

Cytokines in the pathogenesis of influenza

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Abstract

Uncomplicated influenza in humans, horses or swine is characterized by massive virus replication in respiratory epithelial cells, inflammation and an abrupt onset of general and respiratory disease. There is now growing evidence that the so-called early cytokines produced at the site of infection mediate many of the clinical and pathological manifestations. Among these cytokines are interferon- α (IFN- α), tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1) α and β , interleukin-6 (IL-6), interleukin-8 (IL-8) and monocyte-attracting chemokines. This paper reviews: (1) *in vivo* examinations of the cytokine profiles during influenza in mice, humans or swine; (2) *in vivo* data on the probable role of these cytokines; and (3) selected *in vitro* data on cytokine induction by the influenza virus. Examination of respiratory secretions of experimentally infected humans or animals revealed a brisk and concurrent rise in several of the cytokines mentioned. Moreover, peak cytokine levels directly correlated with virus replication and disease. In the mouse model, specific anti-cytokine strategies have further confirmed the role of cytokines in body temperature changes, anorexia and lung inflammation. However, cytokines were clearly not the only factor contributing to disease, and they seemed to be essential for resolution of the infection. Though influenza virus was shown to induce cytokines in cell culture, *in vitro* experiments have also revealed conflicting data. Furthermore, the viral genes or products that are responsible for cytokine induction are unknown. Exactly this information would make important contributions to our understanding of the genetic basis of viral virulence. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Influenza A viruses cause severe epidemics of febrile respiratory illness in humans, horses and swine. Virus transmission readily occurs by the airborne route or by direct

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contact. In non-immune individuals, the virus multiplies rapidly in epithelial cells along the entire respiratory tract, whereas extrapulmonary spread of virus is extremely rare. Virus shedding and disease usually begin within 18–72 h after infection. The available data suggest that the lungs are even more susceptible than the upper respiratory tract, and very high titres (up to 10^9 50% egg infectious doses (EID₅₀)/ml) have been recovered from fatal cases of human influenza pneumonia. Nasal and bronchial biopsy specimens reveal degeneration and desquamation of epithelial cells and, initially, a marked infiltration of neutrophils in the airways. These neutrophils play an important role in the pathophysiology of influenza, as they cause narrowing of terminal bronchi/bronchioli and correlate with the onset of fever in experimental animal models. Characteristic clinical signs involve both the upper and lower respiratory tracts and include nasal obstruction and discharge, sore throat, cough and breathing difficulties. Predominant, however, are fever, chills, headache, myalgias, malaise and anorexia. Degeneration of the respiratory tract epithelium and the onset of disease are extremely rapid, but so is regeneration with clinical recovery and viral clearance frequently occurring within 7 days.

Despite extensive clinical and pathological descriptions of influenza, our understanding of the mechanisms of disease development is still incomplete. There is, however, growing evidence that the so-called ‘early’ cytokines are the cause of many of the clinical signs. Early cytokines are produced by (non-immune) cells at the site of infection and they are responsible for local inflammatory reactions, as well as some systemic effects. Interferon- α (IFN- α), tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1) α and β theoretically stand most proximal in the early cytokine cascade. These cytokines are rapidly followed by IL-6 and a number of chemotactic cytokines, such as the neutrophil-attracting interleukin-8 (IL-8), the macrophage inflammatory proteins (MIPs), and monocyte chemoattractant proteins (MCPs) (reviewed in Bielefeldt-Ohmann, 1995). IFN- α , TNF- α , IL-1 and IL-6 are typical examples of cytokines with multifunctional activities and all of them have been associated with fever, excessive sleepiness and anorexia. TNF- α and IL-1 are well known for their profound stimulating effects on neutrophil and macrophage functions. Both cytokines strongly upregulate leukocyte adhesion molecules on the vascular endothelium, thereby mediating the first essential step for sequestration of neutrophils and/or macrophages into the respiratory tract. True chemokines then give the signal for the directed migration of neutrophils or macrophages into the tissues.

Many of these early response cytokines have overlapping and synergistic activities, and can even induce their own production or that of other cytokines. TNF- α and IL-1, for example, stimulate the release of IL-6 and some chemokines. Both, the production and actions of cytokines are thus critically dependent on the context in which they occur. This review concentrates on the cytokines produced during acute influenza and their possible significance in pathogenesis.

