JCI The Journal of Clinical Investigation

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J Clin Invest. 1998;101(3):643-649. https://doi.org/10.1172/JCI1355.

Research Article

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Local and Systemic Cytokine Responses during Experimental Human Influenza A Virus Infection

Relation to Symptom Formation and Host Defense

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Abstract

To further understand the role of cytokine responses in symptom formation and host defenses in influenza infection, we determined the levels of IL-1β, IL-2, IL-6, IL-8, IFN-α, TGF-β, and TNF-α in nasal lavage fluid, plasma, and serum obtained serially from 19 volunteers experimentally infected with influenza A/Texas/36/91 (H1N1) and correlated these levels with various measures of infection and illness severity. We found that IL-6 and IFN-α levels in nasal lavage fluids peaked early (day 2) and correlated directly with viral titers, temperature, mucus production, and symptom scores. IL-6 elevations were also found in the circulation at this time point. In contrast, TNF-α responses peaked later (day 3 in plasma, day 4 in nasal fluids), when viral shedding and symptoms were subsiding. Similarly, IL-8 peaked late in the illness course (days 4–6) and correlated only with lower respiratory symptoms, which also occurred late. None of IL-1β, IL-2, or TGF-β levels increased significantly. These data implicate IL-6 and IFN-α as key factors both in symptom formation and host defense in influenza. (*J. Clin. Invest.* 1998. 101:643–649.) Key words: influenza • interleukin-6 • tumor necrosis factor-α • interferon-α • interleukin-8

Introduction

Influenza is an acute respiratory infection with high morbidity and significant mortality, primarily because of its complications in very young or very old persons (1). Its symptoms are often quite memorable because of their severity, and consist typically of the sudden onset of malaise and fever, followed by upper and lower respiratory manifestations as well as myalgia and headache. In adults, the fever and other systemic features usually last 3 d, whereas respiratory symptoms, particularly cough, may persist for 1–2 wk (2, 3).

The factors that account for the symptoms of influenza are incompletely understood. While it is attractive to assume that the respiratory symptoms result from direct cytopathic effects of the virus on respiratory epithelial cells and the systemic symptoms from the elaboration of cytokines, it is more likely that considerable overlap exists and that both the virus itself and the cytokines evoked by the infection participate in local and general effects to varying degrees (2). Furthermore, it has not been determined which cytokines contribute most to symptom formation. While previously, IFN-α was shown to be present in nasal lavage fluid and in acute-phase sera of individuals with naturally acquired influenza infection (4), it has been shown more recently that purified influenza virus neuraminidase induces macrophages to secrete IL-1 and TNF-α (5), and whole influenza virions induce PBMC to produce IL-1, IL-6, and TNF-α (6). Therfore, it is quite clear that influenza virus has the potential to bring about the production of several cytokines, any combination of which could be responsible for symptoms.

In recent pilot studies of volunteers experimentally infected with influenza A/Texas/36/91 (H1N1) virus, we documented increased plasma levels of IL-6 and TNF-α on day 4 after virus infection (our unpublished data). These studies informed us that a fuller kinetic analysis of cytokine levels would be necessary to assess their role in both symptom formation and host defenses in influenza. Accordingly, we infected a group of normal volunteers with the same influenza A virus challenge strain used in the pilot study and determined a broad range of cytokine responses in nasal lavage fluids, plasma, and serum collected serially from these individuals. To correlate these cytokine responses with clinical outcome, the infected subjects were also monitored for quantitative virus replication in the upper respiratory tract and defined symptoms. The data obtained indicate that cytokine levels, particularly those of the IL-6 and IFN-α, strongly correlate both statistically and chronologically with acute symptom occurrence in influenza infection, and are thus likely to be playing a major role in symptom formation. In addition, they indicate that these cytokines are important in the early and usually decisive phase of host defense against influenza infection.

Methods

Subjects. 20 healthy susceptible male and female volunteers [hemagglutination-inhibition (HI)[1] antibody titers ≤ 1:8 to influenza A/Texas/36/91 (H1N1) virus] were recruited for participation in the study.

F.G. Hayden and R.S. Fritz contributed equally to this work.

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Received for publication 30 July 1997 and accepted in revised form 1 December 1997.

The Journal of Clinical Investigation
Volume 101, Number 3, February 1998, 643–649
http://www.jci.org

1. *Abbreviations used in this paper:* HI, hemagglutination-inhibition; NK, natural killer; RT, room temperature; $TCID_{50}$, median tissue culture infective dose.

Written informed consent was obtained from each participant in a form approved by the University of Virginia Human Investigation Committee and the National Institute of Allergy and Infectious Diseases Institutional Review Board. All the volunteers were studied at one time. No medications, except acetaminophen for treatment of severe symptoms, were permitted. Subjects were compensated for their participation in this study.

