Clozapine Regulates Cytokines, T-cell Subsets and Immunoglobulins Serum Levels in MK-801-Evoked Schizophrenia Rat

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ABSTRACT

An association between inflammatory abnormalities and schizophrenia has been found repeatedly. In the current study, schizophrenia was induced by MK-801 (0.35 mg kg⁻¹, i.p.) twice a day for 2 weeks in rat pups. We found serum levels of IL-2, IL-6 and immunoglobulins were significantly higher than the normal level but TNF-α levels were significantly suppressed after the treatment of MK-801 in neonatal rats. Clozapine or haloperidol treatment could reverse immunoglobulins and TNF-α levels. However, clozapine, not haloperidol, did not recover serum levels of IL-2 and IL-6. T-cell subsets in peripheral blood of model rats were significantly increased in adulthood. On the contrary, the rate of CD4⁺/CD8a⁺ T cells was reduced significantly. After the treatment of clozapine or haloperidol, the levels of T-cell subsets were restored. However, clozapine, not haloperidol, has no effect on the rate of CD4⁺/CD8a⁺ T cells. These results suggested that the dysfunction of cytokine system and immune disturbance may be involved in the pathogenesis of MK-801-induced rat model of schizophrenia. The effects of clozapine and haloperidol on cytokines production, T-cell subsets switch and immunoglobulins presented in this study indicated that immunomodulatory therapy may be a potential strategy in the treatment of schizophrenia.

Key words: MK-801, Schizophrenia, T-cell subsets, immunoglobulins, cytokines

INTRODUCTION

The etiological explanations of schizophrenia were focused on disturbances of the dopaminergic neurotransmission. Unfortunately, the researches on dopamine system dysfunction did not achieve convincing results and antipsychotic antidopaminergic drugs still show unsatisfactory therapeutic effects (Horvath and Mirnics, 2014). New concepts in the pathophysiology research of schizophrenia are required. Recently, epidemiological, genetic, transcriptome, postmortem, peripheral biomarker and therapeutic studies of schizophrenia all pointed towards immune system abnormalities in patients suffering from schizophrenia and it is likely that these immune changes actively contribute to disease symptoms (Muller et al., 1999).

The abnormalities of immune system in schizophrenia, including dysregulation of cytokines production, T-cell subsets and immunoglobulins, have been one of the more enduring findings in the field over the last few decades, albeit with significant heterogeneity in the results, including negative studies (Rao et al., 1985; Villemain et al., 1989; Maes et al., 2000; Erbagci et al., 2001). Cytokines act as key players in the coordinate responses of cells in the innate immune system and the adaptive immune system (Potvin et al., 2008).

The most frequently studied cytokines in schizophrenia are serum interleukin (IL)-1 β, IL-2, IL-6 and Tumor Necrosis Factor (TNF)-β (Shintani et al., 1991; Zhang et al., 2004; Soczynska et al., 2009; Gray and Bloch, 2012; Munkholm et al., 2013). Beside the results on IL-6 levels is with more consistency (Rao et al., 1985; Akiyama, 1999; Arolt et al., 2000), level of serum interleukin (IL)-1 β, IL-2 and TNF-α mentioned by many authors are still controversial (Cazzullo et al., 1998; Sperner-Unterweger et al., 1999; Ebrinc et al., 2002; Miller and Buckley, 2012). There are
also alterations in T-lymphocyte subsets in the peripheral blood of schizophrenic patients in acute psychosis, abnormal lymphocyte proliferative responses to mitogens and soluble antigens in vitro and changes in the numbers and percentages of T-cells, as well as T-cell subsets (Theodoropoulou et al., 2001; Haberny et al., 2002; Kocahan et al., 2013). Additionally, the alterations of IgG, IgA and IgM antibodies are still detected and most studies revealed that immunoglobulins levels had a change in schizophrenia rats and patients (Delisi et al., 1981; Reichelt and Landmark, 1995; Steiner et al., 2013), even though other studies showed that there was no significant difference of IgG levels between schizophrenia patients and controls (Emelia et al., 2012; Masdeu et al., 2012).

Clozapine, a dibenzodiazepine developed in 1961, is a unique antipsychotic, specifically indicated in the management of treatment resistant schizophrenia (Muller and Schwarz, 2010). However, it is not considered as a rest-line drug due to several potentially life threatening side-effects, including agranulocytosis, aspiration pneumonia, ileus, type II diabetes, myocarditis and cardiomyopathy (Viberg et al., 2008; Cunha et al., 2015). These effects seem to be associated with an immune response and the dysregulation of inflammatory cytokines such as IL-2, IL-6 and TNF-α (Sandberg et al., 2015).

