



Investigation of Serum Cytokine Levels and Cytokine Production in Whole Blood Cultures of Paranoid Schizophrenic Patients

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Abstract. There is some evidence that the pathophysiology of schizophrenia is related to changes in the innate and adaptive immune systems. In an attempt to define a potential immunological dysfunction in schizophrenia, we measured the serum levels of several cytokines in the sera of 24 patients with paranoid schizophrenia and investigated the cytokine production in whole blood assays after stimulation *in vitro* with virus (Newcastle disease), phytohemagglutinin (PHA) or bacterial lipopolysaccharide (LPS) and compared them with healthy, normal controls. A significant increase of interleukin 6 (IL-6), IL-8 and interferon γ (IFN- γ) levels, but a decreased IL-10 level were observed in the sera of patients with schizophrenia. No significant changes in the serum levels of IL-2, IL-4, IFN- α and tumor necrosis factor α (TNF- α) were detected in these patients. When cytokine production *in vitro* was examined, a significant defect in PHA-induced IL-2, IL-4 and IFN- γ , and in virus-induced IFN- α production, but no significant alterations in LPS-induced IL-6, IL-10 and TNF- α production were observed. In summary, increased serum levels of some cytokines such as IL-6, IL-8 and IFN- γ indicate an activation of the inflammatory response in schizophrenia, while the *in vitro* assay indicates significant changes in the Th1 (decreased production of IL-2 and IFN- γ) and Th2 (decreased production of IL-4) cell system responses. The role of the defective IFN- α production in the regulation of the imbalance between Th1 and Th2 cell system responses is suggested.

Key words: paranoid schizophrenia; cytokine production; IL-2; IL-4; IL-6; IL-8; IL-10; IFN- α ; IFN- γ ; TNF- α .

Introduction

Immune alterations in schizophrenia have been described for decades. Both the non-specific and specific arms of the immune system seem to be involved in the dysfunction of the immune system in schizophrenia. Recently, MÜLLER et al. ³⁷, on the basis of experimental data formulated the hypothesis that in unmedicated schizophrenic patients the non-specific “innate” im-

mune system shows signs of an overactivation, while several parameters of the specific cellular immune system, especially Th1-related immune parameters are blunted. However, recent evidence concerning Th1 cytokine response in schizophrenia are rather inconsistent^{6, 20, 44, 49}.

Cytokines are known as soluble mediators that have many critical interactions among cells of the immune system. Abnormalities in their production reflect the

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presence of disturbances in immune processes. Activation of the non-specific immune system in schizophrenia is indicated by signs of an acute phase (AP) response and increased concentrations of interleukin 1 (IL-1) in plasma and culture supernatants of mononuclear cells, increased plasma IL-1 receptor antagonist (IL-1RA), and increased plasma concentrations of IL-6 and its soluble receptor (sIL-6R) ^{1, 11, 17, 18, 20, 28-33, 39, 48, 49}.

The cellular arm of the adaptive immune system is largely activated by Th1 cells, which produce IL-2 and IFN- γ . The humoral arm of the adaptive immune system is mainly activated by Th2 cells, which produce IL-4 and IL-10. Some findings indicate an imbalance between the Th1 and Th2 cell systems in schizophrenia. Several authors^{4, 9, 10, 21, 51} have described decreased *in vitro* production of IL-2, which was interpreted as the consequence of an exhaustion of the lymphocytes after *in vivo* overproduction of IL-2, or a reduced capacity of lymphocytes to produce this cytokine, while other authors^{6, 7} have reported a higher production of this cytokine by the blood cells of patients with schizophrenia. A significant increase of IL-2 in the serum and cerebrospinal fluid of schizophrenic patients has been observed by some authors^{27, 34}, but not confirmed by others¹². A similarly decreased ability of lymphocytes to produce IFN- γ has also been described^{44, 45, 51}. The Th2 cell system seems to be activated in schizophrenic patients. Besides an increased serum level of IL-6 which is not only the product of macrophage/monocytes but also Th 2 cells, increased levels of IL-10 and IL-4 in the cerebrospinal fluid of schizophrenics has been described^{7, 35, 50}.

The aim of this study was to examine the hypothesis concerning the activation of the innate immune system in schizophrenia and the imbalance between the Th1 and Th2 cell systems through: 1) measurements of the serum levels of such cytokines as IFN- α , TNF- α , IL-6 and IL-8, which are mainly products of cells involved in innate immunity, and of such cytokines as IL-2 and

IFN- γ , which were produced of the Th1 system, and IL-4, a product of the Th2 cell system; 2) measurements of above-mentioned cytokines produced in whole blood cell cultures induced *in vitro* by Newcastle disease virus (NDV), bacterial lipopolysaccharide (LPS) or phytohemagglutinin (PHA).