Study outline. Screening assessments were begun within 60 d of the scheduled viral inoculation. Volunteers were confined to individual rooms in an isolation unit 1 d before the day of inoculation, and remained in isolation for 8 d thereafter. The participants were inoculated intranasally (0.25 ml per nostril) with $\sim 10^5$ median tissue culture infective doses ($TCID_{50}$) of influenza A/Texas/36/91 (H1N1) virus on day 0. Nasal washings (6–8 ml return per washing) were collected (days 0, 1, 2, 3, 4, 5, 6, 7, 8, and 23) for virus isolation and titration by standard methods and for cytokine determinations. Serum (days 0, 1, 1.5, 2, 2.5, 3, 4, 8, and 23) and plasma (days 0, 2, 4, 8, and 23) were also collected for urea and cytokine determinations. Both fluids were tested because we were uncertain which would prove more sensitive to changes in cytokine levels. Acute and convalescent sera (2–3 wk after the virus challenge) were assayed for the presence of HI antibody titers to the challenge virus.

Oral temperatures were measured four times daily. Fever was defined as an oral temperature > 37.7°C. Symptom assessments were performed by the volunteers twice daily on a four-point scale (0–3 corresponding to absent to severe). The symptoms assessed were nasal stuffiness, runny nose, sore throat, cough, sneezing, earache/pressure, breathing difficulty, muscle aches, fatigue, headache, feverish feeling, hoarseness, chest discomfort, and overall discomfort. The total symptom score for each time point was obtained by adding the individual symptom scores for that particular time point. The individual symptoms contributing to the total symptom score were divided into three subgroups: systemic symptoms (muscle aches, fatigue, headache, and fever), upper respiratory symptoms (nasal stuffiness, earache/pressure, runny nose, sore throat, and sneezing), and lower respiratory symptoms (cough, breathing difficulty, hoarseness, and chest discomfort). Nasal discharge weights were determined throughout the isolation period by previously described methods (7).

Sample preparation. Nasal washes were collected and mixed thoroughly with a syringe and placed on wet ice. The washes were clarified by centrifugation (1,000 *g* for 10 min at room temperature [RT]), aliquoted, and frozen at −70°C. Blood was collected for plasma in tubes containing acid citrate dextrose anticoagulant Solution A and in serum separator tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ). The blood in the serum separator tubes was allowed to clot (30 min at RT), and the serum was isolated by centrifugation (1,300 *g* for 15 min at RT). The serum was then aliquoted, frozen, and stored in vapor phase liquid nitrogen. The plasma in the acid citrate dextrose tubes was separated from the formed elements by centrifugation (1,300 *g* for 15 min at RT). The plasma was transferred to 15-ml polypropylene tubes (Becton Dickinson Labware, Lincoln Park, NJ) and subjected to a second centrifugation under the same conditions. After the second centrifugation, the plasma was aliquoted, frozen, and stored in liquid nitrogen.

Cytokine determinations. Cytokine levels in nasal lavage fluid, plasma, and serum for each time point were determined using commercially available ELISA kits and the manufacturer's protocols as follows: IL-2, IL-6, and IFN-α (Endogen, Inc., Cambridge, MA); IL-1β, IL-8, and TNF-α (R & D Systems, Inc., Minneapolis, MN); and TGF-β_1 (Genzyme Corp., Cambridge, MA). The limits of sensitivity of these assays supplied by the manufacturers were as follows: IL-1β (0.3 pg/ml), IL-2 (< 6 pg/ml), IL-6 (< 1 pg/ml), IL-8 (18.1 pg/ml), IFN-α (< 3 pg/ml), TGF-β_1 (0.05 ng/ml), and TNF-α (< 0.18 pg/ml).

Determination of serum and nasal urea nitrogen. The total protein recovered during nasal lavage varies from patient to patient. In repeated lavage procedure, variation occurs secondarily to inconsistent recovery of the washings. Kaulbach et al. reported that the urea concentration of serum and epithelial lining fluid are equivalent (7). Therefore, the serum and nasal urea concentrations (in milligrams per deciliter) can be used to calculate the dilution of epithelial lining fluid by lavage fluid according to the following equation:

$$\text{Dilution factor} = [\text{Urea}]_{\text{serum}} / [\text{Urea}]_{\text{nasal}}.$$

This dilution factor was used to correct the concentration of cytokines in the nasal lavage fluids. The urea concentrations of serum and nasal lavage fluid samples were determined by Quest Diagnostics, Inc. (Baltimore, MD).