Blockade of NMDA receptor during early neonatal life can cause long-term alterations in the anatomical, neurochemical, neurophysiological and behavioral properties of rodents (Haberny et al., 2002; Viberg et al., 2008; Kocahan et al., 2013). Therefore, blockade of the NMDA receptor with MK-801 during the early postnatal period has been proposed to be an experimental model of schizophrenia. However, there is unclear on the immunological alterations of animal models of schizophrenia induced by MK-801 during the early postnatal period and the immunological regulatory effects of clozapine are still understood. The aim of the current study was to assess the immunologic alteration in MK-801-evoked schizophrenia rats and to detect the effects of clozapine treatment on immunological parameters.

MATERIALS AND METHODS

Ethics statement: The experiments were performed at the Department of Anatomy of Anhui Medical university. The protocol was approved by the local ethics committees of Anhui Medical university. All efforts were made to minimize the number of animals used and to ameliorate any distress.

Housing: All rats were housed in ambient temperature (approx. 20-23°C) and humidity (approx. 60%) controlled vivarium. Food and water were available ad libitum, except during testing, when no food was provided. In all experiments, animals were allowed to habituate to the housing conditions for at least 1 week before behavioral testing. All testing was performed during the light phase of the day/night cycle.

Animal administration: Sprague-Dawley pups that were fostered by their real mothers (n = 8) and there were 5-6 offspring per litter. The pups were randomly divided into four groups, MK-801 (M group), MK-801+Clozapine (CL group), MK-801+Haloperidol (H group) and normal control group (N group) and each group included eleven Sprague-Dawley pups. All pups from a given litter received the same treatment and were randomly assigned to the groups, so that mean body weight in each group was almost equal on PND 7. The pups of M group, CL group and H group received i.p. injections of the NMDA receptor antagonist dizocilpine (MK-801; 0.35 mg kg⁻¹; SIGMA) and N group received i.p. injections of vehicle (0.9% NaCl) twice a day within 8:00-10:00 am and 14:00-16:00 pm of the light Phase from Postnatal Days (PND) 6-21 of age. The animals were weaned at day 21. The animals of CL group and H group were treated with the administration of clozapine (1 mg kg⁻¹ SIGMA), or Haloperidol (0.1 mg kg⁻¹, Hunan Dongting Pharm. Co. Ltd.) and N group and M group received an equal volume of vehicle (0.9% NaCl) once a day within 14:00-15:00 pm on PND 43 and PND 57 for two weeks.

Flow cytometry: Animals were anesthetized by 10% hydral on PND 90. After anesthesia, the common carotid arteries were isolated from the rats and 4 mL blood sample were collected from rat common carotid artery in tube anticoagulated with heparin. According to the protocol of Ficoll-Paque TM PLUS (Catalog No. 07957, StemCell Technologies), the mononuclear cells were isolated from peripheral blood. The blood were Diluted with Phosphate Buffered Saline plus (DPBS) (volume ratio 1:1) and then carefully layered onto the medium to minimize mixing of blood with ficoll. The cells were harvested by carefully pipetting them from the liquid interface after the centrifugation at room temperature (15-25°C) at 2000 rpm for 20 min and then washed in DPBS and centrifuged at 1500 rpm for 20 min. The supernatant was then abandoned and the cells were resuspended in 300 μL DPBS. Flow cytometry (FACScalibur, BD Biosciences) was performed with the following antibodies, FITC anti-rat CD3 0.5 μL (201403, Biolegend), APC anti-rat CD4 1.25 μL (201509, Biolegend) and PE anti-rat CD8α 1.25 μL (200607, BioLegend). Data was analyzed with WinMDI 2.9 software.

ELISA analysis: Two milliliter blood samples were collected and serum was got after centrifugation at 4 , 4000 rpm for 20 min and stored at -80°C. To assess plasma levels of IL-6, IL-1 β, IL-2, TNF-α, IgA, IgM and IgG, Enzyme-Linked Immunosorbent Assay (ELISA) kits are used according to the manufacturer’s instructions. The details of ELISA kits was shown in Table 1.

