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Serial Mitogen-Stimulated Cytokine Production from Continuously Ill Patients with Schizophrenia

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Aberrant activation of the immune system has been implicated in an increasingly large number of disease states and can influence cognition, mood, and memory. There is a long and controversial history of reports of immune activation associated with schizophrenia. In this study, we measured mitogen-stimulated cytokine levels serially in 100 medication-stabilized continuously ill subjects with schizophrenia and compared and contrasted them with mitogen-stimulated cytokine levels from 51 normal volunteers. The subjects with schizophrenia had consistently higher mitogen-stimulated IL-2 levels and lower IL-6 levels than the normal volunteers. These effects could not be explained by medications, smoking, or other clinical variables. We conclude that continuously symptomatic medication-stabilized subjects with schizophrenia have a mitogen-stimulated cytokine expression pattern that is suggestive of ongoing immune activation.
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INTRODUCTION

There is a resurgence of interest in the role that the immune system might have in the pathogenesis of schizophrenia (Potvin *et al*, 2008). Some of the impetus for this renewed revival is reports that immune activation occurs in disorders ranging from peptic ulcer disease to cardiovascular and neurological disorders. A second stimulus of this renewed curiosity are preclinical findings showing that cytokines mediate metabolic pathways for dopamine, serotonin, norepinephrine, and *N*-methyl-D aspartate (NMDA) (Dunn, 2006; Müller and Schwarz, 2006a; Müller and Schwarz, 2007). Müller and colleagues postulate that an imbalance between T-helper-cell type 1 (TH-1) and T-helper-cell type 2 (TH-2) activation leads to the production of kynurenic acid (KYN-A), an endogenous NMDA-receptor antagonist, and quinolinic acid, an endogenous NMDA-receptor agonist, which in high doses is a neurotoxin (Müller and Schwarz, 2006a; Müller *et al*, 2004; Stone and Behan, 2007). They conclude that acutely ill patients with schizophrenia have TH-2 immune activation that resolves with treatment (Müller and Schwarz, 2006b). In general, studies of mitogen-stimulated cytokine levels from acutely ill individuals with schizophrenia report decreased interleukin-2 (IL-2) production which could be interpreted as being consistent with the aforementioned hypothesis (Arolt *et al*, 2000; Bessler *et al*, 1995; Cazzullo *et al*, 2002; Cazzullo *et al*, 2001; Ganguli *et al*, 1995; Ganguli *et al*, 1989; Kim *et al*, 2001; Kim *et al*, 2004; Potvin *et al*, 2008; Rothermundt *et al*, 2000; Villemain *et al*, 1987).

Our group consistently has reported that patients with schizophrenia have increased serum levels of the soluble IL-2 receptor (sIL-2r). We have postulated that this is a subgroup effect and have shown that this finding is present in medication naive, acutely ill, and chronically ill individuals, as well as individuals with schizophrenia from different ethnic groups (Rapaport *et al*, 1997; Rapaport and Lohr, 1994; Rapaport *et al*, 1994; Rapaport *et al*, 1993; Rapaport *et al*, 1991b; Rapaport *et al*, 1989). Increased serum sIL-2r levels are more commonly observed with immune activation (Rubin *et al*, 1990). In an attempt to extend our work and clarify findings from other groups, we have characterized a large cohort of continuously ill, medically stable patients with schizophrenia. We were interested in determining whether repeated sampling would find a consistent pattern of cytokine production from mitogen-stimulated leukocyte cultures. We postulated that this group of continuously ill subjects with schizophrenia would have an increase in mitogen-stimulated cytokine production favoring a TH-1 immune response. We further postulated that these findings would be stable over 6 weeks.

MATERIALS AND METHODS

Subjects

The study was approved by the Institutional Review Boards of the Veterans Affairs Medical Center, San Diego, and the

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University of California at San Diego. All subjects signed written informed consent to participate in the study. Subjects consisted of 100 patients who met the DSM-IV (American Psychiatric Association, 1994) diagnostic criteria for schizophrenia (men/women = 72/28; mean age = 41.1 ± 8.2 years) and 51 healthy control subjects (men/women = 41/10; mean age = 34.5 ± 11.4 years). Schizophrenic subjects were outpatients without any other current Axis I diagnosis receiving stable doses of medications. Healthy control subjects were recruited by advertisement concurrent with subjects with schizophrenia. Control subjects were non-smokers, medication-free, had never taken psychotropic medications nor had a mental disorder. A Structured Clinical Interview (DSM-IV) was performed by a trained clinician on all subjects (First, 1997). Symptom severity of subjects with schizophrenia was evaluated using the Positive and Negative Symptoms Scale (Kay *et al*, 1987). All subjects had a complete medical history and physical examination including a complete blood count, chemistry panel, urine toxicology screen and urinalysis. Subjects with acute medical or immunological disorders or positive urine toxicology screens were excluded.

