Marco Cosentino et al /J. Pharm. Sci. & Res. Vol.4(2), 2012, 1697-1702

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

The Neurotoxins Rotenone, Paraquat and Manganese Exert Different Effects on Human Isolated Lymphocytes

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Abstract

Aims: To examine the effects of the neurotoxins rotenone, paraquat and manganese on human circulating lymphocytes.

Methods: Lymphocytes were isolated by density-gradient centrifugation from peripheral venous blood. Cell viability, proliferation, interferon (IFN)-γ and interleukin (IL)-4 production, proteasome 20S and caspase 3 and 9 activity were measured in cells at rest and after stimulation with anti-CD3/anti-CD28 antibodies.

Results: Rotenone and manganese concentration-dependently reduced anti-CD3/anti-CD28-induced cell proliferation and IFN-γ production. Manganese also inhibited IL-4 production while paraquat had no major effect on proliferation or on IFN-γ or IL-4 production. Proteasome 20S activity was reduced by paraquat and manganese but not by rotenone, while caspase 9 activity was extensively inhibited by rotenone and manganese and only slightly affected by paraquat, and caspase 3 activity was not affected by any of the neurotoxins tested.

Conclusions: Rotenone is widely used in animal models of neurodegeneration and its immune effects should be therefore further assessed *in vivo*. In addition, circulating lymphocytes warrant further evaluation as early markers of manganese exposure.

Key Words: human lymphocytes, rotenone, paraquat, manganese.

INTRODUCTION

The etiology of the vast majority of cases of neurodegenerative diseases involves not only genetic but also environmental factors [1-4]. Many environmental agents may indeed cause or contribute to neurodegeneration and there is strong need for extensive characterization of their toxic potential [5-7]. The best known example of toxin leading to neurodegeneration is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); following systemic administration, MPTP is converted to the active metabolite MPP+, which selectively targets central dopaminergic neurons, causing substantial cell death in the nigrostriatal system. The resemblance of MPTP and MPP+ to the widely used herbicide paraquat has prompted the hypothesis –supported by experimental and epidemiological data - that pesticide exposure may contribute to neurodegeneration [8-10]. Among pesticides associated with neurodegeneration, rotenone has attracted extensive attention [10]. Systemic administration to rats has been shown to induce nigrostriatal degeneration [11]. This first observation has subsequently led to the extensive use of this toxin (as well as of paraquat) to reproduce neurodegenerative lesions in animals [9,12-14]. At variance with pesticides, manganese is a well known environmental neurotoxin that damages the striatum and pallidum, without affecting the substantia nigra pars compacta or other areas (reviewed in 15). Manganism is, therefore, considered a separate entity, with a distinct pattern of neuropathologic features [15-16].

The central nervous system is considered the main target of neurotoxins, however looking for biomarkers of neurodegeneration research increasingly focussed on peripheral blood cells. In patients with neurodegenerative disease, these cells express many of the biomolecular changes affecting neurons, such as: oxidative stress [17-18], reduced proteasomal activity [19], pro-apoptotic and phosphorylative changes [20], as well as alterations of dopamine-dependent signal transduction, involving cAMP and Ca^{++} [21]. Exposure of human lymphocytes to toxic levels of neurotoxins has been regarded as a convenient strategy to obtain interesting clues towards the understanding of cell death in neurodegenerative disease [22-26]. So far however few studies aimed at the systematic characterization of the immune effects of neurotoxins, which would also contribute to the assessment of circulating cells as biomarkers of environmental exposure to neurotoxitc agents. The present study was therefore undertaken to examine the effects of non-toxic concentrations of the neurotoxins rotenone, paraquat and manganese on human lymphocytes.

