

ORIGINAL RESEARCH

Immune modulatory oligonucleotides in the prevention and treatment of OVA-induced eustachian tube dysfunction in rats

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BACKGROUND: Otitis media with effusion (OME) is often associated with allergies. Immune modulatory oligonucleotides (IMO) mediate allergic inflammation and may therefore be efficacious in the treatment of airway inflammation.

OBJECTIVE: To evaluate the role of an IMO via transtympanic mucosal application in prevention and treatment of ovalbumin-induced OME.

DESIGN: Forty brown Norway rats were divided into control and treatment groups. Eustachian tube dysfunction was evaluated by passive opening pressures, passive closing pressures, active clearance of negative pressure, and mucociliary clearance transit time.

RESULTS: Rats who underwent IMO treatment required 50% less pressure to open and close the eustachian tube ($P < 0.05$) and were able to actively clear 50% more negative pressure than the ovalbumin-control rats ($P < 0.001$). The treatment rats' mucociliary clearance time was half that of the control group ($P < 0.001$).

CONCLUSION: IMO via transtympanic application can prevent and treat allergy-induced eustachian tube dysfunction in rats. IMO may offer substantial promise in the future management of OME.

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Otitis media with effusion (OME) is the chronic inflammation of the middle ear mucosa with presence of fluid in the middle ear. Persistent fluid in the middle ear results in decreased mobility of the tympanic membrane and is a barrier to sound conduction. OME continues to be the leading cause of hearing loss and impaired speech development in children today.¹

The cause of OME is multifactorial. Allergy has been suggested as a potential predisposing factor for OME.² Epidemiologic studies have shown over 50% of patients with otitis media have allergic rhinitis, whereas 21% of allergic rhinitis patients have otitis media.³ There is support for the theory that allergy-induced inflammation of the middle ear mucosa and eustachian tube dysfunction (ETD) are contributing factors to the pathogenesis of

OME.⁴ The eustachian tube plays a key role in middle ear function by regulating air pressure inside the ear.⁵ Fireman⁵ identified four potential mechanisms for the role of allergy in OME: 1) middle ear mucosa as a target organ, 2) inflammatory swelling of the eustachian tube, 3) inflammatory obstruction of the nose and nasopharynx, and 4) reflux of allergic nasopharyngeal secretions into the middle ear cavity.

In the united airways concept, the middle ear space is an anatomic extension of the airway via the eustachian tube.¹ Like mucosa elsewhere in the respiratory tract, the mucosa that lines the eustachian tube contains mucus-producing cells, ciliated cells, plasma cells, and mast cells.⁶ To date, no consistent differences between the inflammatory profiles of the upper and lower airway have been identified. Previous studies suggest that the allergic response in OME is a result of an increased number of eosinophils, mast cells, and T_H2 lymphocytes similar to patients with asthma. T_H2 cells are T lymphocytes that synthesize and secrete interleukin (IL)-4, IL-5, IL-9, and IL-13. These cytokines promote IgE production, eosinophil growth, T_H2 cell development, and mucous production. In contrast, T_H1 cells antagonize allergic inflammation.⁷

New strategies for altering this inflammatory response use immune modulatory oligodeoxynucleotides (IMO) that contain synthetic immunostimulatory motifs.⁸ These oligonucleotides activate cellular signaling pathways through the toll-like receptor 9, which ultimately initiates an immunomodulatory cascade that results in the activation of B and T lymphocytes, natural killer cells, monocytes, macrophages, and dendritic cells.^{8,9} IMOs significantly shift the antigen-induced T_H2 immune response towards a T_H1-type response in antigen-sensitized and challenged mice (Fig 1).^{10,11} Through enhancement of the T_H1-biased immunity, IMOs effectively abate early and late phase allergic airway responses by reducing

