THE ROLE OF CYTOKINES IN THE IMMUNE RESPONSE TO INFLUENZA A VIRUS INFECTION

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Summary. – Influenza A virus is one of the most important causes of respiratory tract diseases. It replicates in epithelial cells and leukocytes resulting in the production of immune mediators – cytokines, substances with various biological effects. Cytokines, as a part of innate immunity, favor the development of antiviral and T_H1-type immune responses. Cytokines also affect the adaptive immune response and disease manifestation. In the organism, the virus infection results in the production of chemotactic [a regulated upon activation, normal T cell-expressed and -secreted cytokine (RANTES), monocyte chemoattractant proteins (MCP) MCP-1, MCP-3, macrophage inflammatory protein 1α (MIP-1α), interferon γ-induced protein 10 (IP-10), and interleukin 8 (IL-8)], pro-inflammatory [IL-1β, IL-6, IL-18, and tumor necrosis factor α(TNF-α)] and antiviral [interferon (IFN) α/β] cytokines. Whilst knowledge of the mechanisms underlying host and tissue specificity has advanced significantly, we still know relatively little about the function of cytokines released from different cells following influenza infection. In this review we deal with the role and mode of possible impact of cytokines on the disease pathogenesis and host immune response.

Key words: cytokines; immunity; influenza; Influenza A virus; pathogenesis

Contents:

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Abbreviations: APC = antigen-presenting cells; BALs = broncho-alveolar lavage fluids; CNS = central nervous system; CSF = colony-stimulating factors; IL = interleukin; INF = interferon; IFN-α/β = IFN-α and/or IFN-β; IP-10 = IFN-γ-induced protein; ISRE = interferon-stimulated regulatory element; JAK = Janus tyrosine kinase; MCP = monocyte chemoattractant proteins; MIP-1α = macrophage inflamatory protein 1α; MLN = mediastinal lymph node; NF-kappa B = nuclear factor kappa B; NK = natural killer; OAS = (2'-5')-oligoadenylate synthase; p.i. = post infection PKR = protein kinase R; RANTES = regulated upon activation, normal T cell-expressed and -secreted cytokine; RIG-I = retinoic acid-inducible protein I; TNF = tumor necrosis factor; TYK = tyrosine kinase

1. Introduction

Influenza A virus (the virus) is the causative agent of the most common and highly infectious human disease. This cosmopolitan pathogen is responsible for a significant morbidity and mortality in humans as well as animals.

Influenza viruses belong to three genera of the family *Orthomyxoviridae*, namely *Influenzavirus A, Influenzavirus B* and *Influenzavirus C* (Fauquet *et al*., 2005). These genera contain corresponding single species, *Influenza A virus, Influenza B virus* and *Influenza C virus*. Influenza viruses are enveloped spherical, sometimes pleomorphic particles

of 80–120 nm in diameter containing a segmented single-stranded (ss) genomic RNA of negative polarity. They are unique for their site of RNA synthesis in the cell; RNA replication and transcription take place in the nucleus (Lamb and Krug, 1996).

Since influenza attacks numerous warm-blooded animal species including birds and mammals, it can be also characterized as a zoonosis. In fact, the virus responsible for influenza is highly contagious for horses, pigs and humans. Moreover, it can infect a wide variety of birds, mostly aquatic and shore ones (Suarez and Schultz-Cherry, 2000). Replication of the virus in mammals is mostly restricted to epithelial cells of the respiratory tract, while in birds it takes place commonly in the gastrointestinal tract. Influenza B virus appears to infect solely human population; it has been rarely isolated from seals. The natural hosts for Influenza C virus are humans and pigs. Influenza virus strains may be distinguished predominantly according to antigenic differences of their nucleoproteins and matrix proteins (Zambon, 1999; Webster and Bean, 2000; Krug *et al*., 2003).

From the medical and epidemiological point of view the most critical is Influenza A virus that causes frequent and widespread human epidemics and pandemics (Cox and Subbarao, 1999; van Reeth, 2000).

Despite extensive clinical and pathological studies on influenza, our understanding of the mechanisms of disease development is still incomplete. Besides the local respiratory symptoms, caused directly by the virus, influenza is typical also with a number of complications due to the immune mediators – cytokines. Their analysis is an important aspect of the study of proinflammatory reactions that has revealed many immunopathological issues.

2. Immune response

All of the vertebrates defend themselves against invading pathogens by not only physiological responses (e.g. cough reflex, mucociliary clearance, and anti-microbial mucosal surface) but mainly by complicated immune ones (Strieter *et al*., 2002). Evolution has provided the mammalian host with two major forms of host defense – innate and adaptive. The innate resistance is non-specific with respect to particular pathogen and has no immunological memory. It represents a "gate-keeper" of the immediate host defense against invading microorganisms. The innate immunity provides a first line of defense against viral infection and influences the subsequent adaptive response (Biron, 1999; He *et al*., 2004). The adaptive immunity depends on two classes of specialized lymphocytes, T- and B-cells, whose specific receptors are generated in response to antigen presentation by professional antigen-presenting cells (APC) (dendritic cells, macrophages and mature B-cells). Finally, this process causes clonal expansion of T- and B-cells resulting in long-term humoral and cell-mediated immune memory.