MATERIALS AND METHODS

Substances - Manganese chloride tetrahydrate, paraquat dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloride) and rotenone were from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of peripheral blood mononuclear cells (PBMCs) - Whole blood was allowed to sediment on dextran at 37°C for 30 min. Supernatant was recovered and PBMCs were separated by density-gradient centrifugation using Ficoll-Paque Plus (Pharmacia Biotech, Uppssala, Sweden). Cells were then washed two times in NaCl 0.15 M and finally resuspended in appropriate media for subsequent experiments. Aliquots were centrifuged and dry pellets containing 2 x 10^6 PBMCs were stored at -80°C for evaluation of proteasome and caspase activites (see below). Typical PBMCs preparations contained about 80% lymphocytes and 16% monocytes, as assessed by flow cytometry. Cell viability, assessed by the trypan blue exclusion test, was always >99%.

Cytotoxicity assay - Cytotoxicity of test substances was assessed by means of the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) reduction method [27].

PBMCs proliferation assay - PBMCs were re-suspended at the final concentration of 1x10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin/streptomycin (all from Sigma-Aldrich, Milan, Italy), at 37°C in a moist atmosphere of 5% CO2. Cells were cultured alone or in the presence 0.5 μg/ml mouse anti-human CD3/anti-human CD28 monoclonal antibodies (Becton Dickinson Biosciences, San Diego, CA, USA) and proliferation was measured after 2 days of culture by using a colorimetric immunoassay for the quantification of cell proliferation, based on the ELISA measurement of BrdU incorporated during DNA synthesis (Amersham, Buckinghamshire, UK). The optical density of the samples was determined by means of a spectrophotometer (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) with wavelength set at 450 nm, and finally expressed as the difference between BrdU-positive and negative samples, in arbitrary units (AU).

Measurement of interferon (IFN)-γ and interleukin (IL)-4 - IFN-γ and IL-4 concentrations in culture supernatants were determined by using commercial ELISA assays (Amersham).

Measurement of proteasome 20S and caspase 3 and 9 activity - The day of the assay, pellets were re-suspended in ice-cold PBS and homogenized by ultrasounds (Vibra Cell, Sonics & Materials, Inc., USA). Homogenates were centrifuged at 15.000 g for 10 min at room temperature, and resulting supernatants were used for the biochemical determinations. The chymotrypsin-like proteolytic activity of purified 20S proteasome was evaluated with the BIOMOL AK-740 QuantiZyme Assay System (Plymouth, PA, USA), which detects the release of free 7-amino-4-methylcoumarin (AMC) fluorophore, upon Suc-LLVY-AMC fluorogenic peptide substrate cleavage. We also used fluorometric assays for the determination of the activity of caspase-3 (Molecular Probes, Inc., USA) and caspase-9 (Oncogene, San Diego, CA, USA). For caspase-3, which has a substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD), the assay was based on the proteolytic cleavage of AMC-derived substrate Z-DEVD AMC, which yields a fluorescent product; for caspase-9 activity, we used a method based on the specificity of the enzyme for cleavage after aspartate residues in a particular peptide sequence (LEHD). The LEHD substrate is labelled with fluorescent 7-amino-4-trifluoromethyl coumarin (AFC) and reaction is monitored by a blue to green shift in fluorescence upon cleavage of the AFC fluorophore. Positive controls were used for all determinations, as expressly foreseen by the assay procedures; standard samples treated with a proteasome inhibitor (lactacystin) or pro-apoptotic agents (camptothecin or actinomycin D) were always added to batches when proteasome 20S or caspases 3 and 9 activities were measured. All readings were performed using a fluorometric microplate reader (Spectramax Gemini XS, Molecular Devices, USA).

Statystical analysis - Data are presented as means±SD. Statistical significance of the differences between groups was assessed by two-tailed Student's *t* test for paired or unpaired data, as appropriate. Calculations were performed using a commercial software (GraphPad Prism version 4.00 forWindows; GraphPad Software, San Diego, CA).

RESULTS

Cytotoxicity - In preliminary experiments, rotenone significantly reduced the viability of resting PBMCs when added at the concentration of 100 μM (62.9±6.6% vs 100.0±6.2%, n = 5; $P<0.05$ vs control), while paraquat and manganese both affected cell viability only when added at the concentration of 1000 μM (86.7±12.2% and 72.0±11.1%, respectively, n = 5; $P<0.05$ vs control). Lower concentrations (rotenone: 0.1-10 μM; paraquat: 0.1-10 μM; manganese: 10-100 μM) did not significantly affect cell viability (data not shown) and were therefore employed in subsequent experiments.