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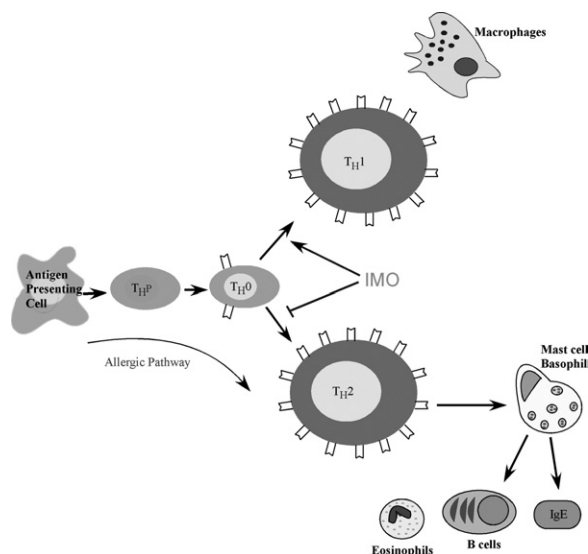


Figure 1 Exposure to an allergen causes activation of the allergic pathway, causing an increase in T helper 2 cells (T_H2)s leading to increased number of eosinophils, rapid degranulation of mast cells, and IgE production. IMO causes naive T helper cells (T_H0) to prefer the T helper 1 (T_H1) cell pathway toward activation of natural killer cells and macrophages.

eosinophils, IL-4, IL-5, IL-9, and IL-13 levels in both serum and bronchoalveolar lavage specimens.¹⁰

Previous studies demonstrated only the prevention of ovalbumin (OVA)-induced ETD when IMOs are given subcutaneously before allergen exposure in the brown Norway rat.¹² Our current study differs from the previous study because we investigate the use of a mucosal IMO application both before and after an allergen exposure. If the IMO proves effective via a mucosal absorption, it may change future applications and means of treatment.

MATERIALS AND METHODS

All rats were handled and housed according to the standards and protocols of the Institutional Animal Care and Use Committee in accordance with the requirements described by the National Institutes of Health Committee on Care and Use of Laboratory Animals. Forty nonallergic, male, brown Norway (BN) rats that weighed between 150 to 300 grams were randomly assigned into one of four groups: I, nonsensitized control (phosphate-buffered saline [PBS] control); II, OVA-sensitized control, untreated (OVA control); III, OVA-sensitized with IMO pretreatment (IMO pretreatment); and IV, OVA-sensitized with IMO treatment (IMO treatment).

IMO Synthesis and Purification

IMO (5'-TCTGACRTTCT-X-TCTTRCAGTCT-5', wherein R and X stand for 2'-deoxy-7-deazaguanosine and glycerol linker, respectively) was synthesized, purified, and analyzed as previously described.¹³ The purity of full-length IMO was over 93% with the rest being shorter by one or two nucleotides (n-1 and n-2) as determined by capillary gel electrophoresis and/or denaturing polyacrylamide gel electrophoresis. IMO contained less than 0.2 EU/mL of endotoxin as determined by the Limulus assay (Bio-Whittaker).

Experimental Protocol Timeline

The protocol timelines for this project are described in Figure 2.

PBS solution control protocol. Ten BN rats in the nonsensitized PBS control group received sterile phosphate-buffered saline (PBS) solution, pH 7.4 (1 X PBS-Cell Culture Grade; Lineberger Tissue Culture Facility, Chapel Hill, NC) on all sensitization, challenge, and treatment days.