Blood was drawn between 0900 and 1200 hours. Control subjects had blood drawn at one time point whereas subjects with schizophrenia had blood samples collected at baseline and again 6 weeks later (41 ± 1 days). Pharmacotherapy was held constant over the span of the study.

In Vitro Cytokine Production Analysis

Immediately after collection, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood using commercially available Ficoll-Paque density gradients (Pharmacia, Bridgewater, NJ). The number of viable cells was determined by a trypan blue exclusion assay. The PBMCs were resuspended in RPMI-1640 media at a concentration of 10^6 cells per ml, containing 1 unit/ml penicillin, 1 ug/ml streptomycin sulfate, 2 mM glutamate, 10 mM HEPES buffer, and 10% heat-inactivated fetal bovine serum. Cells were cultured with 10 ug/ml phytohemagglutinin (PHA) (Sigma-Aldrich, St Louis, MO) at 37°C and 5% CO_2. The cell-free supernatant was collected 48 hours later and frozen in aliquots at –80°C. Supernatant from PHA-free cell cultures served as a negative control.

Cytokine concentrations were determined using a sandwich ELISA using antibody (IL-2, IL-6, IL-10, IFN-γ) and standards purchased from Endogen (Rockford, IL). Standard curve concentrations were 35–1500 pg/ml for IL-2 assays, 10–2000 pg/ml for IL-6, 15–600 pg/ml for IL-10, and 25–1000 pg/ml for IFN-γ. The sensitivity of all assays is <5 pg/ml and the intra- and inter-assay coefficients of variation are <10%. Determinations of cytokine levels were performed in a masked manner, in duplicate with all subjects' samples assayed at the same time.

Statistical Analysis

Demographic characteristics for schizophrenic subjects and normal volunteers were analyzed by χ^2-tests for discrete variables or *t*-tests for continuous variables. Continuous data not normally distributed as assessed by a Kolmogrov–Smirnov test were log-transformed before analysis. Mixed model regressions were used to compare cytokine concentrations between groups (patients with schizophrenia or control subjects) and visit number, while controlling for the demographic covariates of age, gender, and race, with the random effects of each subject over time and an unstructured covariance. Where a difference existed between schizophrenic and control subjects, separate mixed effect modeling was conducted within the group of subjects with schizophrenia for each cytokine with additional fixed predictor variables of PANSS total scores, duration of illness, subtype of schizophrenia, types and dosages of pharmacotherapy, presence of comorbidities, comorbid medications, BMI, and smoking status. For all analyses, significant differences were declared when $p < 0.05$. Data are presented as means ± SD or percent frequency where appropriate. All analysis was performed using SAS statistical software (version 9.1, SAS Institutes, Cary, NC).

RESULTS

Table 1 presents the demographic characteristics of the subjects. While there was no difference in gender, subjects with schizophrenia were significantly older ($t = 3.72$, 78 df, $p < 0.01$) and had a different racial distribution, with a greater proportion of subjects being of Caucasian background ($\chi^2 = 10.1$, 4 df, $p = 0.04$). However, neither age nor race was a significant predictor in any subsequent analysis.

Subjects with schizophrenia met criteria for the following subtypes: paranoid type ($n = 53$), disorganized type ($n = 5$), catatonic type ($n = 4$), undifferentiated type ($n = 6$), residual type ($n = 3$), or NOS ($n = 29$). The average age of onset was 21.3 ± 0.7 years and the mean duration of illness was 19.8 ± 1.0 years. Neuroleptic medication was not standardized and pharmacological treatment was individualized. Subjects with schizophrenia were treated with typical antipsychotic medications ($n = 31$), atypical medications ($n = 40$) or a combination therapy ($n = 29$) with an average daily medication dosage of 1057 ± 942 mg of chlorpromazine equivalence units. Some of the subjects with schizophrenia were also receiving anticholinergic medications ($n = 66$), antidepressant medications ($n = 24$), mood stabilizers ($n = 27$), or benzodiazepines ($n = 10$). The subjects with schizophrenia had medical comorbidities of hypertension ($n = 32$), asthma ($n = 14$), chronic