Cell proliferation - Treatment of PBMCs with anti-CD3/anti-CD28 monoclonal antibodies induced a strong proliferative response (resting cells: 0.006±0.004 AU, n = 5; stimulated cells: 1.221±0.184 AU, n = 5; $P<0.01$ vs resting).

In the presence of either rotenone (0.1-10 μM) or manganese (10-100 μM) anti-CD3/anti-CD28-induced proliferation of PBMCs was concentration-dependently reduced, down to less than 25% of control values, while in the presence of paraquat (0.1-10 μM) only a slight reduction of proliferation values was observed, without a clear concentration-response relationship, down to 71-85% of control values (Figure 1).

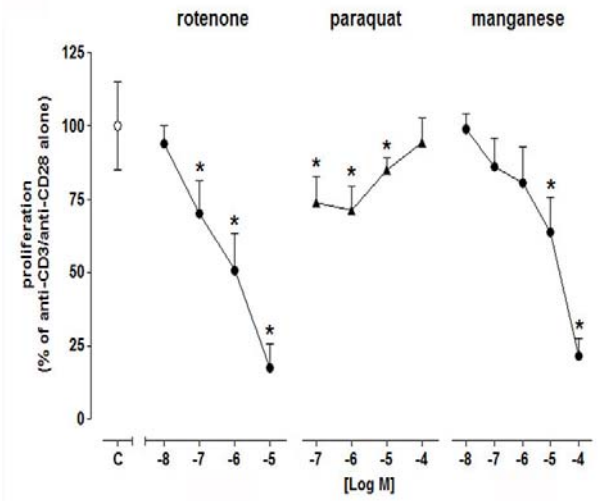

Figure 1. Effect of rotenone, paraquat and manganese on anti-CD3/anti-CD28-induced proliferation of human PBMCs. Each point is the mean±SEM values of 5 separate experiments. * = $P<0.05$ and ** = $P<0.01$ vs control (C).

Production of IFN-γ and IL-4 - In resting PBMCs, spontaneous production of IFN-γ and IL-4 were respectively 4.33±2.24 pg/ml (n = 4) and 0.15±0.08 pg/ml (n = 4). Stimulation with anti-CD3/anti-CD28 monoclonal antibodies significantly increased the levels of both IFN-γ (up to 2575.14±56.61 pg/ml, n = 4; $P<0.01$ vs resting cells) and IL-4 (up to 3.88±0.97 pg/ml, n = 4; $P<0.05$ vs resting cells). The ratio IFN-γ/IL-4 after stimulation was therefore 883,8±524,9 (n = 4).

Incubation with rotenone (1-10 μM) reduced IFN-γ in a concentration-dependent fashion, while it had no effect on IL-4 (Figure 2). As a consequence, the ratio IFN-γ/IL-4 was significantly reduced down to 28.4±5.4% of control values (n = 4) with rotenone 1 μM and to 16.4±5.5% of control values (n = 4) with rotenone 10 μM ($P<0.05$ vs control values in both cases).

Manganese (10-100 μM) reduced the production of both IFN-γ and IL-4 (Figure 2), without significantly affecting the IFN-γ/IL-4 ratio (data not shown).

Paraquat had no significant effect on either IFN-γ and IL-4 production (Figure 2) or on the IFN-γ/IL-4 ratio (data not shown).

