

Received: 2003.07.02
Accepted: 2003.09.26
Published: 2004.02.25

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

Neuroleptics modulate cytokine and reactive oxygen species production in blood leukocytes of healthy volunteers

Agnieszka Szuster-Ciesielska^{1ABCDEF}, Maria Słotwińska^{2B}, Anna Stachura^{2B},
Halina Marmurowska-Michałowska^{3A} and Martyna Kandefer-Szerszeń^{1ADEFG}

¹Department of Virology and Immunology, Maria Curie-Skłodowska University, Lublin, Poland

²Department of Cell Biology, Maria Curie-Skłodowska University, Lublin, Poland

³Department of Psychiatry, University Medical School, Lublin, Poland

Source of support: self financing

Summary

Introduction:

There have been several reports indicating that schizophrenia is related to the activation of the inflammatory response system (IRS), characterized by increased serum concentrations of several cytokines, and that antipsychotic drugs may have immunosuppressive or immunoregulatory effects. The aim of the present study was to examine the effects of neuroleptics on cytokine and reactive oxygen species production *in vitro*, in blood leukocytes.

Materials and Methods:

We studied the effect of haloperidol, chlorpromazine and clozapine on the unstimulated and stimulated (phytohemagglutinin+lipopolysaccharide – PHA+LPS) production of some cytokines which are known to be mainly products of T lymphocytes and monocytes (IL-2, lymphotoxin, IFN- γ , IL-12, IL-4, IL-10 and TGF- β) in peripheral blood mononuclear cells (PBMC) of healthy subjects. We also compared the effect of neuroleptics on superoxide anion and hydrogen peroxide production in blood neutrophils.

Results:

All three antipsychotic drugs significantly increased PHA+LPS-stimulated production of anti-inflammatory cytokines such as IL-10 and TGF- β as well as unstimulated production of IL-10, but they did not influence IL-12 production. In the same *in vitro* conditions they inhibited PHA+LPS-stimulated production of IL-2 and lymphotoxin. IL-4 production was inhibited by haloperidol and chlorpromazine, but not by clozapine. IFN- γ production was inhibited by haloperidol and chlorpromazine, but stimulated by clozapine. All neuroleptics examined at a high (100 μ M) concentration, but not at a 1 μ M concentration, significantly inhibited superoxide anion production by phorbol ester (PMA)-stimulated neutrophils *in vitro*.

Conclusions:

The results indicate that *in vitro*, typical antipsychotic drugs, such as haloperidol and chlorpromazine, and atypical ones, such as clozapine, modulate cytokines which are known to be produced by monocytes as well as by T helper (Th)1 and Th2 subpopulations.

Key words:

peripheral blood mononuclear cells • neutrophils • cytokines • reactive oxygen species • haloperidol • chlorpromazine • clozapine

Abbreviations:

γ GT – γ -glutamyl transferase, ALT – alanine transferase, AOE – antioxidant enzymes, AST – aspartate amino transferase, CC16 – Clara cell protein 16, DMSO – dimethylsulfoxide, ELISA – enzyme-linked immunoassay, HBSS – Hank's balanced salt solution, HRPO – horseradish peroxidase, IFN – interferon, IL – interleukin, IL-R – interleukin receptor, IRS – inflammatory response system, LPS – lipopolysaccharide, MEM – minimal essential medium, PBMC – peripheral blood mononuclear cells, PHA – phytohemagglutinin, PMA – phorbol-12-myristate 13 acetate, PUFAs – polyunsaturated fatty acids, ROS – reactive oxygen species, SCID – Schedule for Clinical Interview, TGF – tumor growth factor, Th – T helper, TNF – tumor necrosis factor

Full-text PDF:

http://www.aite-online.org/pub/aite/vol_52/no_1/4462.pdf

Author's address:

Martyna Kandefer-Szerszeń, Department of Virology and Immunology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland, fax: +48 81 537 59 59, e-mail: KANDEM@BIOTOP.UMCS.LUBLIN.PL