Data analysis. Analyses were based on data collected from all infected (culture-positive and/or a fourfold or greater rise in HI antibody titer) individuals. Measures of infection and illness (virus titer, symptom assessments, temperature, and nasal discharge weight) and cytokine levels were analyzed using the Wilcoxon signed rank test (Instat2; GraphPAD Software for Science, San Diego, CA). Comparisons between the measures of illness and cytokine levels were made using the Spearman rank correlation test (Instat2; SAS version 6.08; SAS Institute, Inc., Cary, NC).

Results

Influenza infection

In the study described, 20 normal volunteers were inoculated intranasally with influenza virus [A/Texas/36/91 (H1N1)] and then followed serially to determine the relationship between the occurrence of symptoms and the development of cytokine responses. One subject was found to be shedding a picornavirus before inoculation, and is excluded from the analysis. Influenza infection was documented in the remaining 19 volunteers by seroconversion and by recovery of virus from the nasal lavage fluid. The majority of these 19 subjects were women (74%) and the median age of the group was 21.0 yr (range, 19–40 yr). Virus was recovered from 14 of the 19 volunteers (74%) on day 1 of challenge. As shown in Fig. 1, the mean viral titers in nasal washings peaked on day 2 and declined gradually thereafter. Viral shedding persisted in individuals for as long as 7 d.

Symptom pattern of the study group

All of the 19 infected volunteers developed one or more symptoms, and, as a group, exhibited symptom scores that peaked on day 2 and returned to normal by day 8 after viral inocula-

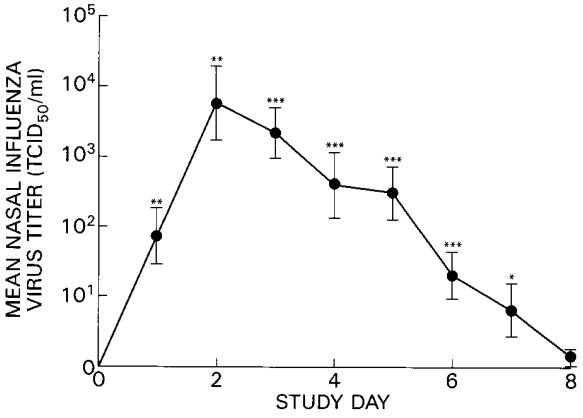

Figure 1. Mean nasal lavage fluid virus titers after experimental influenza A/Texas/36/91 (H1N1) infection. The mean $TCID_{50}$/ml±SEM is shown for each day of the study. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, Wilcoxon signed rank test.

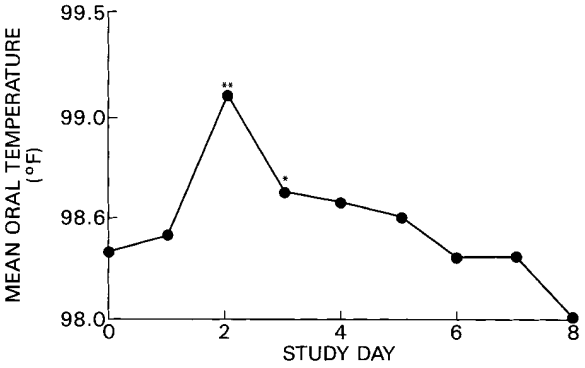

Figure 2. Mean oral temperatures of volunteers experimentally infected with influenza A/Texas/36/91 (H1N1). Oral temperatures were determined four times a day for the duration of the study. Values represent the mean for the 19 volunteers in the study. $*P \leq 0.05$, $**P \leq 0.01$, Wilcoxon signed rank test.

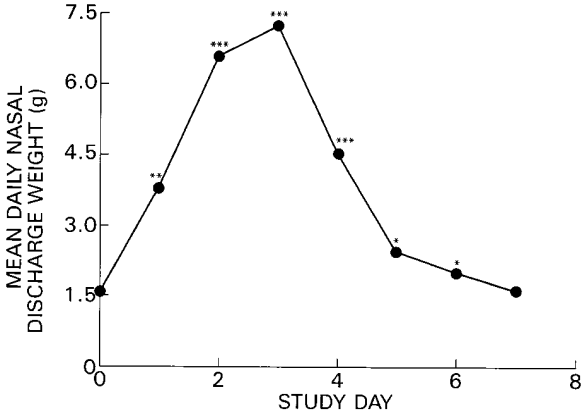

Figure 4. Mean nasal discharge weights of volunteers experimentally infected with influenza A/Texas/36/91 (H1N1). Nasal discharge weights were determined daily using preweighed tissues. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, Wilcoxon signed rank test.

tion. 12 of the 19 volunteers (63%) developed fever (oral temperature > 37.7°C) and, as shown in Fig. 2, the highest mean temperatures were detected on day 2.

