

Comparison of the nasal and otologic responses following intranasal challenge with influenza A virus and rhinovirus type 39

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Episodes of otitis media (OM) in children are commonly preceded by, or concurrent with, viral upper respiratory tract infections (URTIs). Epidemiologic studies suggest differences among the common upper respiratory viruses in relation to their propensity to cause OM.^{1,2} Experimental studies in adult volunteers reported increased nasal mucociliary transit times (MCT), eustachian tube dysfunction, abnormal middle ear (ME) pressures, and OM following infection with either influenza A virus³ or rhinovirus type-39 (RV-39).⁴⁻⁶ In this presentation, the extent and frequency of nasal and otologic signs are compared between groups of adult volunteers experimentally infected with influenza A virus and RV-39. The null hypothesis is that there are no differences in the nasal and otologic expression of disease caused by infection with those viruses.

Materials and methods

The protocols and general methods for these experiments have been described in detail previously.³⁻⁶ The subjects included in this study consisted of a total of 211 adult volunteers experimentally infected with influenza A virus ($n = 60$, 2 cohorts) or RV-39 ($n = 151$, 3 cohorts). The protocols for the studies were approved by the Human Rights Committee of the Children's Hospital of Pittsburgh, and written informed consent was obtained from all subjects prior to enrollment. In this analysis, only the data for subjects with pre-challenge specific antibody titres of less than or equal to 4 for RV-39 ($n = 94$) and of less than or equal to 10 for influenza A virus ($n = 48$) were included.

In all experiments, healthy, adult volunteers (≥ 18 years of age) of either sex were enrolled. The subjects in the five different cohorts were cloistered at the same hotel, but at different times, for a 6-(RV-39) or 8-(influenza A) day period. With the exception of birth control pills and tylenol, which were supplied as needed, the subjects were not permitted to take over-the-counter or prescription medications during the cloister period. The first 24 hours of cloister (day 0) served as a baseline for signs, symptoms, secretion weights, and other physiologic measures. The subjects were then challenged with the virus as per group assignment, and followed throughout the period of cloister

using the same methods of assessment as those for day 0. Viral inoculation was performed with a safety-tested clinical isolate of either influenza A/Kawasaki/86 (H1N1) virus ($\sim 10^7$ TCID₅₀) or rhinovirus type-39 (~ 100 TCID₅₀) by intranasal instillation as course drops. Subjects were monitored by daily physician assessments of general health, nasal signs, and otologic signs, and by completing a self-reported daily symptom diary where eight specific symptoms characteristic of colds/flu were rated on a 4 point scale (0 = none, 3 = severe). Also, the expelled nasal secretion weight (SCW) and the saccharine-dye MCT were determined for each study day as previously described.³ Middle ear pressure (MEP) was measured bilaterally three times per day using tympanometry, and daily nasal lavages were performed for culture of the challenge virus. Twenty-one days after the virus challenge, all subjects had convalescent blood samples drawn for assay of specific antibody titers as previously described.^{3,4}

In this analysis, the results for the SCW, MCT, MEP, and otoscopic examinations were compared between viruses over study days 0 through 5. MEP was defined as abnormal if the recorded pressure was less than -99 mmH₂O or greater than 49 mm H₂O. Otoloscopic observations were classified dichotomously as the presence or absence of OM as defined previously.³ Summary statistics for the two groups were computed at each observation time as the mean and standard deviation for the continuous variables after correction for baseline values by subtraction (MCT and SCW), and as the frequency of responders for the dichotomous variables (MEP and otoscopy).³ These were plotted as a function of time, to allow for a visual appreciation of the difference between viruses in the time course and magnitude of the response. To evaluate the significance of any observed differences in response between RV-39 and influenza A infected groups, response variables were defined for each continuous variable as the area under the response-time curve (AUC), and for each dichotomous variable as the sum of all abnormal observations during the 5-day postchallenge study period.³ The average values of these response variables were compared between groups using a Student's t-test.

Results

One hundred thirty-eight of 142 subjects became infected with the challenge virus (influenza A— $n = 48$, 100%; RV-39— $n = 90$, 96%) as defined by either virus shedding on at least one post-inoculation day, or a fourfold rise in virus specific antibody titer (convalescent titer/pre-inoculation titer).

