ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. LIX, N 2, 133 SECTIO D 2004

Department of Virology and Immunology, Maria Curie-Skłodowska University
Department of Psychiatry, Skubiszewski Medical University of Lublin

HALINA MARMUROWSKA-MICHAŁOWSKA,
AGNIESZKA SZUSTER-CIESIELSKA,
MARTYNA KANDEFER-SZERSZEŃ, HALINA DUBAS-ŚLEMP

The influence of carbamazepine on cytokine and superoxide anion production in blood leukocytes of healthy volunteers

There are data that epileptic seizures may modify cytokine secretion in patients suffering from epilepsy. In non-medicated epileptic patients, the secretion of IL-1, IL-2 and IL-6 in peripheral blood mononuclear cells (PBMC) was found to be enhanced (8), but other authors did not observe any differences between patients before CBZ therapy and a healthy control group. In experimental animals, after the induction of seizures, enhanced concentrations of IL-1 and TNF inside the CNS were found (3).

Carbamazepine (CBZ), a widely used anticonvulsant, is associated with a wide range of adverse effects including agranulocytosis, aplastic anemia and drug-induced lupus (11). It has also been reported to alter immune functions in a variety of ways. Oral CBZ, at 5–15 mg/kg of body weight, significantly diminishes cellular and humoral immune response in mice (2). In man, it suppresses total lymphocyte count (14). In mice, CBZ acts as an immunosuppressive agent and significantly decreases lymphocyte CD4+ and NK cell counts (3). In contrast to that, the treatment of epileptic children and adolescents with CBZ for one year caused a significant increase in interleukin-1 (IL-1), IL-6, IL-2 and monocyte chemoatractant protein 1 (MCP-1) production by *ex vivo* cultivated PMBC stimulated with phytohemagglutinin (PHA) (15).

It has been demonstrated that CBZ in blood is oxidized by activated neutrophils to several metabolites, which also exert a negative effect on the immune cell functions such as lymphocyte transformation and mitogenesis (7, 9). Neutrophils of epileptic patients medicated with CBZ have impaired neutrophil chemotaxis (4). Other authors (5) did not confirm the negative influence of CBZ on chemotaxis, however, they detected that CBZ administration enhanced phagocytosis and oxidative metabolism ($O_2^{\cdot}$ production) but inhibited the killing of microorganisms. When some neutrophil functions of healthy volunteers were examined *in vitro*, CBZ (42-168 μM) inhibited chemotaxis of neutrophils, but did not influence phagocytosis frequency and resting superoxide production (4). Literature concerning the influence of CBZ on immune cell functions presents very limited and contradictory data, and there is no study of CBZ influence on cytokines production which are known to originate mainly from T lymphocytes.

The aim of this paper is to evaluate whether CBZ may influence *in vitro* production of Th1 cytokines such as IL-2, lymphotoxin, IFN- γ, Th2-like cytokines such as IL-4, IL-10 and others such as IL-12 and TGF- β in PBMC of healthy volunteers. We also tested if CBZ influences resting and PMA-induced "oxidative burst" in blood neutrophils.

MATERIAL AND METHODS

Subjects. Blood samples were collected from 16 healthy volunteers. There were 8 females and 8 males aged 34.2 ± 8.1. All subjects gave written informed consent. None of the subjects had a history of psychiatric disorder (axis I psychiatric disorder). None reported a history of using anticonvulsive medications or had used dibenzoazepins during the previous 3 months. All the subjects had normal values of blood parameters such as haematological measures, blood renal tests (urea and creatinine) and normal liver tests (SGOT, SGPT, γGT levels). All the healthy volunteers had been free from acute infections or allergic reactions for at least 3 weeks prior to the study. No one was addicted to alcohol. The subjects abstained from caffeine, alcohol and nicotine at least 12 hrs before blood sampling.