Materials and Methods

Subjects. Fifty-two subjects participated in this study, consisting of 28 healthy volunteers (20 men and 8 women) and 24 patients with schizophrenia (17 men and 7 women), who had been admitted in an acute state to the Psychiatric Clinic of the Medical University in Lublin. None of the patients was neuroleptic-naïve, but before admission to hospital they received low doses of various neuroleptics, such as: perphenazine <16 mg/day, fluphenazine <4 mg/day, perazine <200 mg/day, trifluoroperazine <10 mg/day, or haloperidol <5 mg/day. Nearly all patients had relapsed due to self-discontinuation of neuroleptics. None of them had received a depot neuroleptic injection within half a year prior to the protocol. All patients and control subjects gave informed written consent on a form approved by the Ethical Committee. The patients were diagnosed according to the DSM IVR criteria² as having a paranoid type of illness. We excluded patients with a history of a concomitant psychiatric illness, such as drug or alcohol abuse, or abnormal laboratory findings, e.g. blood-hemoglobin, hematocrit, erythrocyte sedimentation rate, leukocyte, serum electrolyte, renal function test, liver function test, urine analysis, venereal disease test, chest X-ray of the lung, heart electrocardiogram (EKG) and electroencephalogram (EEG). A psychopathological evaluation was performed by an experienced psychiatrist using the Positive and Negative Syndrome Scale (PANSS)¹⁹. The results are shown in Table 1. The healthy volunteers, recruited from the University staff,

Table 1. Demographic and psychopathological characteristics of schizophrenic and control subjects

Variables	Patients with schizophrenia	Normal controls
Number of subjects	24	28
Men/women	17/7	20/8
Mean age (years)	27.8 $\pm$ 14.3	32.6 $\pm$ 10.2
Duration of illness:		
< 1 year	2/24	—
>1 year	22/24	—
PANSS total $\pm$ SD	125.37 $\pm$ 22.54	—
G (general)	64.54 $\pm$ 12.33	—
N (negative)	33.61 $\pm$ 9.82	—
P (positive)	26.78 $\pm$ 5.43	—

All results are shown as mean $\pm$ SD.

were free of any medication for at least 4 weeks prior to blood sampling. None of the volunteers was a regular drinker or had ever taken psychotropic drugs. Controls were excluded who had a present, past or family history (first degree relatives) of psychiatric disorders or autoimmune diseases. All control subjects had normal results on physical examinations and normal values from blood and urine tests.

Methods. Following overnight fasting, blood samples were always drawn into glass tubes without anticoagulant and into glass tubes with heparin (Heparinum, Polfa 20 U/ml) at between 6.30 and 9.30 a.m. The blood without anticoagulant was allowed to clot at room temperature, and the serum was separated by centrifugation at 3000 rpm for 10 min. The serum was then collected and stored at -20°C before cytokine examination (not longer than 3 months).

Peripheral blood leukocytes (PBL) of the patients and controls were cultivated by a whole blood cell technique²². Briefly, heparinized blood was diluted in Eagle's Minimal Essential Medium (MEM) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin to obtain a leukocyte density of 1×10^6 cells/ml. The blood suspension was distributed (2 ml/well) into 24-well plastic plates (Falcon, Bedford, MA) and induced to cytokine production with NDV, Radom strain 5 TCID₅₀/ leukocyte, with 50 $\mu\text{g}/\text{ml}$ PHA (Sigma, St. Louis MO), 10 $\mu\text{g}/\text{ml}$ or LPS from *E. coli* 0111: B4 (Sigma), and incubated at 37°C in 5% CO₂ in air. Supernatants were collected after 24 h of incubation (blood samples induced with NDV for IFN- α or LPS for IL-1, IL-6, IL-10 and TNF- α) or after 72 h (after induction with PHA for IL-2, IL-4 and IFN- γ production) and stored at -20°C before cytokine assay.

Cytokine concentrations were determined by the ELISA technique. Each concentration was measured in duplicate. IFN- α concentrations were measured by ELISA kits from Endogen Inc., Woburn, MA, and IL-2, IL-4, IL-6, IL-8, IL-10, TNF- α and IFN- γ were

measured using ELISA kits from Predicta Genzyme, Cambridge, MA, according to the manufacturers' instructions. The lowest level of detection was 4 pg/ml for IL-2, 6 pg/ml IL-4, 2 pg/ml IL-6, 1 pg/ml IL-8, 5 pg/ml IL-10, 3 pg/ml TNF- α , 3 pg/ml IFN- α and 3 pg/ml IFN- γ . Values of cytokine concentrations below the level of detection were assigned a the value of 0 for purposes of analysis.