The activation of macrophages is one of the first responses in natural immunity against viral and other intracellular infections, and this reaction leads to the cytokine and chemokine production, phagocytes activation and further inflammatory responses. Crucial elements of immune response to viral infections are predominantly natural killer (NK) cells, T_C-lymphocytes and IFN-α and/or IFN-β (IFN-α/β) (Sareneva *et al*., 1998; Biron, 1999).

Influenza viruses in the organism induce a cascade of several immune functions, such as phagocytosis, NK cell and cytotoxic T-lymphocyte activities, and production of antibodies and some cytokines (Han and Meydani, 2000; O´Neill *et al*., 2000; Gerhard, 2001). It has been established that T_C-lymphocytes play a central role in the recovery from influenza infection but helper T-lymphocytes (T_H-lymphocytes), virus-neutralizing antibodies and IFN-γ also contribute significantly (Kostense *et al*., 1998).

Apart from human model, mice (very commonly) and ferrets and pigs (less frequently) are the excellent organisms for study of immune response to influenza viruses. Maximal virus titers in the mouse lungs were observed by days 2–4 post infection (p.i.) and remained high until day 5, then started do decline (Hennet *et al*., 1992; Mori *et al*., 1995; Peper and van Campen, 1995; Gerhard, 2001; Landolt *et al*., 2003). A virus-specific immune response is first detectable by days 3–5 days p.i. and infectious virus is usually cleared within days 7–10 (Stevenson and Doherty, 2000). Experiments on ferrets showed the influx of proinflammatory cells to the upper respiratory tract evidently correlated with the onset of fever about 24 hrs p.i. (Price *et al*., 1997).

2.1. Cytokines as a part of innate immune response

Cytokines are "low-molecular-mass local hormones" secreted by numerous cell types, mostly lymphocytes, less professional APC, monocytes, endothelial cells and fibroblasts. As they can transfer information among the cells, they serve as soluble mediators of innate and adaptive immune response. Cytokines are responsible for the activation, proliferation and differentiation of immune cells. Moreover, they also modulate cell processes – survival, apoptosis and inflammation. However, cytokine effects are mostly influencing whole immune system; they are capable to affect functions of several organs, tissues and systems (Alcami *et al*., 2002; Giulietti *et al*., 2001).

Cytokine secretion can be explained as a relative rapid self-limited event. Regularly, cytokines exert their major effects on the neighbor cells (the paracrine effect), but when

over-produced they can have even systemic (endocrine) effects. Although their consequences are very specific, the cytokines may overlap (the redundant effect) or collaborate (the synergistic effect) in their activities; some of them may work either in antagonistic or inhibitory manner. As the majority of cytokines do not influence just one type of cells, we can regard them generally as clearly pleiotropic (Buc, 2001).

Production of distinct immune mediators is determined by the inducing signal, cell type, location, developmental stage and particular gene spectrum induced by transcription and translation factors within the cell (Sareneva *et al*., 1998). Cytokines can contribute to disease pathogenesis directly or indirectly (by regulating subsequent immunopathological events). Some cytokines with multifunctional activities like IFN-α, TNF-α, IL-1 and IL-6 are associated with certain clinical manifestations, e.g. fever, sleepiness and anorexia (Neuzil and Graham, 1996; Swiergel and Dunn, 1999; van Reeth, 2000).

2.1.1. Cytokine classification. Chemokines

Individual cytokines occupy several families with distinguishable properties such as IL, IFN, colony-stimulating factors (CSF), TNF, tumor growth factors (TGF), and chemokines (Giulietti *et al*., 2001). The chemokine family consists of more than 40 chemotactic cytokines. They are produced by a variety of cells including monocytes, alveolar macrophages, neutrophils, eosinophils, thrombocytes, mast cells, T- and B-lymphocytes, NK cells and epithelial cells (Julkunen *et al*., 2001). Chemokines function as chemoattractants of immune cells; their role is to control leukocyte migration through the body and recruit proinflammatory cells (neutrophils, macrophages, T-lymphocytes, endothelial cells, eosinophils, and thrombocytes) (Ferenčík *et al*., 2001; Alcami *et al*., 2002). Chemokines bind to their specific receptors on the surface of these cells, thus leading to rapid changes needed for binding to vascular endothelial cells. Subsequently, proinflammatory cells are allowed to migrate from blood vessels through the vascular endothelium into the site of inflammation (Julkunen *et al*., 2000). Recently, additional activities of chemokines, namely participation in angiogenesis, collagen production and proliferation of precursor hematopoetic cells have been documented (Buc, 2001).

Regarding the position of two N-terminal cysteines in chemokine four groups of chemokines are recognized: CC and CXC (α-chemokines), XC (γ-chemokines), and CX_3C (δ-chemokines). CXC chemokines can be further divided into two subgroups on the basis of presence or absence of a structure/function domain consisting of Glu-Leu-Arg (ELR motif). ELR^+ CXC chemokines are chemoattractants for neutrophils and act as potent angiogenic factors. In contrast, ELR^- CXC chemokines serve as chemoattractants for monocytes (Strieter *et al*., 2002).