The baseline-corrected, average values of SCW and MCT for the two groups are shown as a function of time in Figure 1A and 1B. Increases in both measures and in both groups were observed following viral inoculation and

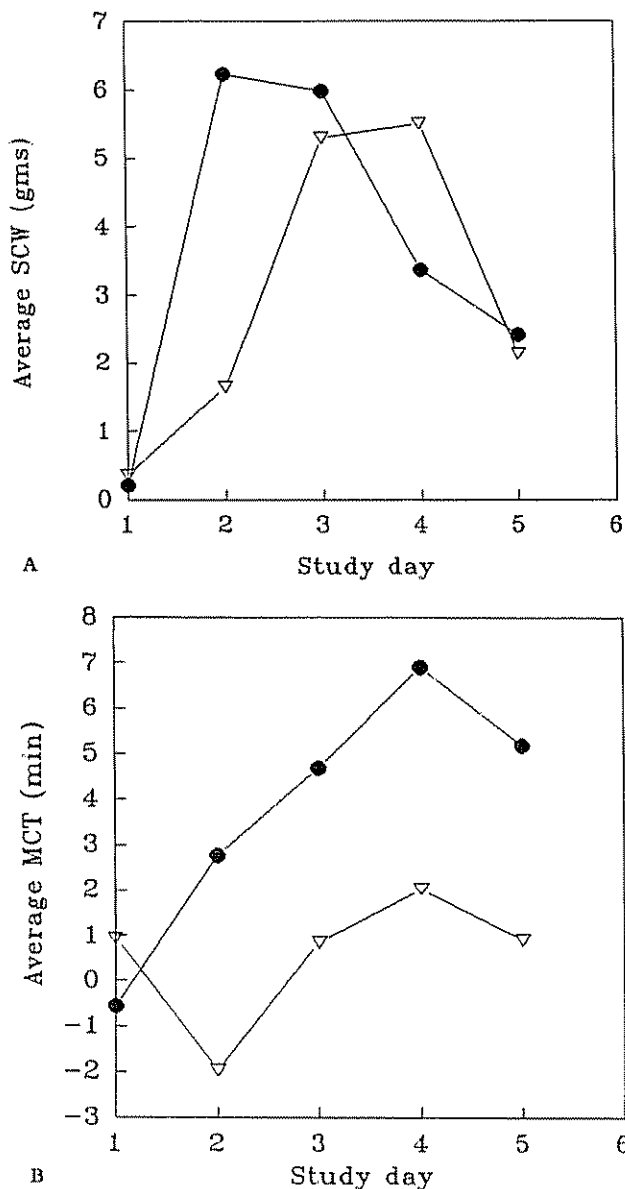


Figure 1 Average baseline-corrected daily values of (A) expelled secretion weight (SCW) and (B) mucociliary transit time (MCT) for the RV-39 (●) and the influenza A (▽) infected groups

infection. Average SCW peaked on day 2 in the RV-39 group (6.2 ± 12 g) as compared to day 4 in the influenza A group (5.5 ± 12 g). Average MCT reached a maximum on day 4 in the RV-39 group (6.9 ± 10 minutes) as compared to a maximum on day 4 in the influenza A group (2.0 ± 11 minutes). Statistical analysis (Table 1) of the between-group differences in the response variables for these measures was only significant for MCT ($p < .05$).

The frequencies of abnormal MEP as a function of time for the two groups of subjects are shown in Figure 2. Prior to inoculation, 10 (6%) ears in 10 (11%) RV-39 challenged subjects had at least one abnormal MEP as compared to 11 (12%) ears in 8 (17%) influenza A challenged subjects. Following challenge, the frequency of abnormal MEP increased in both groups and reached a maximum on day 3 for the RV-39 challenge group (53% subjects; 37% ears), and a maximum on day 5 for the influenza A challenge group (79% subjects; 64% ears). In both groups, the frequency of abnormal pressures showed a trend toward recovery by the end of the observation period. Analysis of the response variable for MEP (Table 1) showed a significant difference between the RV-39 and influenza A challenge groups ($p < .05$).