INTRODUCTION

There have been several reports indicating that schizophrenia is related to the activation of the inflammatory response system (IRS), characterized by increased serum concentrations of interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α), which are mainly the products of activated monocytes/macrophages, increased IL-6 receptor (IL-6R) and IL-1 receptor antagonist (IL-1RA), but low serum concentrations of inhibitory Clara cell proteins (CC16)^{14, 15, 23}. Some findings also indicate that a moderate T cell activation may occur in some patients with schizophrenia, as soluble IL-2R, which is an unequivocal index of T cell activation, increases in the blood. Moreover, an increased IL-2 concentration in the cerebrospinal fluid of schizophrenic patients was detected, accompanied by decreased *in vitro* production of IL-2 by stimulated peripheral blood mononuclear cells (PBMC). This blunted *ex vivo* IL-2 production may be explained by *in vivo* overproduction of this cytokine, the exhaustion of T cells and, consequently, hyporesponsiveness of PBMC *in vitro*². Recently, elevated serum levels of IL-12 and IL-18 in schizophrenic patients were described^{16, 39}. Both cytokines play a pivotal role in T helper (Th)1 response; therefore it seems likely that the Th1/Th2 balance is disturbed in schizophrenia. According to a hypothesis of Müller et al.³², schizophrenia is characterized by a decrease in the Th1 cell-specific type of cellular immunity and Th1/Th2 imbalance with a shift towards the Th2 system. If this is correct, one can expect that neuroleptics may improve Th1/Th2 balance by enhancing Th1-cytokine response and inhibiting Th2-derived cytokine production. The data from the literature are, however, very conflicting and depend on patient selection, medication, and the methodology used *in vitro*¹¹.

In vivo, typical antipsychotic drugs, such as haloperidol, suppressed IL-6 and IL-6R in the plasma of schizophrenic patients^{22, 25}. At the same time, treatment with an atypical antipsychotic, i.e. clozapine, induced an increase in plasma concentrations of IL-6, interferon γ (IFN- γ), CC16, IL-1RA and TNF- α ^{23, 34}, but a decreased IL-2R level^{23, 29, 32}.

In *ex vivo* studies, IL-1 β and TNF- α release from lipopolysaccharide (LPS)-stimulated monocytes, isolated from the blood of schizophrenic patients or LPS-stimulated whole blood cell cultures, was significantly inhibited by haloperidol and perazine¹⁷.

In vitro, Leykin et al.²⁰ demonstrated that both clozapine and haloperidol at 1–50 μ M concentrations inhibited phytohemagglutinin (PHA)-stimulated production of several cytokines, such as IL-2, IL-4 and IFN- γ , in lymphocytes of healthy subjects as well as lymphocyte pro-

liferation measured by ³H-thymidine uptake. In whole blood cell cultures, clozapine used at a therapeutic dose (10⁻⁶ M) significantly increased PHA-stimulated IFN- γ and IL-2 production, but both clozapine and haloperidol enhanced the production of IL-1RA, which is an inhibitor of IL-1 biological activity^{36, 38}. Chlorpromazine inhibited the accumulation of mRNA specific for IL-2, IFN- γ and TNF- α in human thymocytes, but not mRNA for the α chain of IL-2R³⁷. Chlorpromazine also inhibited TNF- α production in the liver and lungs of mice treated with LPS⁵.

It seems likely that typical antipsychotic agents such as haloperidol and chlorpromazine are immunosuppressive, as they stimulate IL-1RA production and suppress the production of proinflammatory cytokines such as IL-6, IFN- γ and TNF- α . Atypical antipsychotics such as clozapine appear to share both immunostimulatory and immunosuppressive activities, mainly dependent on the dose used.

There is increasing evidence that free radical-mediated neuronal dysfunction is involved in the pathophysiology of schizophrenia because of an increased catecholamine turnover and an altered activity of antioxidant enzymes (AOE) such as superoxide dismutase (SOD)³¹ as well as the total antioxidant status measured in the plasma of schizophrenic patients⁴⁴. Moreover, an increased amount of lipid peroxidation products in the cerebrospinal fluid and a reduced level of membrane polyunsaturated fatty acids (PUFAs) in the brain and red blood cells were detected²⁴. Also neutrophils derived from the peripheral blood of schizophrenic patients produced more superoxide anion (O₂⁻) than neutrophils of healthy people³⁵.

Haloperidol, a classic neuroleptic and a dopamine receptor antagonist, did not exert a direct influence on AOE activity, but after haloperidol therapy the improvement in clinical symptoms was connected with the normalization of these enzymes' activity⁴³. Clozapine, a non-classic neuroleptic, significantly inhibited reactive oxygen species (ROS) production by monocytes from neuroleptic-resistant schizophrenic patients treated with clozapine¹³. Moreover, chlorpromazine negatively affected the ability of macrophages to produce O₂⁻ during phagocytosis, additionally acting as a O₂⁻ scavenger^{12, 42}. In contrast to that, haloperidol showed no ability to scavenge peroxy radicals and inhibit lipid peroxidation¹². Generally, there are very few papers concerning the effect of neuroleptics on free radical metabolism in blood cells.

In order to test the hypothesis of Müller et al.³², the aim of the present study was to examine the effects of haloperidol, chlorpromazine and clozapine on the unstimulated and stimulated production of some Th1-

–derived cytokines, i.e. IL-2, IFN- γ and lymphotoxin, as well as Th2-derived IL-4 and IL-10. Especially, we were interested in the examination of the influence of neuroleptics on the production of IL-12, a cytokine which influences the balance of the Th1/Th2 cytokine response. We also examined the effect of neuroleptics on the production of antiinflammatory cytokines such as tumor growth factor β (TGF- β) in PBMC isolated from the blood of healthy subjects. As neutrophils and monocytes of schizophrenic patients exhibited an enhanced ability to produce O_2^- , and some neuroleptics inhibited the “oxidative burst”, the aim of this paper was also to compare haloperidol’s, chlorpromazine’s and clozapine’s ability to regulate O_2^- and hydrogen peroxide (H_2O_2) production in neutrophils isolated from the blood of healthy subjects.

