Evidence for Cytokine Mediation of Disease Expression in Adults Experimentally Infected with Influenza A Virus

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The roles of interleukin (IL)–6 and IL-8 in mediating the symptoms and signs of influenza A infection were examined. Adults were intranasally inoculated with a rimantadine-sensitive strain of influenza A HlNl virus and treated with rimantadine or placebo. Viral shedding, secretion weights, symptom scores, and concentrations of IL-6 and IL-8 in nasal lavage fluids were compared between treatment groups. Viral shedding was associated with increases in local and systemic symptoms, in expelled secretion weights, and in levels of IL-6 and IL-8. Compared with placebo, rimantadine treatment reduced viral shedding, systemic symptoms, and levels of IL-8. Days of viral shedding and IL-6 but not IL-8 concentrations were significantly correlated with the other measures of symptoms and signs. These data support a causal relationship between viral replication, cytokine production, and symptom expression, and they suggest that IL-6 may have a role in mediating symptom and sign expression during influenza A infection.

Viral upper respiratory tract infections (URIs) are extremely common in children and adults [1]. The mechanisms underlying the expression of the local and systemic symptoms and the development of secondary complications are not well understood. Early research implicated roles for cytopathology, cellular infiltration, and inflammatory mediators (histamine, bradykinin) in disease pathogenesis, but these factors are now recognized as late events with specific and limited contributions to disease expression [2–8].

More recently, it was shown that respiratory epithelial cells and different leukocyte populations elaborate biologically active cytokines, including interleukin (IL)–1, tumor necrosis factor–α, IL-6, and IL-8, when exposed to or infected with rhinovirus, adenovirus, respiratory syncytial virus, measles virus, or influenza virus [9–18]. These cytokines were also detected in the blood or secretions (or both) of patients infected with those viruses [8, 19–25] and were shown to provoke symptoms and signs consistent with those of a viral URI when administered systemically or locally [26–28]. For these reasons, it is tempting to suggest that the similarities in symptom presentation for URIs of different viral etiology reflect a generalized profile of proinflammatory cytokine elaboration. In support of this, two recent studies of experimental rhinovirus and influenza A virus infection in adults reported a correlation between expressed symptoms and signs and IL-8 and IL-6 concentrations, respectively [24, 25].

In the current study, the effect of rimantadine treatment on the local concentrations of IL-6 and IL-8 during a confirmed influenza A virus infection was determined, and the correlations between the concentration of these cytokines and symptom severity were defined. These results were examined for supportive evidence that cytokines do not mediate symptom expression during influenza A virus infection.

Materials and Methods

Adult subjects (≥18 years) were screened and excluded if presenting with a history of nasal or otological surgery; pulmonary, cardiovascular, renal, or other serious disease; or seizures; a symptomatic URI within the last 30 days, ear disease within the last 6 months, concurrent pregnancy, nasal or otological signs and symptoms, abnormal clinical profiles, seropositivity for human immunodeficiency virus, or a hemagglutination inhibition antibody titer of >1:10 to the challenge strain.

The design was a double-blind, placebo-controlled, randomized trial of rimantadine treatment for influenza A virus infection. Healthy, susceptible adults were enrolled and studied in 3 cohorts (n = 30, 32, and 43). For each cohort, subjects were cloistered in individual rooms of a local hotel for 8 days (study days 0–7). Twenty-four hours after admission to the cloister, patients were intranasally administered coarse drops of a safety-tested, rimantadine-sensitive clinical isolate of influenza A/Kawasaki/9/86 (HlNl) virus (wild type, lot E-262). Forty-eight hours after inoculation (0.25 mL/nostril; total dose = 10^7 median TCID), subjects were given their first dose of medication according to code (100

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Informed consent was obtained from subjects, and human experimentation guidelines of the US Department of Health and Human Services and the Human Rights Committee of Children's Hospital of Pittsburgh were followed.

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mg rimantadine HCl [Flumadine; provided by Forest Pharmaceuticals, St. Louis] or placebo), with repeat dosing at 12-h intervals for a total of 8 days. The subjects were not permitted to take alcohol or over-the-counter or prescription medications, with the exception of birth control pills.