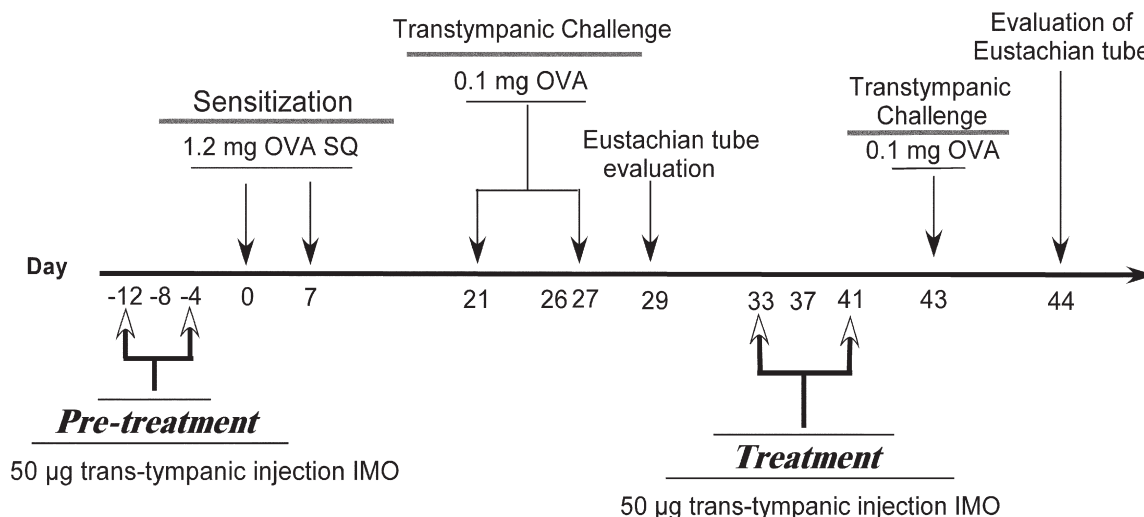


Figure 2 Experimental protocol timeline. All OVA-sensitized rats received subcutaneous injections of ovalbumin on days 0 and 7. Rats in the IMO pretreatment group were given transtympanic injections of IMO (50 µg) on days 12, 8, and 4. Rats in the IMO treatment group were given transtympanic injections of IMO (50 µg) on days 33, 37, and 41. Rats in the control groups received sham transtympanic PBS injections on these days. All rats were challenged on day 43 with evaluation of the eustachian tube on day 44.

OVA-sensitization of rats. BN rats in the OVA control, IMO pretreatment, and IMO treatment groups were sensitized to OVA (A5503; Ovalbumin, Grade V, Sigma Chemical Co, St Louis, MO) via a subcutaneous injection of 1.2 mg OVA in 0.6 mL PBS, pH 7.4 mixed with 5.14 mg aluminum hydroxide (AX0696-1 Aluminum Hydroxide Gel Dried Powder USP; EM Science, Gibbstown, NJ) in 0.6 mL PBS as a local adjuvant on days 0 and 7.

OVA control protocol. Ten BN rats received OVA sensitization as described above. This group also received OVA transtympanic challenges, but received sham injections of PBS on all treatment days.

IMO pretreatment protocol. Ten BN rats received three transtympanic injections (50 $\mu\text{g}/\text{injection}$) of IMO dissolved in sterile PBS, pH 7.4. The IMO was administered 12, 8, and 4 days before initial OVA sensitization.

IMO treatment protocol. Ten BN rats received three transtympanic injections (50 $\mu\text{g}/\text{injection}$) of IMO dissolved in sterile PBS solution. The IMO was administered 33, 37, and 41 days after OVA sensitization.

Transtympanic Challenge

Rats were anesthetized with 0.1 mL/100 gm body weight of a 1:1 mixture of ketamine (20 mg/mL):xylazine (100 mg/mL) and placed in the left lateral recumbent position. The depth of anesthesia was tested by applying joint pressure. All rats were given 80 $\mu\text{L}/\text{min}/\text{kg}$ of 300 mOsm Krebs Ringer solution (140 mmol/L Na^+ , 120 mmol/L Cl^- , 5.2 mmol/L K^+ , 25 mmol/L HCO_3^- , 1.1 mmol/L Ca^{2+} , 1.2 mmol/L Mg^{2+} , 0.4 mmol/L HPO_4^{2-} , and 5.6 mmol/L glucose) subcutaneously to maintain blood pressure and renal perfusion during the experiment. To maintain body temperature at 37°C, a thermistor-controlled heating pad was used each time anesthesia was administered.