MATERIALS AND METHODS

Subjects

Blood samples were collected from 16 healthy volunteers. There were 8 females and 8 males aged 34.2 ± 8.1 years. All subjects gave written informed consent on a form approved by the Ethics Committee. A standardized interview (SCID – Schedule for Clinical Interview for DSM-IV⁷) was used to assess the mental health status of the subjects. None of the subjects had a history of psychiatric disorder (axis I psychiatric disorder). None reported a history of using antidepressants or neuroleptic medications or had used benzodiazepines during the previous 3 months. All the subjects had normal values of blood parameters, such as hematological parameters, blood renal tests (urea and creatinine) and normal liver tests (AST, ALT and γ GT levels). All the subjects had been free from acute infections or allergic reactions for at least 3 weeks prior to the study. None were addicted to alcohol. The subjects abstained from caffeine, alcohol and nicotine at least 12 h before blood sampling.

Isolation of granulocytes and PBMC

After an over-night fast, blood samples were taken in the morning (7.00 – 9.00 a.m.) into tubes with heparin (Heparinum, Polfa, Warszawa, Poland 20 U/ml) and immediately delivered to the laboratory. Granulocytes were separated according to the Sigma procedure: a gradient was formed by layering an equal volume of Histopaque-1077 over Histopaque-1119 (both from Sigma, St. Louis, MO, USA). The whole blood was carefully layered onto the upper Histopaque-1077. The tubes were then centrifuged at $700 \times g$ for 30 min. Granulocytes were isolated from the Histopaque-1077/1119 inter-phase and washed 3 times with Hank’s balanced salt solution (HBSS), centrifuged ($350 \times g$ for 15 min) and suspended in HBSS. Granulocytes repre-

sented 97–98% of the isolated cells, as estimated by May–Grünwald–Giemsa staining. PBMC were separated from plasma–Histopaque-1077 inter-phase, washed 2 times with Eagle’s minimal essential medium (MEM) supplemented with 10% fetal calf serum and suspended in the medium at 2×10^6 cells/ml. The viability of cells determined by the trypan blue exclusion test was above 98%. Mononuclear cells (lymphocytes and monocytes) represented 96% of the cells (determined after staining with May–Grünwald–Giemsa).

Induction of cytokines

The PBMC suspension was distributed into 24-well plastic plates (Falcon, Bedford, MA, USA), 2×10^6 cells/well, and stimulated or not (spontaneous release) with PHA-M (Sigma) at a final concentration of 5 μ g/ml together with LPS (from *E. coli* 0111:B4, Sigma), with a final concentration of 25 μ g/ml. At the time of stimulation with polyclonal activators, neuroleptics dissolved in MEM, chlorpromazine at a final concentration of 1 μ M, or haloperidol and clozapine dissolved in dimethylsulfoxide (DMSO) and diluted in MEM to a final concentration of 1 μ M, were added (10 μ l volume) to the PBMC in wells. MEM alone or DMSO at a concentration which corresponded to its dilution in the respective sample served as controls. Samples were incubated for 72 h in a humidified atmosphere with 5% CO_2 at 37°C. After incubation, supernatants were collected, centrifuged and frozen immediately at –20°C and kept for no longer than 3 weeks before cytokine titration.

Cytokine measurements

Cytokines were quantified in duplicate by means of the ELISA method using commercially available kits. IL-2, IFN- γ , IL-10, IL-4, IL-12 were detected using kits from Endogen, (Woburn, MA, USA), and TNF- β or TGF- β using kits from Quantikine, R&D System, (Minneapolis, MN, USA). The intensity of color developed was measured by a microtiter plate reader (E-max, Molecular Devices Co, Menlo Park, CA, USA) at 450 nm (correction at 550 nm or 540 nm). All samples for each cytokine were assayed at the same time in a single run with a single lot number of reagents. The detection limits were IL-2 > 2 pg/ml, IFN- γ > 2 pg/ml, TNF- β > 7 pg/ml, IL-10 > 3 pg/ml, IL-4 > 2 pg/ml, TGF- β > 7 pg/ml and IL-12 > 3 pg/ml. Intra-assay variations were less than 5%.