2.1.2. Cytokines as mediators of immune response to virus infection

Influenza virus infection results in production of different cytokines: proinflammatory (e.g. IL-1β, IL-6, IL-18, IFN-α/β, and TNF-α), antiviral (e.g. IFN-α/β), and others (e.g. RANTES, MIP-1α/β, MCP-1, MCP-3, and IP-10) (Julkunen *et al*., 2000). The importance of cytokines in the infection was known already earlier; it has been proved that IL-6 and IFN-α in the infection evidently correlate with systemic disease symptoms (Hayden *et al*., 1998; Masuyama *et al*., 2002).

Nevertheless, to define the contribution of particular cytokines to the disease pathogenesis is not easy. The interplay of various cytokines is illustrated in Fig. 1.

IFN, one of crucial components of immune system, was discovered in 1957 (Issacs and Lindemann, 1957). Its name was derived from the ability to interfere with viral replication. Later on, various kinds of IFN have been described. In accordance with diverse antigenic structure, producers and activity we presently recognize type I and II IFNs. IFNs I play role predominantly in the regulation of natural immunity, while IFNs II coordinate mainly the cellular immunity (Buc, 2001). IFN-α (secreted by the cells of lymphoid lineage) and IFN-β (produced by epithelial and fibroblastoid cells), both belonging to IFN I, evidently increase the lytic potential of NK cells. IFN-γ (type II) is secreted by different cells including T- and B-lymphocytes, NK cells and macrophages (Price *et al*., 2000). Besides other functions, it induces MHC I and MHC II expression and activates mononuclear phagocytes (Neuzil and Graham, 1996).

The virus is an undoubted inducer of IFN-α/β, which works as a direct antiviral substance with additional antiproliferative and immunomodulating activities (Neuzil and Graham, 1996; Julkunen *et al*., 2001). IFN-α/β are usually induced within hours following viral infection (Wang *et al*., 2000; Tamura and Kurata, 2004). After secretion from infected cells IFN-α/β interact with a common receptor on the surface of infected as well as neighbor cells, the IFN-α/β receptor (IFNAR). The IFN binding induces dimerization of the receptor resulting in activation of tyrosine kinases JAK1 (Janus tyrosine kinase 1) and TYK2 (tyrosine kinase 2). The activated kinases induce tyrosine phosphorylation of the receptor. Subsequently, latent transcriptional factors (STAT1, STAT2) become activated, form heterodimers, and are attached to the complex. Next, after recruiting IRF-9 protein, it is translocated to the cell nucleus, where it can bind to specific DNA sequences containing an IFN-stimulated regulatory element (ISRE-6) (Salvatore and