Table 1 Demographic Data as Means ± SD or Percentages

	Control ($n = 51$)	Schizophrenia ($n = 100$)
Age, years	34.5 ± 11.4	41.1 ± 8.2*
Male	80%	72%
Ethnicity		
Caucasian	43%	71%*
African-American	22%	11%
Hispanic	14%	12%
Asian	12%	4%
Other	10%	2%

*Significantly different from controls ($p < 0.05$).

Table 2 Group Means ± SD of Measured Cytokine Levels

	Controls ($n=51$)	Subjects with schizophrenia					
	Visit 1	All ($n=100$)		Smokers ($n=69$)		Non-smokers ($n=31$)	
		Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2
IL-2	23.8 ± 17.9	35.3 ± 22.3*	40.3 ± 52.2*	33.7 ± 18.1*	42.2 ± 61.5*	38.7 ± 29.7*	36.1 ± 19.3*
Il-6	1680.8 ± 600.5	1327.7 ± 431.8*	1216.5 ± 532.8*	1299.9 ± 441.8*	1173.4 ± 555.7*	1389.6 ± 408.6*	1312.5 ± 472.4*
IL-10	388.6 ± 288.2	309.3 ± 240.2	312.2 ± 295.6	286.2 ± 247.1	279.0 ± 280.9	360.9 ± 219.3	386.0 ± 318.3
IFN-γ	59.4 ± 106.4	77.3 ± 163.8	46.9 ± 91.1	76.5 ± 163.4	55.2 ± 106.0	78.9 ± 167.3	28.5 ± 37.4

*Significantly different from controls ($p<0.05$).

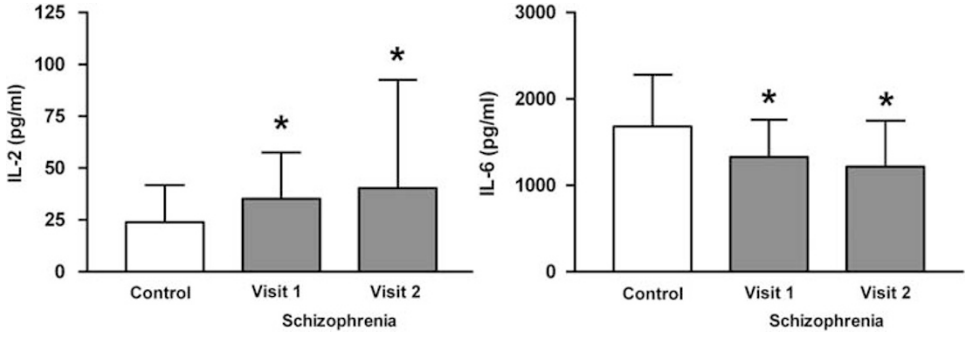

Figure 1 *In vitro* cytokine levels. Means ± SD. *Significantly different from controls ($p<0.05$).

heartburn ($n=8$), hypothyroidism ($n=5$), hyperlipidemia ($n=5$), or diabetes ($n=1$). While all control subjects were non-smokers, 72% of the subjects with schizophrenia were current smokers. Over the 6 weeks of the study, subjects with schizophrenia had a statistical, but not clinically significant, decrease in total PANSS scores with a score of 82.1 ± 18.2 at visit 1 and 78.1 ± 17.6 at visit 2 ($t=2.64$, 98 df, $p=0.01$).

As shown in Table 2, subjects with schizophrenia had significantly higher mitogen-stimulated IL-2 levels ($F=23.5$, 1,149 df, $p<0.01$), lower IL-6 levels ($F=17.2$, 1,149 df, $p<0.01$), but no difference in IL-10 ($F=1.9$, 1,149 df, $p=0.17$) or IFN-γ ($F=1.0$, 1,149 df, $p=0.31$) levels when compared with normal volunteers. Over the 6 weeks of the study, cytokine production levels remained stable in subjects with schizophrenia for IL-2 ($F=0.2$, 1,149 df, $p=0.67$), IL-10 ($F=1.1$, 1,149 df, $p=0.29$) and IFN-γ ($F=2.1$, 1,149 df, $p=0.15$) whereas IL-6 production decreased slightly ($F=5.9$, 1,149 df, $p=0.02$; Figure 1).