Apoptosis measurements

Apoptosis of cells was measured by two different routine methods¹. A PBMC suspension was incubated with neuroleptics (1 μ M haloperidol, chlorpromazine and cloza-

pine) for 24 h, centrifuged ($350 \times g$ for 10 min) and resuspended in MEM. The cell suspension (1×10^5 cells/ml) in 25 μ l of the medium was incubated for 3 min at room temperature with 1 μ l of a solution of orange acridine (100 μ g/ml) with ethidium bromide (100 μ g/ml) in phosphate-buffered saline (PBS). The suspension was placed on a microscope slide and covered with a coverslip. The cells were examined with a fluorescence microscope (Olympus, Japan). They were scored and categorized into normal (green fluorescence), apoptotic (green with fragmented chromatin and apoptotic bodies formed) and necrotic (red stained nucleus). A total of 500 cells were examined and the percentages of apoptotic, necrotic and normal cells were calculated.

Cell apoptosis induced with antidepressants was also measured by cytochrome C release from mitochondria by using a cytochrome C ELISA kit from Bender Med System (MedSystem Diagnostic GmbH, Vienna, Austria) according to the manufacturer's instructions. PBMC, after treatment with neuroleptics, were centrifuged, washed in PBS, resuspended in Lysis Buffer, and incubated for 1 h at room temperature. After centrifugation at $1000 \times g$ for 15 min, the concentration of cytochrome C was measured in supernatants. Briefly, samples of supernatant were transferred into wells coated with the monoclonal antibody specific for cytochrome C, washed, and incubated with the biotinylated second antibody. After the incubation and washing, a streptavidine-horseradish peroxidase complex was added. After washing, a substrate solution was added to the wells. Color development was stopped by sulphuric acid and the intensity of color was measured at 450 nm (correction at 610 nm). The limit of cytochrome C detection was 0.08 ng/ml.

Measurement of O_2^- production by cytochrome C reduction assay

HBSS (177.5 μ l), 12.5 μ l of cytochrome C solution in HBSS (a final concentration of 75 μ M), 5 μ l of either SOD solution (a final concentration of 60 U/ml) or 5 μ l of distilled water, and 50 μ l of a neutrophil suspension (a final density of 2.5×10^5 cells/well) were added into each well on a 96-well plate. After 3 min of incubation, the neutrophils were treated or not with neuroleptics (100 and 1 μ M concentrations of haloperidol, chlorpromazine or clozapine in 5 μ l). The microplate was incubated at 37°C for 60 min and transferred to the microplate reader. The absorbance values at 550 nm (the differences in the optical density between samples with and without SOD) were converted to nanomoles of O_2^- based on the extinction coefficient of cytochrome C: $\Delta E_{550} = 21 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. The results were expressed as nanomoles of O_2^- per 1×10^6 cells per 60 min. In some experiments neutrophils were preincubated with neu-

roleptics for 24 h and then activated with 12-myristate-13-acetate (PMA, Sigma) at a final concentration of 1 μ g/ml, and after 60 min of incubation, absorbance at 550 nm was read in the microplate reader²⁵.

Measurement of H_2O_2 production

The assay was based on horseradish-dependent peroxidation (HRPO) of phenol red by H_2O_2 leading to the formation of a compound that exhibits absorbance at 600 nm. A neutrophil suspension (4×10^6 cell/ml of HBSS) was distributed into wells (50 μ l/well) on a 96-well microplate. The cells were covered with 50 μ l/well of assay solution. The assay solution was prepared on the day of the experiment and consisted of HBSS, phenol red (Sigma, a final concentration of 0.56 mM), HRPO (Serva, Heidelberg, Germany, a final concentration of 20 U/ml) and a 1 μ M concentration of neuroleptics. In some experiments, except for neuroleptics, the assay solution also contained PMA (Sigma, a final concentration of 1 μ g/ml). To the control wells an assay solution without PMA or without neuroleptics was added. The plate was incubated at 37°C for 60 min and then the reaction was stopped by adding 10 μ l/well of 1 N NaOH. After 3 min of incubation, the plates were read at 600 nm in the microplate reader. The results were expressed as nanomoles of H_2O_2 per 10^6 cells per 60 min based on the phenol red extinction coefficient ($\Delta E_{600} = 19.8 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$)³³.

Statistics

The values of cytokine concentrations and O_2^- or H_2O_2 which were produced under the influence of haloperidol, chlorpromazine or clozapine by each cell type were calculated as means $\pm$ SD. The differences in the cytokine or ROS concentrations between the cells treated with neuroleptics and the respective controls were analyzed with the Student's paired *t*-test using Statistica software. All results were considered significant at $p < 0.05$. The relations between variables were assessed by Pearson's product moment correlation coefficient.