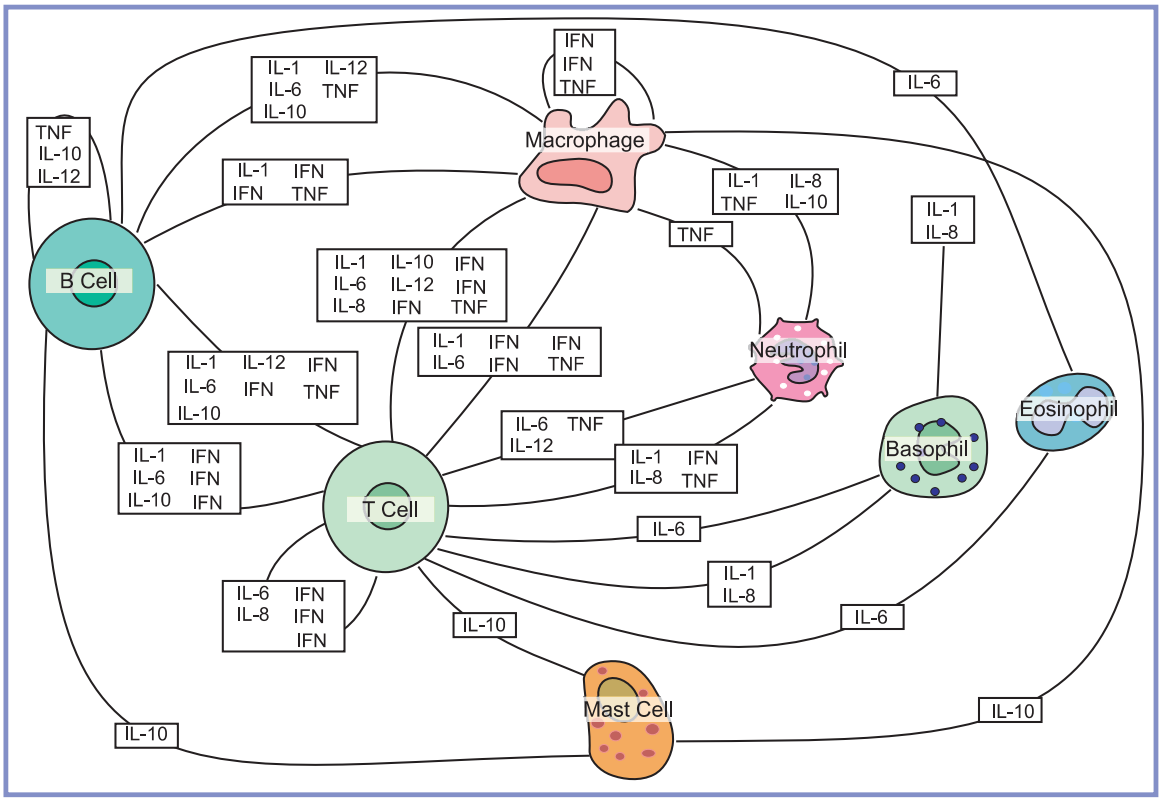

Fig. 1

Interplay of cytokines

Modified according to http://meetings.cshl.org/tgac/tgac/images/h_cytokinePathway.gif.

Garcia-Sastre, 2001). Thus IFN-α/β secretion stimulates promoters containing ISRE sequences, resulting in transcriptional induction of hundreds of genes, including those encoding proteins, which inhibit viral replication (Garcia-Sastre, 2002; Krug *et al*., 2003). *In vitro* studies on mouse embryo fibroblasts with targeted deletion of nuclear factor kappa B (NF-kappa B) have revealed a negative regulatory role of NF-kappa B in IFN-induced gene expression and biological activities of IFN, which might lead to enhanced therapeutic effectiveness of IFN in influenza infection (Wei *et al*., 2006).

In the host cell, there takes place induction of expression of viral resistance mediators called IFN-inducible proteins, namely proteinkinase-R (PKR), (2'-5')-oligoadenylate synthase (OAS) and Mx proteins, which have antiviral activity. PKR, in the presence of double-stranded RNA (dsRNA) structures arising during viral replication, is able to inhibit host cell protein synthesis by activating endoribonuclease L (RNase L) that degrades mRNA (Restifo, 2000). Furthermore, PKR can phosphorylate the translation initiation factor eIF2 and thus block its recycling from inactive form (Biron, 1998; Bergmann *et al*., 2000). IFN produced by virus-infected cells induces also a family of proteins designated Mx. The central role of such proteins is probably in conferring a high degree of resistance to the virus. They reside either in the nucleus or in the cytoplasm. Murine Mx1 protein, a nuclear one, selectively inhibits Influenza A virus and other orthomyxoviruses at the level of primary transcription. Human MxA protein occurring in the cytoplasm interferes with the virus expression in the posttranscriptional or translational phase, so that there is a reduced number of new viral particles (Han and Meydani, 2000; Suarez and Schultz-Cherry, 2000).

IFN-α/β seem to be important cofactors in the development of T_H1-type immune response. It can upregulate the expression of some chemokine genes (MCP-1, MCP-3 and IP-10), which results in further recruitment of monocytes/macrophages and T_H1-type cells to the site of infection. They are also involved in stimulation of the IL-12 receptor expression, enhancement of IFN-γ gene expression in human NK cells and T-lymphocytes, and augmentation of dendritic cells response (Price *et al*., 2000; Julkunen *et al*., 2000).

IFN-γ is a pleiotropic cytokine with scores of immunoregulatory functions. It is produced by NK cells, lymphocytes and macrophages as a response to stimulation with different antigens (Wyde *et al*., 1982; Baumgarth and Kelso, 1996; Bot *et al*., 1998). It has predominantly indirect antiviral effects; it inhibits cell growth and upregulates MHC I and MHC II expression on the surface of APC (Graham *et al*., 1993). This cytokine is an essential regulator of the *in vivo* immune response – it can stimulate cell-mediated immunity, activate alveolar macrophages and NK cells, and control the chemokine gene expression (Biron, 1998; Monteiro *et al*., 1998; Sareneva *et al*., 1998; Oh *et al*., 2002). During the immune response it participates in the control of Ig class switching (IgM to IgG2a); many authors suppose that IFN-γ might have a role in processing of antigens (Graham *et al*., 1993; Han and Meydani, 2000; Price *et al*., 2000). It stimulates the production of IL-1 and TNF. In the presence of IL-2, IL-12, IL-18, and IFN-α/β, their synthesis can be enhanced (Buc, 2001).

Although it has been shown on a mouse model that IFN-γ is strongly induced in the lung parenchyma, some data suggest this cytokine is not necessary for recovery from influenza infection. In addition, several authors doubt its importance in influencing the *in vivo* effector activity of T_H- a T_C-lymphocytes in the respiratory tract (Graham *et al*., 1993; Baumgarth and Kelso, 1996). However, the latter experiments confirmed that absence of IFN-γ reflected not only a reduced survival of experimental animals but also a diminished ability of organism to completely clear the pulmonary tract from the virus (Bot *et al*., 1998). It has been reported that IFN-γ is not required for recovery from primary infection with the virus but is critical for later heterosubtype immunity (Nguyen *et al*., 2000).