Statistical analysis. Differences in the cytokine levels in serum or these induced *in vitro* in blood cell cultures between the control subjects and the patients were evaluated using one-way analysis of variance (ANOVA). The relationships between the variables were assessed by Spearman's rank order correlation coefficients. The independence of the classification systems was verified by means of an analysis of contingency (χ^2 test). Differences with $p < 0.05$ were considered significant.

Results

Table 1 shows the demographic data of the subjects in the present study. There were no significant differences in age ($F=2.18$, $df=1/49$, $p=0.14$) or men/women ratio ($\chi^2=0.01$, $df=1$, $p=0.94$). There were no significant correlations between age or gender and serum cytokine levels in the schizophrenic patients and the control group, except a positive correlation between age and TNF- α serum level in control group ($r=0.46$, $p=0.05$).

Table 2 shows that schizophrenic subjects had significantly higher IL-6, IL-8 and IFN- γ levels, but significantly lower IL-10 concentrations in sera. Differences in serum IL-2, IL-4 and IFN- α levels were not statistically significant. Correlation analysis pooled over the two groups showed a positive correlation ($r=0.40$, $p=0.03$) between IL-6 and IL-8 levels and an inverse relationship between serum IL-10 and IFN- γ level ($r=-0.42$, $p=0.022$). The level of IFN- γ also

Table 2. Measurements of cytokine serum levels in 24 schizophrenic patients and 28 normal controls

Cytokine	Schizophrenics	Normal controls	F	Degrees of freedom	p
IL-2	4.16 $\pm$ 4.25 ^a	3.86 $\pm$ 4.90 ^a	0.078	1/40	0.92
IL-4	1.46 $\pm$ 2.89	0.27 $\pm$ 0.49	1.65	1/32	0.21
IL-6	5.05 $\pm$ 3.05	2.54 $\pm$ 2.51	7.91	1/37	0.007*
IL-8	104.38 $\pm$ 160.15	6.12 $\pm$ 16.89	6.37	1/28	0.017*
IL-10	0.34 $\pm$ 1.38	11.04 $\pm$ 14.94	8.64	1/33	0.005*
IFN- α	0.33 $\pm$ 1.36	0.87 $\pm$ 2.91	0.48	1/33	0.48
IFN- γ	8.52 $\pm$ 8.29	2.35 $\pm$ 4.62	8.87	1/40	0.005*
TNF- α	2.31 $\pm$ 3.43	5.43 $\pm$ 7.18	2.76	1/34	0.105

^aAll results are shown as mean concentration of cytokine in pg/ml $\pm$ SD.

* Significantly different in comparison with control.

Table 3. Measurements of cytokine levels induced *in vitro* in whole blood cell cultures of schizophrenic patients and normal controls

Cytokine	Schizophrenics	Normal controls	F	Degrees of freedom	p
IL-2	1155.30±1416.2 ^a	2115.83±700.18 ^a	6.92	1/39	0.01*
IL-4	33.23±26.64	71.50±51.64	8.15	1/35	0.007*
IL-6	1870.18±374.78	1962.80±499.02	0.28	1/29	0.59
IL-8	n.d.	n.d.	—	—	—
IL-10	1021.76±369.21	875.83±413.34	1.21	1/33	0.27
IFN- α	373.90±159.82	839.32±443.91	19.63	1/40	0.00007*
IFN- γ	1443.00±402.32	1703.35±173.11	7.47	1/35	0.0097*
TNF- α	447.37±170.41	398.08±133.64	0.76	1/30	0.38

^a All results are shown as mean cytokine concentration in pg/ml $\pm$ SD.

* Significantly different in comparison with control.

n.d. – not done.

correlated positively with G value (general psychopathology scale scores) ($r=0.42$, $p=0.05$). In the patients group, IL-2 correlated negatively with TNF- α ($r=-0.58$, $p=0.01$) and IL-8 correlated positively with TNF- α ($r=0.54$, $p=0.05$). In the control group, IL-8 correlated negatively with IFN- γ ($r=-0.57$, $p=0.02$).

