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Effects of Mancozeb and Metribuzin on in vitro proliferative responses and oxidative stress of human and rat spleen lymphocytes stimulated by mitogens

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ABSTRACT

Pesticides have been shown to possess marked immunotropic activity. The aim of this work was to study the in vitro effects of different concentrations (1–100 μM) of Mancozeb (fungicide) and Metribuzin (herbicide), on the proliferative responses of human and rat spleen lymphocytes stimulated by concanavalin A (ConA, mitogen), the Th1- (IL-2, INFγ) and Th2- (IL-4) cytokine secretion and on the intracellular oxidative status. The results showed that Mancozeb significantly reduced ConA lymphocyte proliferation in a dose-dependent manner in both humans and rats. It also decreased IL-2, INFγ and IL-4 secretion with a a shift away to Th1 phenotype. Metribuzin at low concentrations (1–10 μM) resulted in activation of ConA stimulated lymphocyte proliferation and cytokine production in both human and rat spleen cells. However, at high concentrations (25–100 μM), Metribuzin induced a dose-dependent inhibition of lymphocyte proliferation and cytokines. Changes in intracellular levels of reduced Glutathione, hydroperoxides and carbonyl proteins and in the activities of catalase and SOD were observed after Mancozeb and Metribuzin exposure reflecting oxidative stress and DNA damage specially at high concentrations.

In conclusion, Mancozeb and Metribuzin had significant immunomodulatory properties with oxidative stress induction at high concentrations.

1. Introduction

Pesticides are used in intensive agriculture worldwide. An important target organ of pesticide exposure is the immune system [1]. Lymphocyte proliferation assays and cytokine expression have been widely used for immunotoxicity assessment [2]. Ability of pesticides to stimulate or suppress lymphocyte proliferation and cytokine production, and to induce genotoxic activity and chromosomal aberrations in cultured lymphocytes were proposed as possible mechanisms explaining their adverse effects on immune system [3–6]. Cytokines, the important regulators of immune function play a crucial role in activation, proliferation and differentiation of lymphocytes. Cytokine production was altered in response to pesticide exposure [7]. It has been shown that serum IL-2, IL-4 and TNF-α levels are significantly raised with a decrease in IFN-γ level after pesticide exposure [7,8]. Other studies indicated that exposure to pesticide inhibited IL-2 production by rat and mice splenocytes [9,10]. IL-2, IFN-γ and IL-10 secretion were reduced while IL-4 secretion was not altered in cultured lymphocytes in the presence of pesticides [11].

Several studies have also provided evidence for the involvement of reactive oxygen species and oxidative stress in pesticide immunotoxicity [12,13]. Some pesticides induced peroxisomal β-oxidation increasing the intracellular production of H_2O_2 and other reactive oxygen species, leading to alterations in cell signaling pathways involved in cell growth and cell death [13–15].

Mancozeb and Metribuzin are the most widely used pesticides in Algeria. Mancozeb is a dithiocarbamate fungicide used against fungal diseases of field crops and fruits. Mancozeb was found to have toxic effects in experimental animals including histopathological changes in the liver and adrenal gland, renal necrosis, inflammatory cell infiltration, chromosomal aberrations and skin tumors [16–18]. Immunomodulatory effects of Mancozeb were observed with an increase in T-lymphocyte proliferation and PHA-induced IL-2 production in Mancozeb-exposed agricultural workers [19]. Mancozeb inhibited TNF alpha production by interfering with LPS-induced ROS generation [20], and induced an oxidative stress in rats [17,18]. However, no study has reported immunomodulatory effects of Mancozeb on ConA-induced Th1 and Th2 cytokine expression. Given the key role of T helper (Th)1-type and Th2-type cytokines in mounting appropriate immune responses to pathogens and also in diseases, it seems important to understand more fully the influence of pesticides in modulating the production of both Th1-type and Th2-type cytokines.

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Metribuzin is a selective triazinone herbicide used mostly on vegetable crops. There is evidence from animal studies that Metribuzin may cause adverse health effects, such as liver enzyme activities, histopathological changes, kidney alterations, endocrine modifications and fetus toxicity [21,22]. No animal studies have addressed the immunotoxic effects of Metribuzin although immunomodulator effects were seen with other herbicides such as atrazine [23].

Therefore, in the current study we investigated the in vitro effects of Mancozeb and Metribuzin on mitogen-stimulated proliferation, Th1- and Th2-type cytokine production and oxidant/antioxidant status of human blood and rat spleen lymphocytes. Both proliferation and cytokine release are considered to be very relevant to investigate the toxicity towards the immune system [2]. The in vitro micronucleus test was used to test if Mancozeb and Metribuzin induce DNA damage.