IL-1 is an important molecule of inflammatory reaction. It has both direct and indirect effect on the chemotaxis of neutrophils, the main representative of inflammation (Buc, 2001). The IL-1 family of cytokine consists of two agonists, IL-1α and IL-1β. These are encoded by two different genes and represent two protein isoforms. IL-1α is mostly membrane-associated, while IL-1β is secreted. The both of them bind to the same receptor expressed on target cells (Strieter *et al*., 2002).

An enhanced production of IL-1β during the virus infection was observed besides monocytes, macrophages and keratinocytes also in T-lymphocytes, neutrophils and endothelial cells. IL-1β is expressed in inactive form, which is activated after proteolytical cleavage by caspase-1 (Julkunen *et al*., 2001). Although IL-1 appears not to influence the killing of virus-infected cells, it enhances IgM antibody response and stimulates recruitment of T_H-lymphocytes to the site of infection (Schmitz *et al*., 2005).

TNF-α is a homotrimeric cytokine produced largely by monocytes/macrophages but also by T- and B-lymphocytes, NK cells, astrocytes, epithelial and endothelial cells (Seo and Webster, 2002). This mediator binds to two distinct cell-surface receptors, p55 and p75. The former, like a related Fas receptor, contains a 60-aa domain known as "death domain", essential for signal transduction of an apoptotic signal (Strieter *et al*., 2002). TNF-α mediates a wide spectrum of multiple proinflammatory and immunological functions. In the course of infection TNF-α can induce the release of IL-1 and IL-6, which are important for influenza infection and most likely cooperates with other immunoregulators (IL-2, IL-8 and IFN-α/β). During the post-infection tissue-reparating process it can stimulate the growth of fibroblastoid and endothelial cells. It is capable to activate and recruit neutrophils, macrophages, T_C-lymphocytes and NK cells (Peper and van Campen, 1995; Biron, 1999; Buc, 2001).

An increase of TNF-α in influenza infection was found in nasopharyngeal lavage fluid on day 1 and its maximum on day 4 p.i. in correlation with fever (Kaiser *et al*., 2001). *In vitro* studies have shown that TNF-α can inhibit viral replication by at least two different mechanisms: direct lysis of virus-infected cells or induction of a selective anti-viral state in uninfected cells (Peper and van Campen, 1995). Recent data suggest that this cytokine has a crucial position in resolving influenza infection in the host respiratory tract (Seo and Webster, 2002). Using an *in vitro* system with human lung epithelial A549 cells, Matikainen *et al*. (2006) have found that TNF-α enhances the expression of retinoic acid-inducible protein I (RIG-I)-signaling pathways. RIG-I is a central regulator of the virus-induced expression of antiviral cytokines in human lung epithelial cells.

Both IL-1β and TNF-α are well known for their profound stimulating effects on neutrophil and macrophage function. They strongly upregulate leukocyte adhesion molecules (MCP-1 and MCP-3) on the vascular endothelium and in this way mediate the first essential step for sequestration of neutrophils and macrophages into the respiratory tract (Neuzil and Graham, 1996; van Reeth, 2000). IL-1β and TNF-α are involved in maturation of tissue macrophages and dendritic cells (Julkunen *et al*., 2001).

Many studies have confirmed increased levels of multifunctional cytokine IL-6, produced by stimulated monocytes, possibly also by granulocytes and lymphocytes, during the influenza infection (Gentile *et al*., 1998; Buc, 2001). IL-6 serves as a co-stimulating factor for T-lymphocytes and a growth factor for B-lymphocytes. It can induce terminal differentiation of B-lymphocytes to plasma cells and subsequent antibody production, stimulates mucosal IgA response and may augment T-lymphocyte-dependent IgE production (Neuzil and Graham, 1996). It is supposed that IL-6 regulates the immune and acute-phase responses and hematopoiesis (Han and Meydani, 2000; Kaiser *et al*., 2001). Recently, it has been suggested that a high IL-6 concentration

in plasma or just its presence in cerebrospinal fluid could be a reliable indicator of the disease progression and of clinical outcome (Ito *et al*., 1999; Togashi *et al*., 2001; Sugaya, 2000).

IL-11 has effects that evidently overlap those of IL-6. This cytokine activates B-lymphocytes and, like IL-6, is involved in hematopoiesis. Moreover, IL-11 affects lipid metabolism and induces macrophage differentiation (Neuzil and Graham, 1996). Generally, IL-11 is a major component of the immune response to many viral but not bacterial infections (Oh *et al*., 2002).

IL-18 is a product of infected macrophages and dendritic cells. It shows similar properties to IL-1 in biochemical functions and to IL-12 in biological functions (Liu *et al*., 2004). Like IL-1β, IL-18 is expressed in inactive form that is activated after proteolytical cleavage by caspase-1 (Julkunen *et al*., 2001). IL-18 can stimulate the overall function of immune cells either indirectly, by decreasing IL-10 production, or directly by stimulation of activated T-lymphocyte proliferation. This cytokine is capable to induce differentiation of T_H0-lymphocytes toward the T_H1-type, which obviously profiles the organism to favor cell immunity (Buc, 2001). It can also provoke the production of IL-1β, TNF-α and other chemokines. IL-18 in synergy with IL-12 or IFN-α/β activates the IFN-γ production in human T-lymphocytes. IL-18 is a key factor in the early antiviral host response by upregulation of NK cell-killing activity. Most likely it exerts proinflammatory effects, but, on the other hand, it also regulates an excessive inflammatory response (Sareneva *et al*., 1998; Liu *et al*., 2004).