2. Cytokine profiles in the respiratory tract during influenza

Several investigators have tried to establish a correlation between cytokines and disease during influenza. For this purpose, they have used various animal models and experimental approaches, each of which has its specific advantages and limitations.

The mouse remains the most common model for studying influenza pneumonia. After intranasal or droplet inoculation with mouse-adapted A/Puerto Rico/8/34 (H1N1) virus, mice develop a lower respiratory tract infection with lung consolidation and interstitial pneumonia. Clinical signs include anorexia, sleepiness and lethargy. Several independent studies documented an early rise of IFN- α , TNF- α , IL-1 α and β and IL-6 in bronchoalveolar lavage (BAL) fluids or lung homogenates, in temporal association with symptom formation and lung pathology (Vacheron et al., 1990; Hennet et al., 1992; Peper and Van Campen, 1995; Kurokawa et al., 1996). In one study, disease and mortality as well as cytokine production were virus-dose dependent (Conn et al., 1995). Systemic cytokine levels were generally lower than those in the lungs or undetectable, indicating highest local production of cytokines. One major drawback of these studies is that the clinical picture in mice differs somewhat from that seen in the natural hosts. For example, mice of most mouse strains show a drop in body temperature instead of fever, and the infection usually is lethal.

In human volunteers, intranasal inoculation with wild type H1N1 or H3N2 viruses produces a characteristic virus shedding pattern and febrile upper respiratory illness. Virus is usually detected in nasal secretions within 24 h after inoculation, rapidly rises to a peak of $10^{3.0}$ – $10^{7.0}$ 50% tissue culture infectious doses/ml on day 2, and is no longer detectable after 5–7 days of shedding. The quantities of virus shed correlate temporally with the typical symptoms, such as fever, muscle aches, fatigue, runny nose and sore throat. Nasal lavage fluids were recently shown to contain elevated levels of (pro)inflammatory cytokines and chemokines, such as IFN- α , IL-6, TNF- α , IL-8, MIP-1 α , MIP-1 β and MCP-1 (Gentile et al., 1998; Hayden et al., 1998; Fritz et al., 1999; Skoner et al., 1999). All cytokines peaked within 2 or 3 days after inoculation and correlated directly with nasal virus titres and illness. Here again, most cytokines occurred at much lower levels in plasma or serum than in respiratory tract secretions. IFN- α and IL-6, in particular, seem to be responsible for much of the early symptom formation (Hayden et al., 1998; Skoner et al., 1999). Other cytokines, such as RANTES (regulated on activation, normal T-cell expressed and secreted) and IL-1 β , remained at low or undetectable levels and are probably of minor importance. However, these human volunteer-challenge studies fail to reproduce the severe lower respiratory tract disease often observed in natural influenza. In addition, assessment of the virological and cytokine responses is limited to the upper respiratory tract.

We used a gnotobiotic pig model to reproduce the pneumonia and lower respiratory symptoms that are so typical of 'swine flu'. These pigs are inoculated with an H1N1 swine influenza virus (Sw/Belgium/1/83) directly into the trachea, so that the entire virus dose reaches the lungs. Within 18 to 24 h, titres in the lungs reach up to $10^{8.2}$ – $10^{9.7}$ EID₅₀/g tissue, and the characteristic pathology and symptoms develop. At that time, there is excessive lung epithelial desquamation and massive infiltration of bronchi and bronchioli with inflammatory cells. Neutrophils, which are virtually absent in virus-negative control pigs, account for $\approx 50\%$ of the total lung lavage cells. Clinically, there is lethargy, shivering, anorexia, tachypnoea, laboured abdominal respiration and coughing. Two and 3 days after inoculation, virus titres decrease and neutrophils have almost disappeared. The airways are then filled with exudates containing necrotic debris and macrophages, and the pigs already start to recover. We found a strong temporal

association between IFN- α , TNF- α and IL-1 levels in BAL fluids and virus titres, neutrophil infiltration and disease (Van Reeth et al., 1998). As clinical symptoms and lung inflammation subsided, IFN- α levels diminished and TNF- α and IL-1 became undetectable. In our opinion, pigs are valuable animal models for the study of cytokines in influenza virus pneumonia. The paucity of assays and reagents for porcine cytokine detection, on the other hand, is a major handicap for studies in pigs.