2. Materials and methods

2.1. Materials, blood and spleen samples

Human peripheral blood cells and rat splenocytes were used as the test system, and the commercial fungicide Mancozeb and herbicide Metribuzin (obtained from INRAA, Algeria) as the test substances (Nos. CAS mancozeb 8018-01-7 and CAS metribuzine 21087-64-9).

Peripheral blood was obtained from eight healthy non-smoking male (aged 25 years) donors, under no medication or food supplements intake and free of any known exposure to genotoxic agents. Fasting venous blood samples were collected in heparinized tubes. These samples were used for immediate lymphocyte isolation. The purpose of the study was explained to the volunteer subjects and their consent was obtained. The protocol was approved by the ethical committee of the Tlemcen-University Hospital.

Adult Wistar rats, 10–12 weeks of age and weighing 250–320 g, were obtained from IFA-CREDO (Lyon, France). They were housed in wood-chip bedded plastic cages with free access to food and water with a 12/12 light–darkness cycles and at ambient temperature of 20–25 °C. Eight male rats were anesthetized by intraperitoneal administration of sodium pentobarbital (0.2 ml/100 g body weight). The abdominal cavity was opened, whole blood was drawn from the abdominal aorta, and spleens were removed. The study was conducted in accordance with the national guidelines for the care and use of laboratory animals.

2.2. Lymphocyte proliferation assay

Peripheral blood lymphocytes were isolated from heparinized venous blood using differential centrifugation (400*g* for 40 min) on a density gradient of Ficoll-Paque (Pharmacia Biotech, Sweden, UK). The peripheral blood lymphocytes (PBL) at the interface of plasma and Ficoll-Paque were collected and washed twice with RPMI 1640 culture medium (Gibco, USA). After washing and counting, the cells were resuspended in a tissue culture medium at 4×10^6 cells/ml concentration. For proliferation assay, 4×10^5 cells were cultured in triplicate in 200 μl of medium RPMI 1640 supplemented with 25 mM Hepes buffer, 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), penicillin (100 UI/ml) and streptomycin (100 μg/ml) with or without mitogen. Concanavalin A (ConA, Sigma, St. Louis, MO, USA), a T-cell specific mitogen was used at 5 μg/ml final concentration. Cultures were grown in 96 flat-bottomed microtiter plates (Nunc, Paris, France) and maintained at 37 °C in a 5% CO_2 humidified atmosphere for 48 h. In order to determine the effects of pesticides, lymphocytes were incubated with different concentrations of mancozeb and Metribuzin. The pesticides were initially dissolved in DMSO (final solvent concentration <1%) and prepared immediately before use. The concentrations of each pesticide were adjusted in complete RPMI 1640 culture medium to yield the appropriate final concentration (1–100 μM). The doses were chosen, referring to the the ADI (admissible daily intake) and below NOAELs (no-observed adverse effect levels) to mimic human exposure to pesticides [24,25]. After incubation, cells were harvested by washing with RPMI 1640 medium. Cell viability was controlled by using a trypan blue exclusion test, and was unaffected by the pesticide concentrations used in our experiments (over 80%). Proliferation was monitored by MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) assay as described by Mosmann [26]. The absorbance of each sample and control (ConA-free medium, with DMSO) was read on a spectrophotometer at 565 nm.Stimulation index (PI) was calculated as follows:

SI = (optical density of mitogen-stimulated cells/optical density of non-stimulated cells) × 100 [27]. SI was expressed as pourcentage of the control.

2.3. Isolation and preparation of splenic T-cells

The removed spleens were immediately transferred to the petri dishes containing complete RPMI 1640. The spleens were teased apart using a wire gauge. The number of viable cells was determined using the trypan blue exclusion test. After centrifugation (2000*g* for 5 min), the cells were resuspended in phosphate-buffered saline (PBS), pH 7.4, and placed in a sterile petri dish for 1 h at 37 °C to remove the macrophages by adherence. The unadhered cells were decanted and centrifuged (2000*g* for 5 min) once with PBS-containing bovine serum albumin (BSA) (2 g/l BSA) and were transferred to the petri dishes, which were previously coated with antirat immunoglobulin (IgG) (37.5 mg/15 ml) overnight at 4 °C. Hence, selective depletion of B-lymphocytes was accomplished because they adhered to the substratum of the petri dishes. After an incubation of 1 h at 4 °C, T-lymphocyte-rich supernatant was decanted and centrifuged (200*g* for 5 min) twice with PBS–BSA and resuspended in the complete RPMI 1640 at 4×10^6 cells/ml concentration, as previously described [28]. The T-cell viability was again checked by employing the trypan blue test. Spleen lymphocytes were cultured with or without Concanavalin A, in the presence of pesticides using the same protocol used for human lymphocytes.

