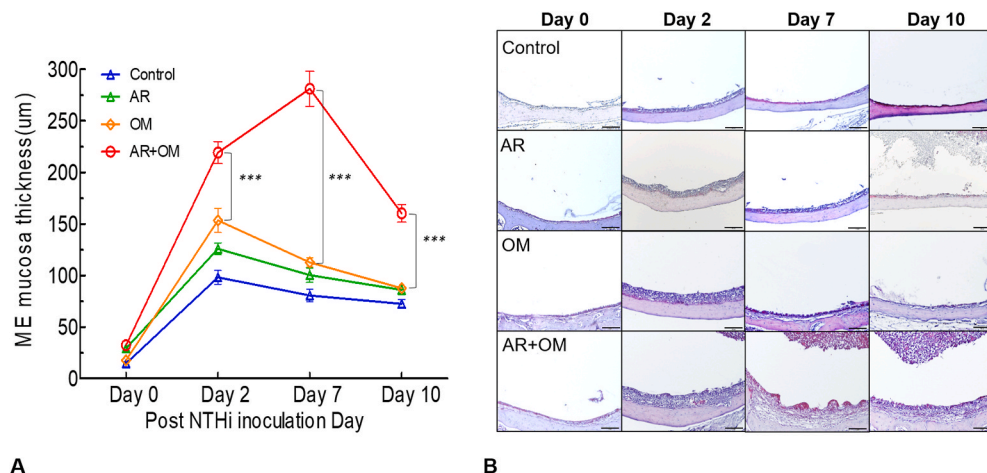


Fig. 4. Measured middle ear (ME) mucosa thickness and ME mucosa with H&E staining ($\times 200$ magnification). The ME mucosa of all groups increased after NTHi or PBS inoculation. The mucosa thickness of the AR + OM group was significantly thicker than the ME mucosa of the OM group on post-inoculation days 2, 7, and 10 (A). The OM group showed peak thickness on post-inoculation day 2 and then a gradual decrease, whereas the ME mucosa thickness of the AR + OM group increase to day 7 (A and B). The mean $\pm$ 1 SEM is shown; ***, $p < 0.001$; Scale bar = 50 μ m.