Upper respiratory illness, defined by the presence of symptoms such as runny nose and sore throat, occurred in 15 of the 19 volunteers (79%) and was also maximal on day 2 after viral inoculation. In contrast, as shown in Fig. 3, lower respiratory symptoms such as cough and hoarseness peaked later, on day 5, and were milder in severity: group scores on day 5 were not significantly different from those on day 0, and only 6 of 19 volunteers (32%) experienced cough of any magnitude on two or more days. Scores for systemic symptoms such as muscle aches and fatigue also peaked on day 2. Finally, as shown in Fig. 4, nasal discharge weights were elevated significantly by day 2, peaked on day 3, and declined thereafter.

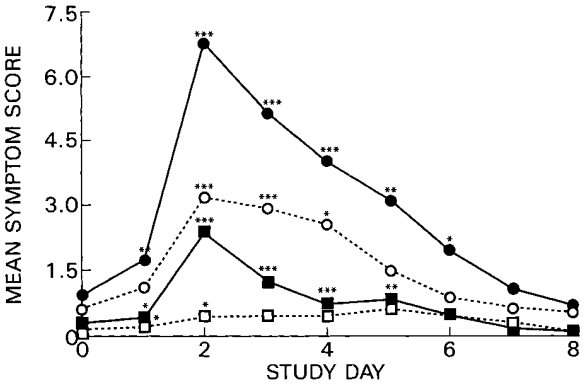

Figure 3. Mean symptom scores of volunteers experimentally infected with influenza A/Texas/36/91 (H1N1). Symptom assessments were performed by the volunteers twice daily on a four-point scale (absent to severe). The score for each symptom group was obtained by adding the individual symptom scores for that particular group. The daily symptom score was obtained by taking the average of the two symptom scores for that particular day. The mean score for total (●), upper respiratory (○), systemic (■), and lower respiratory (□) symptoms are shown for each study day. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, Wilcoxon signed rank test.

Cytokine responses of infected volunteers

Cytokine levels in nasal lavage fluid. Nasal lavage fluids collected on days 0, 1–8, and 22 were assayed for IL-1β, IL-2, IL-6, IL-8, IFN-α, TGF-β and TNF-α. The resulting cytokine concentrations obtained were normalized for the dilution factor as described in Methods.

As shown in Fig. 5, serial nasal lavage fluids obtained from the 19 volunteers during the course of experimental influenza infection exhibited increased levels of most of the various cytokines studied, but the magnitude and time course of change for individual cytokines varied. One pattern was seen with IL-6 and IFN-α, both of which displayed an early biphasic increase, with a 16-fold increase in IL-6 on day 2 and a lesser, 14-fold increase on day 5. 12 of 19 subjects showed increases in IL-6, and both peak levels were significantly greater than baseline ($P < 0.01$ and $P < 0.001$, respectively). In the case of IFN-α, 5 of 19 subjects had detectable levels in their nasal lavage fluids, and only the initial peak level was elevated significantly ($P < 0.05$) when the average level was 52 times preinfection level ($P < 0.05$). A second pattern was exhibited by TNF-α and IL-8: both manifested a somewhat delayed peak on day 4 which dropped off rapidly in the case of TNF-α and was sustained through day 6 in the case of IL-8. However, it should be noted that significant elevations in TNF-α levels were found on days 2–6 ($P < 0.05$–$P < 0.001$), with a maximal 59-fold average increase on day 4, and significant elevation in IL-8 levels was noted on days 5 and 6 ($P < 0.05$–0.01); thus, whereas TNF-α was clearly elevated early on (day 2), IL-8 was not clearly elevated until later (days 5 and 6). A third and different pattern was observed with IL-1β, IL-2, and TGF-β, none of which showed any substantive rise in the nasal lavage fluid over the course of the experimental infection.

Cytokine levels in plasma and serum. Plasma levels of the various cytokines discussed above were measured on days 0, 2, 4, and 8, whereas serum levels were measured on days 0, 1, 1.5, 2, 2.5, 4, and 8. Both IL-6 and TNF-α manifested small (2.5-fold) but statistically significant elevations in plasma levels at time points corresponding to their nasal lavage fluid peak elevations (days 2 and 4, respectively). In contrast, plasma IFN-α and IL-8 were not detected during the course of the study, and