Isolation of granulocytes and peripheral blood mononuclear cells (PBMC). After an overnight 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). 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 diluted whole blood was carefully layered onto the upper Histopaque-1077. The tubes were then centrifuged at 700 x g for 30 min. Granulocytes were isolated from the Histopaque-1077/1119 inter-phase and washed three times with HBSS (Hanks' Balanced Salt Solution), centrifuged (350 x g for 15 min) and suspended in HBSS. Granulocytes represented 97–98% of the isolated cells as estimated after May-Grünwald-Giemsa staining. Peripheral blood mononuclear cells (PBMC) were separated from plasma-Histopaque-1077 inter-phase, washed two times with Eagle's Minimal Essential Medium (MEM) supplemented with 10% foetal calf serum (FCS) and suspended in the medium at $2x10^6$cells/ml. The viability of cells determined by 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. PBMC suspension was distributed into 24-well plastic plates (Falcon, Bedfors, MA, USA) 2 $x10^6$cells/well and stimulated or not (spontaneous release) with PHA (Sigma) at a final concentration of 5 μg/ml together with LPS (from *E.coli* 0111:B4, Sigma), a final concentration 25 μg/ml. At the time of stimulation with polyclonal activators, CBZ dissolved in DMSO and diluted in MEM to a final concentration of 1 μM was added (10 μl volume) to the PBMC in wells. MEM alone or DMSO at a concentration which corresponded to its dilution in the appropriate sample served as controls. Samples were incubated for 72 hrs in a humidified atmosphere at 37°C, 5% CO_2. 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 by using kits from Endogen, Woburn, MA, USA, and TNF-β or TGF-β by using kits from Quantikine, R&D System, Minneapolis, MN, USA. Each kit employed a specific monoclonal antibody immobilized on a 96-well microtiter plate that bound one of these cytokines in the aliquot and a second enzyme-conjugated specific polyclonal antibody. Following several washings in order to remove unbound substances and antibodies, a substrate solution was added to the wells. Colour development was stopped by sulphuric acid and the intensity of colour 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 limit was 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%.

Measurement of superoxide anion (O_2^-) production by cytochrome c reduction assay (9). HBSS (176 µl), 12.5 µl of cytochrome c solution in HBSS (a final concentration 75 µM), 5 µl of either SOD solution (a final concentration 60 U/ml) or 5 µl of distilled water, and 50 µl of neutrophil suspension (a final density of 2.5 x 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 CBZ (100 µM and 1 µM concentration in 5 µl). 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 OD between samples with and without SOD) were converted to nanomoles of O_2^- based on the extinction coefficient of cytochrome c: $\Delta E_{550} = 21 \times 10^3 M^{-1}cm^{-1}$. The results were expressed as nanomoles of O_2^- per 1 x 10^6 cells per 60 min. In some experiments neutrophils were preincubated with CBZ for 24 hrs and after that activated with 12-myristate-13 acetate (PMA, Sigma) at a final concentration of 1 µg/ml, and after 60 min of incubation, absorbance at 550 nm was read in microplate reader.

Statistics. The values of cytokine and superoxide anion concentrations produced under the influence of CBZ by each cell type were calculated as mean ± S.D. The differences in the cytokine or superoxide anion concentrations between the cells treated with CBZ and the respective controls were analysed in Wilcoxon test using Statistica software. All results were considered significant at $p < 0.05$. The relations between variables were assessed by Spearman's rank order correlation coefficient.

RESULTS AND DISCUSSION

Peripheral blood mononuclear cells (PBMC) from healthy volunteers stimulated *in vitro* with PHA+LPS produced several cytokines, as detected by ELISA test (Table 1). When the cells were

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

Cytokine	Conditions	Control (DMSO)[a]	Carbamazepin 1µM
IL-2	UN	9.2 ± 0.7	8.2 ± 0.4
	ST	416 ± 44	193 ± 167*
IFN-γ	UN	2.3 ± 1.3	2.5 ± 0.1
	ST	1481 ± 108	1454 ± 174
Lymphotoxin	UN	3.4 ± 1.6	4.6 ± 1.1
	ST	5155 ± 912	3827 ± 756*
IL-4	UN	0	0
	ST	17.3 ± 6.1	6.1 ± 4.1
IL-10	UN	8.3 ± 1.2	22.1 ± 5.4*
	ST	1371 ± 283	2405 ± 820*
IL-12	UN	0	0
	ST	73.6 ± 31.1	49.9 ± 44.6
TGF-β1	UN	220 ± 11	199.6 ± 9.1
	ST	1812 ± 993	2530 ± 1270*

[a]The level of cytokines induced in the presence of carbamazepine was compared to DMSO control as drugs were dissolved in DMSO. The results obtained with PBMC treated with carbamazepine were compared to the control

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

treated with 1 μM carbamazepine (CBZ) for 72 hrs, significant changes in cytokine production were detected. CBZ caused a decrease in IL-2 and lymphotoxin as well as IL-4 production, while it enhanced the production of IL-10 and TGF-β. The levels of IFN-γ and IL-12 were not changed. Spearman's rank order correlation analysis revealed a positive correlation between cytokines which are known to be produced mainly by Th1 subpopulation, i.e. between IL-2 and lymphotoxin, but negative between IFN-γ and IL-10 or IFN-γ and TGF-β. IL-4 also correlated negatively with TGF-β (Table 2).