The ferret has also been used in influenza pathogenesis studies. This animal suffers a predominantly upper respiratory tract infection, with abundant epithelial cell desquamation and neutrophil infiltration in the nasal mucosa, nasal discharge, fever, listlessness and anorexia (Smith and Sweet, 1988). Unlike in the previously mentioned species, the cytokine profile in the respiratory tract of infected ferrets has not yet been examined. Studies to investigate the possible role of cytokines in ferret influenza are discussed below.

Though studies in mice, humans and pigs dealt with different experimental conditions and clinico-pathological outcomes, all of them assert a role of multiple cytokines in influenza symptoms and pathology. It is even reasonable to assume that additive and synergistic interactions between these cytokines will occur. On the other hand, production of most cytokines during influenza is transient and particularly short-lasting. Cytokines involved in the down-regulation of the inflammatory response, notably IFN- γ and IL-10, have also been demonstrated early in murine and human influenza (Hennet et al., 1992; Fritz et al., 1999). It seems that in uncomplicated influenza at least, upregulation of early cytokines is tightly controlled and escalation of the cytokine response is an exception rather than the rule.

3. Further analysing the role of cytokines in influenza

Further insights in the significance of specific cytokines during influenza come from experiments in the murine model, using either specific cytokine antagonists or cytokine gene knock-out mice. Injection of TNF- α antibodies at the time of influenza inoculation reduced lung lesion severity and prolonged survival by 24 h (Peper and Van Campen, 1995). Antiserum injection experiments have also implicated IFN- α and IL-1 α in the fever response during influenza (Kurokawa et al., 1996). Another consequence of anti-IFN- α treatment was a reduction of IL-1 levels, supporting the importance of the cytokine network in influenza. Treatment with a naturally occurring IL-1 receptor antagonist produced a small but statistically significant increase in food intake and survival rates (Swiergiel et al., 1997). In knock-out mice deficient for either IL-1 β or IL-6, influenza virus inoculation had less effect on body temperatures than in wild type mice, but depression or cachexia were unaffected (Kozak et al., 1995, 1997). Finally, mice lacking the MIP-1 α gene showed less mononuclear cell infiltration and pulmonary edema than wild-type mice (Cook, 1996). These studies have thus confirmed the involvement of cytokines in influenza pathogenesis, but the effect of blocking individual cytokines was also partial and in some cases very modest. One plausible explanation is the substantial redundancy between cytokines, such as IFN- α , TNF- α , IL-1 and IL-6. Due to this phenomenon, the lack of one specific cytokine may be compensated for by another cytokine with overlapping activities.

Of necessity, alternative and less specific strategies have been tried to confirm the role of cytokines in human and ferret influenza. Treatment of human volunteers with the neuraminidase inhibitor zanamivir prevented both experimental influenza virus infection and the production of seven cytokines tested for (Fritz et al., 1999). Cytokines, therefore, are probably useful indicators of influenza severity and suitable for monitoring the efficiency of preventive and therapeutic measures. Studies in ferrets, on the contrary, failed to demonstrate a role for pyrogenic cytokines in fever (Jakeman et al., 1991; Price et al., 1997a). In the ferret model, virulent and attenuated reassortant influenza viruses clearly differ in fever-producing capacity. Also, when nasal inflammatory cells from infected ferrets are further incubated *in vitro*, more 'pyrogenic substances' are liberated with virulent than with attenuated viruses. Still, no correlation could be established between IL-1 and IL-6 levels in the supernatants and pyrogenicity, and the attenuated viruses produced as much of these cytokines as the virulent viruses. Cytotoxic and apoptotic activities in cell culture, on the other hand, were significantly higher with virulent than with attenuated strains (Price et al., 1997b). Based on these findings, it has been hypothesized that differences in viral damage to the respiratory epithelium *in vivo* may account for a differential cytokine profile and resulting differences in constitutional symptoms. Direct *in vivo* examinations of the pathologic and cytokine responses will be needed to test this hypothesis.