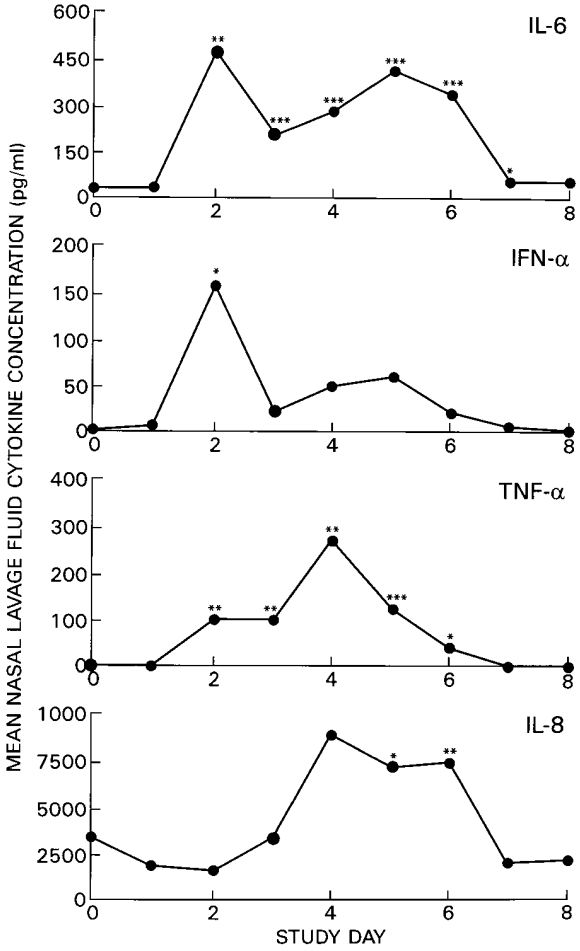

Figure 5. Nasal lavage fluid cytokine levels in volunteers experimentally infected with influenza A/Texas/36/91 (H1N1). Cytokine levels for each day of the study were determined using commercially available ELISA kits. $*P \leq 0.5$, $**P \leq 0.01$, $***P \leq 0.001$, Wilcoxon signed rank test.

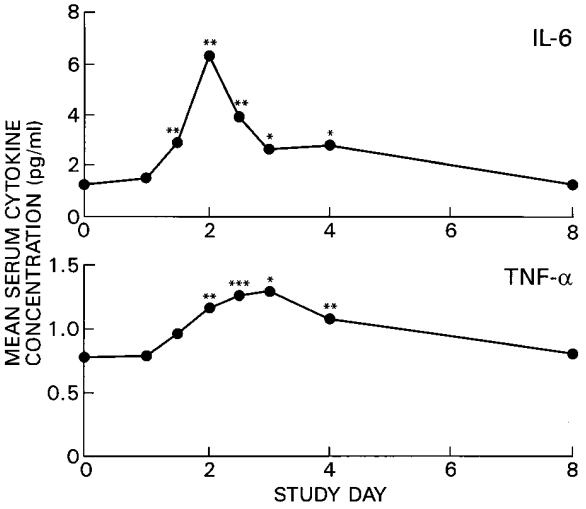

Figure 6. Serum IL-6 and TNF-α levels of volunteers experimentally infected with influenza A/Texas/36/91 (H1N1). Cytokine levels were determined on days 0, 1, 1.5, 2, 2.5, 3, 4, and 8 using commercially available ELISA kits. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, Wilcoxon signed rank test.

IL-1β, IL-2, and TGF-β were detected but did not change during the course of the study (plasma data not shown).

Serum levels of the various cytokines were similar to those of plasma. Thus, as shown in Fig. 6, serum levels of IL-6 peaked on day 2 (a fivefold increase above baseline), whereas TNF-α peaked on day 3 (a 1.7-fold increase above baseline), and statistically significant increases were noted for both of these cytokines on days 1.5–4 for IL-6 and days 2–4 for TNF-α. The serum levels of TGF-β were detectable but did not change, and serum levels of IL-1β, IL-2, IL-8, and IFN-α were undetectable during the course of the study.

Correlation of cytokine levels with measures of infection and symptoms in experimental influenza infection

To further relate cytokine responses to viral titers and symptoms, we subjected the data obtained from all 19 volunteers undergoing experimental influenza infection to Spearman rank correlation analyses.

Viral titer. As shown in Table I, viral titers in nasal lavage fluids of the 19 volunteers were significantly correlated with fever, total symptoms, systemic symptoms, and upper respiratory symptoms on day 2. In addition, viral titers correlated with total symptoms on day 5, and with total and upper respiratory symptoms on day 8. With respect to cytokine elaboration, viral titers were significantly correlated with IL-6, IFN-α, and TNF-α on day 2, and with IL-6 and TNF-α on day 5 (Table II).

