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Analysis of peripheral immune activation in schizophrenia using quantitative reverse-transcription polymerase chain reaction (RT-PCR)☆

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ABSTRACT

Immune system abnormalities in schizophrenia include a shift from a Type 1 (cellular) to a Type 2 (humoral) immune response. To characterize the activation status of the immune system in schizophrenia, we examined the pattern of gene expression in peripheral blood cells for three Th1 cytokines (interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2)), and one Th2 cytokine (interleukin-10 (IL-10)). In a cross-sectional study, we used quantitative reverse-transcription polymerase chain reaction (RT-PCR) to compare the mRNA levels of IFN-γ, TNF-α, IL-2, and IL-10 in peripheral blood mononuclear cells (PBMCs) between 15 schizophrenia patients and 15 matched healthy controls. Expression of IFN-γ and TNF-α was significantly reduced in patients with schizophrenia compared with normal controls. No differences in IL-2 and IL-10 gene expression were observed. These results are consistent with impaired Type 1 cellular immunity in schizophrenia. While the data illustrate the potential utility of mRNA-based approaches for the identification and analysis of immune biomarkers for neuropsychiatric disorders, correlation of gene expression with direct measures of cytokine concentrations is required.

1. Introduction

A consistent laboratory finding in schizophrenia has been a shift from a Type 1 (cellular) to a Type 2 (humoral) immune response, which results in diminished cytotoxic T cell-mediated activity in favor of B cell-mediated antibody responses (Mueller et al., 2000). An impaired Type 1 response might explain the negative association observed between schizophrenia and rheumatoid arthritis, the latter resulting from an inappropriately self-reactive cytotoxic response (Oken and Schulzer, 1999). Furthermore, an intact Type 1 response is necessary for the control of many viral and intracellular parasitic infections (Torrey et al., 2007), clearance of which depend on robust cytotoxic T cell activities. Impaired Type 1 immune responses could result in a failure to sufficiently limit the deleterious neurological effects of certain infections, such as Toxoplasma gondii, both in utero and later in life. Loss of Type 1-mediated immune function, or enhanced Type 2 activity, might explain the increased prevalence of Toxoplasma gondii serum antibodies that has been observed in schizophrenia patients compared with controls (Torrey and others, 2007). In a recent case–control study, Riedel et al. (2007) found decreased skin reactivity to seven different antigens administered intracutaneously in schizophrenia patients compared with age- and sex-matched healthy controls, providing direct functional evidence for an attenuated Type 1-mediated cellular immune response in schizophrenia patients.

Type 1 immunity is characterized by the expression of particular cytokines, the most notable being interferon-γ (IFN-γ) (Mosmann and Sad, 1996). IFN-γ is produced by T cells, and natural killer (NK) cells and it functions as a central mediator of the Type 1/Type 2 immune balance. Tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2) are other key proinflammatory cytokines that activate the cellular arm of immunity, and both cytokines play important roles in an effective immune response to pathogens. IFN-γ and TNF-α have direct anti-pathogen activity, and mice with deficient activity of either protein are highly susceptible to infection by intracellular microbes (Kamijo et al., 1993; Ruby et al., 1997). IL-2 is produced predominantly by CD4+ T cells and is essential for the rapid proliferation of all T lymphocytes during the early stages of an immune response (Smith, 1988).

Interleukin-10 (IL-10) expression is a hallmark of Type 2 immunity (Moore et al., 2001; Vicari and Trinchieri, 2004). IL-10 activity suppresses cell-mediated immune responses by hindering T cell proliferation and by altering the activation potential of professional antigen

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Table 1
Schizophrenia patient and control subject characteristics.

	Patients ($n=15$)	Controls ($n=15$)
Age in years*	41 (S.D. 12.4)	40 (S.D. 7.9)
Gender*		
Male	13 (87%)	13 (87%)
Female	2 (13%)	2 (13%)
Clinical characteristics		
PANSS total score	66.1 (range 37–92; S.D. 13.4)	
Mediation status		
Unmedicated**	2 (13%)	
Clozapine	4 (27%)	
Other Antipsychotic	9 (60%)	

PANSS denotes Positive and Negative Syndrome Scale.
* Differences between schizophrenia patients and control subjects were not statistically significant ($P>0.05$).
** Unmedicated with an antipsychotic but not antipsychotic-naïve.

presenting cells (APC), such as dendritic cells and macrophages (O'Garra et al., 2008). IL-10 also promotes the proliferation of B cells and thereby enhances humoral immunity.