Both abnormal ME underpressures (< -99 mm H₂O) and overpressures ($> +49$ mm H₂O) were observed in the two challenge groups following viral inoculation. The frequency of abnormal underpressures reached a maximum on day 3 in the RV-39 infected subjects (39% subjects; 26% ears) as compared to day 5 in the influenza A infected subjects (71% subjects; 52% ears). The between-group difference in the response variable for abnormal underpressures was statistically significant ($p < .05$). In contrast, abnormal overpressures peaked on day 4 in the RV-39 challenge group (19% subjects; 14% ears), and on day 5 (25% subjects; 17% ears) in the influenza A challenge group. The between-group difference in the re-

Table 1 Mean and standard deviations of response variables

Response variable	Rhinovirus-39	Influenza A	p value*
MCT (min)	19.0 ± 35.9	5.4 ± 38.0	$< .05$
SCW (gm)	18.8 ± 37.9	14.9 ± 21.8	NS
MEP < -99 mm H ₂ O			
ears	1.7 ± 3.2	4.2 ± 4.2	$< .05$
subjects	2.6 ± 3.9	6.0 ± 4.7	$< .05$
MEP $> +49$ mm H ₂ O			
ears	0.5 ± 2.2	0.8 ± 1.9	NS
subjects	1.1 ± 2.3	1.6 ± 2.2	NS
Total abnormal MEP			
ears	2.4 ± 3.5	5.0 ± 4.2	$< .05$
subjects	3.56 ± 4.2	7.2 ± 4.4	$< .05$

MCT = mucociliary transit time; SCW = secretion weight; MEP = middle ear pressure

NS = not significant

* Student's *t*-test

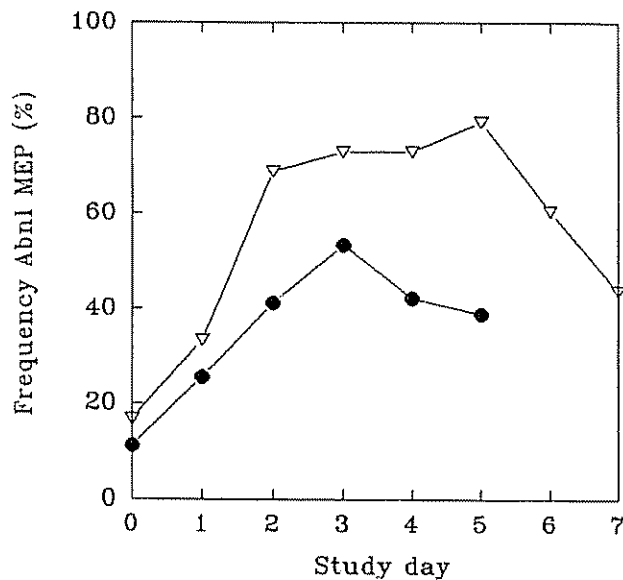


Figure 2 Frequency of subjects with at least one abnormal (Abnl) MEP measurement on each study day for RV-39 (●) and influenza A (▽) infected groups.

sponse variable for abnormal positive MEP was not statistically significant (Table 1).

Otoscopic evidence of OM occurred in only 5 (6%) RV-39 infected subjects as compared to 9 (19%) influenza A infected subjects ($p < .05$). All cases of OM in the RV-39 group were asymptomatic and had a serous appearance. By contrast, 2 of the 9 subjects who developed OM following influenza A infection developed otalgia and otoscopic evidence of purulent OM, one of which was confirmed by myringotomy. The other subject with purulent OM also complained of transient vertigo which spontaneously resolved. All subjects with OM were treated with appropriate antimicrobial therapy. All cases of OM resolved by the final follow up examination without evidence of complications.

Discussion

This and previous virus challenge studies in adult volunteers have shown altered signs and pathophysiologies following intranasal inoculation with either influenza A or RV-39.³⁻⁶ Specifically, experimental infection with either of these two viruses reproducibly results in prolongation of the MCT, increases in SCW, abnormal MEPs, as well as otoscopic evidence of OM. In the present study, comparative analyses demonstrated that RV-39 infection has a significantly greater effect on the MCT when compared to influenza A infection. However, no differences between viruses were noted for the magnitude of the nasal secretions provoked by infection. In relation to otologic changes, the frequency and severity of abnormal MEP was significantly greater for influenza A infected subjects

Also, OM occurred more often (6% RV-39 vs 19% influenza) in the influenza A infected subjects, and symptomatic, purulent OM was observed only in that group.

Viral URTIs are the commonest diseases which affect the human population. Of the viruses responsible for URTIs, the rhinoviruses are the commonest, accounting for up to 40 percent of episodes. Epidemiologic and experimental evidence suggest a causal role for viral URTIs in the pathogenesis of OM.¹⁻⁶ During naturally occurring cases of OM in children, disparity exists between virus isolation rates for influenza and the rhinoviruses, with the former playing a more prominent role. Application of the findings of the present study would suggest that this epidemiologic difference may, in part, be due to an increased otologic virulence of influenza relative to the rhinoviruses; i.e., although rhinoviruses account for most of the viral-mediated URTIs in the population, these infections rarely result in significant otologic complications or sequelae.