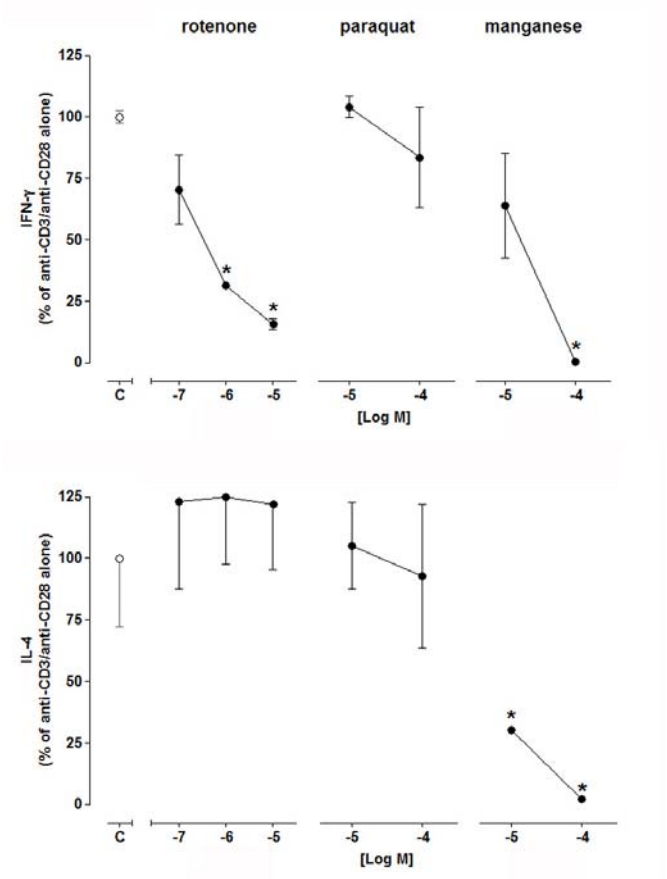

Figure 2. Effect of rotenone, paraquat and manganese on anti-CD3/anti-CD28-induced production of IFN-γ and IL-4 in human PBMCs. Each point is the mean±SEM values of 5 separate experiments. * = $P<0.05$ and ** = $P<0.01$ vs control (C)

Activity of proteasome 20S and of caspase 3 and 9 - The activity of proteasome 20S and of caspase 3 and 9 was detected in all PBMCs preparations. Stimulation with anti-CD3/anti-CD28 monoclonal antibodies significantly increased activity of caspases 3 and 9, but had no effect on proteasome 20S (Table 1).

Proteasome 20S activity was not significantly affected by rotenone either in resting or in stimulated PBMCs, and was reduced only at the highest concentration (100 μM) of paraquat (in resting cells only) and manganese (in stimulated cells only) (Table 1).

Caspase 3 activity was not significantly modified by rotenone, paraquat or manganese in either resting or stimulated cells (Table 1). On the contrary, caspase 9 activity was extensively inhibited in stimulated PBMCs by rotenone and manganese, the latter affecting also resting cells. Paraquat did not affect caspase 9 activity in stimulated cells and slightly reduced its activity in resting cells, however only at the concentration of 10 μM (Table 1).

Table 1. Effect of rotenone, paraquat and manganese on the activity of proteasome 20S, caspase 3 and caspase 9 in human PBMCs. Data are expressed as pmol of Z-DEVD-R110/10^6 cells (mean±SEM values of 4 separate assays). # = $P<0.01$ vs resting cells; * = $P<0.05$ and ** = $P<0.01$ vs respective control.

Cells	*Proteasome 20S*		*Caspase 3*		*Caspase 9*	
	resting	*anti-CD3/anti-CD28*	*resting*	*anti-CD3/anti-CD28*	*resting*	*anti-CD3/anti-CD28*
control	2.90±0.82	2.90±0.43	632.0±128.7	1127.9±157.7#	1085.0±228.0	5348.8±1308.6#
rotenone						
10^{-7} M	2.79±0.84	3.40±0.60	605.0±65.4	1004.3±171.3	857.9±13.8	5498.7±635.4
10^{-6} M	1.75±0.50	2.31±0.04	745.4±3.4	1092.8±82.8	1126.2±46.1	3120.8±80.4*
10^{-5} M	1.61±1.12	2.52±0.80	768.8±69.9	1003.5±67.9	1019.3±56.6	2769.1±380.8**
paraquat						
10^{-5} M	2.90±0.81	1.55±0.93	490.8±3.8	1229.2±4.8	671.1±42.9**	6294,5±1123.0
10^{-4} M	2.90±0.81	1.06±0.55*	653.4±46.4	1197.5±20.0	833.9±15.5	4639,1±1369.6
manganese						
10^{-5} M	2.30±0.15	3.43±1.28	550.0±3.9	1226.3±1.4	730.9±34.0*	4321.2±631.1
10^{-4} M	1.32±0.49*	1.35±0.28	467.9±2.8	649.3±75.6	678.7±81.3**	925.4±99.1**