Table 1: Details of ELISA kits

<table>
<thead>
<tr>
<th>ELISA kits</th>
<th>Lot No.</th>
<th>Manufacturers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat IL-6</td>
<td>ELR-IL6-001</td>
<td>Raybiotech</td>
</tr>
<tr>
<td>Rat IL-1 beta</td>
<td>ELR-IL1beta-001</td>
<td>Raybiotech</td>
</tr>
<tr>
<td>Rat IL-2</td>
<td>ELR-IL2-001</td>
<td>Raybiotech</td>
</tr>
<tr>
<td>Rat TNF-alpha</td>
<td>ELR-TNFalpha-001</td>
<td>Raybiotech</td>
</tr>
<tr>
<td>Rat IgA</td>
<td>88-50400-22</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Rat IgM</td>
<td>88-50540-22</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Rat IgG</td>
<td>88-50490-22</td>
<td>eBioscience</td>
</tr>
</tbody>
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IL: Interleukin, TNF: Tumor necrosis factor, ELISA: Enzyme linked immunosorbent assay
Statistical analysis: All data analysis was conducted using SPSS 16.0 for Windows. The values was presented as the Means±SD. All data of water maze was analyzed with repeated measures ANOVA, followed by Tukey-kramer HSD post hoc test to compare means of interest. The two study groups were compared for continuous variables by an independent t test. The pre- and post- treatment data were compared by the paired t test. Two-tailed p<0.05 was considered significant.

RESULTS

Serum levels of cytokines in schizophrenia rats: As shown in Fig. 1, after the treatment of MK-801 in neonatal rats, serum levels of IL-2 (Fig. 1d) and IL-6 (Fig. 1c) were significantly increased compared with normal level but TNF-α levels were significantly down regulated in M group (p<0.01) (Fig. 1b). Of note, IL-1β serum levels had no significant difference between N group and M group (p>0.05) (Fig. 1a).

Effects of clozapine and haloperidol on cytokines in schizophrenia rats: Interestingly, after treated with clozapine, serum concentrations of IL-2 and IL-6 were increased significantly compared to M group (p<0.01) (Fig. 1c-d). However, the levels of TNF-α were only increased to normal level (p<0.05) (Fig. 1b). Similarly, IL-2, IL-6 and TNF-alpha concentrations were all recovered to normal level in haloperidol group compared to N group (p<0.01) (Fig. 1b-d). Serum level of IL-1β had no changes after the treatment of clozapine or haloperidol (Fig. 1a).

T-cell subsets of peripheral blood in schizophrenia rats: T-cell subsets in peripheral blood of model rats were detected by flow cytometer and results showed that, compared with N group, CD3 (+), CD8a (+), CD4 (+) T cell levels in M group were significantly increased (p<0.01) (Fig. 2a-c). Similarly, the levels of CD3 (+) CD8a (+) and CD3 (+) CD4 (+) double-positive T cells were also high expressed in M group compared with N group (p<0.01) (Fig. 2d-e). In contrast, the rate of CD4 (+)/CD8a (+) T cells was reduced significantly in M group compared with N group (p<0.01) (Fig. 2f).

Effects of clozapine and haloperidol on T-cell subsets in schizophrenia rats: In comparison with M group, significant low-expression of CD3 (+), CD4 (+), CD8a (+), CD3 (+) CD8a (+) and CD3 (+) CD4 (+) T-cell subsets were detected in peripheral blood after clozapine treatment (p<0.01) (Fig. 2a-e). Moreover, compared with N group, the levels of CD3 (+), CD3 (+) CD8a (+) and CD3 (+) CD4 (+) T-cell subsets of CL group were recovered (p>0.05) but CD4 (+) and CD8 (+) in CL group were still significantly higher than that in N group.

Fig. 1(a-d): Effects of clozapine and haloperidol on serum cytokines in schizophrenia rats (n = 11), N: Normal group, M: Model group, CL: Clozapine group, H: Haloperidol group. Compared with N group, **p<0.01, *p<0.05, Compared with M group, #p<0.01, ip<0.05, Comparison between CL groups and H group, Δp<0.01, Δp<0.05, (a) IL-1β, (b) TNF-α, (c) IL-6 and (d) IL-2
Fig. 2(a-f): Effects of clozapine and haloperidol on T-cell subsets in schizophrenia rats (n = 11), (a) and (c) T-cell subsets in peripheral blood, (b) and (d) CD4 (+)/CD8a (+) T cells in peripheral blood. N: Normal group, M: Model group, CL: Clozapine group, H: Haloperidol group, compared with N group, **p<0.01, *p<0.05, Compared with M group, ##p<0.01, #p<0.05, Comparison between CL groups and H group, ΔΔp<0.01, Δp<0.05

In N group (p<0.05) (Fig. 2a-e). However, the rate of CD4 (+)/CD8a (+) T cells in peripheral blood of CL group was still lower than N group (p<0.01) (Fig. 2f).

