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Tumor necrosis factor-α/interleukin-10 balance in normal and cystic fibrosis children

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***Background*: The balance between tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) is important for immune homeostasis maintenance. Exuberant production of TNF-α contributes to overwhelming inflammatory response and tissue damage. But, commonly, increase in TNF-α is counterbalanced by simultaneous synthesis of an anti-inflammatory cytokine IL-10, which suppresses production of many activating and regulatory mediators.**

***Aims*: In the present study, the relationships between TNF-α and IL-10 in the plasma of healthy schoolchildren and cystic fibrosis (CF) patients have been investigated.**

***Methods*: Blood samples were obtained from 12 CF patients with chronic pulmonary disease and 18 healthy schoolchildren vaccinated with live attenuated rubella vaccine. IL-10 and TNF-α were determined in the plasma samples using commercially available enzyme-linked immunosorbent assay kits.**

***Results*: Before vaccination, most healthy children (13 of 18) demonstrated superiority of pro-inflammatory TNF-α over anti-inflammatory IL-10 (TNF-α/IL-10 Â 1). In these subjects, a significant positive linear association between the cytokine values has been found. Vaccine challenge resulted in a marked reduction of TNF-α/IL-10 ratios. In addition, a disappearance of correlation between the cytokine values was observed. Such disturbance was related to exuberant elevation of the IL-10 levels after inoculation. On the contrary, in CF individuals, plasma cytokine values remained in strong linear association independently of TNF-α or IL-10 predominance. No spikes in the plasma levels of IL-10 in CF patients during a 6-month observation period have been revealed.**

***Conclusions*: There were no fundamental differences between CF and healthy children in the regulation of TNF-α and IL-10 secretion. Thus, immune quiescence seemed to be associated with the predominance of TNF-α, whereas immune disturbance was characterized by IL-10 superiority. The only abnormality that was found in CF patients consisted of their inability to produce unlimitedly IL-10 in response to antigen stimuli.**

Key words: Tumor necrosis factor-α, Interleukin-10, Inflammatory response, Cystic fibrosis, Vaccination

Introduction

Challenge with infectious agents or their products provoke early secretion of regulatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10). These cytokines are produced by the activated macrophages and play opposite roles in both innate and specific immune response.[1,2] TNF-α upregulates production of other pro-inflammatory cytokines by the immune and nonimmune cells[3–6], augments leukocyte adhesion and promotes cell migration into the tissue space.[7] It facilitates antimicrobial activity of neutrophils and macrophages but, in addition, potentiates their tissue-damaging properties.[8] Overzealous production of the cytokine may have serious adverse consequences such as systemic inflammation and septic shock.[9,10] But, commonly, rapid increase in TNF-α is counterbalanced by early and sustained expression of anti-inflammatory IL-10.[11] This cytokine suppresses production of important activating and regulatory mediators (including TNF-α),[2,12] inhibits the leukocyte recruitment to sites of inflammation,[13] decreases HLA-DR expression by monocyte/macrophages and reduces their Ag-presenting capacity.[14] In addition, IL-10

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directly inhibits leukocyte bactericidal activity and downregulates tissue injury.[15,16]

Until recently, there was very little existing documentation on changes and regulation of the balance of TNF-α/IL-10 secretion under both basal and immune-challenge conditions. The relationships between the cytokines have been most widely studied in the context of sepsis syndrome. There are a lot of reports providing a protective effect of IL-10 and deteriorative TNF-α action during systemic inflammatory response syndrome.[17,18] However, several uncommon studies have suggested that overwhelming expression of IL-10 may contribute to sepsis-induced immunosuppression and predispose the host to the development of a variety of nosocomial infections; in particular, bacterial infection of the lung.[19,20] In this regard, change in TNF-α/IL-10 ratio might be predictive of complications in patients with inflammatory diseases. Indeed, the recent study focusing on TNF-α and IL-10 production in patients with, or at risk of developing, adult respiratory distress syndrome (ARDS) has demonstrated that there was a larger ratio of TNF-α to IL-10 in the bronchoalveolar lavage fluid from patients with ARDS, favoring a pro-inflammatory process.[21] Similarly, a significant decrease of IL-10 content in the lung of cystic fibrosis (CF) patients with chronic pulmonary disease has been noticed.[22]