Nasal lavage fluid cytokine levels. As shown in Table II, nasal fluid IFN-α, IL-6, and TNF-α levels of the 19 volunteers correlated with fever and total and systemic symptom scores on day 2. These cytokines also correlated with other categories of symptoms in a variable fashion on days 4, 5, and 6. It is noteworthy in this regard that IFN-α and IL-6 levels correlated with lower respiratory symptoms on days 5 and 6, respectively, and, as mentioned above, the scores for these symptoms peaked at the same time. TNF-α levels also correlated with an array of symptoms on days 2 and 4, but IL-8, the cytokine for which peak levels were the most delayed of the various cyto-

Table I. Correlations between Nasal Virus Titers, Clinical Features, and Nasal Cytokine Levels in Experimental Influenza A Virus Infection

	Virus titer		
Feature	Day 2	Day 5	Day 8
Temperature	0.64 (0.007)*	—‡	—
Symptoms			
Total	0.69 (0.004)	—	0.52 (0.03)
Systemic	0.53 (0.02)	—	—
Upper respiratory	0.67 (0.005)	0.51 (0.03)	0.59 (0.01)
Nasal cytokine			
IFN-α	0.70 (0.003)	—	—
IL-6	0.74 (0.002)	0.66 (0.005)	—
TNF-α	0.79 (< 0.001)	0.56 (0.02)	—

*Spearman rank correlation coefficient (*P* value). ‡NS ($P > 0.05$).

Table II. Correlations between Plasma and Nasal Cytokine Levels and Clinical Features of Experimental Influenza A Virus Infection

	Plasma levels				Nasal lavage fluid levels					
	IL-6		IFN-α		IL-6			TNF-α		IL-8
Feature	Day 2	Day 4	Day 2	Day 5	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6
Temperature	0.58 (0.01)*	0.58 (0.01)	0.61 (0.01)	—‡	—	—	—	0.52 (0.03)	—	—
Symptoms										
Total	0.77 (< 0.001)	0.65 (0.003)	0.66 (0.002)	0.61 (< 0.001)	0.73 (0.003)	0.64 (< 0.001)	0.77 (0.005)	0.62 (0.005)	0.55 (0.02)	0.46 (0.05)
Systemic	0.69 (0.004)	0.46 (0.05)	0.69 (0.001)	0.52 (0.02)	0.61 (0.006)	—	—	0.57 (0.01)	—	—
Upper respiratory	0.56 (0.02)	0.69 (0.003)	—	0.56 (0.01)	0.68 (0.001)	0.60 (0.007)	0.59 (0.008)	0.50 (0.03)	0.56 (0.01)	
Lower respiratory	—	—	—	0.60 (0.007)	—	—	0.74 (< 0.001)	—	—	0.52 (0.02)

*Spearman rank correlation coefficient (*P* value). ‡NS ($P > 0.05$).

kines, only correlated with total symptom scores and lower respiratory scores on day 6.

Plasma and serum cytokine levels. As also shown in Table II, plasma IL-6 levels correlated with total, systemic, and upper respiratory symptom scores on both days 2 and 4, but did not correlate on any day with lower respiratory symptom scores. No significant correlations were observed between plasma IFN-α, IL-8, or TNF-α levels and symptoms on any day. In contrast, serum IL-6 levels correlated with temperature elevations on day 4, but not with any of the symptom groups on any day; in addition, serum TNF-α levels correlated both with temperature elevations on days 2 and 3 and with total and upper respiratory symptom scores on days 1 and 2 (data not shown).

Discussion

Influenza virus infection and replication in the respiratory tract directly injures the nasal and tracheobronchial epithelium, possibly as a result of virus-induced cellular apoptosis. The resulting loss of respiratory epithelial cells is one major reason for several of the symptoms that accompany infection, such as cough, depressed tracheobronchial clearance, and altered pulmonary function (2, 3). Infection also elicits a cascade of host immune defenses leading to mucosal inflammation and the influx of polymorphonuclear cells, lymphocytes, and macrophages into the respiratory mucosa. While this response leads to resolution of the infection and protection against reinfection, it is likely that it also contributes to the development of local and systemic symptoms. To evaluate this possibility, we determined the magnitude and pattern of cytokine responses in human influenza infection and related these responses to both the time course of viral replication and the rise and fall of symptoms.

Our approach was to infect normal volunteers with a defined, virulent strain of influenza A virus and then to quantitate levels of a broad range of proinflammatory cytokines occurring locally in nasal lavage fluid, i.e., at the site of infection, as well as systemically in the plasma and serum. In this way we could relate each cytokine level both to the course of infection as assessed by viral shedding and to the occurrence of a set of defined and uniform systemic and local symptoms which were semiquantitated as symptom scores. The major finding was that of the spectrum of cytokines studied, IL-6 and IFN-α stand out as the two cytokines whose kinetics and magnitude in the 19 volunteers were most closely associated with both infection and the occurrence of symptoms. The levels of two other cytokines, TNF-α and IL-8, also rose in the nasal lavage fluid, but in both cases the increases occurred after the peak of symptoms. Measurements of cytokine responses in plasma and serum complemented and confirmed the nasal lavage fluid findings. Again, IL-6 levels were more closely associated with symptoms than were TNF-α levels. Finally, levels of IL-2, IL-1β, and TGF-β did not increase to a significant degree either locally or systemically, suggesting that these cytokines may be less important to symptom formation, though they may still contribute to host response.