Similarly, haloperidol treatment was also able to reduce the numbers of CD3 (+), CD4 (+), CD8a (+), CD3 (+) CD8a (+) and CD3 (+) CD4 (+) T-cell subsets in peripheral blood (p<0.01) (Fig. 2a-e). However, compared with CL group, the levels of CD4 (+) and CD8 (+) T cells in H group were decreased substantially (p<0.05) (Fig. 2b-c). The levels of T-cell subsets were all recovered to normal compared with N group (p>0.05) (Fig. 2a-e). The rate of CD4 (+)/CD8a (+) T cells was investigated as well in haloperidol group. Although the ratio in H group was still lower than normal levels, it was significantly higher than that in M and CL group (p<0.01) (Fig. 2f). The results suggested that, treatment with haloperidol, unlike clozapine, had partially restored the rate of CD4 (+)/CD8a (+) T cells in schizophrenia rats.

Serum levels of immunoglobulins in schizophrenia rats:
Serum levels of immunoglobulins were measured by ELISA and results showed, compared to N group, concentrations of IgA, IgM and IgG were significantly up regulated in M group (p<0.01) (Fig. 3a-c).

Effects of clozapine and haloperidol on immunoglobulin in schizophrenia rats: In comparison with M group, serum concentrations of IgA, IgM and IgG were markedly decreased after clozapine and haloperidol treatment (p<0.01) (Fig. 3a-c). IgA IgM and IgG serum concentrations in H group had no significant difference compared to N group (p>0.05) (Fig. 3a-c). However, after the treatment of clozapine, IgG and IgM serum concentrations in CL group decreased more significantly than that in H group (p<0.05) (Fig. 3b-c).
DISCUSSION

Schizophrenia (SZ) is a chronic and severe CNS disease affecting ~1% of the population (Perala et al., 2007). Recently, there is a growing body of evidence that points to dysfunction of the immune system in patients with SZ (Pathmanandavel et al., 2013). Up to now, although clozapine is also the most effective drug in schizophrenia treatment on clinic, its use is limited because of the high incidence of adverse reactions (Jules et al., 2012). However, the pathophysiology of SZ and the mechanism of clozapine-induced adverse reactions remain mysterious. In this study, we aimed to explore the immunologic alteration in MK-801-evoked schizophrenia rats during the clozapine-treated process.

Cytokines: Cytokines are pleotrophic proteins that coordinate the host response to infection as well as mediate normal, ongoing signaling between cells of non-immune tissues, including the nervous system (Deverman and Patterson, 2009). The production and release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α are typically intended to prevent further damage to CNS tissue, they may also be toxic to neurons and other glial cells, which might be involved in the pathogenesis and pathophysiology of schizophrenia (Boulanger and Shatz, 2004; Drexhage et al., 2011). In particular, interleukins are involved in a variety of neural processes and have a relationship with psychiatric disorders (Malek-Ahmadi, 1996; Boulanger and Shatz, 2004; Nawa and Takei, 2006). However, series of studies found that abnormal expression of interleukins in the plasma, brain tissues, or cerebrospinal fluid of SZ patients (Theodoropoulou et al., 2001; Potvin et al., 2008; Soderlund et al., 2009). However, there has been significant heterogeneity for cytokines expression among studies (Watanabe et al., 2010). Our results showed serum levels of IL-1β were not affected in schizophrenia rats with or without clozapine treatment, which was consistent with most of studies (Baker et al., 1996; Toyooka et al., 2003; Potvin et al., 2008), although Song et al. (2009) found that IL-1β serum levels and mRNA expression in peripheral blood mononuclear cell were increased in rest-episode schizophrenia patients.