Table 3 shows that when cytokine levels induced in whole blood cell cultures were examined, a significant defect in the ability of PBL to produce IL-2, IL-4, IFN- α and IFN- γ was observed. The differences between the patient group and the control in the amounts of the other cytokines, e.g. IL-1 α , IL-6, IL-10 and TNF- α , produced *in vitro* were statistically insignificant. Correlation analysis over the two pooled groups revealed a significant correlation between IL-10 and P values (positive scale) ($r=0.47$, $p=0.05$) and a negative correlation between IFN- γ and IL-10 levels ($r=-0.60$, $p=0.01$) and between IL-2 and TNF- α ($r=-0.61$, $p=0.001$).

Discussion

The schizophrenic patients examined in this study were admitted to hospital in a very acute state. Clinically, they were all infection-free, with hematological parameters within the normal range. However, in regard to the mean serum level of IL-6, the schizophrenic patients differed from the control, exhibiting a significantly higher level of this cytokine. Our finding is in accordance with the results of other authors^{1, 11, 18, 20, 29, 39}, who detected increased IL-6 levels in the plasma of schizophrenic patients, accompanied by increased levels of sIL-6R, which act as an enhancer of IL-6-receptor interactions. Moreover, in our study we detected a significantly increased level of IL-8, which is produced by monocytes/macrophages and endothelial cells and acts as a chemotactic and activating factor for neu-

trophils^{13, 36}. In the organism, IL-6 is also produced mainly in monocytes/macrophages and endothelial cells³; therefore, its overproduction, together with IL-8 overproduction, seems to indicate activation of the proinflammatory response in schizophrenia, especially connected with non-specific immunity.

This supposition was not, however, confirmed when another proinflammatory cytokine, TNF- α , was examined. In our study we detected normal levels of TNF- α in the sera of schizophrenic patients, which is contradictory to the previous observations by those authors who detected an increased activity of this cytokine in schizophrenia^{38, 49}, but in agreement with others^{14, 46} who did not detect any changes in TNF- α level in the sera of schizophrenic patients. An increased TNF- α level in the sera of some patients was, according to HAACK et al.¹⁴, considered the consequence of therapy with benzodiazepines. The patients examined in our study did not receive therapy with benzodiazepines, so we indirectly confirmed this supposition. We also detected normal levels of IFN- α in the sera of schizophrenic patients, which is inconsistent with the findings of those authors who detected increased IFN activity in the sera and cerebrospinal fluid of schizophrenic patients^{26, 41}. The reason for these discrepancies may be the methods used for IFN detection. In the above-mentioned publications, IFN activity was detected using biological methods, by which total IFN activity, i.e. IFN- α , IFN- γ , IFN- β , IFN- ω and also acid-labile IFN activity, can be detected. The ELISA method used in this study allowed us to detect only IFN- α , but not other types of IFNs. We can speculate that the increased IFN activity present in the sera of the schizophrenic patients observed by other authors was connected rather with IFN- γ than IFN- α .

Another major finding in our study was a significant increase in IFN- γ serum level. As IFN- γ is a key proinflammatory cytokine that activates several aspects of

cell-mediated immunity and, in contrast to IL-6 and IL-8, is produced mainly by Th1 lymphocytes⁴⁷, this observation indicates that the specific immune response can be activated in schizophrenia. The activation of the Th1 response is also suggested by a slight, statistically insignificant increase in IL-2 serum level. It is worth mentioning that an increased serum IL-2 level in schizophrenic patients has already been described by other authors^{20, 21}.

The production and action of IFN- γ is antagonized by IL-10, which is a product of the Th2 cell system²³ and also produced by monocytes/macrophages. As we detected a significantly decreased IL-10 level in the sera of schizophrenic patients, the overproduction of IFN- γ can be considered as the effect, at least in part, of IL-10 deficiency and an imbalance between the Th1 and Th2 cell systems. However, a strong relationship between an increased IL-10 level in cerebrospinal fluid and negative symptoms of schizophrenia was observed⁵⁰. This inconsistency can be explained by the possibility that IL-10 can be overproduced locally in the brain but not in the other organs. It is worth mentioning that in contrast to a low IL-10 concentration in sera, the blood leukocytes of the schizophrenic patients in our study produced normal levels of IL-10.

Another cytokine, almost exceptionally produced by Th2 cells, is IL-4. An IL-4 level increase in cerebrospinal fluid of schizophrenic patients was reported by MITTLEMAN et al.³⁵. The production of IgE is also a sign of activation of the Th2 cell immune response. An increased level of IgE in the sera of schizophrenic patients compared with controls was also reported, which indirectly suggests an IL-4 production increase⁴². In our study we detected a slightly higher level of this cytokine in the sera of some schizophrenic patients in comparison with the healthy controls. This observation suggests that IL-4 production seems to be enhanced in the organism of schizophrenic patients.