CF is an autosomal recessive genetic disorder, which occurs with mutation in the CF transmembrane conductance regulator (CFTR) gene. Abnormal function of CFTR results in obstructive pulmonary process due to accumulation of thick, viscous mucus, which leads to impaired mucociliary clearance.[23] During the first years of life, young children with CF are colonized and develop pneumonia secondary to *Staphylococcus aureus, Haemophilus influenzae* or, less commonly, *Klebsiella pneumoniae*.[24,25] Later, the patients become infected with *Pseudomonas aeruginosa*. Colonization with the pathogens initiates exuberant host immune response characterized by a marked influx of neutrophils into the lung, and elevation in inflammatory mediators such as TNF-α, IL-1β, IL-6, IL-8, and leukotriene B_4.[26,27] In this regard, restricted production of IL-10 in CF patients may contribute to exuberant immune response and lung tissue damage.

In the present study, the relationships between TNF-α and IL-10 in the plasma of healthy teenagers and CF children with chronic lung disease have been investigated.

Material and methods

Patient assessment

Twelve CF patients (mean age, 11.9 years) from the Department of Cystic Fibrosis of the Research Center for Medical Genetics (Moscow) were enrolled in the study. Cystic fibrosis was diagnosed by increased chloride concentrations (> 60 mmol/l) in a sweat test and typical clinical symptoms of the disease, and/or detection of mutation in both CFTR alleles. All children were pancreatic insufficient and suffered from progressive suppurative pulmonary disease. The patients were treated with basic therapy (mucolytics, multivitamins, high calorie diet, microspheric enzymes) and nonsteroidal anti-inflammatory drug nimesulide in the daily dose of 3 mg per kg of body weight. In the case of acute pulmonary exacerbation, antibiotics were prescribed. Individuals with *P. aeruginosa* infection were treated by cephalosporins of third generation in combination with aminoglycosids or ciprofloxacin. The patients were seen every third month at the Department of Cystic Fibrosis where clinical data and bacteriology of bronchopulmonary infections had been recorded. The following pulmonary function tests were performed: forced expiratory volume in 1 sec (FEV_1), and forced vital capacity (FVC).

Blood samples were collected at the beginning of the study, and then again 3 and 6 months later. Seven patients (group A) with chronic *P. aeruginosa* infection and poor lung function (FVC and FEV_1 < 70% predicted) were evaluated after a 2-week routine antibiotic course. Five patients (group B) who demonstrated relatively good lung function (FVC and FEV_1 > 70% predicted) were examined at a time of well being during ordinary visit to the Department. The study was approved by the Ethics Committee of the Federal Children Hospital (Moscow, Russia).

Healthy schoolchildren

Blood samples were obtained from healthy schoolchildren at Moscow Gabrichevsky Research Institute of Epidemiology and Microbiology. The study group included 18 children, aged 11–13 years, with a negative history of rubella. All subjects had routine physical examination and rubella serotesting. None of the participants were seropositive. Children were vaccinated with live attenuated rubella vaccine Rudivax® (Pasteur Merieux). No vaccine-related adverse effects were noted. Heparinized blood was collected before vaccination then again 1 week and 1 month later. The subjects had no personal or immediate history of CF and no evidence of illness at the start of or during the study. The investigation has been performed as a part of the special research program 'Vaccination against rubella' and supported by the Ministry of Health of Russian Federation.

Blood collection

Blood was collected in tubes with heparin (25 IU/ml) by venopuncture.

Cytokine assay

Plasma samples were harvested and analyzed for IL-10 and TNF-α by enzyme-linked immunosorbent assay (ELISA) techniques with commercially available kits, which were designed to measure the 'total' (bound and unbound) amount of the cytokines (Cytimmune Science Inc, MD, USA). The lower limits of detection for the both assays were 0.195 ng/ml.

Statistical analysis

Statistical analysis was performed using non-parametric Wilcoxon tests. Relationships between TNF-α and IL-10 were investigated by multiple regression analysis. The TNF-α concentration was considered as the independent variable, and the IL-10 concentration as the dependent one. Student's *t*-test was used to evaluate the regression coefficient.

Results

TNF-α and IL-10 in plasma of CF patients and healthy children

Fig. 1 illustrates the individual variations in TNF-α and IL-10 levels measured from normal children and CF patients. As expected, we found a marked elevation in plasma cytokine concentrations in healthy individuals following rubella vaccination. In CF subjects, plasma TNF-α levels were situated within a similar range as compared with healthy children before immunization.