Moreover, we also found that the serum level of TNF-α was decreased in schizophrenia rats and recovered to normal after treatment of clozapine and haloperidol. Similarly, Haack drew a same conclusion that TNF-α concentrations were lower in schizophrenia patients than controls (Haack et al., 1999). On the contrary, elevated serum or plasma levels of TNF-α are one of the most frequently confirmed immunological features associated with schizophrenia (Akiyama, 1999; Garcia-Miss et al., 2010; Kubistova et al., 2012; Song et al., 2013). Alzbeta (Kubistova et al., 2012) also reported that the
plasma level of TNF-α did not show any decrease after antipsychotics treatment in patients with drug naive, rest episode schizophrenia while Miller et al. (2011) found TNF-α levels remained elevated in acute exacerbations and following antipsychotics treatment. Interestingly, although results from many investigations indicated that serum IL-6 level is elevated or decreased after antipsychotics treatment in patients (Akiyama, 1999; Garcia-Miss et al., 2010; Kubistova et al., 2012; Song et al., 2013), our results detected a higher expression of TNF-α in model group of rats while treatment of haloperidol decreased but treatment of clozapine significantly increased its expression.

The IL-2 is a T-cell growth factor and has been shown to modulate a range of psychiatric manifestations. Some reports (Ganguli et al., 1995; Singh et al., 2011; Liu et al., 2012) showed that a lower levels of IL-2 were observed among the schizophrenia patients but others (Ebrinc et al., 2002; Zhang et al., 2002; Muller and Schwarz, 2006; Potvin et al., 2008; Zhang et al., 2009) found that increased IL-2 levels were observed in the serum, plasma and cerebrospinal fluid of schizophrenic patients. Moreover, another study (Kim et al., 2000) has also found close relationships between IL-2 and schizophrenic symptomatology. In our study, we found that elevated serum level of IL-2 in M group and it was recovered to normal after haloperidol treatment but increased significantly after clozapine treatment.

T-cell subsets; T Cell-mediated immunity is an adaptive process of developing antigen-specific T lymphocytes to eliminate viral, bacterial, or parasitic infections or malignant cells. Its functions depend on the total T lymphocytes (CD3+) and its subsets (CD4+, CD8+) relative composition (Broere et al., 2011). Treg subsets are presented within both the CD4+ and the CD8+ T cell compartments. It has been characterized that the CD8+ Treg alterations in autoimmune diseases (Filaci et al., 2011). The enumeration of CD4 and CD8 positive cells, surrogate markers for HIV disease progression, is helpful in management and follow up of immunocompromised patients (Ray et al., 2006). Nyland et al. (1980) reported that both immune mechanisms and the neuroleptic drug treatment may be of importance for the observed decrease in T lymphocyte numbers in blood from patients with acute schizophrenia. The animal studies showed that peripheral T cell deficit could lead to cognitive and behavioral impairment, highlighting the importance of properly functioning adaptive immunity in the maintenance of mental activity and in coping with conditions leading to cognitive deficits (Kipnis et al., 2004). Our current study detected a significant increase of CD3+CD8a+CD4+CD3+CD8a+ and CD3+CD4+ T cells but a lower ratio of CD4+/CD8a+ T cell in peripheral blood of model rats. However, the T cell subsets were all recovered except for the ratio of CD4+/CD8a+ T cells after clozapine and haloperidol treatment, indicating that clozapine treatment were able to attenuate the disturbances in T cell subgroup immunity that was occurred in schizophrenia rats.

Immunoglobulin: The present findings of elevated IgG, IgA and IgM antibodies in schizophrenia rats are in agreement with Steiner et al. (2013). Similarly, the previous studies also found a higher levels of IgA in patients with schizophrenia above the upper normal limits (Reichelt and Landmark, 1995). On the contrary, Delisi et al. (1981) detected a generalized reduction of IgG, IgA and IgM serum levels in the schizophrenic patients, which was not consistent with our data. However, other studies showed that there was no significant difference of IgG levels between schizophrenia patients and controls (Emelia et al., 2012; Masdeu et al., 2012). In our study, we also found that levels of IgG, IgA and IgM were markedly decreased after clozapine and haloperidol treatment.

The main finding of this study was demonstration that neonatal exposure to MK-801 led to long-lasting immunological disturbances in adult, evident already in 28 days old male rats. This clearly indicates that immunological changes in this animal model precede the behavioral deficit and suggests functional engagement of the immune system in the development of schizophrenia-like behavioral symptoms. Furthermore, Clozapine treatment was able to reverse some immunological parameters, has no significant impact on other indexes. These might be related to its therapeutic effects and adverse reactions.

In summary, this study demonstrated that peripheral immunological changes in the MK-801-evoked model of schizophrenia were long-lasting and attenuated by clozapine treatment. Moreover, the disturbances in T cell-mediated immunity as well as cytokine production. Thus, our data support the hypothesis that prenatal immune over activation can be a causative factor in pathogenesis of schizophrenia and effects of clozapine may be associated with immunological regulation.

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