That IL-6 levels peaked in the circulation on day 2 after initiation of experimental infection nicely accords with findings from previous studies of human influenza infection. Skoner et al. found that after influenza virus inoculation, infected subjects had ~ 15-fold higher nasal lavage IL-6 levels than uninfected subjects (8). In addition, their data agrees with findings obtained in a recent study we conducted on community-acquired influenza infection (our unpublished data). In that study, eight patients who were enrolled within 48 h of onset of symptoms manifested significantly increased plasma IL-6 levels on day 1 of the study (corresponding to days 2–3 of infection in the study reported here). Thus, in both this and previous studies of experimental and naturally acquired influenza infection, IL-6 appears to play a major role in symptom formation.

The cytokine responses in the plasma and serum were not as large as those documented in the nasal lavage fluid, and in some instances, such as in the case of IFN-α and IL-8, no systemic increases were discerned despite the fact that increases in these cytokines were observed in the nasal lavage fluid. This

is probably due to the fact that cytokines associated with influenza infection are both produced and consumed in the respiratory mucosa, and cytokines that do enter the circulation from the infected mucosa are subject to massive dilution and/or rapid clearance. In this regard, the quantity of a cytokine in the circulation does not necessarily reflect the amount produced in the mucosa, since some cytokines may be bound and cleared more rapidly and completely at mucosal sites than others.

A variety of cells in the respiratory mucosa are likely sources of the proinflammatory cytokines we detected. Cultured human monocytes/macrophages exposed to influenza A virus produce IL-1β, IL-6, TNF-α, and IFN-α/β, and human bronchial cells infected with the virus produce IL-6, IL-8, and RANTES (for regulated upon activation, normal T cell expressed and secreted) (5, 6, 9–11). $CD4^+$ and $CD8^+$ T cells from influenza-immune mice respond to viral antigens with the production of IL-2, IL-4, and IFN-γ (12). These data suggest that lymphoid cells participating in the immune response to influenza infection and the infected epithelial cells themselves are both responsible for the cytokines that elicit symptoms.

Studies of experimental murine influenza infection provide information on in vivo cytokine production that is compatible with that obtained here in experimental human infection. Thus, in the murine studies, one also sees an early rise in the level of several proinflammatory cytokines in the broncheoalveolar lavage fluid (13–15). In addition, in one study, while a number of cytokines peaked within 3 d of infection, IL-6 remained elevated throughout the infection, again suggesting a primary role for this cytokine in influenza infection of the respiratory mucosa (15). However, it should be noted that in the murine studies, elevations in an expanded range of cytokines were seen, including elevations in IL-1α and IL-1β levels. This may reflect the fact that in mice, infection of the lower respiratory tract is more severe than in human volunteer studies, and, in addition, in the murine studies one is sampling cells from lower in the respiratory tract, including the alveolar macrophages (1, 12–15). These findings suggest that the range of cytokines we identified as being associated with symptom formation in human influenza may have to be expanded somewhat in the context of naturally occurring infections of humans with more severe, lower respiratory involvement, the type of complication that leads to the excess mortality associated with this disease (2, 3).

IL-6 is a multifunctional molecule which shares with TNF-α and IL-1β a variety of activities associated with the inflammatory response (16), including a particular ability to stimulate hepatic synthesis of acute phase proteins and to induce terminal B cell differentiation. Recently, it was shown that IL-6 is an important cofactor for the induction of Th2 T cell responses which are necessary for the production of cytokines that support humoral immune responses and for the production of antibodies (17). This may be directly relevant to influenza infection, since formation of protective antibodies is an important mechanism in preventing reinfection with this virus. IL-6 is also a potent pyrogen, and its administration to human subjects induces an acute febrile illness with systemic symptoms like those observed in influenza (18). Thus, it is not unexpected that IL-6 responses would be observed in patients with a variety of viral upper respiratory infections in addition to influenza, as shown here (19, 20).

That we found a correlation between the peak fever and the magnitude of the IL-6 response indicates that it, and not TNF-α, is a main cause of fever in influenza infection. This, plus the fact that the IL-6 response evolved within a short time after viral challenge and in the relative absence of another broad spectrum proinflammatory cytokine, IL-1β, points to a special role for IL-6 in the pathogenesis of influenza. The similar early evolution of IL-6 levels in the absence of an IL-1β rise and the primacy of IL-6 in mediating glucocorticoid induction were noted recently in studies of murine cytomegalovirus infection (21).