RESULTS

Table 1 shows that haloperidol, chlorpromazine and clozapine significantly enhanced the unstimulated production of IL-10 in human PBMC isolated from the blood of healthy subjects. All the three neuroleptics examined had a significant influence on PHA+LPS-induced cytokines produced by the Th1 and Th2 subpopulations. Haloperidol inhibited the production of IL-2, lymphotoxin (Th1 cytokines), and IL-4 (a Th2 cytokine), but stimulated the production of both inflammation inhibitory cytokines, i.e. IL-10 and TGF- β . Chlorpromazine, besides IL-2, lymphotoxin and IL-4,

Table 1. The influence of neuroleptics on unstimulated (UN) and PHA+LPS-stimulated (ST) cytokine production by PBMC of healthy volunteers

Neuroleptic	Conditions	Cytokines pg/ml						
		IL-2	IFN- γ	lymphotoxin	IL-12	IL-4	IL-10	TGF- β
Haloperidol ^a	UN	4.56 $\pm$ 0.5	0	0	0	0	25.0 $\pm$ 8.6*	198 $\pm$ 18
	ST	191 $\pm$ 138*	1141 $\pm$ 294*	2237 $\pm$ 775*	60.5 $\pm$ 68.5	2.9 $\pm$ 2.9*	2304 $\pm$ 778*	2461 $\pm$ 1069*
Chlorpromazine	UN	4.64 $\pm$ 0.4	0	0	0	0	20.3 $\pm$ 4.8*	235 $\pm$ 2
	ST	179 $\pm$ 119*	789 $\pm$ 491*	3972 $\pm$ 680*	50.4 $\pm$ 34.9	4.9 $\pm$ 2.3*	2347 $\pm$ 744*	2715 $\pm$ 1353*
Clozapine ^a	UN	4.87 $\pm$ 0.6	0	0	0	0	26.2 $\pm$ 6.57*	198 $\pm$ 20
	ST	161 $\pm$ 119*	1928 $\pm$ 150*	3357 $\pm$ 831*	46.6 $\pm$ 25.6	12.5 $\pm$ 7.1	2253 $\pm$ 382*	2349 $\pm$ 139*
Control	UN	4.05 $\pm$ 0.5	0	0	0	0	8.2 $\pm$ 0.8	229 $\pm$ 22
	ST	416 $\pm$ 44	1481 $\pm$ 108	5155 $\pm$ 912	73.6 $\pm$ 31.1	17.3 $\pm$ 6.1	1371 $\pm$ 283	1812 $\pm$ 993
Control DMSO	UN	4.21 $\pm$ 0.7	0	0	0	0	8.3 $\pm$ 1.2	220 $\pm$ 11
	ST	411 $\pm$ 24	1501 $\pm$ 42	5194 $\pm$ 511	74.0 $\pm$ 24.0	17.9 $\pm$ 5.4	1380 $\pm$ 190	1900 $\pm$ 550

PBMC at the density of 2×10^6 cells/ml were incubated for 72 h in the presence of neuroleptics at 1 μ M concentration and with PHA-M (5 μ g/ml) mixed with LPS (25 μ g/ml). In unstimulated cultures only neuroleptics were added.

^a The level of cytokines induced in the presence of haloperidol and clozapine was compared with the DMSO control as drugs were dissolved in DMSO. The results obtained with PBMC treated with chlorpromazine were compared with the control.

* Statistically significant difference compared with the respective control at $p < 0.05$.

also inhibited the production of IFN- γ , while it enhanced the production of IL-10 and TGF- β . Clozapine modulated *in vitro* Th1- and Th2-like cytokines: it inhibited IL-2 and lymphotoxin, but enhanced IFN- γ production. IL-4 production was not influenced by clozapine, while IL-10 was enhanced. Moreover, TGF- β production was also enhanced by clozapine. None of the neuroleptics examined significantly influenced the production of IL-12. Pearson's correlation coefficient showed significant positive correlations among cytokines which are the products of the Th1 subpopulation and between IL-12 and

Th1 cytokines. Negative correlations were observed between cytokines which are known to be the products of Th1 and Th2 subpopulations, as well as between Th1 cytokines and TGF- β and between Th2 cytokines and TGF- β (Table 2).

The inhibitory effect of neuroleptics on cytokine production was not caused by the induction of necrotic or apoptotic death of a specific cell clone, as none of the neuroleptics used at a 1 μ M concentration significantly influenced the number of apoptotic or necrotic cells stained with orange acridine and ethidium bromide or increased the level of cytochrome C released from mitochondria (Table 3). Only chlorpromazine was slightly toxic for lymphocytes, increasing the number of necrotic cells.

The influence of neuroleptics on O_2^- or H_2O_2 release from neutrophils isolated from the blood of healthy persons was also examined. As can be seen from Table 4, the effect of haloperidol, chlorpromazine and clozapine on PMA-stimulated superoxide production was inhibitory when the neuroleptics were used at high doses (100 μ M). At lower, 1 μ M doses the neuroleptics did not influence O_2^- production. When used at a high dose, clozapine also inhibited unstimulated (spontaneous) O_2^- production in neutrophils. None of the neuroleptics used significantly influenced H_2O_2 production (data not shown).