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

Cytokines	r	p
IFN-γ/IL-10	-0.63	< 0.007
IFN-γ/TGF-β	-0.72	< 0.001
IL-2/lymphotoxin	0.53	< 0.032
IL-2/ TGF-β	-0.52	< 0.037
IL-4/ TGF-β	-0.73	< 0.001

An unexpected decrease in the production of "stimulatory" cytokines such as IL-2, lymphotoxin and IL-4 after a short treatment of PBMC with CBZ *in vitro* differs significantly from the results of other authors. In their study they detected a significant increase in proinflammatory cytokine production in PBMC isolated from epileptic patients after one-year medication with CBZ, and stimulated *ex vivo* with mitogen. Concentration of such cytokines as IL-2, IL-1 and IL-6 was significantly higher than at the beginning of the therapy (15) and regarded by authors as a consequence of CBZ therapy. When the results of *in vitro* and *ex vivo* experiments were compared, the reason for such discrepancies was still unclear. However, one can speculate that CBZ may exert an inhibitory effect after short treatment, while long-term therapy produces a stimulatory effect, which can be considered an adaptive effect of regulatory mechanisms.

Table 3. The influence of carbamazepine on unstimulated and PMA-stimulated superoxide anion (O_2^-) production by neutrophils isolated from blood of healthy subjects

Dose of carbamazepine μM	O_2^- nmol/10^6 of neutrophils/h	
	unstimulated	PMA-stimulated
100	6.0 ± 0.9	25.1 ± 1.97*
1	6.7 ± 0.2	21.7 ± 0.6*
(DMSO control)	5.02 ± 0.04	18.9 ± 1.2

* Statistically significant in comparison to control

Other data from literature concerning the influence of CBZ on immune cytokine function indicated its immunosuppressive activity. Andrade-Mena et al. (2) described that oral CBZ treatment significantly diminished cellular and humoral immune response in mice. Also Silverman et al. (14) detected that CBZ therapy suppressed total lymphocyte counts in man. Such immunosuppressive effects of CBZ can be, at least in part, caused by its negative influence on IL-2 and IL-4 production.

These cytokines are necessary growth factors for lymphocytes T and B, respectively. Moreover, CBZ was described to inhibit IL-6 production in human prostate cancer cells, which are sensitive to its anti-proliferative effect (1). As in our study CBZ was a stimulator of IL-10 and TGF-β production, we suppose that the changes in cellular and humoral immune response can be also considered the effect of an enhanced production of "inhibitory" cytokines. Another possible explanation for such discrepancies in the literature is that epileptic patients have temporal over-expression of some cytokines in the brain and also in blood (8).

In our study CBZ at high (100 μM) and low (1μM) concentrations did not influence resting (not induced) superoxide (O_2^-) production in blood neutrophils of healthy volunteers, however, after PMA stimulation, CBZ enhanced "oxidative burst" in neutrophils. These results are in agreement with the results of other authors (4, 5) who described that CBZ medication enhanced phagocytosis and oxidative metabolism, but impaired chemotaxis and the killing of microorganisms by neutrophils of epileptic patients.

There are no data in the literature explaining how CBZ influence the activity of b558 cytochrome, which is considered an essential component of the superoxide-generating system in phagocytes (6, 7), but it has been detected that myeloperoxidase system and several P450 cytochrome isoforms, among others CYP2E1 and CYP3A4, present also in neutrophils (12), are involved in the formation of CBZ metabolites. CYP2E1 is well known to generate reactive oxygen species (ROS) in several cells, both *in vitro* and *in vivo* (13). It is possible that this isoform of cytochrome P450 is also involved in superoxide anion formation in neutrophils treated with CBZ. It has been described that in the blood of epileptic patients treated with CBZ, glutathione adducts of haemoglobin were detected. They correlated with low activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) indicating the presence of oxidative stress in the blood of patients (10). It seems likely that CBZ may enhance oxidative metabolism of neutrophils not only *in vitro* but also *in vivo*.

In conclusion, short (72 hrs) treatment of peripheral blood mononuclear cells (PBMC) of healthy volunteers with carbamazepine (CBZ) caused several changes in cytokine production. IL-2, IL-4 and lymphotoxin production was decreased, while the production of anti-inflammatory cytokines such as IL-10 and TGF-b was enhanced. Our results can, at least partially, explain the mechanism involved in immunosuppressive effect of BCZ on humoral and cellular immunity observed by other authors (6). CBZ can stimulate PMA-induced oxidative metabolism of neutrophils and participate in the development of oxidative stress in the organisms of CBZ-medicated epileptic patients.

REFERENCES

1. Abdul M., Hoosein N.: Inhibition by anticonvulsants of prostate-specific antigen and interleukin-6 secretion by human prostate cancer cells. Anticancer Res., 21, 2045, 2001.
2. Andrade-Mena C. E. et al.: Effect of carbamazepine on murine humoral and cellular immune response. Epilepsia, 35, 205, 1994.
3. Bonhomme-Faivre L. et al.: Chronic administration of verapamil, ketoconazole and carbamazepine: impact on immunological parameters. Int. J. Pharmac., 238, 133, 2002.
4. Caldiroli E. et al.: Effect of carbamazepine on human PMN function: possible role of peripheral benzodiazepine receptors. Ann. N.Y. Acad. Sci., 15, 130, 1997.
5. Cosentino M. et al.: Assessment of lymphocyte subsets and neutrophil leukocyte function in chronic psychiatric patients on long-term drug therapy. Prog. Neuro-Psychopharmacol. Biol. Psychiatry, 20, 1117, 1996.
6. Fuji H. et al.: Modulation of the heme environment of neutrophil cytochrome b558 t a "cytochrome P 450-like" structure by pyridine. J. Biol. Chem., 270, 3193, 1995.