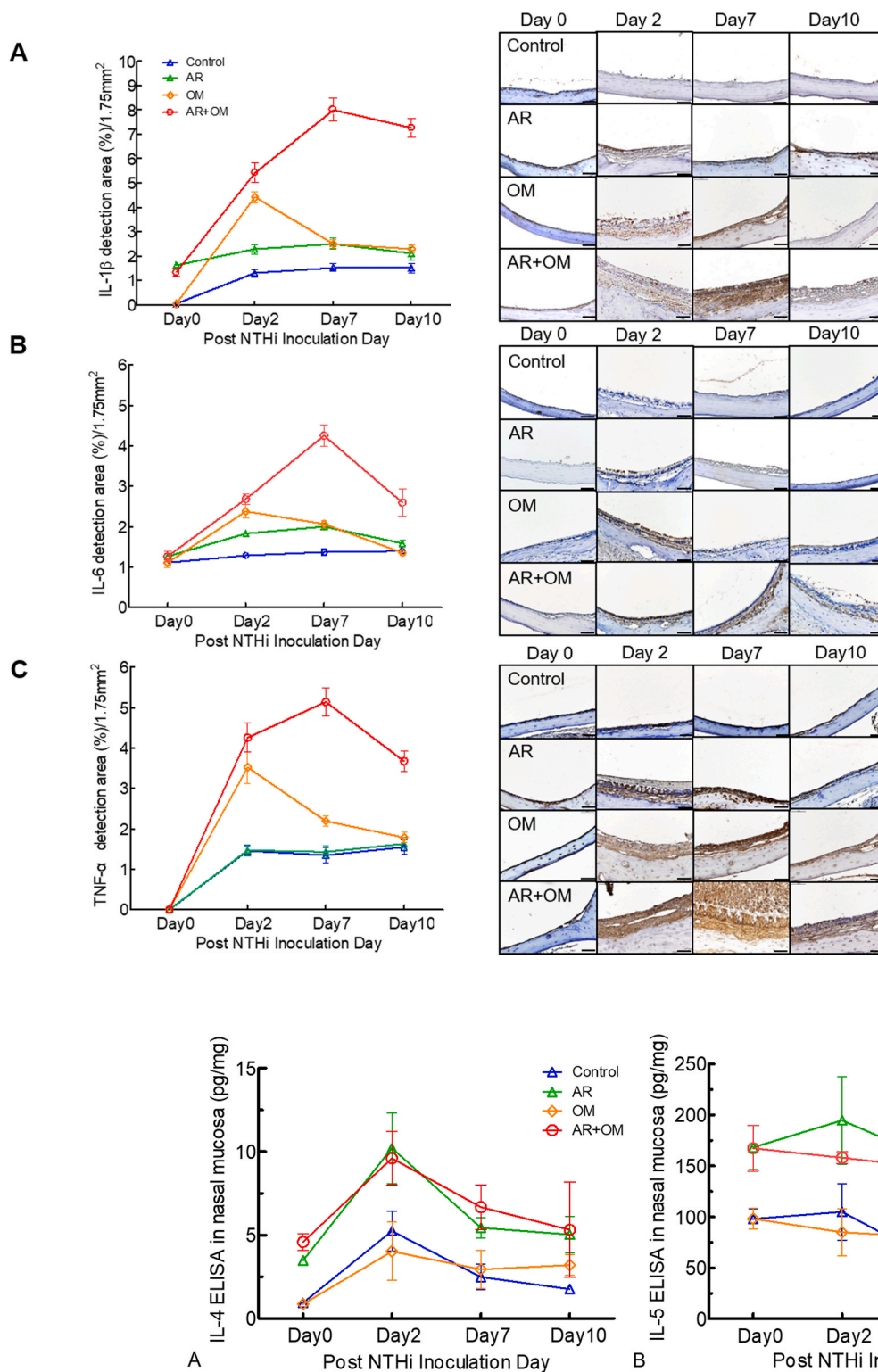


Fig. 6. Cytokine profile from the nasal mucosa for each group. Concentrations of IL-4 (A) and IL-5 (B) were more elevated in the AR and AR + OM groups than in the OM and control groups. The mean $\pm$ 1 SEM is shown.

group still had OM by post-NTHi inoculation day 10, while only 16.7% of OM group had OM in OM group. The ME mucosa of all groups increased, and the ME mucosa of the AR + OM group was significantly thicker than that of the other groups. The OM group showed peak thickness on post-inoculation day 2 and thereafter a decrease, whereas the ME mucosa thickness of the AR + OM group continued to increase to day 7. The expression of proinflammatory cytokines (IL-1 β , IL-6, and

Fig. 5. Expression of proinflammatory cytokines in the middle ear. Immunohistochemical staining of IL-1 β (A), IL-6 (B), and TNF- α (C) shows that expression of IL-1 β , IL-6, and TNF- α at the mucosal epithelium was elevated on post-inoculation day 2 in the OM and AR + OM groups. The OM group showed a peak on post-inoculation day 2 and then a gradual decrease. In the AR + OM group, levels increased until day 7 and then decreased. This group exhibited a higher maximum value and a slower rate of decrease than the OM group. The mean $\pm$ 1 SEM is shown; Scale bar = 50 μ m.

TNF- α) in the ME also showed similar patterns to that of the ME mucosa thickness. In the AR + OM group, the expression of cytokines increased until day 7 and then decreased. In this group, a higher maximum value was exhibited than in the OM group, which showed a peak on day 2 and then a gradual recovery. The inflammatory reaction to NTHi was more intense and lasted longer in the allergic group.

We observed the most proliferative mucosal response and

inflammatory changes two days after bacterial inoculation in earlier studies with the NTHi-induced OM rat model [18,32]. The expression of proinflammatory cytokines as well as mucosal thickness showed peaks by day 2 and decreased gradually, normalizing 7–10 days after infection [18,32]. In this study with a mouse model, the OM group showed consistent results with previously reported research. Mucosal thickness and detection area of IL-1 β , IL-6, and TNF- α were also increased in the PBS-injected control and AR groups, as well as the OM and AR + OM groups, compared to that on day 0 (Figs. 4 and 5). NTHi or PBS inoculation was performed surgically. We considered that surgical dissection to approach the ME cavity (bullae) and bony bulla wall breaks by injecting PBS might have induced subsequent inflammation of adjacent mucosa and soft tissue around the bulla opening sites, even in the control group and AR group and, consequently, an increase in the expressions of inflammatory cytokines. However, differences were clear in the elevation of cytokine level were observed between the NTHi-injected and PBS-injected groups.