FIG. 1. Cytokine concentrations in the plasma of healthy children and CF patients. Plasma samples were assayed for TNF-α (A) and IL-10 (B) by ELISA. The individual values of CF patients are the means of all results during 6 months of the observation period. Median values are indicated by the lines. Each dot represents one subject. A two-sample Wilcoxon test was used to compare the cytokine levels in healthy group before and after vaccine administration: TNF-α (A), $p_{1,2} = 0.32$, $p_{1,3} = 0.001$; IL-10 (B), $p_{1,2} = 0.27$, $p_{1,3} = 0.038$. Differences between CF patients and healthy children were analyzed using the unpaired Wilcoxon test: TNF-α (A), $p_{1,4} = 0.55$; IL-10 (B), $p_{1,4} = 1$). Note that in response to rubella vaccination, both cytokines in the plasma of healthy subjects were elevated and significantly exceeded the levels measured from CF patients: TNF-α (A), $p_{2,4} = 0.037$, $p_{3,4} = 0.003$; IL-10 (B), $p_{2,4} = 0.0001$, $p_{3,4} = 0.0006$.

Table 1. TNF-α/IL-10 ratios in healthy children and young cystic fibrosis patients

Healthy children				CF patients	
Subject number	Before vaccination	7 days after	30 days after	Patient number	
1	1.56	0.89	1.26	1	0.52
2	9.33	0.65	0.43	2	0.79
3	0.14	0.77	0.43	3	1.31
4	1.60	0.62	0.48	4	0.69
5	6.44	0.97	0.48	5	1.24
6	4.03	0.32	0.06	6	1.02
7	0.99	0.83	0.56	7	1.03
8	0.64	0.94	0.77	8	1.82
9	1.12	0.26	ND	9	1.11
10	0.69	ND	0.90	10	1.06
11	1.46	0.24	0.56	11	1.73
12	0.32	0.27	0.63	12	2.37
13	1.22	0.28	0.45		
14	6.79	65.69	ND		
15	0.20	0.30	0.27		
16	23.33	0.67	4.86		
17	3.84	ND	ND		
18	13.03	ND	ND		
Median	1.51	0.65	0.52		1.09
p		0.05	0.02		0.341 0.003* 0.004**

The individual parameters of CF patients are the median values of the results during 6 months of observation.
* Compared with the parameters of healthy children 1 week after rubella vaccination.
** Compared with healthy subjects on day 30 after inoculation.

Mean values of the cytokine were 1.10 ± 0.21 ng/ml in children with CF and 1.61 ± 0.30 ng/ml in healthy individuals; no significant difference was noted between the groups ($p = 0.32$). With regard to IL-10, mean values were 0.83 ± 0.06 ng/ml in CF and 2.58 ± 0.84 ng/ml in healthy children; no difference between the groups was detected ($p = 1$). At the same time, both TNF-α and IL-10 levels in CF children were much lower than the cytokine values found in the plasma of healthy subjects after rubella vaccination (all $p < 0.04$).

Ratio of TNF-α to IL-10 in the plasma of healthy individuals and CF patients

The relative concentrations of plasma TNF-α and IL-10 in healthy children and CF patients were calculated (Table 1). Prior to vaccination, the cytokine ratios in healthy individuals were widely varied ranging from 0.14 to 23.33 (median value, 1.56). After inoculation, the ratio of TNF-α/IL-10 was found to be significantly decreased. Median values of the cytokine ratio were 0.65 on day 7 and 0.52 on day 30 following vaccination ($p = 0.05$ and 0.02, respectively). In CF patients, the ratio of TNF-α/IL-10 was at intermediate value (1.09), which was not significantly different to that of the healthy children before vaccination ($p = 0.34$). At the same time, in comparison with vaccinated children, the cytokine ratio in CF subjects was significantly elevated (both $p < 0.004$). Table 2 displays the semi-annual changes of TNF-α/IL-10 ratio in CF patients. Dependent on pulmonary function failure and disease severity, the patients were divided into two groups. Group A ($n = 7$) showed poor lung function (FVC and FEV_1 < 70% predicted) and severe pulmonary disease. Group B ($n = 5$) demonstrated relatively good lung function (FVC and FEV_1 > 70% predicted) and mild disease severity. A significant difference in the cytokine ratio between the groups has been observed ($p = 0.03$). In group B, all the ratios were > 1 except for a case associated with vaccina-