Experimental evidence from both human¹⁻⁶ and animal^{7,8} studies suggests that influenza A-mediated URTIs disrupt normal eustachian tube function, which alters MEP regulation, causing ME underpressures and consequent development of OM. These physiologic alterations appear to result from a combination of cytopathic, inflammatory, and immunomodulating effects induced within the mucosa of the upper respiratory tract during influenza infection. Unfortunately, similar functional and pathologic correlations are lacking for rhinovirus-mediated URTIs making the understanding of disease progression less well understood. Additional studies using this model, animal models, and epidemiologic methods are necessary to validate these findings, to better elucidate the pathophysiologic alterations caused by different viruses, and to develop mechanistic descriptions of disease pathogenesis. With that understanding, rationale for the prophylaxis or treatment of OM caused by viral URTIs can be designed and tested for therapeutic efficacy.

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Influenza A virus infection and changes of lectin binding patterns in the nasopharyngeal mucosa in mice

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Otitis media is thought to occur when bacteria adhere to the mucosal surface of the nasopharynx, enter the middle ear via the eustachian tube, and replicate in the middle ear space. Bacteriologic studies of children suffering from otitis media reveal the consistent finding that *Haemophilus influenzae* is among frequently isolated pathogens. However, viral infection may also be related to this pathogenesis,¹ since it is suggested that influenza infection predisposes the host to bacterial colonization and infection by enhancing the adherence of some bacteria to virus-infected respiratory cells.² Cell surface glycoconjugates are known to play an important role in cellular communication, migration, and adherence.³ Recently, it has been reported that the terminal glycosylation sequences of glycoconjugates mediate biologic recognition, such as receptors in bacterial adherence.⁴ Viral infection is considered to be one of the causes for changing the terminal glycosylation sequence. In this study, we examined the effect of influenza A virus infection on glycosylation of the nasopharyngeal epithelial cells, as well as the effect of the virus on the colonization of *H. influenzae* on the nasopharynx.

Materials and methods

Healthy BALB/c male mice 5 weeks old were used in this study. Eighteen mice were used for the lectin histochemistry, and the bacterial colonization study was carried out in 16 animals.

Lectin histochemistry

Animals were intranasally inoculated with 50 μ L of the suspension, which contained approximately 3.7×10^9 PFU/mL of influenza A virus (PR8, H1N1). Three animals were sacrificed respectively at 1, 3, 5, 7, and 9 days after the inoculation. The animals were anesthetized with

intraperitoneal pentobarbital injection and fixed by intracardial perfusion with 10 percent neutral buffered formalin. Heads of the mice were removed, immersed in the same fixative for 2 hours, and decalcified in 10 percent EDTA for 2 weeks. The tissue was subsequently dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin. Serial horizontal sections were cut at 6 μ m for each specimen and processed for lectin histochemistry, using limax flavus agglutinin (LFA), wheat germ agglutinin (WGA), succinyl WGA, sambucus nigra agglutinin (SNA), and peanut agglutinin (PNA). All lectins were obtained from EY laboratories (San Mateo, CA). Sections that contained the pharyngeal orifice of the eustachian tube were deparaffinized, rinsed in 0.1 M phosphate-buffered saline (PBS), and treated with 3 percent H_2O_2 in absolute methanol. Sections were then exposed to a 2 percent solution of bovine serum albumin (BSA) in PBS and incubated for 12 hours at room temperature with one of the biotinylated lectins, which was diluted with 1 percent BSA-PBS. Sections were rinsed with PBS and incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) for 1 hour. Sites of bound lectins were visualized by development for 8 minutes in 0.05 percent diaminobenzidine 0.01 percent H_2O_2 substrate medium in 0.1 M phosphate buffer.

Three animals that were intranasally challenged with PBS, were processed as control. Some of the sections from the control animals were incubated with neuraminidase from Newcastle disease virus (Oxford Glycosystems, Rosedale, NY) for 18 hours at 37°C before the lectin histochemistry.

Bacterial colonization study

Twelve mice were intranasally inoculated with 50 μ L of the virus suspension. At 1, 5, and 9 days after the inocu-