Additional analysis was then performed to discern biological confounds that might explain the differences in IL-2 and IL-6 noted between controls and subjects with schizophrenia. Mixed model regressions were performed for IL-2 and IL-6 levels with PANSS-total scores, duration and subtype of schizophrenia illness, types and dosages of pharmacotherapy, presence of comorbidities, BMI, and smoking status as fixed predictor variables in the model. *In vitro* IL-2 levels were found to be significantly higher in those subjects with schizophrenia treated for hyperlipidemia ($F=10.1$, 1,61 df, $p<0.01$) and significantly lower in those subjects treated for hypothyroidism ($F=5.6$, 1,61 df, $p=0.02$) compared to subjects with schizophrenia not taking these types of medications. *In vitro* IL-6 levels were found to be inversely correlated with PANSS total scores ($F=5.7$, 1,61 df, $p=0.02$); a 1 unit increase in total PANSS scores corresponds to a 5.4 unit decrease in IL-6 production levels. However, anticholinergic medication usage was associated with a significant increase in IL-6 levels. Subjects with schizophrenia taking anticholinergic medications had significantly higher IL-6 levels ($n=65$; visit 1: 1419 ± 382; visit 2: 1302 ± 470 pg/ml) than those not taking anticholinergic agents ($n=35$; visit 1: 1150 ± 471; visit 2: 1050 ± 611 pg/ml; $F=8.8$, 1,61 df, $p=0.01$). Aside from these findings, there was no significant effect of the other variables investigated.

As a confirmatory analysis of the lack of significance regarding the effects of smoking status on these cytokine measures, secondary analysis was performed to examine the differences between controls and non-smoking schizophrenics (Table 2). Results from mixed model regressions found no difference between groups in IL-10 ($F=0.3$, 1,80 df, $p=0.59$) or IFN-γ levels ($F=0.7$, 1,80 df, $p=0.42$) whereas controls had significantly lower mitogen-stimulated IL-2 levels than non-smoking subjects with schizophrenia of IL-2 ($F=21.4$, 1,80 df, $p<0.01$) and significantly higher mitogen-stimulated IL-6 levels than non-smoking subjects with schizophrenia ($F=5.7$, 1,80 df, $p=0.02$). Control subjects also had no difference in levels of IL-10 ($F=3.7$, 1,118 df, $p=0.06$) or IFN-γ ($F=0.9$, 1,118 df, $p=0.35$) than subjects with schizophrenia that smoked, but had lower mitogen-stimulated IL-2 levels than smoking subjects with schizophrenia of IL-2 ($F=17.0$, 1,118 df, $p<0.01$)

and significantly higher mitogen-stimulated IL-6 levels ($F = 16.0$, 1,118 df, $p < 0.01$).

DISCUSSION

This study is the largest investigation of serial mitogen-stimulated cytokine levels ever performed in a well-characterized cohort of continuously ill outpatients with schizophrenia. We observed increased mitogen-stimulated IL-2 production and decreased mitogen-stimulated IL-6 production but did not find consistent, statistically significant alterations in mitogen-stimulated IL-10 or IFN-γ levels. It is interesting to note that the general pattern of mitogen-stimulated leukocyte cytokine production is more consistent with TH-1-mediated response since IL-6 and IL-10 trend downward and IL-2 and IFN-γ in general, trend upward. Our second important finding was that mitogen-stimulated cytokine production was relatively constant across the two time points in this sample. This suggests that immune dysfunction might represent a biomarker that could define a subset of patients with schizophrenia. Our preliminary analysis of the relationship between PANSS scores and cytokine levels suggested an inverse relationship between severity of illness and IL-6 production.

Although speculative, our findings may explain a previous paper we published showing that augmentation with celecoxib, a cyclooxygenase-2 (Cox-2) inhibitor, was ineffective in continuously ill medication-stabilized patients with schizophrenia (Rapaport *et al*, 2005). As a major function of selective COX-2 inhibitors is to decrease levels of prostaglandin-E2 (PGE2), and PGE2 has been shown to be responsible for increasing IL-1 and IL-6 levels, this lack of responsiveness to COX-2 inhibitor augmentation is not surprising (Müller and Schwarz, 2008; Stolina *et al*, 2000).