Table 2. Semi-annual changes of TNF-α/IL-10 ratios in CF patients

CF patients	Patient number	TNF-α/IL-10 ratio			
		Month 0	Month 3	Month 6	Median
Group A	1*	0.52	0.60	0.47	0.52
(FVC < 70%	2**	0.79	0.92	0.76	0.79
predicted)	3	2.14	0.88†	1.31	1.31
	4	1.47	0.69	0.57	0.69
	5	0.57	1.49	1.24	1.24
	6	0.89	1.02	1.47	1.02
	7	1.03	0.83‡	1.50	1.03
Mean ± m		1.06 ± 0.22	0.92 ± 0.11	1.05 ± 0.16	0.94 ± 0.11
Group B	8	1.56	1.82	1.92	1.82
(FVC > 70%	9	0.83‡	1.11	1.32	1.11
predicted)	10	1.20	1.06	0.79†	1.06
	11	6.04	1.73	1.68	1.73
	12	2.8	1.60	2.37	2.37
Mean ± m		2.49 ± 0.95	1.46 ± 0.16	1.62 ± 0.27	1.62 ± 0.18
p		0.10	0.02	0.07	0.03

* The patient demonstrated a significant lung function failure in the end of the study.
** The patient died 2 months after finishing the study.
† Children were examined 2 weeks after acute lung exacerbation.
‡ Subjects were evaluated 2 weeks after vaccination against influenza (group A) and poliomyelitis (group B).

tion and an incidence related to acute virus respiratory infection. In group A, individual values of TNF-α/IL-10 ratio fluctuated about 1 and were clearly lower than in group B.

Relationships between plasma levels of TNF-α and IL-10 in healthy children and CF patients

The data indicate that the cytokine ratios were at much reduced levels in vaccine-challenge subjects (including two vaccinated CF patients) as well as in CF patients after acute lung exacerbation. At the same time, these parameters were significantly elevated in the most healthy children before vaccination (see Table 1) and in CF subjects during periods of well being (see Table 2). In the following, two different situations are considered. The first is associated with the incidences when TNF-α/IL-10 > 1 (immune quiescence), the second related to cases when TNF-α/IL-10 < 1 (immune disturbance). Multiple regression analysis has been performed to examine the relationships between the plasma cytokine levels. IL-10 was considered as a dependent parameter, whereas TNF-α was independent. As can be seen in Fig. 2A, there was a significant positive linear association between the plasma cytokine levels in 13 healthy children, who showed TNF-α/IL-10 > 1, before vaccine application ($r = 0.94$, $p = 0.000002$). A similar result has been obtained in CF patients with high cytokine ratios ($r = 0.62$, $p = 0.002$). At the same time, no correlation has been noted in healthy children after immunization (Fig. 2B). However, when we did not take into account subject number 6, who had demonstrated the only spike in IL-10 plasma level, a strong positive association between the cytokine values was found in other 11 children 1 month after vaccination ($r = 0.65$, $p = 0.03$). With regard to CF patients with low cytokine ratios, they showed a significant positive linear association ($r = 0.89$, $p = 0.0002$).

Discussion

In recent years, it has become evident that pro-inflammatory cytokines are involved in the induction of IL-10 by stimulated monocyte.[28,29] It has been suggested that TNF-α is able to primarily regulate IL-10 transcription through stimulation of activating protein-1 recognizing by IL-10 promoter.[30] Later, Foey *et al.* hypothesized that IL-10 production requires at least two signals; the first is provided by lipopolysaccharide (or its physiologic equivalent), and the second by endogenous TNF-α and/or IL-1β.[31] Furthermore, circulating TNF-α and IL-1β are powerful inducers of brain-mediated anti-inflammatory response. They can directly stimulate the hypothalamic–pituitary–adrenal axis and enhance sympathetic nerve system activity, resulting in glucocorticoid and catecholamine secretion that limits pro-inflammatory cytokine production and stimulates IL-10 releasing.[32,33]

Changes in TNF-α/IL-10 ratios that have been observed in healthy children after rubella vaccination are in accordance with the current concept of inflammatory response control. Thus, before vaccination, most healthy children (13 of 18) exhibited relatively high cytokine ratios (TNF-α/IL-10 > 1), demonstrating predominance of pro-inflammatory TNF-α over anti-inflammatory IL-10. In these subjects, a significant positive linear association between the cytokine levels has been found. Rubella vaccination and associated stress were bound to result in catecholamine and corticosteroid releasing that promoted IL-10 synthesis.[32] Besides, auxiliary sources of anti-inflammatory IL-10 (such as liver, spleen and brain as well) might be activated.[17,34] Thereafter, on day 7 after immunization, a rise in IL-10 passed ahead of TNF-α expansion. In this context, a significant decrease in TNF-α/IL-10 ratios and the absence of the correlation between the cytokines have been observed. On day 30 after rubella vaccination IL-10 still predominated over TNF-α, but the association between the cytokine concentrations tended to recover and was described again by linear regression model.