Support for the role of a dysregulated immune system in schizophrenia comes from treatment trials with the anti-inflammatory agent, celecoxib, which has been reported to be of therapeutic benefit (Mueller et al., 2002; Akhondzadeh et al., 2007). This finding is consistent with the hypothesis that immune activation could play a role in the etiopathogenesis of some of the symptoms of schizophrenia. However, while a variety of immune system abnormalities have been observed in schizophrenia patients, many of the reported findings are inconsistent (Mueller and Schwarz, 2007). This may be due, in part, to reliance on the detection of immune markers in serum or to the wide variety of in vitro assays employed to characterize the immune system. New technologies to collect and stabilize total RNA from peripheral blood provide an opportunity to characterize the activation state of the peripheral immune system on the basis of cytokine gene expression. This method may be less prone to the inherent variability associated with protein-based assays that results from limited sensitivity, effects of sample handling, and dynamic aspects of cytokine release and absorption by the respective cytokine receptors.

In a cross-sectional study of 15 schizophrenia patients, we used quantitative reverse-transcription polymerase chain reaction (RT-PCR) to measure the mRNA expression of four cytokines involved in modulation of cellular immunity, IFN-γ, TNF-α, IL-2, and IL-10 in peripheral blood samples. Based on previous reports, we hypothesized that these genes would show altered expression in at least a subgroup of patients, consistent with a dysregulated cellular immune response.

2. Methods

2.1. Patients and controls

Fifteen stable outpatients with schizophrenia were recruited from the Massachusetts General Hospital Schizophrenia Program. Patients were selected to represent the complete spectrum of schizophrenia with regards to disease severity, illness duration, and treatment status. Psychiatric diagnosis was confirmed via clinical interview and chart review by a research psychiatrist (OF) using DSM-IV criteria. Patients were excluded if they suffered from an infection or immune disorder or were taking anti-inflammatory medication, as determined by clinical interview and chart review. Fifteen healthy controls were matched for age and gender.

Patients and controls had their blood drawn and processed as outlined below; patients in addition were clinically characterized with the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987).

The study was approved by the responsible Institutional Review Boards, and all patients and controls provided written, informed consent.

2.2. Isolation and quantification of RNA

Whole blood samples were collected using Paxgene Blood RNA tubes (Becton Dickenson) and stored at −80 °C. Total RNA was extracted using the Paxgene Blood RNA kit (Qiagen) according to the manufacturer's protocols. Complementary DNA was generated from 1 μg of total RNA using oligo dT primers and Powerscript RT enzyme (BD Clontech) at 42 °C for 60 min in a total volume of 20 μl. Two μl of cDNA (approximately 0.1 μg) was used to quantify gene expression by real-time PCR on an Mx3000P thermocycler (Stratagene) using Full Velocity SYBR Green 2× mix (Stratagene) and gene-specific primers for human hypoxanthine guanine phosphoribosyltransferase 1 (HPRT1/NM_000194; F:gac ccc acg aag tgt tgg at; R:ggc gat gtc aat agg act cca; 209 nt), interferon-γ