Because of concerns about the role that medications, medical comorbidities, subtype of schizophrenia, smoking, age, and sex might have on our results, we performed mixed effect longitudinal analyses which take into account these factors. Our findings could not be explained by any of these potential confounding factors. The literature studying the effects of antipsychotic medication on immune parameters in psychiatric patients and normal volunteers is limited and inconclusive. Most studies investigate the effects of initiating antipsychotic medications in acutely ill, hospitalized patients. The majority of these small studies measure serum cytokine and/or cytokine receptor levels at admission and then periodically for up to 3 months. Comparing and contrasting the acute effects of haloperidol and clozapine can serve as an illustration of the complexity of the extant literature. Acute *in vivo* exposure to haloperidol does not effect serum cytokine and cytokine receptors, whereas acute exposure to clozapine therapy increases serum levels of TNF-α, the TNF-α receptor, and sIL-2r (Haack *et al*, 1999; Maes *et al*, 2000; Maes *et al*, 1997; Monteleone *et al*, 1997; Müller *et al*, 1997; Pollmacher *et al*, 1997; Pollmacher *et al*, 1996; Rapaport *et al*, 1991a). This example reminds us that the acute effects of antipsychotic medication may be dramatically different. Thus, the conclusions of the recently published meta-analysis by Potvin *et al*. (2008) suggesting that serum sIL-2r elevations and decreased mitogen-stimulated IL-2 production may be medication mediated, need to be tempered by considerations about potential individual differences in the effect of antipsychotic medications on immune function as well as potential differences between the acute and chronic effects of medications. Another factor that confounds our understanding of the literature is the inability to discern the differences between the acute effects of medication on immune function *vs* the impact that acute treatment has on mitigating effects of an acute exacerbation of psychosis and the subsequent stress of hospitalization has on immune function.

As discussed above, our data indicate that the class of antipsychotic medication, use of combinations of typical and atypical antipsychotic medications, anticonvulsant medication augmentation, and antidepressant medication augmentation do not account for alterations in mitogen-stimulated cytokine levels. However, secondary analyses did reveal that schizophrenic subjects who received anticholinergic medication had higher mitogen-stimulated IL-6 production than schizophrenic subjects who were not receiving anticholinergic medication. (Both groups had lower supernatant IL-6 levels than the normal control sample.) This intriguing preliminary finding is consistent with newly emerging animal studies. Preclinical work investigating pancreatitis and endotoxin-induced inflammation and shock suggest that nicotinic cholinergic activity has anti-inflammatory effects that are reversed by anticholinergic medication. In fact, pretreatment with anticholinergic compounds enhance IL-6 production in rodent models of inflammation (Giebelen *et al*, 2007; van Westerloo *et al*, 2006; Matsunaga *et al*, 2001). As all medications were held constant during the course of this study, a change in anticholinergic medication usage does not explain the 100 pg/ml difference in IL-6 levels between the two sampling time points.

The association between statin use for hyperlipidemia and increased mitogen-stimulated IL-2 production is intriguing for two reasons. First, as we did not find a relationship between BMI and IL-2 production, this finding is unlikely to be an artifact of a lipocyte-mediated proinflammatory cytokine production (Wisse, 2004). Secondly, statins are known to decrease proinflammatory cytokine production and so an increase in mitogen-stimulated IL-2 production may be thought to be a counter-intuitive result (Cheng *et al*, 2008; Nissen *et al*, 2005). As is the case with our finding that thyroid replacement therapy is associated with lower mitogen-stimulated IL-2 production, these secondary analyses must be replicated in prospective data sets.

The reasons why our findings differ from other reports in the literature fall into two broad categories: differences in study population and differences in assay methodology. We deliberately chose to study continuously ill individuals who are stable but minimally responsive to current pharmacotherapy. An advantage of studying this group is that our findings are not confounded by the stress of an acute psychotic exacerbation (Arolt *et al*, 2000; Hornberg *et al*, 1995; Maes *et al*, 1994; Rothermundt *et al*, 2000; Villemain *et al*, 1987). Some investigators have postulated that acutely ill subjects with schizophrenia have such profound activation of the immune system that it leads to an 'exhaustion' epiphenomenon…, that is, as the leukocytes are already activated, they respond less robustly to

mitogen challenge than leukocytes from normal volunteers subjected to mitogen challenge (Arolt *et al*, 2000; Hornberg *et al*, 1995; Maes *et al*, 1994; Rothermundt *et al*, 2000; Villemain *et al*, 1987). As we were interested in collecting a representative 'real world' sample, our subjects were medically stable, but treated with a wide array of psychotropic medications. This is distinctly different than most published studies that control for medication. Another difference between our study and most of the other ones is that our subjects were outpatients rather than inpatients. Thus, this sample is different from most studies in the published literature and one we believe is more likely to include subjects with chronic immune activation.