IL-12 is a 72 K heterodimeric cytokine composed of a heavy and a light chain. In the course of infection it is primarily produced by APC and neutrophils (Biron, 1999; Mbawuike *et al*., 1999). In the organism, IL-12 directs the differentiation of T_H-0 to T_H-1 cells, inhibits the T_H-2 type immune response, and induces the cell-mediated immune response (Kostense *et al*., 1998). It represents a growth factor for NK cells and contributes to early production (on days 3–5 p.i.) of IFN-γ by these cells (Monteiro *et al*., 1998). Moreover, IL-12 is characteristic for enhancement of proliferation and cytotoxicity of activated T-lymphocytes (Kostense *et al*., 1998; Mbawuike *et al*., 1999). Monteiro *et al*. (1998) have suggested that the presence of viral antigen is not enough to induce the release of IL-12 and rather active virus replication is essential.

IL-10 can be found as a homodimer in extracellular areas and reminds IFN-γ due to its tertiary structure. It is produced most commonly by monocytes and macrophages but also by T_H- and B-lymphocytes. Generally, the production of IL-10 is a late sign of inflammatory process during the primary response; a maximum prevalence is reached usually on day 10 p.i. (Carding *et al*., 1993). IL-10 also prevents an excessive response of organism to proinflammatory cytokines (predominantly IFN-γ, IL-1, IL-6, TNF, and IL-12, and CXC and CC chemokines) by blocking their expression and stimulating the synthesis of their specific inhibitors. However, it should be emphasized that IL-10 is not an exclusively inhibitory and anti-inflammatory cytokine. Moreover, it is able to stimulate the proliferation and differentiation of B-lymphocytes and to augment mononuclear phagocyte and neutrophil recruitment to the site of infection (Sarawar and Doherty, 1994; Strieter *et al*., 2002). It is strongly believed that IL-10 is responsible for an increased susceptibility of the organism to secondary bacterial infections by a so far unknown mechanism (van der Sluijs *et al*., 2004).

Throughout the virus infection, there appear besides the cytokine mediators of immune response also chemokines. Infected bronchial epithelial cells secrete RANTES, MCP-1, MCP-3, IP-10, and IL-8. IL-8, a typical chemoattractant, activates neutrophils and draws them to the site of infection. Apart from this, it is able to attract and activate T-lymphocytes (Adachi *et al*., 1997; Arndt *et al*., 2002). Monocytes and macrophages contribute to the response to the virus infection with production of chemokines stimulating mononuclear leukocytes (MIP-1α, MIP-1β, MIP-3α, RANTES, MCP-1, MCP-3, and IP-10) (Bussfeld *et al*., 1998; Kaufmann *et al*., 2001). MIP-1α affects recruitment and degranulation of eosinophils and basophils and occurs in the nasopharyngeal secrets during the virus infection (Oh *et al*., 2002).

2.1.3. Spectrum of cytokines in various hosts during influenza infection

Different viruses apparently use the various mechanisms of cytokine induction (van Reeth, 2000). In case of influenza infection the most widely postulated trigger for cytokine induction is the dsRNA produced in cells during the viral replication (Brydon *et al*., 2005). Type I IFNs (IFN-α/β) are the crucial cytokines produced in the course of the virus infection. As it has been proved that infected macrophages produce IL-18 and dendritic cells secrete relatively high levels of IL-12 in response to influenza infection, it appears that tissue macrophages and dendritic cells represent the basic cell types responsible for the production of antiviral and immunostimulatory cytokines during the virus infection of humans (Julkunen *et al*., 2000). It is believed that IFN-α/β and IFN-γ have protective effects against infection, while IL-1, TNF-α and IL-6 rather seem to be involved in the inflammatory phase of the infection (Gentile *et al*., 1998; Han and Meydani, 2000). The latter cytokines have some overlapping activities: endogenous pyrogenic effect, activation of macrophages and co-stimulation of T-lymphocytes (Konstantinos and Sheridan, 2001).