Once anesthetized, left-sided tympanic membranes (TM) were assessed with an operating microscope to exclude obvious middle ear pathoses. A small myringotomy was placed in the anteroinferior quadrant of the TM. All OVA-sensitized rats then received a transtympanic (TT) challenge with a solution of 0.1 mg OVA in PBS solution, total volume 35 μL . Rats were given five days of repose and were reanesthetized to evaluate the TM under the operating microscope and graded for the presence or absence of middle ear effusion (day 26). If an effusion was present, the effusate was aspirated before a second TT OVA injection. After 24 hours, a third TT injection of ovalbumin was given on day 27. A final TT OVA injection was performed on day 43. The TM and middle ear were examined and any residual fluid was aspirated before the assessment of the eustachian tube's ventilatory and clearance function was performed (days 29 and 44).

Assessment of Eustachian Tube (ET) Function

This procedure has been described previously.^{12,14} Briefly, while under anesthesia with the xylazine/ketamine mixture, rats were placed in the left lateral recumbent position. The tympanic membrane was visualized with an operating microscope, and a myringotomy was made in the posteroinferior and anteroinferior quadrants. The myringotomies functioned as a port for the application of positive and negative pressure to the middle ear.

Passive opening and closing pressures of the ET were measured with a technique first described by Flisberg et al.¹⁵ A pressure measurement apparatus was created by inserting the end of a 6 French latex pediatric Foley catheter with a 3 mL balloon (Willy Rusch, AG; Duluth, GA) under direct visualization into the external auditory canal and inflating the balloon to create an airtight seal. The catheter was connected to a water manometer and created a closed system, which allowed for the application of positive pressure via a Harvard infusion pump. Slow uniform pressure (20 mL/min) was infused until the ET opened, marked by the meniscus of the water column that changed from convex to concave. This "breaking point" was designated the passive opening pressure (POP). At this time, the infusion was discontinued and pressure escaped through the opened ET and caused the meniscus of the water column to descend. The point at which the column stopped descending and stabilized was considered the closure of the ET. This point was designated as passive closing pressure (PCP).

To assess the capability of the ET to actively clear negative middle ear pressure, rats were then placed in the recumbent position. Once the catheter balloon was inserted and inflated in the external auditory canal, 14 cm H_2O of pressure was gradually withdrawn from the middle ear by the Harvard infusion pump. This process created negative pressure within the middle ear. Rats were then stimulated to swallow with polyethylene tubing, which caused the tensor veli palatini to contract and actively open the ET. Each swallow allowed for ventilation of the middle ear and caused the water column to rise (effectively decreasing the negative pressure). Rats were stimulated to swallow until negative pressure could no longer be cleared (the point at which the water column no longer changed after five stimulated swallows). The difference in the pressures between these two points were divided by 14 cm H_2O to compute the percent capability to actively clear negative pressure (ACNP).

To assess the mucociliary clearance function of the ET, rats were deeply anesthetized to prevent swallowing and active opening of the ET. The rats were placed in the supine position and a standard nasal speculum was used to aid in the visualization of the soft palate. A small midline incision (5 mm) was made in the soft palate posterior to the posterior border of the hard palate. Hemostasis and anesthesia were maintained by swabbing the incision site with xylocaine (lidocaine HCl 1% and epinephrine 1:100,000; AstraZeneca LP, Wilmington, DE). A miniature hand mirror was inserted

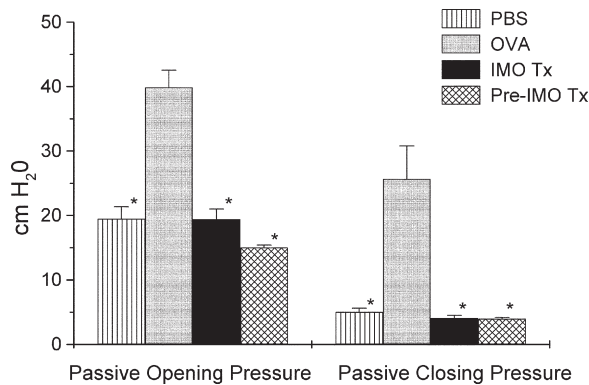


Figure 3 Comparison of mean passive opening and closing pressures in cm H₂O for groups. Error bars represent the standard error of the mean (* $P < 0.001$).