On a daily basis, the subjects had physical examinations, rated their symptoms, and completed a battery of tests as previously described [29]. Each morning, the nasal cavities of the subjects were lavaged, and the recovered fluids were stored at −70°C for later virus and cytokine assays. Convalescent blood samples were obtained ~2 weeks after patients were dismissed from the cloister and assayed for hemagglutination inhibition antibody titer, by use of standard techniques. Samples of lavage fluids were inoculated onto Madin Darby canine kidney cell monolayers in triplicate, and virus was identified by hemadsorption. Cytokine ELISAs were obtained from Endogen (Woburn, MA) and had an interassay and intraassay variability of <10%. The lower limit of detection was 1 pg/mL for IL-6 and 2 pg/mL for IL-8.

The effect of rimantadine treatment on the signs, symptoms, and pathophysiologies caused by experimental influenza A virus infection in this population has been described [29]. Herein, we report the effects of infection and of rimantadine treatment on the expression of IL-6 and IL-8. The sample consisted of all subjects ($n = 72$) in cohorts 2 and 3 who had sufficient nasal lavage fluids for the IL-6 and IL-8 assays. Of the 576 possible samples for each cytokine, 8 for IL-6 and 13 for IL-8 were missing from the data set. Values for these were estimated as the average of the two bordering observations.

All continuous variables on study days 1–7 were adjusted by subtracting the corresponding value recorded on study day 0 (baseline adjusted). For each subject and study day, two influenza-related summary symptom scores were constructed corresponding to upper respiratory symptoms (sum of baseline-adjusted scores for rhinorrhea, nasal congestion, sneezing, sore throat, and cough) and systemic symptoms (sum of baseline-adjusted scores for malaise, headache, chilliness, muscle ache, joint pain, sweats, and fever). On each day, the total expelled weight of nasal secretions was measured as previously described, and these data were baseline-adjusted prior to analysis [29].

The hypotheses tested were that (1) cytokine levels are increased by influenza A infection, (2) rimantadine treatment reduces the two summary symptom scores, the expelled weight of secretions, and the local concentration of the two measured cytokines, and (3) the cytokine levels correlate with symptom scores and secretion weight. To test those hypotheses, we compared symptom scores, secretion weights, and cytokine levels for groups of persons defined on the basis of documented viral shedding. The 2 treatment groups of the population that shed virus were compared for those measures, and the correlation coefficients between cytokine levels and symptom scores were calculated. The primary analysis was a between-group comparison of the area under the measure-time curve (AUC; summed over the pretreatment or treatment periods) using a 1-tailed Mann-Whitney U test evaluated at $\alpha = .05$. Statistical analysis of dichotomous data between groups was made by use of Fisher's exact test and evaluated at $\alpha = .05$. Relationships between variables were evaluated by use of the Spearman rank order correlation coefficient (ρ). Data represent the mean ± SD.

Results

Seventy-two subjects (1 Hispanic, 7 black, 64 white) were included in the analysis; 34 were men, and the average age was 31 ± 11 years (range, 18–50). Thirty-four subjects were treated with rimantadine and 38 with placebo. The average number of days of viral shedding was 1.6 ± 1.6 for rimantadine-treated subjects and 3.1 ± 2.1 for placebo-treated subjects ($P < .01$). Seventeen subjects (6 placebo treated, 11 rimantadine treated) did not shed virus on any day. IL-8 was detected in all samples; IL-6 was detected in about half of the samples.

The results are presented for 3 comparison groups: nonshedding subjects (group 1, $n = 17$), placebo-treated subjects who shed virus (group 2, $n = 32$), and rimantadine-treated subjects who shed virus (group 3, $n = 23$). Table 1 shows the mean (± SD) for pretreatment (days 1 and 2) and posttreatment (days 3–7) AUCs for IL-6, IL-8, secretion weight, nasal symptom score, and systemic symptom score. With the exception of IL-8 levels, all measures for both periods were significantly greater in the 2 groups that shed virus than in the group that did not shed virus. For the pretreatment period, there were no significant differences between groups 2 and 3 in any of the measures, but both the IL-8 and systemic symptom AUCs for the treatment period were significantly less in group 3 than group 2.

Figure 1 shows the pattern of change with time in the average baseline-adjusted lavage IL-6 and IL-8 concentrations, nasal secretion weight, nasal symptom score, and systemic symptom

Table 1. Average (± SD) area under the pretreatment and treatment period response curves for interleukin (IL)–6, IL-8, mucus weight, nasal symptom score, and systemic symptom score.