In the studies carried out on individuals infected with influenza experimentally or naturally, the virus was detected

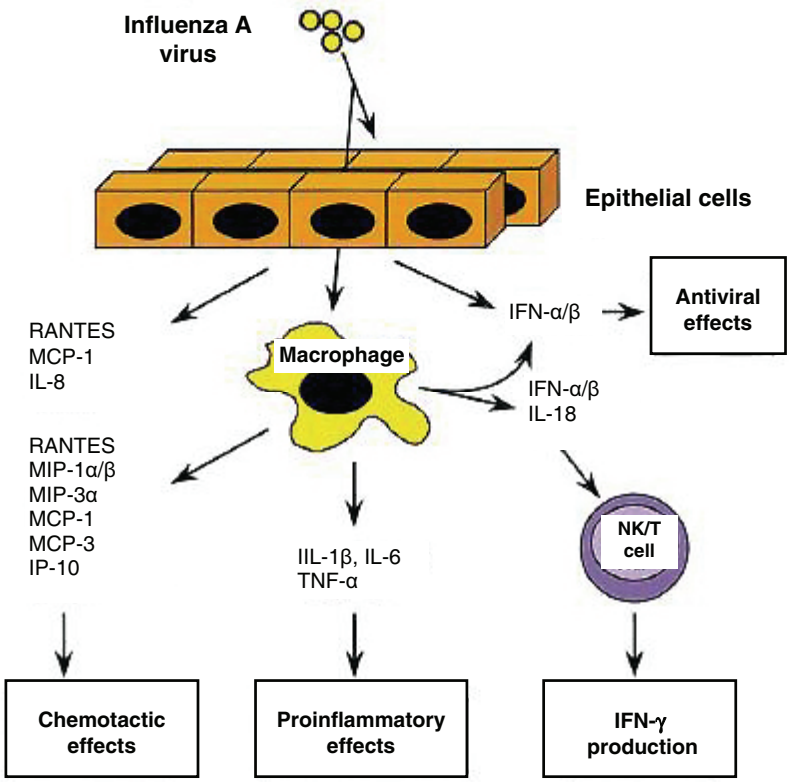

Fig. 2

Cytokine production in Influenza A virus-infected epithelial cells and macrophages

According to Julkunen *et al.* (2001).

in nasal secrets already within 24 hrs p.i. and in the lungs a little later. The bronchoalveolar lavage fluids (BALs) from infected persons showed increased levels of IL-6, TNF-α, IFN-α, IFN-γ, IL-8, IL-10, MCP-1, and MIP-1α/β (Hayden *et al*., 1998; Fritz *et al*., 1999; Skoner *et al*., 1999; Kaiser *et al*., 2001; Seo and Webster, 2002). In most studies on human volunteers experimentally infected with influenza, cytokines reached maximum on days 2–3 p.i. and their levels, predominantly of IL-6 and IFN-α, directly correlated with the intensity of viral replication and respiratory and systemic symptoms too (Zambon, 1999; Kaiser *et al*., 2001). However, in general, levels of systemic cytokines are usually lower than those of lungs cytokines, which are at times almost undetectable; these findings indicate the highest production of local cytokines (van Reeth, 2000; Brydon *et al*., 2005). There is growing evidence that the so-called "early" cytokines, produced by the cells at the site of infection, are the cause of numerous clinical and pathological signs manifested already during the first 18–72 hrs p.i. In case of fever, there has been found out no correlation between its duration and the levels of individual cytokines. A few studies indicate that febrile patients have high levels of serum IFN-α (van Reeth, 2000; Masuyama *et al*., 2002).

The results of *in vitro* experiments are to certain extent inconsistent with those obtained *in vivo*. Lines of human epithelial cells of lung origin respond to influenza infection just by very poor production of IFN-α/β, IL-1, IL-6 and TNF-α. On the contrary, infected monocytes and macrophages secrete significantly higher levels of cytokines, mainly IFN-α/β, TNF-α, IL-1β, IL-6, and IL-18 (Sareneva *et al*., 1998; Kaufmann *et al.,* 2001; Cheung *et al*., 2002; Pauligk *et al*., 2004). Human phagocytes incubated *in vitro* with the virus produced TNF-α, IL-1β, IL-6, IFN-γ, IL-8 and MIP-1α (Brydon *et al*., 2005). In this context, a remarkable observation was made on human macrophages: the virus of subtype H5N1 induced more strongly the expression of proinflammatory cytokines (mainly TNF-α and IFN-β) as compared to other virus subtypes (Cheung *et*

al., 2002). A similar finding was obtained with human bronchial and alveolar epithelial cells by Chan *et al*. (2005): virus strains of H5N1 subtype were stronger inducers of cytokines (IFN-β, IL-6, RANTES, and IP-10) than those of H1N1 subtype.

A comparable cytokine profile, like in human population, also exists in the mouse model. Levels of IFN-α, TNF-α, IL-1α, IL-1β, IL-2, and IL-6 in BALs and lung homogenates rise soon after infection (Neuzil and Graham, 1996; Brydon *et al*., 2005). Moreover, in some cases also an increased quantity of IFN-γ was observed in BALs and mediastinal lymph nodes (MLN) (Sarawar and Doherty, 1994). In studies on kinetics of mouse cytokines, levels of immune mediators (IFN-γ, TNF-α, IL-1α, and IL-1β) peaked between 36 hrs and day 3 p.i. with influenza virus A/PR/8/34. Particularly, IL-1α in the lungs increased already by 24 hrs, peaked by 48 hrs, and returned to normal by 72 hrs p.i. (Hennet *et al*., 1992); IL-6 behaved similarly. Apart from these cytokines, release of IL-12 from mouse alveolar macrophages has been observed *in vitro* (Tamura and Kurata, 2004). Moreover, in general, correlations among the post-infection cytokine response, reduced locomotor activity and food intake decrease have been found (Swiergiel and Dunn, 1999).