When cytokine production was induced in whole blood cell cultures of schizophrenic patients in our study by using such strong inducers as virus, mitogen and bacterial LPS, we also detected several changes in comparison with the controls: a significant reduction in amounts of IL-2, IL-4 and IFN- γ and a slight enhancement (statistically insignificant) of IL-10 production. Our results partially confirm the findings of other authors, who described the immunological dysfunction in schizophrenic patients and significant reduction in the production of IL-2 and IFN- γ by blood leukocytes^{4, 21, 44, 45, 51}. Both cytokines are products of the Th1 cell system, and the defect in their production *in vitro* has often been interpreted as a consequence of an exhaus-

tion of the lymphocytes after overproduction of cytokines *in vivo* or by a reduced capacity of lymphocytes to produce cytokines. As in our study a statistically significant increase in IFN- γ serum level and a slight (insignificant) increase in IL-2 serum concentration were detected, the decreases in production *in vitro* of both cytokines seem to support the possibility of an exhaustion of the lymphocytes after prolonged activation *in vivo* by unknown factor/factors, which are also involved in the activation of monocyte/macrophages. It is also worth mentioning that in our study IL-4 production *in vitro* was significantly depressed, while its serum level was slightly (insignificantly) higher than in healthy controls. In contrast to the significantly depressed production of IL-2, IL-4 and IFN- γ as the consequence of cell hyporeactivity, the production of IL-10 *in vitro* seemed to be slightly enhanced (difference statistically insignificant), while in serum its concentration was significantly lower in comparison with the controls. An increase in IL-10 production in blood leukocytes of schizophrenic patients compared with healthy control has already been reported by CAZZULLO et al.⁷.

These observations strongly indicate a considerable imbalance between the Th1 and Th2 cell systems in schizophrenic patients.

When IFN- α was induced *in vitro* by virus in whole blood cell cultures of schizophrenic patients, a statistically significant defect in its production in comparison with controls was observed. A decreased ability of the leukocytes of schizophrenic patients has already been described by several authors^{15, 43}. As both IFN serum levels and IFN levels induced *in vitro* were lower than those of the controls in our study, it seems likely that an inherited or acquired defect in IFN- α production is characteristic for schizophrenia. The acquired defect can be the consequence of a chronic stimulation of IFN- α production, which induces the very deep state of hyporeactivity observed as low concentrations of IFN- α present in serum and reduced ability of blood leukocytes to produce IFN after viral infection *in vitro*. In 1980 CANTELL et al.⁵, studied the effect of IFN- α therapy on a small number of schizophrenic patients and found a transient improvement. Thereafter, a larger group of patients was treated with IFN- α ²⁵. Such therapy improved the mental stage in some patients and worsened it in others, in whom IFN therapy increased the susceptibility to neuroleptics. These results indicate a heterogeneity of schizophrenia and suggest a role of IFNs in the ethiopathology of this illness. In recent publications the role of IFNs in affective disorders was also considered by other authors¹⁶.

The role of type I IFNs (IFN- α , IFN- β , IFN- ω) in

the induction of Th1 cell development via activation of the transcriptional factor STAT 4 has been indicated in several publications^{8, 40, 47}. It is now clear that the differentiation of Th1 cells is crucial for the effective host response to intracellular parasites, but this cell subset is also thought to contribute to the pathogenesis of a variety of autoimmune disorders, including multiple sclerosis. Many hypotheses have been presented which seek to explain schizophrenia as an interaction of a person's genetic endowment and various environmental influences. One of the most controversial of these is that schizophrenia is related to autoimmune disorders²⁴. Therefore, studies on the role of type I IFNs in schizophrenia should be of special interest.

In conclusion, our data have confirmed and extended the previous findings that paranoid schizophrenia is characterized by activation of the proinflammatory response connected with increased serum levels of IL-6, IL-8 and IFN- γ , but with decreased serum levels of anti-inflammatory IL-10. Decreased IL-2 and IFN- γ production in blood leukocytes of the schizophrenic patients observed in our study may be associated with increased IL-2 and IFN- γ serum levels and the exhaustion of cytokine-producing cells. It seems likely that a defect also in IL-4 production *in vitro* may be caused by its local overproduction *in vivo*. Moreover, as type I IFNs were recently shown to induce selectively Th1 type immune response, the defect in the leukocytes of schizophrenic patients in IFN- α production can, at least partially, be responsible for changes in cytokines which are products of Th1 cells, as well as the imbalance between the Th1 and Th2 cell responses.

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