When only AR was induced before NTHi inoculation into the bulla, ME effusion already occurred in about 10% of ears. These may result from the eustachian tube dysfunction caused by AR. Kim et al. reported that OM with effusion was observed in 75% of the allergic animals [33]. There is a discrepancy between our findings and those from the previous study. The previous study only evaluated a few ears (eight ears) and used an acute AR model (only one week of nasal OVA challenge) [33], whereas this study used a chronic AR model with a five-week nasal challenge for stable AR induction during a long experiment time. Possible mechanisms by which AR could be responsible for the occurrence of OM with effusion have been previously suggested [25]. The first conceivable cause is that inflammatory swelling or obstruction of the eustachian tube creates negative pressure in the ME cavity and causes OM with effusion. Aspiration of bacteria-laden allergic nasopharyngeal secretions into the ME cavity could also be a cause. It has also been suggested that the ME can function as a “shock organ” of allergy [25]; this is an interesting hypothesis for clinicians to test. If so, such cases of OM can be handled in a different way from the traditional observation or treatment, and more aggressive allergic control can be used. Several studies have reported increased Th2 cytokine levels in ME effusions and mucosa [26–28,34]. Serum Ig E levels showed a significant correlation with the Ig E levels of ME secretion in patients with acute/chronic suppurative OM [35]. Other studies demonstrated higher IL-4 and eosinophils in ME effusion of atopic patients with OM than non-atopic patients with OM. Higher IL-4 levels were observed in the ME effusion of AR patients than in non-AR subjects in a human study [28]. Other authors have also suggested that Th2 cytokines such as IL-4 may be related to the pathogenesis of OM with effusion in AR patients, whereas Th1 cytokines potentially are related in OM patients unrelated to allergies [36].

In this regard, this study evaluated IL-4 and IL-5 levels in the ME mucosa of all four groups, although our study induced additional bacterial infection on allergies and was not suitable to observe the effects of allergies alone. IL-4 and IL-5 expression in the ME mucosa of AR and AR + OM groups tended to be higher than those in the control and OM groups; however, the measured amount of cytokines in ME was too small to compare the difference statistically.

To determine whether bacterial infection itself affects AR, the changes in IL-4 and IL-5 expression in the nasal mucosa, which are associated with allergies, were evaluated (Fig. 6). The results of this study were inconclusive. Levels of expression were higher in the AR induction group (AR and AR + OM groups) than in the non-AR groups. IL-4 expression tended to increase in all groups on post-inoculation day 2; however, there was no statistical difference after bacterial infection. Elevation was also observed even in PBS-injected groups (control and AR groups). IL-5 expression showed no statistical difference among days after NTHi inoculation.

There were several limitations in interpreting this study. As mentioned before, this study used a chronic AR model for stable AR

induction. AR induction groups (AR and AR + OM group) showed higher OVA-sIgE levels than non-AR groups for all experimental periods. However, mice sacrificed on day 10 showed significantly lower OVA sIgE than subjects evaluated on days 2 and 7 (Fig. 2). These differences may affect the results. The present study only focused on acute OM with comorbid AR. OM with effusion in AR patients may be related to infection initially; however, the majority may not be related to bacterial infection, and they may have different mechanisms. Thus, our results cannot apply all the cases of OM subjects with AR. To elucidate OM development and recovery in AR subjects, further studies investigating the role of allergy and eustachian tube dysfunction in OM are needed. This study induced AR locally (intranasal OVA challenge), though systemic sensitization with OVA was performed initially. Serum levels of the Th1 and Th2 cytokine were not investigated in the AR + OM complex model. It will be interesting to study whether regeneration in the progression and recovery of acute OM can be mediated via local or systemic mechanisms.

Despite these limitations, these results are meaningful because experimental studies on the relationship between AR and bacterial OM are rare. Most previous studies about AR and OM (majority was OM with effusion) were epidemiologic studies and focused on allergic casual effects on effusion. Little research about the effect of AR on development, progression, and recovery of acute OM has been reported before. Some insights have been made into the pathophysiological changes underlying this association. The results of this study showed a significant association between AR and bacterial OM and could provide data that support the hypothesis that AR could influence the progression and recovery of acute OM caused by bacterial infection histopathologically. In addition, the AR + OM combined mouse model, which was technically difficult for experimenters, was successfully generated. In particular, this is the first AR + OM model applying transbullae bacterial inoculation, which is a more typically physiological route for OM infection, rather than injecting the bacteria through the eardrum simply. This animal model may help to serve as a basic model for immunologic and pathological studies in various comorbid conditions with AR and/or OM in the future. We demonstrated that pathological changes in the ME mucosa derived from acute bacterial OM were more severe and needed longer recovery times in mice with comorbid AR compared to those without AR. This indicates that AR affects the progression and subsequent recovery of acute bacterial OM. In consultation with AR children and their parents, this study will provide more detailed information on the importance of AR control in OM occurrence and recovery. However, we do not yet understand the relationship between Th1 and Th2 cytokines in the pathogenesis of acute OM and AR and whether OM affects AR. Further, studies are needed to evaluate the mechanism and relationship between AR and OM. These studies will contribute to the advancement in the understanding and treatment of childhood OM with AR.