There are two important differences in assay methodology between our study and some of the other published works in the literature. We investigated mitogen-stimulated cytokine production employing purified leukocytes rather than a whole blood assay (Arolt *et al*, 2000; Bessler *et al*, 1995; Cazzullo *et al*, 2002; De Groote *et al*, 1992; Ganguli *et al*, 1995; Kim *et al*, 1998; O'Donnell *et al*, 1996; Rothermundt *et al*, 1998; Wilke *et al*, 1996). A technical advantage to this approach is that one has greater precision across assays because absolute leukocyte numbers are actually counted rather than estimated from CBC results, as is the standard practice with whole blood assays. A second methodological difference between our study and many previous studies is that we chose to employ a single mitogen—PHA—rather than a combination of mitogens (Arolt *et al*, 2000; Hornberg *et al*, 1995; Maes *et al*, 1997; Rothermundt *et al*, 2000; Wilke *et al*, 1996). There are advantages to both approaches, but evaluating a single mitogen is simpler and enhances precision because one does not need to assure lot consistency of two mitogens. Furthermore, interpreting data about the interactions between two mitogens on leukocyte function is more complex than is frequently appreciated in the psychiatry literature.

We acknowledge that investigating complex syndromes like schizophrenia by measuring peripheral markers of immune function may not be the most exciting approach; however, data clearly show that activation of the peripheral immune system has profound effects on cognition, appetite, sleep, mood, anxiety, and psychosis (Dunn, 2006; McAfoose and Baune, 2009; Müller and Schwarz, 2006a; Myint *et al*, 2009). Cytokine interactions in the brain effect neurotransmitters and neurohormones involved in learning, memory, and emotions (Dunn, 2006; McAfoose and Baune, 2009; Myint *et al*, 2009). Müller and colleagues have synthesized disparate literature and developed a cogent hypothesis that proposes immune activation stimulates the kynurenine pathway and shunts tryptophan away from production of serotonin and toward the production of kynurenine that can be metabolized to KYN-A by astrocytes or quinolinic acid by microglia (Müller and Schwarz, 2006a; Müller *et al*, 2004). As discussed previously, KYN-A is a naturally occurring NMDA-receptor antagonist and α-7 nicotinic receptor antagonist whereas quinolinic acid is a potent NMDA-receptor agonist. They postulate that certain forms of schizophrenia are the result of immune activation that favors a TH-2 response and increases the catabolism of kynurenine by tryptophan 2,3-dioxygenase to KYN-A (Müller and Schwarz, 2006a). Although our findings do not directly support this postulate, our work is consistent with observations that immune activation may be associated both with treatment-resistance and a more chronic course of illness (Maes *et al*, 2000; Narayan *et al*, 2008; Zhang *et al*, 2005).

In conclusion, data from 100 well-characterized, chronically ill patients with schizophrenia who were serially sampled, suggest there is an increase in mitogen-stimulated IL-2 production and a concomitant decrease in mitogen-stimulated IL-6 production. These data require further exploration, particularly in light of newer findings suggesting that some patients with schizophrenia may have an immune mediated component to their illness.

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DISCLOSURE

Dr Rapaport is a consultant to and has received honoraria for presentations from Cyberonics, Forrest Labs, Roche, Pfizer, Sanofi Synthelabo, Solvay, Sumitomo, Wyeth, GlaxoSmithKline, Janssen Pharmaceutica, Neurocrine Biosciences, Eli Lilly, and Novartis; has received research support from Astra Zeneca, Pfizer, Janssen Pharmaceutica, GlaxoSmithKine, Forrest Labs, Eli Lilly, Abbott Laboratories, Corcept Therapeutics, Cyberonics, Novartis, Pharmacia Upjohn, Sanofi Synthelabo, Solvay, Wyeth, and UCB Pharma; and is a stockholder in Forrest Labs. Ms Bresee has no biomedical financial interests or potential conflicts of interest.

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