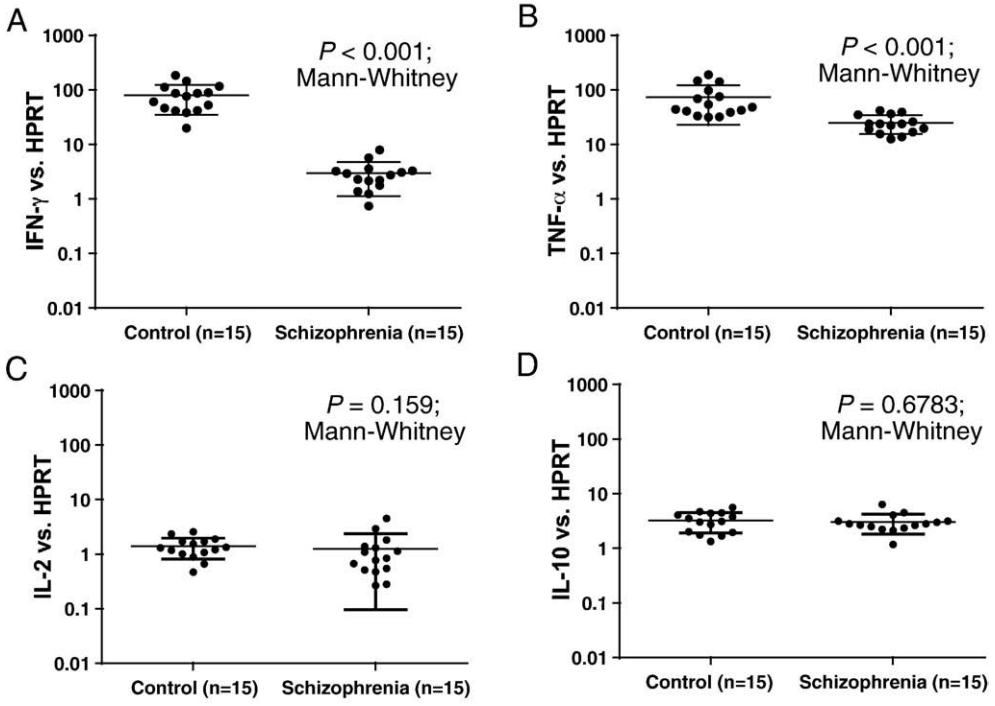

Fig. 1. Panels show the mRNA expression of (A) interferon-γ (IFN-γ), (B) tumor necrosis factor-α (TNF-α), (C) interleukin-2 (IL-2), and (D) interleukin-10 (IL-10) in peripheral blood mononuclear cells (PBMCs) of normal controls and schizophrenia patients. mRNA expression levels are normalized to a housekeeping gene, human hypoxanthine guanine phosphoribosyltransferase 1 (HPRT). Note the logarithmic scale on the *y*-axis. Bars indicate mean and standard deviation.

(IFN-γ/IFNG/NM_000619; F:agg cat ttt gaa gaa ttg gaa ag; R:cac act ctt ttg gat gct ctg g; 121 nt), tumor necrosis factor-α (TNF-α/TNF/NM_000594; F:ctt ctg cct gct gca ctt tg; R:cct cag ctt gag ggt ttg ct; 158 nt), interleukin-2 (IL-2/IL2/NM_000586; F: caa act cac cag gat gct cac; R: gca ctt cct cca gag gtt tga; 107 nt), and interleukin-10 (IL-10/IL10/NM_000572; F: cgt gga gca ggt gaa gaa tg; R: aga gcc cca gat ccg att tt; 195 nt). Following denaturation (10 min, 95 °C), DNA was amplified using 40 cycles at 95 °C, 60 °C, and 72 °C (20 s each). Primer pairs were designed to span intron boundaries and were tested to verify specificity for mRNA products and not genomic DNA. All primers were purchased from Invitrogen. IFN-γ, TNF-α, IL-2, and IL-10 results were normalized to HPRT1 using a $2^{(\Delta\Delta Ct)}$ method and are displayed as relative expression values in arbitrary units.

2.3. Statistical analysis

Gene expression results are displayed as the mean ± S.D. for each gene. Data were analyzed for significance using the non-parametric Mann–Whitney *U*-test in Prism 4.0 software (GraphPad Software, Inc.). *P*-values less than 0.05 were considered significant and all tests were two-tailed.

3. Results

3.1. Patients and controls

Patients and controls were well matched for age and gender. Their demographics and the clinical characteristics of patients are shown in Table 1. All patients except for two were medicated with an antipsychotic at the time of the blood draw; the two unmedicated patients were antipsychotic-free at the time of the study but not antipsychotic-naïve.