5. Conclusion

The inflammatory reaction to NTHi was more intense and lasted longer in the AR + OM group in mice. These findings suggested that bacterial OM in allergic subjects can develop to a more severe stage and is resolved more slowly.

Financial disclosures/conflict of interest

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. NRF-2017R1C1B5017129 to J.H. Park). The funding bodies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

- [1] S. Ahmed, N.L. Shapiro, N. Bhattacharyya, Incremental healthcare utilization and costs for acute otitis media in children, *Laryngoscope* 124 (2014) 301–305.
- [2] M.M. Rovers, A.G. Schilder, G.A. Zielhuis, R.M. Rosenfeld, Otitis media, *Lancet* 363 (9407) (2004) 465–473.
- [3] D.W. Teele, J.O. Klein, B. Rosner, Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study, *J. Infect. Dis.* 160 (1) (1989) 83–94.
- [4] R. DeAntonio, J.P. Yarzabal, J.P. Cruz, J.E. Schmidt, J. Kleijnen, Epidemiology of otitis media in children from developing countries: a systematic review, *Int. J. Pediatr. Otorhinolaryngol.* 85 (2016) 65–74.
- [5] N. Shirai, D. Preciado, Otitis media: what is new? *Curr. Opin. Otolaryngol. Head Neck Surg.* 27 (6) (2019) 495–498.
- [6] M. Al Khabori, R. Khandekar, The prevalence and causes of hearing impairment in Oman: a community-based cross-sectional study, *Int. J. Audiol.* 43 (8) (2004) 486–492.
- [7] R. Mittal, J. Kodyan, R. Gerring, et al., Role of innate immunity in the pathogenesis of otitis media, *Int. J. Infect. Dis.* 29 (2014) 259–267.
- [8] B. Yang, C.D. Brook, The role of allergy in otologic disease, *Otolaryngol. Clin.* 50 (6) (2017) 1091–1101.
- [9] J.M. Coticchia, M. Chen, L. Sachdeva, S. Mutchnick, New paradigms in the pathogenesis of otitis media in children, *Front Pediatr* 1 (2013) 52.
- [10] D.J. Lim, H. Birk, Ultrastructural pathology of the middle ear mucosa in serous otitis media, *Ann. Otol. Rhinol. Laryngol.* 80 (6) (1971) 838–853.
- [11] M. Tos, P. Caye-Thomasen, Mucous glands in the middle ear - what is known and what is not, *ORL J Otorhinolaryngol. Relat Spec* 64 (2) (2002) 86–94.
- [12] C.D. Bluestone, J.O. Klein, Otitis media with effusion, atelectasis, and Eustachian tube dysfunction, in: C.D. Bluestone, S. Stool (Eds.), *Pediatric Otolaryngology*, Saunders, Philadelphia, 1983, pp. 356–512.
- [13] H.F. Schuknecht, *Pathology of the Ear*, Lea & Febiger, Philadelphia, 1993.
- [14] M. Hernandez, A. Leichtle, K. Pak, et al., Myeloid differentiation primary response gene 88 is required for the resolution of otitis media, *J. Infect. Dis.* 198 (12) (2008) 1862–1869.
- [15] A. Leichtle, M. Hernandez, K. Pak, et al., TLR4-mediated induction of TLR2 signaling is critical in the pathogenesis and resolution of otitis media, *Innate Immun.* 15 (4) (2009) 205–215.
- [16] A.F. Ryan, J. Ebmeyer, M. Furukawa, et al., Mouse models of induced otitis media, *Brain Res.* 1091 (2006) 1–8.
- [17] C.G. Cho, K. Pak, N. Webster, A. Kurabi, A.F. Ryan, Both canonical and non-canonical NF-kappaB activation contribute to the proliferative response of the middle ear mucosa during bacterial infection, *Innate Immun.* 22 (8) (2016) 626–634.
- [18] G.H. Yu, H.B. Kim, S.H. Ko, et al., Expression of surfactant Protein-A in the Haemophilus influenzae-induced otitis media in a rat model, *Int. J. Pediatr. Otorhinolaryngol.* 112 (2018) 61–66.