Sign of symptom	Treatment period	Group 1	Group 2	Group 3	Significance[a]
IL-6 (pg/mL)	Before	3.1 ± 10.3	21.8 ± 52.1	40.3 ± 59.2	1, 2
	During	3.5 ± 5.2	107.7 ± 157.3	80.2 ± 115.4	1, 2
IL-8 (pg/mL)	Before	143 ± 192	285 ± 464	406 ± 791	
	During	306 ± 269	1710 ± 2044	838 ± 845	1, 3
Mucus, weight (g)	Before	2.9 ± 3.1	5.3 ± 5.8	18.9 ± 29.3	1, 2
	During	5.3 ± 6.3	10.5 ± 13.9	12.9 ± 16.9	1, 2
Nasal symptom score	Before	2.5 ± 2.4	4.7 ± 3.6	5.7 ± 3.7	1, 2
	During	2.7 ± 3.5	7.3 ± 6.4	5.0 ± 4.4	1, 2
Systemic symptom score	Before	1.1 ± 1.7	4.8 ± 6.4	4.1 ± 5.8	1, 2
	During	1.2 ± 1.4	5.3 ± 7.7	2.0 ± 3.1	1, 3

[a] $P < .05$ for group 2 vs. group 1 comparison (1), group 3 vs. group 1 comparison (2), and group 2 vs. group 3 comparison (3); Mann-Whitney U test.