A less common but frequently used model host for study of influenza virus remains pig, in which infected alveolar macrophages have been observed. And when cytokines were assayed in these cells *in vitro*, significant levels of IL-1β, IL-6, IL-8, and TNF-α were found; the latter occurred in largest amounts. In contrast, in *in vivo* experiments, just IL-6 and TNF-α were detected, while the infected pigs showed clinical symptoms similar to those in humans (Seo *et al*., 2004).

2.2. The complications associated with influenza infection and their relation to cytokines

Following the virus infection many cytokines cause not only local but often systemic symptoms too. In humans, several serious signs appear. Some studies have documented a causal relationship between influenza infection and encephalitis (Delorme and Middleton, 1979, Kimura *et al*., 1998). Influenza-associated encephalopathy/encephalitis represents a rare but severe complication, mostly in the childhood. The major symptoms usually include altered consciousness, often coma, brain edema, convulsions, vomiting, fever, and cough. In some cases also a multiorgan dysfunction appears (Sugaya, 2000; Morishima *et al*., 2002; Kawada *et al*., 2003). Generally, influenza-associated encephalitis may have two forms: infectious encephalitis, characteristic for fulminant clinical progress, and post-infectious encephalitis occurring a few weeks after recovery (autoimmune process associated with demyelination) (Hayase and Tobita, 1997). Apart from these facts, influenza infection can be also the reason for particular myelitis (Salonen *et al*., 1997) or special encephalopathy forms with serious consequences (acute necrotizing encephalopathy or the Reye syndrome) (Mizuguchi *et al*., 1995; Studahl, 2003). However, encephalitis and encephalopathy overlap markedly in their symptoms, so that it is frequently not easy to recognize them.

Based on numerous observations, the virus antigens are confined to a very limited part of human brain, especially Purkinje cells in the cerebellum and many neurons in the pons (Takahashi *et al*., 2001). In some studies, it has been suggested that the virus of H3N2 subtype generates more severe illness than that of H1N1 subtype (Studahl, 2003). And although a few reports have described the isolation of the virus (predominantly of H3 subtype) from cerebrospinal fluid or brain tissues, it was rather exceptional than regular (Togashi *et al*., 1997, 2000). However, in general, this issue is controversial. Whereas Fujimoto *et al*. (1998b) take a direct invasion of the virus into the brain for regular, Ito *et al*. (1999) regard the attack of the virus on central nervous system (CNS) as rare. In this context, two pathways of viral penetration from the epithelium of upper respiratory tract into CNS, are proposed: (i) neuronal pathway, via the olfactory and trigeminal nerve system, and (ii) hematogenous pathway (Mori *et al*., 1995; Fujimoto *et al*., 1998; Ito *et al*., 1999; Studahl, 2003).

An interesting issue is the evidence of elevated concentrations of some cytokines and their receptors (TNF-α, sTNF-R1, IL-6) in cerebrospinal fluid of patients with influenza-associated encephalopathy/encephalitis (Morishima *et al*., 2002; Togashi *et al*., 2004). Kawada *et al*. (2003) have found that transcription of TNF-α, IL-6 and IL-10 genes was apparently upregulated in the patients mentioned above as compared to those without these neurological complications. Therefore it is supposed that incident rate of these cytokines is probably a key indicator of the disease progression and also a good marker for the diagnosis of encephalopathy/encephalitis (Ito *et al*., 1999). Sudden onset of cytokines (mainly TNF-α, sTNF-R1, and IL-6), exceptionally high cytokine levels, and histological alterations are the main factors responsible for the generalized impairment of vascular endothelial cells, which most likely plays role in the pathophysiology of this disease (Togashi *et al*., 2001; Ichiyama *et al*., 2004). However, the source of these cytokines is unknown. Proinflammatory cytokines may be produced in the respiratory tract or be commonly secreted by permanently activated cells (Kawada *et al*., 2003). Furthermore, there exists a hypothesis that the cytokine release from virus-stimulated glial cells may be responsible for neurotoxic effect on the brain and a rapid breakdown of the blood-brain-barrier (Yokota *et al*., 2000).

3. Conclusions

Influenza virus usually brings about a typical respiratory disease. Tracheal and bronchial cells of the respiratory tract represent the primary targets of the virus. Besides local symptoms the pathogen can cause many systemic complications. However, these are not the consequences of virus occurrence but rather of immune mediators. To date, the riddles, what molecules activate, regulate and coordinate immune responses, and what substances, apart from the virus, are responsible for the symptoms, course and severity of the disease, remain unsolved. The most prominent candidates for these posts are cytokines. Their analysis is crucial part of the study of inflammatory reactions of organism, which allows us to reveal important immunological facts. It is believed that a better and more substantial knowledge of the proinflammatory response to influenza infection can help us to control better possible disease complications and to prevent massive damage of infected tissues.

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