- [19] A.F. Ryan, A. Catanzaro, S.I. Wasserman, J.P. Harris, Secondary immune response in the middle ear: immunological, morphological, and physiological observations, *Ann. Otol. Rhinol. Laryngol.* 95 (3 Pt 1) (1986) 242–249.
- [20] K. Singh, S. Axelrod, L. Bielory, The epidemiology of ocular and nasal allergy in the United States, 1988–1994, *J. Allergy Clin. Immunol.* 126 (2010) 4778–783.e776.
- [21] W.F. Wu, K.S. Wan, S.J. Wang, W. Yang, W.L. Liu, Prevalence, severity, and time trends of allergic conditions in 6-to-7-year-old schoolchildren in Taipei, *J. Investig. Allergol. Clin. Immunol.* 21 (7) (2011) 556–562.
- [22] H. Byeon, The association between allergic rhinitis and otitis media: a national representative sample of in South Korean children, *Sci. Rep.* 7 (1) (2019) 1610.
- [23] B.C. Pau, D.K. Ng, Prevalence of otitis media with effusion in children with allergic rhinitis, a cross sectional study, *Int. J. Pediatr. Otorhinolaryngol.* 84 (2016) 156–160.
- [24] C. Kwon, H.Y. Lee, M.G. Kim, S.H. Boo, S.G. Yeo, Allergic diseases in children with otitis media with effusion, *Int. J. Pediatr. Otorhinolaryngol.* 77 (2) (2013) 158–161.
- [25] C.D. Bluestone, Eustachian tube function and allergy in otitis media, *Pediatrics* 61 (5) (1978) 753–760.
- [26] E.D. Wright, D. Hurst, D. Miotto, C. Giguere, Q. Hamid, Increased expression of major basic protein (MBP) and interleukin-5(IL-5) in middle ear biopsy specimens from atopic patients with persistent otitis media with effusion, *Otolaryngol. Head Neck Surg.* 123 (5) (2000) 533–538.
- [27] C.H. Jang, Y.H. Kim, Characterization of cytokines present in pediatric otitis media with effusion: comparison of allergy positive and negative, *Int. J. Pediatr. Otorhinolaryngol.* 66 (1) (2002) 37–40.
- [28] S. Kariya, M. Okano, H. Hattori, et al., TH1/TH2 and regulatory cytokines in adults with otitis media with effusion, *Otol. Neurotol.* 27 (8) (2006) 1089–1093.
- [29] D.W. Kim, K.M. Eun, H.R. Jin, S.H. Cho, D.K. Kim, Prolonged allergen exposure is associated with increased thymic stromal lymphopoietin expression and Th2-skewing in mouse models of chronic rhinosinusitis, *Laryngoscope* 126 (8) (2016) E265–E272.
- [30] C.F. Gunville, P.M. Mourani, A.A. Ginde, The role of vitamin D in prevention and treatment of infection, *Inflamm. Allergy - Drug Targets* 12 (4) (2013) 239–245.
- [31] C. Infante-Rivard, A. Fernández, Otitis media in children: frequency, risk factors, and research avenues, *Epidemiol. Rev.* 15 (2) (1993) 444–465, <https://doi.org/10.1093/oxfordjournals.epirev.a036129>.
- [32] C.G. Cho, S.H. Gong, H.B. Kim, et al., Role of group 3 innate lymphoid cells during experimental otitis media in a rat model, *Int. J. Pediatr. Otorhinolaryngol.* 88 (2016) 146–152.
- [33] D.K. Kim, H.E. Park, S.A. Back, et al., Otitis media with effusion in an allergic animal model: a functional and morphological study, *Int. J. Pediatr. Otorhinolaryngol.* 84 (2016) 6–11.
- [34] M. Hotomi, T. Samukawa, N. Yamanaka, Interleukin-8 in otitis media with effusion, *Acta Otolaryngol.* 114 (4) (1994) 406–409.
- [35] A.O. Lasisi, O.G. Arinola, O. Olayemi, Role of elevated immunoglobulin E levels in suppurative otitis media, *Ann. Trop. Paediatr.* 28 (2) (2008) 123–127.
- [36] L.H. Nguyen, J.J. Manoukian, S.E. Sobol, et al., Similar allergic inflammation in the middle ear and the upper airway: evidence linking otitis media with effusion to the united airways concept, *J. Allergy Clin. Immunol.* 114 (5) (2004) 1110–1115.