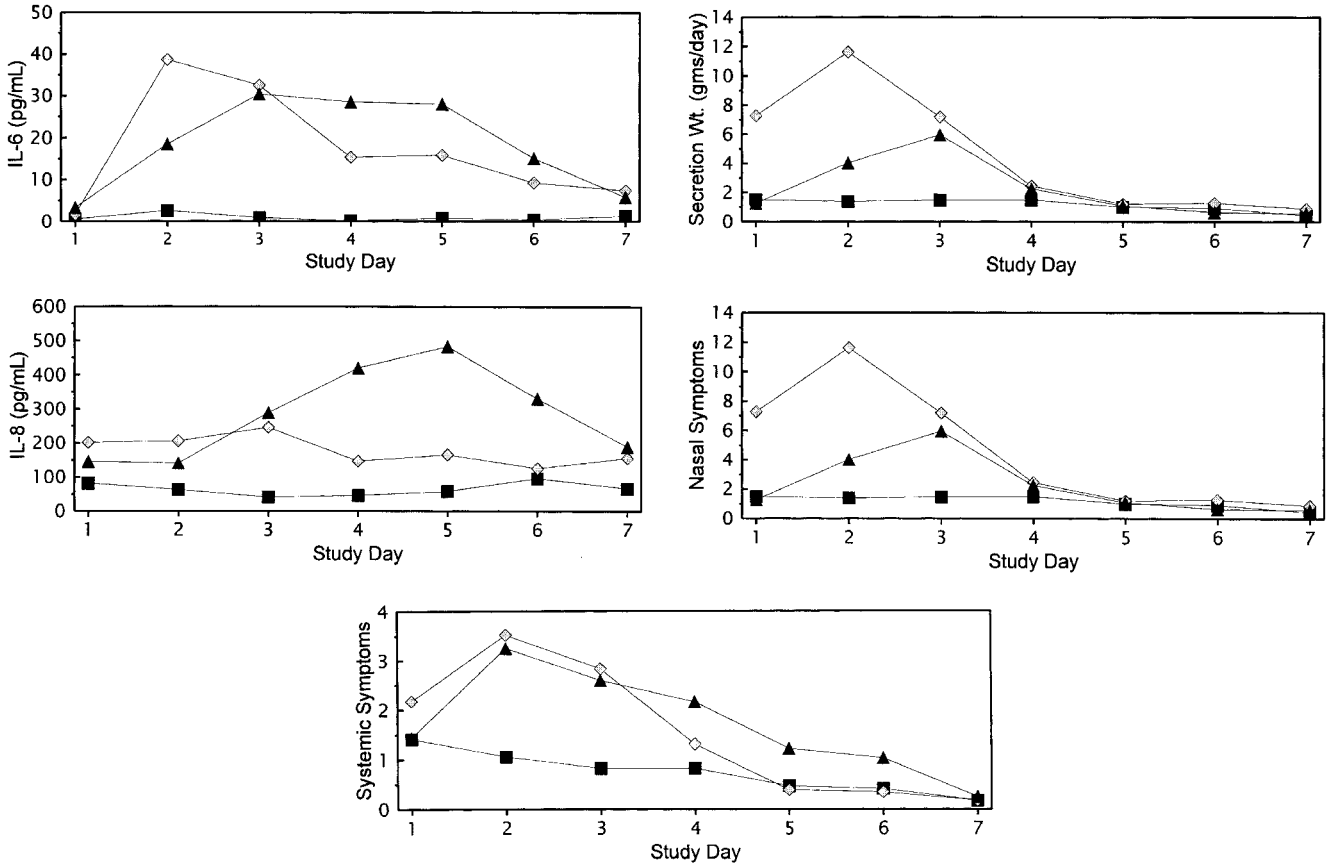

Figure 1. Average baseline-adjusted interleukin (IL)–6 and IL-8 nasal lavage concentrations, secretion weights, nasal symptom scores, and systemic symptom scores as a function of study day for group without viral shedding (group 1, ■) and for groups with viral shedding who were treated with placebo (group 2, ▲) or rimantadine (group 3, ◇).

score for each of the 3 groups. None of these measures for group 1 showed patterned changes after virus challenge. In contrast, average values for IL-6 concentration, secretion weight, nasal symptom score, and systemic symptom score for groups 2 and 3 increased to a peak on the second or third day after challenge and then decreased to approach baseline. With the exception of secretion weight, the rate of the postpeak decrease was greater for the rimantadine treatment group. IL-8 concentration increased only in group 2 and was characterized by a relatively late onset (day 3) and a peak concentration on day 5.

Correlation coefficients between shedding days, the AUCs for IL-6 and IL-8 concentrations, and the AUCs of the three symptom and sign measures for the pretreatment and treatment periods are reported in table 2. These coefficients are reported for the total population, for the total population after controlling for days of shedding (partial correlation), and for each of the 2 treatment groups. In general, days of shedding were significantly correlated with the five other measures, and those relationships were not affected by treatment. For both periods, IL-6 AUCs were correlated with those of the other measures. For the pretreatment period, those correlations were not affected by treatment or by controlling for days of shedding. In contrast, for the treatment period, the correlation coefficients between the IL-6 AUCs and those for IL-8, nasal symptoms, and systemic symptoms were less in the rimantadine (vs. placebo) treatment group and were decreased after controlling for days of shedding. For the pretreatment period, the IL-8 AUC was correlated only with that for nasal symptoms, and this was not affected by treatment or by control for days of shedding. IL-8 AUCs for the treatment period were not correlated with any of the three symptom and sign measures.

Figure 2 shows for each day of study the adjusted (controlling for days of shedding) correlation coefficients between IL-8 or IL-6 concentrations and secretion weight, nasal symptoms, and systemic symptoms. Early after virus challenge, IL-6 but not IL-8 concentrations were positively correlated with all three measures. Significant negative correlations with systemic symptoms were documented for both cytokines during the later period of follow-up.

Discussion

In this study, the nasal concentrations of two proinflammatory cytokines, IL-6 and IL-8, were increased in subjects

Table 2. Matrix of Spearman rank-order correlation coefficients for the pretreatment and treatment period area under the response curve of the six outcome measures.

Period/outcome measure		Virus	IL-6	IL-8	Mucus weight	Symptom score	
						Nasal	Systemic
Pretreatment							
Virus	Total	1.00	0.45	0.06	0.29	0.32	0.33
	Placebo	1.00	0.48	−0.03	0.16	0.34	0.33
	Active	1.00	0.52	0.20	0.49	0.35	0.42
IL-6	Total		1.00	0.22	0.39	0.54	0.44
	Total[a]		1.00	0.21	0.31	0.47	0.34
	Placebo		1.00	0.11	0.30	0.54	0.37
	Active		1.00	0.35	0.44	0.52	0.53
IL-8	Total			1.00	−0.14	0.28	0.18
	Total[a]			1.00	−0.17	0.28	0.17
	Placebo			1.00	−0.34	0.29	0.13
	Active			1.00	0.07	0.23	0.21
Treatment							
Virus	Total	1.00	0.65	0.41	0.35	0.42	0.35
	Placebo	1.00	0.67	0.48	0.30	0.49	0.42
	Active	1.00	0.68	0.17	0.39	0.27	0.11
IL-6	Total		1.00	0.53	0.43	0.35	0.18
	Total[a]		1.00	0.39	0.28	0.12	−0.07
	Placebo		1.00	0.65	0.42	0.31	0.27
	Active		1.00	0.39	0.44	0.50	0.02
IL-8	Total			1.00	0.20	0.08	−0.02
	Total[a]			1.00	0.07	−0.10	−0.20
	Placebo			1.00	0.27	0.01	0.11
	Active			1.00	0.16	0.09	−0.30

NOTE. IL = Interleukin.