3.2. Cytokine gene expression data

Peripheral blood was collected from patients and controls over the course of approximately 6 weeks using Paxgene Blood RNA tubes, and whole blood samples were stored frozen prior to processing. RNA was extracted and cDNA was generated from all samples at the same time to reduce potential variability during sample handling. Quantitative PCR was used to measure IFN-γ, TNF-α, IL-2, IL-10, and HPRT cDNA. All values were normalized to HPRT and are presented as relative levels in arbitrary units. Expression of IFN-γ (Fig. 1A) and TNF-α (Fig. 1B) was significantly lower in patients than controls ($P<0.001$ for each as determined by the Mann–Whitney *U*-test). The mean (± S.D.) value of IFN-γ gene expression was 80.35 (± 45.25) units in healthy controls versus 2.98 (± 1.84) units in the schizophrenia patients (median of 77.38 units and 2.77 units, respectively), a greater than 25-fold difference in RNA levels for this important Type 1 cytokine. The variation in TNF-α gene expression was less pronounced, with mean values of 72.74 (± 49.53) units in healthy controls versus 24.83 (± 9.38) units in the schizophrenia patients (median of 48.84 units and 23.92 units, respectively). No significant differences in gene expression were seen for either IL-2 (Fig. 1C) or IL-10 (Fig. 1D) ($P=0.159$ and $P=0.676$ using the Mann–Whitney *U*-test, respectively). Since all samples were processed at the same time, we believe that it is unlikely that the observed disparities in IFN-γ and TNF-α expression resulted from differences in RNA extraction or quantification methods. In addition, IL-2 and IL-10 gene expression appeared similar between patient and control samples, further arguing against a systematic technical cause for these results. Consistent results were also obtained following normalization to an independent control gene, beta Actin (data not shown).

4. Discussion

We observed a significant reduction in expression of the cytokine IFN-γ in PBMC from all patients with schizophrenia, the median value of which was reduced approximately 25-fold compared with healthy controls. A modest (approximately 2-fold) reduction in TNF-α expression was also observed in these individuals, while no differences were seen for IL-2 or IL-10 expression compared with healthy controls. This pattern of IFN-γ and TNF-α expression is consistent with a deficiency in the Type 1 (cellular) immune response in schizophrenia patients, and the similarity in IL-10 expression between groups argues against similar defects in Type 2 (humoral) immunity.

This study highlights the feasibility of using mRNA expression analysis in peripheral blood cells as a means to characterize the activation state of the immune system in schizophrenia and other mental illnesses. Additional studies are needed to establish that gene expression correlates with direct measures of the gene products (e.g., cytokine concentrations in plasma) and their biological activities on the target organs. Furthermore, it will be necessary to examine cytokine expression by individual cell types in the peripheral blood. Our results suggest that a significant difference in IFN-γ and TNF-α expression will be apparent for at least some cellular subsets. Larger sample sizes and additional Th2 cytokines (e.g., IL-6 and IL-4) are required, however, to conclusively determine that Type 2 immunity is not affected in schizophrenia.

Acknowledging the limitations of gene expression data alone, our results are in agreement with the reduced IFN-γ protein levels described previously in a longitudinal treatment study in schizophrenia (Arolt et al., 2000). In that report, IL-2 protein was also decreased, but this was not the case with IL-2 mRNA expression in our samples. We also observed a significant reduction in TNF-α expression in schizophrenia patients. Since overproduction of this cytokine can result in toxic shock syndrome (Tracey et al., 1987) its expression must be tightly regulated. Therefore, reduced TNF-α expression may indicate either a defect in the induction of inflammatory pathways or active inhibition of this cytokine.

The analysis of gene expression using peripheral blood is advantageous, due to the ease of sample collection and the ability to analyze a large number of individuals or serial samples from the same patient, but several limitations to our cross-sectional study must be acknowledged. First, antipsychotic treatment may have affected our results. The evidence for an antipsychotic drug effect on the immune system is inconsistent and difficult to interpret (Pollmaecher et al., 2000). Antipsychotic drug-related decreases in TNF-α (Moots et al., 1999; Cazzullo et al., 2002) and IFN-γ secretion (Cazzullo et al., 2002) have been described following *in vitro* stimulation. However, other studies found no effect of treatment on serum levels of TNF-α (Hori et al., 2007) or IFN-γ (Rothermundt et al., 2000; Kim et al., 2001). In addition, Kim et al. (2004) found that antipsychotic treatment normalized elevated pre-treatment IFN-γ levels. Findings for serum IL-2 have been equivocal as well, with antipsychotic treatment shown either to decrease IL-2 levels (Zhang et al., 2004; Hori et al., 2007) or to have no effect (Kim et al., 2001). To complicate matters further, disease activity (i.e., actue psychosis) is associated with increased levels of stimulated cytokine production (e.g., TNF-α and IL-6) and subsequent decrease with antipsychotic treatment (Na and Kim, 2007). However, we know of no study that has examined the effects of antipsychotic treatment on cytokine mRNA expression. Second, our study sampled gene expression of only a limited number of cytokines in a cross-sectional design. The cytokines that we selected are representative measures of Type 1 and Type 2 cellular immunity, and cross-sectional assessment of expression for IFN-γ, TNF-α, IL-2, and IL-10 is informative regarding general levels of immune activation. Future studies will be necessary to expand this analysis to many more cytokine genes (e.g., using gene array technology), which should allow further characterization of immune status in schizophrenia. In addition to cytokines genes themselves, key regulatory genes should be added (e.g., TGF-b1, which has been shown to suppress the production of Th1 cytokines). A longitudinal study design will be necessary to capture the dynamic nature of cytokine expression depending on illness stage, disease activity, and treatment status. Third, we used whole blood and are therefore unable to determine the exact cell type implicated by our results. Nevertheless, the robustness of the collection system, which obviates the need for immediate