DISCUSSION

The present study aimed at the investigation of the effects non-toxic concentrations of the neurotoxins rotenone, paraquat and manganese on human lymphocytes. Results show that:

a) rotenone concentration-dependently exerts a profound inhibition of anti-CD3/anti-CD28-stimulated proliferation and IFN-γ production, without however affecting IL-4 production; in addition, rotenone concentration-dependently reduces caspase 9 activity, without affecting proteasome 20S or caspase 3 activity;

b) paraquat has negligible effects on all the examined parameters, with the only exception of proteasome 20S activity, which is significantly reduced in anti-CD3/anti-CD28-stimulated cells;

c) manganese concentration-dependently reduces anti-CD3/anti-CD28-stimulated proliferation as well as IFN-γ and IL-4 production (to about the same extent for both cytokines), and inhibits proteasome 20S and caspase 9 activities.

Results from our cytotoxicity assays showed that significant cell death in human PBMCs was induced by rotenone at 100 μM or higher, and by paraquat and manganese at 10 μM or higher. These data are in agreement with published studies, showing that 100-250 μM but not 1-10 μM rotenone induced moderate mitochondrial depolarization [26]. Similarly, paraquat has been reported to induce apoptosis in human lymphocytes only at 100-1000 μM [25]. Effect of manganese remains controversial as 250 μM manganese induced only 2-6% apoptotic cells [23], while lower concentrations (20-25 μM) were cytotoxic to human lymphocytes and induced clastogenicity as well as DNA damage [28]. In our subsequent experiments we chose to use non-cytotoxic concentration ranges (1-10 μM for rotenone, and up to 100 μM for paraquat and manganese) of the various neurotoxins. The effects reported can, therefore, be considered as mainly pharmacologic. This view is further supported by the lack of effect of all three agents on caspase 3 activity and the inhibitory potential of paraquat and manganese on caspase 9 activity.

Very few information exists regarding the immunomodulating effects of rotenone, paraquat and manganese. In particular, rotenone acts as an effective mitochondrial complex I inhibitor also in human lymphocytes [26], however only at concentrations at least ten times higher than those required for inhibition of lymphocyte proliferation and cytokine production in our experiments., Rotenone has been also reported to be genotoxic in human lymphocytes, at 2.5-5 μM [29], a concentration range which fits well with that used in our study. It cannot be excluded, therefore, that the inhibitory effect we observed may be due to DNA damage. Nonetheless, the effect on cytokine production was specific for IFN-γ, rotenone being ineffective on IL-4, a finding hardly explained just on the basis of generalized DNA damage. We are not aware of any studies investigating the immune profile of people exposed to rotenone, therefore the clinical relevance of our results can be only a matter of speculation. Indeed, inhibition of lymphocyte proliferation and of IFN-γ production may suggest a potential anti-inflammatory profile. Interestingly, anti-inflammatory potential of some plant rotenoids has been reported [30]. The shift of IFN-γ/IL-4 ratio towards IL-4 also deserves some considerations. The IFN-γ/IL-4 ratio is a reliable index of Th1/Th2 balance [31,32]. Th1-polarized immune response play a role in the defense against intracellular pathogens, but are also involved in autoimmune diseases and in chronic inflammation.

On the other side, Th2-polarized responses usually occur during intestinal nematodes infections, however they are also involved in atopic disorders and in autoimmune diseases (reviewed in 33-35). On this basis we would predict that exposure to rotenone could be associated with atopy and/or with exacerbations of Th2-dependent autoimmune disease such as systemic lupus erythematosus, a hypothesis which should be tested in appropriate studies.