[a] With control for days of shedding $P < .05 - .23 > \rho$ or $-.23 < \rho$.

with documented influenza A virus infection but not in those subjects who were exposed but did not shed virus. These data support and confirm previous observations documenting proinflammatory cytokine production in adults and children infected with respiratory viruses [8, 19–25]. Also, the number of days of viral shedding was significantly correlated with the local cytokine concentrations, symptom magnitude, and weight of provoked secretions. These results suggest that the presence of active viral replication initiates and sustains those responses. In vitro studies documented that respiratory epithelial cells and different leukocyte populations infected with common upper respiratory viruses produce IL-6 and IL-8, as well as tumor necrosis factor–α, interferon, and IL-1, indicating that these cell types are likely sources for the cytokines measured in vivo [9–18].

A causal link between cytokines and specific cold or influenza symptoms is suspected but not established. For experimental rhinovirus infection, parallel elevations of IL-1, IL-6, and IL-8 and symptoms have been reported [8, 20, 23–25]. Specifically, increased levels of IL-1 were detected in the nasal secretions of rhinovirus-infected volunteers who were symptomatic, compared with those who were uninfected or infected but asymptomatic [20], and increased IL-8 levels positively correlated with symptom scores [8, 25]. As with rhinovirus infection, experimental influenza virus infection provoked local, sequential elevations in specific cytokines with IL-6 levels related temporally to symptom expression [23, 24].

In the current study, secretion weights, nasal symptoms, and systemic symptoms tracked lavage IL-6 concentrations during the early period after virus infection. Also, the significant correlation between IL-6 concentrations and early but not late symptom scores was preserved after controlling for viral shedding. These data do not exclude a role for IL-6 in initiating the symptoms or signs of influenza A infection but do exclude it as a requisite factor for maintaining those symptoms and signs. In contrast, the onset of change in IL-8 levels was temporally delayed with respect to that for IL-6 and the measured symptoms and signs, and the correlations between IL-8 concentrations and the other measures were not significant. These results exclude IL-8 from a role in initiating or sustaining the measured symptoms and signs of influenza A virus infection.

Rimantadine treatment 48 h after virus exposure decreased the number of days of viral shedding, prevented the increase in lavage IL-8 concentration, and reduced the total systemic symptom load in subjects who shed virus. While not statistically significant, IL-6 levels and nasal symptoms were also decreased by rimantadine treatment. Correlational analysis of these data document significant relationships between days of viral shedding, cytokine levels, and symptoms and signs. These results can be interpreted as evidencing inhibition of viral replication by rimantadine, which in turn decreases the signal required to initiate or sustain symptom and sign expression.

In summary, this study shows that active viral infection is a prerequisite to the elaboration of IL-6 and IL-8 and for the development of symptoms and signs after experimental exposure to influenza A virus. While IL-6 may have a role in initiating the measured symptoms and signs, it is not required for persistence of their expression. Contrary to the results of a recent study of experimental rhinovirus infection [25], IL-8 is not a potential mediator of the measured symptoms and signs of influenza A infection. However, the precise role of cytokines in mediating disease expression cannot be defined fully in correlational studies, but this is amenable to study using pharmacologic probes that suppress or moderate the expression of

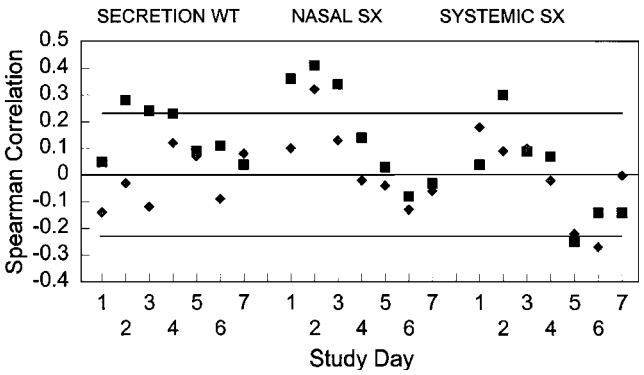

Figure 2. Spearman rank-order correlation coefficients between interleukin (IL)–6 (■) or IL-8 (♦) lavage concentrations and secretion weight (WT), nasal symptom score (NASAL SX), and systemic symptom score (SYSTEMIC SX) for each study day. Solid lines bound threshold values for statistical significance.

specific cytokines [25]. In that regard, NF-κB, a transcription factor that regulates a number of influenza virus–relevant cytokines, including IL-6 and IL-8, may be an attractive target for future studies [30].

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