IFN-α, a second cytokine for which we documented an early rise in the nasal lavage fluid, must also be assumed to be playing a role in the early systemic and local symptoms of influenza infection. This cytokine was the first proinflammatory cytokine associated with influenza infection, found in the sera of individuals with naturally acquired influenza A/Brazil/78 (H1N1) some 15 years ago (4). In this study, we also noted a rise in serum IFN-α levels early in infection, but the increase did not achieve statistical significance. Moreover, elevations in nasal IFN-α levels were detected in only five patients. These may reflect the relatively mild infection induced in volunteers compared with individuals with naturally acquired infection. The ability of IFN-α to contribute to the symptom complex associated with influenza infection is underscored, though, not only by the correlation between the nasal levels and the symptoms in this study, but also by observations in individuals given IFN-α for therapeutic purposes, such as individuals with chronic viral hepatitis who commonly develop fever, malaise, and muscle aches similar to those accompanying influenza infection (22).

Quite aside from the role of IFN-α in symptom causation in influenza is its role as a key component of antiinfluenza host defense. This point has been highlighted recently by Orange and Biron (23), who showed that IFN-α is central to the induction of natural killer (NK) cell activation during certain viral infections, and that, in addition, NK cell activity is the major mechanism by which the host limits viral infections before the point that antigen-specific immune responses can be engaged. In this regard, it is obvious from this study of experimental influenza infection that the symptoms of infection subside long before the individual can mount a primary antigen-specific B or T cell immune response, and this early resolution of infection is best attributed to antigen-nonspecific immune mechanisms such as NK cells. An additional and somewhat unexpected role of IFN-α, as also shown recently by Cousens et al. (24), is that IFN-α can inhibit IL-12 responses and thus the production of IFN-γ. This counterregulatory role of IFN-α may be a critical aspect of the response to influenza infection, since in its absence such infection might trigger an untoward granulomatous inflammatory response associated with prolonged tissue damage. It is relevant here to mention that a preliminary analysis of additional influenza-infected subjects has detected little or no circulating IFN-γ but high levels of nasal IL-10 during the peak of illness (our unpublished observations).

An important finding in this study is that TNF-α levels and IL-8 levels manifest delayed peaks in the nasal lavage fluid. Thus, as alluded to above, while TNF-α is already significantly elevated on day 2 of infection at the time of peak symptom scores, and TNF-α administration can induce the systemic symptoms we observed (25), it cannot be credited as playing the main role in the induction of the symptoms recorded here,

since its highest elevations are seen later, when these symptoms have already subsided. More likely is the possibility that TNF-α functions relatively late in the inflammatory cycle induced by infection, at a time when virus is already being contained and the response is centered on resolution of the inflammation. It is interesting in this regard that TNF-α has been thought of as a "master" proinflammatory cytokine that induces the subsequent production of other proinflammatory cytokines such as IL-6 and IL-1β (26). At least within the context of influenza infection, this does not seem to be the case. While TNF-α levels were already rising and may have permitted the IL-6 response, the cumulative production of IL-6 and IFN-α seems to be independent of the production of TNF-α.

The relatively late response of TNF-α was accompanied by an even later IL-8 response. IL-8 is a chemokine with both chemotactic and proinflammatory properties. Cultured human respiratory epithelial cells release IL-8 in response to influenza A virus infection (27). In this study, its peak secretion occurs at a time that corresponds to the later infiltration of cells into the respiratory mucosa and to an increase in the turbidity of the nasal mucus discharge (2, 3). In addition, the IL-8 rise correlated only with systemic and lower respiratory symptom scores. These various observations suggest that IL-8 and TNF-α are part of a second wave of proinflammatory cytokines that are perhaps more involved in severe influenza infection centered in the lower respiratory tract. In this regard, in the fatal pneumonic mouse model of influenza, the extent of pulmonary pathology correlates directly with peak bronchoalveolar TNF-α levels and was reduced by neutralizing antibodies to TNF-α (28).

In summary, these studies define the cycle of cytokine secretion that accompanies experimental influenza A respiratory infection. To the extent that these cytokines can be shown to account for symptoms but not to be essential for resolution of the infection, it may be useful in the future to devise ways of ameliorating influenza with regimens that diminish one or another component of this cytokine response. Furthermore, these responses provide an objective measure of the effects of intervention, such as antiviral agents, in treating acute influenza infection.

Acknowledgments

The authors wish to thank the nurses and technical staff of the Respiratory Disease Study Unit, University of Virginia Center for Prevention of Disease and Injury. We thank Ms. Sara Kaul for editorial assistance. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

This study was supported in part by an unrestricted gift to the University of Virginia from Glaxo Wellcome Inc.

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