Together, three points regarding the role of cytokines in influenza deserve attention. First, a multitude of cytokines is involved, and it remains to be seen whether some are more important than others. Second, as accepted long ago, direct viral cytopathic effects to the respiratory tract will at least in part account for the pathological changes. Third and most important, the same cytokines that exacerbate inflammation and disease also have a central role in the resolution of the infection. IFN- α , TNF- α , IL-1 and IL-6, for example, are known to participate in non-specific and specific antiviral immune responses (Le and Vilcek, 1987). More specifically, influenza infection produced higher mortalities in IL-1 β knock-out mice than in wild type mice (Kozak et al., 1995). Similarly, MIP-1 α knock-out mice showed a delayed viral clearance (Cook, 1996). These data suggest that the rapid and efficient immune response against SIV may also relate to the production of early response cytokines.

4. Mechanisms of cytokine induction by the influenza virus

Influenza virus has also been shown to induce the production of cytokines in cell culture. Researchers at the Philipps University (Marburg, Germany) reported the production of IFN- α , TNF- α , IL-1, IL-6 and mononuclear cell attracting chemokines (MIP-1 α , MIP-1 β , RANTES) after A/PR/8/34 inoculation of human monocytes, rat alveolar macrophages or a murine macrophage cell line (Nain et al., 1990; Hofmann et al., 1997; Bussfeld et al., 1998). These *in vitro* studies have revealed several interesting characteristics of the influenza virus-induced cytokine response. First, the virus probably uses differential mechanisms for the induction of different cytokines, i.e. TNF- α production could only be induced by infectious virus, whereas IFN release was also stimulated by UV-inactivated virus (Nain et al., 1990). Second, the cytokine

'microenvironment' seems to have a profound effect on the magnitude of the virus-induced cytokine response. Exposure to granulocyte macrophage-colony stimulating factor (GM-CSF), for example, markedly enhanced production of IFN- α , TNF- α , IL-1 and IL-6 in response to influenza virus (Bender et al., 1993). Third, a synergism between influenza virus and lipopolysaccharide from *Escherichia coli* or *Haemophilus influenzae* was detected for TNF- α production (Nain et al., 1990). This finding supports the idea that the complications of combined influenza virus and bacterial infections may be partially due to an excessive TNF- α production.

Caution must be exercised, however, when extrapolating from these in vitro data to the in vivo situation. In vitro experiments fail to reproduce the complex interactions between different cell types and cytokines. In fact, discrepant results have arisen from virus-cytokine induction studies in different in vitro systems. Though TNF- α induction required infectious virus in the study of Nain et al. (1990), other researchers found TNF- α upon contact of murine peritoneal macrophages with purified influenza virus neuraminidase (Houde and Arora, 1990). Another example: IL-8 production was suppressed upon inoculation of human monocytes with A/PR/8/34 (Hofmann et al., 1997), but stimulated by H3N2 influenza virus infection of primary (Choi and Jacoby, 1992) or continuous airway epithelial cells (Adachi et al., 1997). In addition, influenza virus-induced expression of transcription factor genes was highly cell type-specific (Bussfeld et al., 1997). This confirms that the cytokine response can be profoundly influenced by the cell type used. So far, the nature of the cells producing cytokines in vivo remains unknown, and so are the viral genes or products involved in cytokine induction. A careful analysis of cytokine expressing cells in the influenza virus-infected lung would help to develop biologically relevant in vitro models to study the viral mechanisms of cytokine induction.

5. Future prospects

From this review, it will have become clear that we will probably never use anti-cytokine strategies in the treatment of human or animal influenza. The brisk appearance of cytokines and their redundant actions are just two reasons that hamper a timely and efficient suppression of the effects of (pro)inflammatory cytokines. Most problematic, however, is the fact that potentially harmful cytokines can also play an essential role in pathogen clearance. Still, one of the major challenges remains to unravel the genetic basis of viral virulence. As for mammalian influenza viruses, this has proven an extremely difficult task. If we could explain the basic mechanisms of one specific virulence characteristic, such as the production of a given cytokine, this would greatly add to our understanding of the relationship between genetic constitution and the virulence of influenza.

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