In CF patients, the plasma cytokine values seemed to be in linear association independently of IL-10 or TNF-α predominance. Furthermore, no spikes in the levels of IL-10 in CF patients during a 6-month observation period have been presently observed. This is in agreement with previous studies that revealed a significant decrease of IL-10 content in CF lung as well as reduced ability of CF epithelial cells and CD4+ T lymphocytes to produce this cytokine in response to inflammatory stimuli.[22,35,36] One of the possible reasons of these phenomena is a primary CFTR defect resulting in an absent or diminished Cl^- secretory function of the cells in response to cAMP-mediated agonists. The latter is known to exert a dual regulatory function by enhancing IL-10 formation and attenuating TNF-α synthesis.[37]

CFTR or even its first nucleotide binding fold act as Cl^--specific pores in lipid bilayer.[38,39] Triggering of the cAMP pathway (e.g. by the binding of epinephrine to β-adrenergic receptor) stimulates adenylate cyclase to form cAMP that results in the activation protein kinase A, which in turn phosphorylates the regulatory domains of the CFTR Cl^- channels and increases their open probability.[38,40] Another consequence of cAMP pathway triggering is a stimulation of CFTR gene expression.[41] There are segments within the CFTR promoter that resemble AP-1 and AP-2 binding sites, a cAMP-response element, and glucocorticoid response elements.[41,42] In this context, cellular response to various physiological stimuli (including catecholamines and glucocorticoids) might be associated with

FIG. 2. Relationships between plasma TNF-α and IL-10 concentrations in normal children and CF patients. Multiple regression analysis has been performed. IL-10 and TNF-α were supposed as dependent and independent parameters, respectively. (A) In the case of TNF-α/IL-10 > 1 (immune quiescence), plasma cytokine concentration correlated with regression lines, which were described by the equations: IL-10 = 0.76 × TNF-α – 0.21 (line 1, normal children before vaccination) and IL-10 = 0.27 × TNF-α + 0.45 (line 2, CF patients). (B) Under TNF-α/IL-10 < 1 (immune disturbance), regression models are described by the equations: IL-10 = 2.20 × TNF-α + 1.28 (line 3, normal children 1 month after vaccination) and IL-10 = 1.53 × TNF-α – 0.07 (line 4, CF patients). The encircled point demonstrates a spike in IL-10 plasma level of subject number 6 (see text).

the rapid opening of CFTR channels and efflux of Cl^- and/or with the increase in CFTR expression. In CF, reduced Cl^- conductance and, as a consequence, defective signaling through a cAMP-dependent pathway may result in numerous abnormalities including restricted IL-10 production by the immune and nonimmune cells.

According to common opinion, increase in production of IL-10 and decrease in TNF-α secretion should be salutary in CF patients, especially in those of them who have experienced chronic *P. aeruginosa* infection and exuberant inflammatory response. However, our data indicate that predominance of IL-10 over TNF-α in the circulation of CF patients may be considered an unfavorable sign. Thus, TNF-α/IL-10 ratios in patients with chronic *P. aeruginosa* infection and severe lung function failure were clearly lower than in patients with relatively good lung function

(see Table 2). Moreover, two patients (numbers 1 and 2) with extremely poor lung function had TNF-α/IL-10 < 1 for all 6 months of the observation. Patient number 1 experienced a significant lung function failure (more than 20% predicted) and patient number 2 died 1 month after finishing the study.

In conclusion, there were no fundamental differences between CF and healthy children in the regulation of TNF-α and IL-10 secretion under both basal and immune-challenge conditions. Thus, immune quiescence seemed to be associated with the predominance of TNF-α, whereas immune disturbance was characterized by IL-10 superiority. The only abnormality, which was found in CF patients, consisted in their inability to produce unlimitedly IL-10 in response to antigen stimuli. This phenomenon is assumed to be related to primary CF defect, resulting in inadequate cAMP-dependent signaling.

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