7. Furst S. M., Uetrecht J. P.: The effect of carbamazepine and its reactive metabolite, 9-acridine carboxaldehyde, on immune cell function *in vitro*. Int. J. Immunopharmacol., 17, 445, 1995.
8. Lorentz R.: Clinical study by R. Lorenz. Neuroendocrinol. Lett., 2, 330, 2001.
9. Mayo L. A., Curnutte J. T.: Kinetic microplate assay for superoxide production by neutrophils and other phagocytic cells. Meth. Enzymol., 86, 571, 1990.
10. Niketic V. et al.: Activities of antioxidant enzymes and formation of the glutathione adduct of hemoglobin (HbASSG) in epileptic patients with long-term antiepileptic therapy. Pharmacology, 50, 811, 1995.
11. O'Connor C. R. et al.: Evaluation of the mechanisms of antiepileptic drug-related chronic leukopenia. Epilepsia, 35, 149, 1994.
12. Pearce R. E. et al.: Pathways of carbamazepine bioactivation *in vitro*. I. Characterization of human cytochrome P450 responsible for the formation of 2- and 3-hydroxylated metabolites. Drug Metab. Dispos., 30, 1170, 2002.
13. Raucy J. L. et al.: Use of lymphocytes for assessing ethanol-mediated alterations in the expression of hepatic cytochrome P4502E1. Alcohol Clin. Exp. Res., 19, 1369, 1995.
14. Silverman D. A., Chapron D. J.: Lymphopenic effect of carbamazepine in a patient with chronic lymphocytic leukaemia. Ann. Pharmacother., 29, 865, 1995.
15. Verrotti A. et al.: Effect of anticonvulsant drugs on interleukin-1, 2 and 6 and monocyte chemoattractant protein-1. Clin. Exp. Med., 1, 133, 2001.

SUMMARY

The aim of this paper is to evaluate whether carbamazepine (CBZ) may influence *in vitro* cytokine and superoxide anion (O_2^-) production by blood leukocytes of healthy volunteers. Peripheral blood mononuclear cells (PBMC) were induced *in vitro* with phytohemagglutinin (PHA) + lipopolysaccharide (LPS) in the absence or presence of CBZ. Cytokine concentration in culture media was measured by ELISA method. The influence of CBZ on resting (not induced) and phorbol ester (PMA)-induced O_2^- production by neutrophils isolated from the blood of healthy volunteers was also examined. CBZ at 1 μM concentration caused a significant decrease in IL-2, IL-4 and lymphotoxin, but enhanced IL-10 and TGF-β production. CBZ also stimulated PMA-induced O_2^- production. In conclusion, the *in vitro* study revealed that CBZ may exert immunoregulatory activity inhibiting "stimulatory" cytokines, enhancing the production of "inhibitory" ones and enhancing the "oxidative burst" of neutrophils.

Wpływ karbamazepiny na wytwarzanie cytokin i anionorodnika ponadtlenkowego w leukocytach krwi zdrowych ochotników

Celem naszych badań była ocena wpływu karbamazepiny (CBZ) na wytwarzanie cytokin i anionorodnika ponadtlenkowego w leukocytach krwi zdrowych ochotników. Jednojądrzaste komórki krwi obwodowej indukowano *in vitro* fitohemaglutyniną (PHA) z lipopolisacharydem bakteryjnym (LPS) do wytwarzania cytokin w obecności CBZ lub jej braku (kontrola). Poziom cytokin obecnych w płynie hodowlanym określano metodą ELISA. Badano także wpływ karbamazepiny na spoczynkowe (nieindukowane) i indukowane estrem forbolu (PMA) wytwarzanie anionorodnika ponadtlenkowego w neutrofilach izolowanych z krwi zdrowych ochotników. Badania wykazały, że 1 mM CMZ obniża wytwarzanie IL-10, IL-4 i limfotoksyny, lecz zwiększa wytwarzanie IL-10 i TGF-b. Zwiększa także indukowane PMA wytwarzanie anionorodnika ponadtlenkowego. Wykazano, że *in vitro* CBZ działa immunomodulacyjnie, stymulując wytwarzanie cytokin „hamujących", a obniżając wytwarzanie cytokin „stymulujących". Zwiększa także „wybuch tlenowy" neutrofilów.