DISCUSSION

Typical antipsychotic drugs, e.g. phenothiazines such as chlorpromazine or butyrophenons such as haloperidol, may suppress the proliferation of human lymphocytes *in vitro* and inhibit IL-2 production by activated lympho-

Table 2. Pearson's analysis of correlations between cytokines produced in PBMC of healthy volunteers stimulated *in vitro* with PHA+LPS

PBMC treated with	Cytokines	r	p
Control	IFN- γ /TGF- β	-0.77	0.0004
	IL-2/IL-10	-0.53	0.031
	IL-10/IL-12	-0.74	0.001
	IL-4/TGF- β	-0.58	0.018
Haloperidol	IFN- γ /IL-4	-0.54	0.03
	IL-2/IL-12	0.90	0.0001
	IL-10/ TGF- β	-0.66	0.0050
	IL-4/ TGF- β	-0.72	0.0010
	lymphotoxin/ TGF- β	-0.76	0.0004
Chlorpromazine	IFN- γ /IL-10	-0.71	0.002
	IFN- γ / TGF- β	-0.85	0.0001
	IL-2/ TGF- β	-0.73	0.001
	IL-2/IL-12	0.94	0.0001
	IL-4/ TGF- β	-0.66	0.005
	TGF- β /IL-12	-0.59	0.016
Clozapine	IL-2/lymphotoxin	0.60	0.014
	IL-2/IL-12	0.97	0.0001
	IL-10/IL-12	-0.52	0.037
	IL-4/ TGF- β	-0.73	0.001
	lymphotoxin/IL-12	0.55	0.02

Table 3. Induction of apoptosis and necrosis by neuroleptics in PBMC of healthy subjects

Neuroleptic 1 μ M	% of apoptotic cells	% of necrotic cells	Cytochrome C (ng/ml)
Haloperidol	3.0 $\pm$ 2.6	3.8 $\pm$ 2.1	10.4 $\pm$ 4.5
Clozapine	2.9 $\pm$ 2.3	4.8 $\pm$ 2.1	9.9 $\pm$ 2.8
Chlorpromazine ^a	2.6 $\pm$ 2.7	5.1 $\pm$ 1.4*	10.1 $\pm$ 3.4
DMSO control	2.3 $\pm$ 0.5	2.3 $\pm$ 1.5	9.8 $\pm$ 2.9
Control	1.4 $\pm$ 0.7	2.8 $\pm$ 1.1	8.7 $\pm$ 3.6

^a The results obtained with chlorpromazine were compared with the control, but the ones obtained with haloperidol and clozapine with the DMSO control.

* Statistically significant in comparison to respective control at $p < 0.05$.

cytes *in vitro*^{4,37}. Besides the inhibitory influence on Th1-like cytokines, typical antipsychotic drugs may also inhibit other proinflammatory cytokines, such as monocyte/macrophage-derived IL-6, IL-1 β , or TNF- α ^{5, 17, 38}.

Our results corroborate those previously mentioned indicating an immunosuppressive character of typical neuroleptics, as we have detected significantly inhibited PHA+LPS-induced IL-2 and lymphotoxin (Th1-derived cytokines) production in the haloperidol- and chlorpromazine-treated PBMC of healthy people. We have also determined that PHA+LPS-stimulated IFN- γ (another

Table 4. The influence of neuroleptics on unstimulated and PMA-stimulated O_2^- production by neutrophils isolated from blood of healthy subjects

Neuroleptic	Dose μ M	nmol/106 of neutrophils/h	
		unstimulated	PMA-stimulated
Haloperidol	100	3.1 $\pm$ 1.2	9.2 $\pm$ 0.8*
	1	3.2 $\pm$ 0.9	15.0 $\pm$ 1.9
Chlorpromazine ^a	100	2.8 $\pm$ 1.4	6.9 $\pm$ 1.5*
	1	2.7 $\pm$ 0.4	11.2 $\pm$ 0.8
Clozapine	100	1.7 $\pm$ 0.4*	8.9 $\pm$ 0.5*
	1	3.2 $\pm$ 0.4	11.5 $\pm$ 0.9
DMSO control	—	3.1 $\pm$ 1.4	12.9 $\pm$ 2.4
Control	—	3.2 $\pm$ 0.7	12.1 $\pm$ 1.2

^a The results obtained with chlorpromazine were compared with the control, but the ones obtained with haloperidol and clozapine with DMSO control.

* Statistically significant in comparison to respective control at $p < 0.05$.