through the incision to visualize the ET orifice under the operating microscope. Rats were then placed in the left lateral recumbent position. Using a micropipette, 35 μ L of blue dye (Coomassie Brilliant Blue, B-0149; Sigma Chemical Co, St Louis, MO) was injected through incisions in the tympanic membrane into the middle ear. At this time, a stopwatch was started, and the rats were returned to the supine position. The nasopharyngeal orifice of the ET was again visualized through the palate incision with the miniature hand mirror. The time at which dye appeared at the orifice signaled the mucociliary clearance transit time (MCTT) of the tubotympanum. A cutoff was established at 15 minutes based on numerous previous experiments in our laboratory if no dye was seen.

STATISTICAL ANALYSIS

All statistical analyses were performed using SigmaStat Statistical software Version 2.03 (SPSS Inc, Chicago, IL). Sample size and statistical significance was determined before the experiment for P values of <0.05 , a power of 0.80, a expected standard deviation of 0.50, and an anticipated effect size of 40% reduction in eustachian tube pressures. Pressures were analyzed by 1-way analysis of variance with pairwise comparisons of means evaluated by paired t tests. Active clearance of negative pressure was converted to a percentage of total possible pressure for clearance and then analyzed. Graphs were constructed with means and the standard error of the mean (SEM).

RESULTS

OVA-sensitized rats in both the IMO pretreatment and IMO treatment groups required half of the pressure (cm H₂O) to both open ($P < 0.001$) and close ($P < 0.050$) the ET compared with OVA control rats (Fig 3). The average opening pressures for the IMO pretreatment group was 15.0 mm

H₂O and the IMO treatment group was 19.4 mm H₂O; closing pressures were 4.01 mm H₂O and 4.06 mm H₂O, respectively. The OVA control rats demonstrated an average opening pressure of 39.4 mm H₂O and a closing pressure of 25.6 mm H₂O. There were no statistically significant differences between the IMO pretreatment POP ($P = 0.27$), PCP ($P = 0.42$), or IMO treatment groups POP ($P = 0.98$), PCP ($P = 0.52$) compared with the PBS control group. According to the analysis of variance, the differences in the means among and within the IMO groups for both POP and PCP are greater than would have been expected by chance ($P < 0.001$).

IMO pretreatment and IMO treatment substantially improved the capability to actively clear negative pressure (ACNP) from the middle ear compared with the OVA control rats ($P < 0.001$). IMO treatment rats actively cleared 75% of negative middle ear pressure compared with 20% clearance by the OVA control rats (Fig 4). There was no significant difference in the ACNP when comparing the IMO pretreatment, IMO treatment, and PBS control rats.

The OVA control rats' MCTT was twice as long as the IMO pretreatment and IMO treatment groups ($P < 0.001$). The average time for dye to be transmitted through the eustachian tube for the OVA control group was 12 minutes 54 seconds compared with 7 minutes 18 seconds for the IMO pretreatment and 7 minutes 5 seconds for the IMO treatment group (Fig 5). The IMO pretreatment ($P = 0.08$) and IMO treatment ($P = 0.13$) groups also had a significantly longer MCTT when compared with the PBS control.

DISCUSSION

This study indicates that immune modulatory oligonucleotides administered via transtympanic injection to the mucosal epithelium can successfully prevent and treat ETD in rats. The IMO pretreatment and IMO treatment in OVA-sensitized rats also significantly improved the ventilatory function of the eustachian tube compared with OVA-control rats. The capacity to actively clear negative pressure is the most significant and accurate parameter of tubal function.¹²

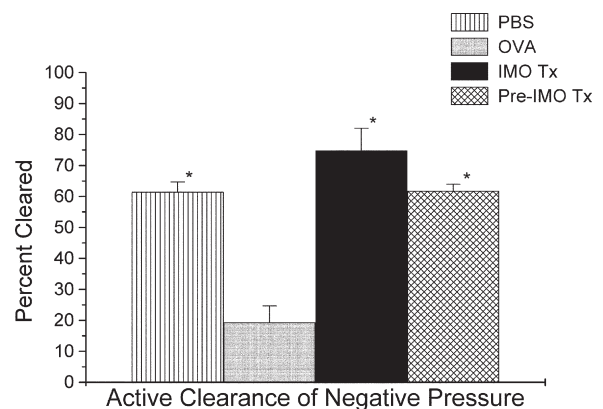


Figure 4 Mean active clearance of negative pressure in cm H₂O. Error bars represent the standard error of the mean (* $P < 0.001$).