processing of samples, is a technical advantage particularly well suited for a clinical setting. While analysis of gene expression in blood cells cannot alone replace the direct measurement of cytokines in the serum, it should be considered as a complement to these assays to examine the function of immune cells. The excellent sensitivity of PCR-based assays may provide an additional advantage to current methods when cell numbers are limited, e.g in cerebrospinal fluid. Further, given similarities in metabolic pathways in lymphocytes and neuronal cells, analysis of peripheral blood might be a suitable model system to estimate gene expression of cells residing in or trafficking to the CNS (Sullivan et al., 2006). The feasibility of this approach has been shown in a study that found different levels of gene transcription patterns for proteins of the mitochondrial respiratory chain in response to energy stress in controls compared to bipolar disorder patients (Naydenov et al., 2007).

In summary, we found significantly decreased gene expression for the cytokines IFN-γ and TNF-α in peripheral blood cells from schizophrenia patients compared to healthy controls, a pattern that is consistent with impaired Type 1 cellular immunity. A larger sample size is needed to examine correlations between clinical symptoms and immune measures. Future studies will further need to 1) establish that cellular immunity is impaired using more distal measures (i.e., correlating gene expression data with protein concentrations and functional immune assays), 2) expand the analysis to additional key cytokines including regulatory cytokines, and 3) clarify the etiology and timing of this difference in cellular immunity (i.e., control for confounding variables such as medication status, concurrent medical illnesses, drug use, illness stage and disease activity) in a longitudinal design. Impaired cellular immunity regardless of etiology would have functional relevance for the control of infections in schizophrenia.

Author disclosure

Role of funding source

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Contributors

Dr. Freudenreich and Dr. Goff designed this study. Dr. Freudenreich wrote the protocol as well as the first manuscript draft. Dr. Brockman analyzed the blood samples using RT-PCR and provided statistical help. Drs. Henderson, Evins, and Fan helped with the overall study design and with subject recruitment. Mr. Walsh maintained the database and helped characterize subjects with regards to psychopathology. All authors contributed to and have approved the final manuscript.

Conflict of interest

Dr. Freudenreich has received support from the Sidney Baer Foundation and Cephalon. Dr. Henderson reports having received lecture fees from Pfizer, INC, Eli Lilly, Janssen, Solvay, Wyeth, Bristol Meyers Squibb and Otsuka Pharmaceuticals, and research funding from Eli Lily, Bristol Meyers Squibb and Pfizer. Dr. Evins reports having received research supplies from Janssen Pharmaceutica and GSK, a research grant from Janssen Pharmaceutica, expects a research grant from Sanofi-Aventis and has applied for a research grant from Pfizer. She has not received any consulting fees, or lecture fees and owns no biomedical related equity. She also receives funding from NIDA on a collaborative project with GSK. Dr. Fan disclosed having received consulting fees from Solvay Pharmaceuticals and Eli Lilly and Company. Dr. Goff has received compensation within the past three years from: AstraZenca, Cephalon, Bristol-Myers-Squibb, Eli Lilly, Glaxo Smith Kline, Janssen Pharmaceuticals, Merck, Organon, Pfizer, Inc., Solvay, Wyeth, XenoPort, Vox, DiMedix, SG Cowen, Advanced Health Media, American Psychiatric Association, Primedia, Behavioral Options, Axio, Verusmed, the Nelson Group, Letters and Science, Centron, Imedex, Oakstone Publishing, Synapse, NARSAD, NIMH, and the Sidney Baer Foundation. Dr. Brockman and Mr. Walsh reported no biomedical financial interests or potential conflicts of interest.

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