Th1-like cytokine) production is inhibited by chlorpromazine and haloperidol. In contrast to our results, Song et al.³⁸, using different concentrations of haloperidol and whole blood cells from healthy volunteers, did not observe any changes in PHA+LPS-induced IFN- γ production. Such a comparison, however, has its limits, as in our experiment mononuclear cells isolated from blood were stimulated *in vitro* for cytokine production. On the other hand, Yaqoob et al.⁴⁵ compared cytokine production in cultures of whole human blood and purified mononuclear cells and detected significant correlations among the production of each cytokine in whole blood and mononuclear cells. We suppose that the reasons of such discrepancies were variations and differences in cytokine production kinetics among individuals. Another limitation of our results is the fact that all cytokines were collected after 72 h of incubation. However, this time of cytokine collection was chosen by us as the time of peak cumulative response for most of the cytokines examined, such as IFN- γ , IL-4, IL-12, lymphotoxin, and IL-10 (preliminary results not shown).

Previously it was found by other authors that chlorpromazine upregulated the secretion of IL-10 in an *in vitro* model of acute superantigen-driven immune activation in mice and that this effect was abolished by a blockade of dopamine-1 but not dopamine-2 receptors⁴¹. In mice, chlorpromazine increased LPS-induced IL-10 secretion in serum, but inhibited TNF- α production²⁵. In our study, chlorpromazine at a 1 μ M concentration also increased unstimulated and PHA+LPS-stimulated IL-10 production, which in experimental conditions was produced by the Th2 subpopulation and by monocytes. Haloperidol exerted a similar effect. Moreover, both antipsychotic drugs enhanced the mitogen-stimulated production of another inhibitory cytokine, monocyte-derived TGF- β . However, they did not influence the production of IL-12, a monokine, which is known to have a stimulatory activity on Th1-like cytokine response³⁰. Thus, the emerging picture is that *in vitro*, haloperidol and chlorpromazine exert a rather immunosuppressive effect, inhibiting the production of IL-2 and lymphotoxin (Th1-like cytokines) and enhancing the production of two inhibitory cytokines i.e. IL-10 and TGF- β .

In the same *in vitro* conditions, production of another Th2-like cytokine, IL-4, was inhibited. Therefore it seems likely that typical neuroleptics modulate the monocyte-derived, Th1- and Th2-derived cytokine response, and in the final effect their action is immunosuppressive.

Clozapine, an atypical antipsychotic drug with mixed dopamine D-1, D-2 and serotonin-2 receptor antagonist properties, has been found to be effective in schizophrenic patients who are refractory to treatment with

typical antipsychotic agents^{8, 27, 28}. It has already been shown that treatment of schizophrenic patients with clozapine induced an increase in the plasma concentration of IL-2R, which may bind IL-2 and thereby compete for the binding of IL-2 to cellular receptors. Clozapine also enhanced the production of inhibitory cytokines such as CC16 and IL-1RA, but at the same time it enhanced the production of proinflammatory cytokines such as IL-6 and TNF- α ^{9, 10, 23, 29, 34}.

In vitro, in whole blood cultures, depending on the concentration used, clozapine (at doses within the therapeutic range) stimulated the production of IL-1RA or (at doses above 1 μ M) inhibited the production of IL-2 and IL-4^{20, 38}. Its influence on IFN- γ production was dose dependent and clozapine at a 1 μ M concentration significantly increased the production of this cytokine, while at a higher concentration inhibited it.

In our study, clozapine at a 1 μ M concentrations inhibited the production of IL-2 and lymphotoxin, but it enhanced IFN- γ production (all Th1 cytokines). It did not influence production of IL-4 (a Th2-like cytokine) and IL-12 (a monokine). It enhanced the unstimulated and PHA+LPS-stimulated production of inhibitory cytokines such as IL-10 and TGF- β . Our results concerning the stimulation of IFN- γ production are in agreement with the data of Leykin et al.²⁰ and Song et al.³⁸

As clozapine, except for IFN- γ , inhibited Th1-like cytokine production, but enhanced the production of inhibitory cytokines such as IL-10⁶ and TGF- β in our experiments, its effect was predominantly immunosuppressive. It is worth mentioning that Pearson's correlation analysis revealed that neuroleptics did not change positive correlations among cytokines which are mainly the products of a certain Th subpopulation, and negative, when cytokines produced by different Th1 or Th2 subpopulations were compared.

One question is how the concentrations employed in the present study compare with plasma levels usually obtained under clinical conditions. Plasma concentrations of haloperidol usually run in the range of 10–100 μ M when the daily doses range from 2 to 100 μ g¹⁹. Thus the haloperidol concentration of 1 μ M used in our experiments was about 10 times higher than therapeutic plasma concentrations obtained during treatment, but it was not toxic for blood leukocytes. Plasma levels of chlorpromazine and clozapine run in the range of 0.5–1.5 μ M when the daily doses range from 100 to 800 mg¹⁸. Thus the therapeutic concentrations of clozapine and chlorpromazine obtained during the treatment of patients are in the 1 μ M range, and such concentrations were used in our study. The supraoptimal concentrations of haloperi-

dol employed in this study correspond, however, to the concentration of neuroleptics usually employed by other researches in *in vitro* experiments which aim to examine the effect of drugs on immune cell function^{20, 38}.