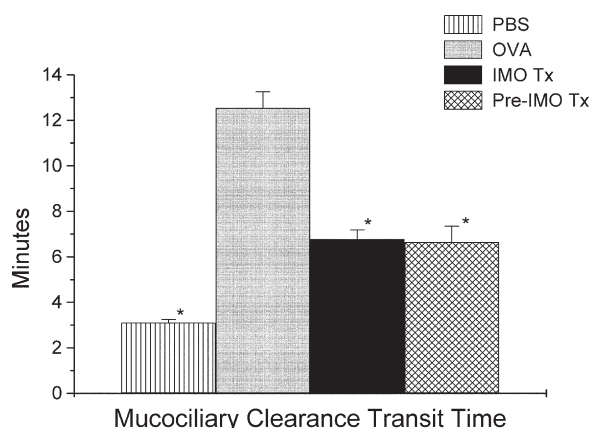


Figure 5 Mean mucociliary clearance transit time in minutes. Error bars represent the standard error of the mean (* $P < 0.001$).

For both the IMO pretreatment and the IMO treatment groups, ACNP demonstrated improved ventilatory function when compared with the OVA control, which suggests that the OVA-induced allergic inflammation of the ETD of the BN rat was effectively ablated. The ciliary function of the ET, measured by the MCTT, in the IMO pretreatment and IMO treatment groups was also significantly lower than the OVA control group, although the ciliary function did not completely return to baseline function. Differences in ciliary transient time in the IMO groups and PBS group can suggest 3 causes: 1) the patency of the ET was not returned to normal, 2) an increase in mucus production, or 3) an affect on ciliary function by the IMO. Because the opening and closing pressure differences in the IMO and PBS groups are not significantly different, it would indicate that patency of the ET has returned to normal. Previous systemic and mucosal studies with IMOs have shown a significant reduction in eosinophilia, mucus secretion, and collagen deposition in the airways.¹⁶ Our laboratory in the past has only studied the effects of IMO after subcutaneous administration. No study to our knowledge has demonstrated any effect of IMO on ciliary function after mucosal application.

Takabayashi et al¹⁷ demonstrated in mice that the induction of mucosal immunity generally requires a local antigen exposure. Their study determined that antigen exposure via the airway can induce immune responses at both systemic and mucosal sites, whereas intradermal exposure generally produces a weak mucosal response. Our study used a mucosal application of OVA to the middle ear epithelium to increase our chance to induce an allergic response and produce OME.

Direct application of immunotherapy to surfaces has been proven to elicit a higher systemic and mucosal immune response. However, systemic immunotherapy for aeroallergen exposure has not been proven effective.¹⁷ It has been shown that immune modulatory oligonucleotides modify the immune response by preventing native T lymphocyte differentiation and attenuation of IgE levels when applied to the treatment of allergic-like airway inflammation and airway hyperresponsiveness in asthma.^{10,11} The application of

the IMO directly to middle ear mucosa may work due to the united airways concept that states the middle ear space is an extension of the airway by way of the eustachian tube.

Our findings demonstrate improved ET ventilatory functions with the application of transtympanic IMO on the middle ear mucosal epithelium in the pretreatment and treatment protocol for OVA-induced eustachian tube dysfunction in the rat. Thus, IMO may offer substantial promise in the future management of OME.

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FINANCIAL DISCLOSURE

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Drs Kandimalla and Agrawal are employees and have stock options in Idera Pharmaceuticals.

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