As for manganese, our results might be in apparent agreement with previous evidence in human tonsillar B cells, in which exposure to manganese reduced stimulated proliferation [36]. In that study, however, inhibition was paralleled by increased apoptosis and both effects were obtained in the 50-100 μM concentration range. In our experiments, significant inhibition of lymphocyte proliferation and cytokine production was already evident at 10 μM and no evidence of apoptotic processes were obtained up to 100 μM. At high concentrations (usually equal or more than 100 μM) manganese promotes neuroinflammation both *in vitro* [37] and in animal models [38]. The cellular and molecular mechanisms involved in the inhibitory effects of manganese on human lymphocytes need therefore clarification in further experiments. In regard to the possible clinical relevance of the present results, it is of interest that a recent study in welders chronically exposed to manganese revealed an inverse correlation between blood manganese concentrations and the total number of lymphocytes as well as of several lymphocyte subsets. In particular, the number of CD3+ and CD8+ (T lymphocytes) and of CD19+ (B lymphocytes) cells were significantly lower in subjects with high blood manganese concentrations [39]. Another study reporting behavioral toxicology in welders with similarly high blood manganese concentrations failed however to identify significant changes in the various lymphocyte subsets [40]. No functional test or cytokine measurements were however performed in either studies.

Paraquat showed little effects on the parameters evaluated in this study; only proteasome 20S activity was profoundly reduced in anti-CD3/anti-CD28-stimulated lymphocytes following treatment with 10 μM paraquat. This finding is in agreement with the reported ability of paraquat to inhibit proteasome 20S in cultured neuronal SH-SY5Y cells [41], and animal models of parkinsonian neurodegeneration [42,43]. In SH-SY5Y cells, however, paraquat also increased the activities of caspase 3 and 9 [41], while in the present study, in lymphocytes caspase 3 activity was not affected and caspase 9 activity was not affected or just slightly reduced. In a recent study the immunotoxicity of paraquat has been systematically assessed after subacute (21 days) exposure in mice [44]. In that study paraquat was immunosuppressive in the 0.1-1 mg/kg dose range, while it had no adverse effects on the immune system at 0.01 mg/kg. Paraquat concentrations in mice were not assessed, however the immunosuppression observed in mice may be in agreement with the slight inhibition of lymphocyte proliferation observed in our experiments. On the other side, the differences observed in the effects of paraquat between lymphocytes and SH-SY5Y cells call for caution when using non-neuronal cells to assess the cellular effects of some neurotoxins.

CONCLUSIONS

In human cultured lymphocytes rotenone exhibited an inhibitory (possibly anti-inflammatory) profile inducing a shift towards IL-4 (Th2)-skewed response. Neurodegenerative disease are increasingly regarded as neuroinflammatory disease in which Th1 immunity is likely detrimental, while Th2 responses might be even protective. Results may thus have implications for rotenone-based animal models of neurodegeneration, in which attention should be devoted also to immunity and its possible relationship with neurodegeneration. Manganese as well exerted an inhibitory effect on human lymphocytes and, also in view of circumstantial evidence in exposed workers [39,40], the relevance of lymphocytes as early markers of exposure should be further assessed. Finally, paraquat had only slight effects on lymphocytes. It is uncertain whether immunity might play a role in paraquat-induced neurodegeneration. In any case caution should be exerted when using non-neuronal cells as a model to assess the effects of some neurotoxins on neurons.

ACKNOWLEDGEMENTS

The study was supported by a grant to EM from IRCCS Neurological Institute “C. Mondino”, Pavia - RF 2004 ex art. 56, Legge 289/2002, Project title: "In vitro evaluation of environmental toxins possibly involved in the pathogenesis of Parkinson's disease". The authors express their gratefulness to Prof. Giovanni Chelazzi, Dr. Davide Rossi and Dr. Simona Cattaneo (Immunohematology and Transfusional Service, Ospedale di Circolo, Varese, Italy) who collaborated in providing human blood. The helpful assistance of Dr. Elena Carcano and of Dr. Anna Loraschi in performing some of the assays is gratefully acknowledged.

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