It should be stressed that this study was performed *in vitro* in PBMC isolated from the blood of normal volunteers. Thus, the findings cannot be generalized to *in vivo* treatment with antipsychotic drugs in schizophrenic patients; however, as the results were obtained with low concentrations of neuroleptics which *in vitro* did not induce necrotic or apoptotic death of mononuclear blood cells, one can speculate that neuroleptics present in the plasma may exert a similar immunoregulatory effect. However, to determine the generalizability of these results, other antipsychotic agents, both typical and atypical, should be examined. Another limitation of our interpretation of the results is the fact that in our study cytokines were produced by a mixture of different types of mononuclear cells, such as T and B lymphocytes and monocytes. Thus, for example, IL-10 was produced not only by Th1 lymphocytes but also by monocytes. Moreover, IL-10 is not only an inhibitory cytokine, but it acts as a survival and differentiation factor for B cells⁶; therefore, the role of neuroleptics-enhanced IL-10 production should be examined.

The oxidant/antioxidant balance is an important determinant of immune cell function, not only for maintaining the integrity and functionality of membrane lipids, cellular proteins and nucleic acids, but also for the control of signal transduction and gene expression in immune cells. The cells of the immune system are particularly sensitive to changes in the oxidant/antioxidant balance because of the relatively higher percentage of PUFAs in their plasma membranes. They are also frequently exposed to changes in this balance because high levels of ROS are produced as part of their normal function. Polymorphonuclear neutrophils play a central role in IRS, and their function associated with phagocytosis and microorganisms killing, including O₂⁻ generation, is implicated in tissue injury. It has already been shown that neutrophils derived from the peripheral blood of schizophrenic patients produce more O₂⁻ than the neutrophils of healthy people. Moreover, an increased amount of lipid peroxidation products in the cerebrospinal fluid and plasma as well as a reduced level of membrane PUFAs in the brain and red blood cells were detected^{24, 35}.

The results of our study performed by the commonly used methods indicated that clozapine used at a high (100 μ M) concentration inhibited unstimulated O₂⁻ production. These results are generally in agreement with the observations of Joffe et al.¹³ despite the differences in the methods used in the measurements of ROS pro-

duction and the origin of the cells (schizophrenic patients or healthy volunteers). In our study, clozapine at a 100 μM concentration also inhibited the production of O_2^- by PMA-stimulated neutrophils isolated from the blood of healthy volunteers. Joffe et al.¹³, considering their results, stressed that this *in vivo* clozapine effect on ROS production may be the marker of monocyte sensitivity to clozapine action and may be predictable for the clinical response to clozapine in neuroleptic-resistant schizophrenia.

In our study, haloperidol and chlorpromazine at high concentrations (100 μM) also exhibited a pronounced effect on O_2^- production in PMA-stimulated neutrophils. Low doses of both neuroleptics were not effective.

It has already been described that both chlorpromazine and haloperidol were scavengers of O_2^- *in vitro* mainly at high concentrations, above 1 mM, but at low concentrations, below 1 μM , they inhibited the ability of macrophages to produce O_2^- during phagocytosis^{12, 42}. Moreover, chlorpromazine, which depresses Ca^{2+} -dependent neutrophil function, being a calmoduline antagonist, used *in vitro* at 5–100 μM concentrations influenced several neutrophil functions, such as motility, chemotaxis, free radical generation, and lactoferrin release^{3, 21, 40}.

We conclude that haloperidol and chlorpromazine, typical neuroleptics, did not exert the expected specificity toward a certain Th subpopulation, influencing exclusively a Th1 or Th2-cytokine response, but they modulated Th1 and Th2-like cytokine response as well as the production of cytokines which are mainly products of monocytes/macrophages. They enhanced the production of inhibitory cytokines such as IL-10 and TGF- β and inhibited the production of IFN- γ , lymphotoxin, IL-2 and IL-4. They did not influence IL-12 production. At high doses, they also inhibited O_2^- production in PMA-activated neutrophils.

The influence of clozapine on some immunological parameters *in vitro* seemed to be even more complex. Clozapine inhibited the production of IL-2 and lymphotoxin (Th1-like cytokines), but at the same time it enhanced IFN- γ production (a key proinflammatory Th1-derived cytokine). It also enhanced the production of inhibitory cytokines such as IL-10 and TGF- β . Clozapine *in vitro* was an inhibitor of the spontaneous and PMA-stimulated “oxidative burst” in neutrophils, but only at a high, 100 μM concentration.

The mechanisms involved in the immunoregulatory activity of neuroleptics are still largely unclear and remain to be explored.

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