2.4. Interleukin-2, -4 and INFγ quantification

Aliquots of culture supernatants were used to quantitate interleukins (IL-2, IL-4) and interferon-γ (INFγ) by using commercially available ELISA kits (R & D System, Oxford, UK), as per instructions furnished with. The results are expressed as pg/ml. The Th1/Th2 ratio was determined as the IFNγ/IL-4 ratio.

2.5. Lymphocyte oxidant/antioxidant markers

2.5.1. GSH measurement

Glutathione (GSH) levels were measured using a Bioxytech GSH-400 kit (OXIS International, Inc., Portland, OR, USA) as per the manufacturer's protocol. Briefly, cells were resuspended 5% (w/v) metaphosphoric acid and were homogenized. After centrifugation of the homogenate at 3000*g* for 10 min, the supernatant was transferred to 200 mM potassium phosphate containing 0.2 mM diethylenetriamine pentacetic acid and 0.025% (w/v) lubrol. Then chromogenic reagent was added, and the mixture was incubated at 25 °C for 10 min in the dark. The absorbance at 400 nm was measured, and the GSH concentration was then determined with the GSH standard curve.

2.5.2. Determinations of lymphocyte antioxidant enzyme activities

After different incubations and cell collection, lymphocytes were washed once, resuspended in chilled 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl, sonicated for 30 s and then centrifuged at 15,000*g* for 20 min. Aliquots of the supernatant fractions were used to determine the activities of catalase and the total activity of Superoxide dismutase (Cu, Zn-SOD and Mn-SOD) according to established methods. Briefly, Catalase (CAT, EC 1.11.1.6) activity was measured by spectrophotometric analysis of the rate of hydrogen peroxide decomposition at 240 nm [29]. Enzyme activity was expressed as U/mg of protein. The activity of the total SOD was measured by the NADPH oxidation procedure [30] and expressed as units of SOD per mg of protein. Protein content was determined using a Lowry protein kit.

2.5.3. Determination of lymphocyte hydroperoxides

To determine markers of lipid peroxidation, hydroperoxides were measured, in sonicated lymphocyte supernatant, by the ferrous ion oxidation-xylenol orange assay (Fox2) in conjunction with a specific ROOH reductant, triphenylphosphine (TPP) using a PeroxiDetect kit (Sigma, St. Louis, MO, USA) according to manufacture's instructions. Calibration was done with standard peroxides such as hydrogen peroxide, measured spectrophotometrically at 560 nm.

2.5.4. Determination of lymphocyte carbonyl proteins

Carbonyl proteins (markers of protein oxidation) were assayed in sonicated lymphocyte supernatant by the 2,4-dinitrophenyl hydrazine reaction as described previously [31].

2.6. Micronucleus (MN) assay

The MN assay is used as a fast and reliable assay for detecting genotoxic effects of the compound investigated. For the MN assay, after 24 h of incubation, cytochalasin B (Sigma) was added to the cultures at a concentration of 6 μg/ml to block cytokinesis. Following additional 24 h of incubation at 37 °C, the cells were collected by centrifugation and treated for 3 min with a mild hypotonic solution (75 mM KCl) followed by fixation with a fresh methanol/acetic acid mixture (3:1 v/v). Cells were stained with Giemsa (pH 6.8). Micronuclei were scored in 2000 binucleated lymphocytes with well-preserved cytoplasm per incubation, following the established criteria for MN evaluation [32].

2.7. Statistical analysis

Data are expressed as means ± SD. Statistical analysis was carried out using STATISTICA, version 4.1 (Statsoft, Paris, France). Multiple comparisons were performed using ANOVA followed by the LSD (least significant difference) test. $P < 0.05$ was considered to represent significant statistical differences.

3. Results

3.1. Effects of pesticides on in vitro human and rat lymphocyte proliferations

The mean mitogen stimulated lymphocyte proliferations as expressed by stimulation index, co-cultured with or without Mancozeb or Metribuzin, are shown in Fig. 1. We observed that Mancozeb had no effect on stimulated human lymphocyte proliferation at the concentration of 1 μM. However, concentrations 5, 10, 25, 50 and 100 μM of Mancozeb induced a significant inhibition of ConA-stimulated human lymphocyte proliferation in a dose-dependent manner. Rat spleen lymphocytes were more sensitive

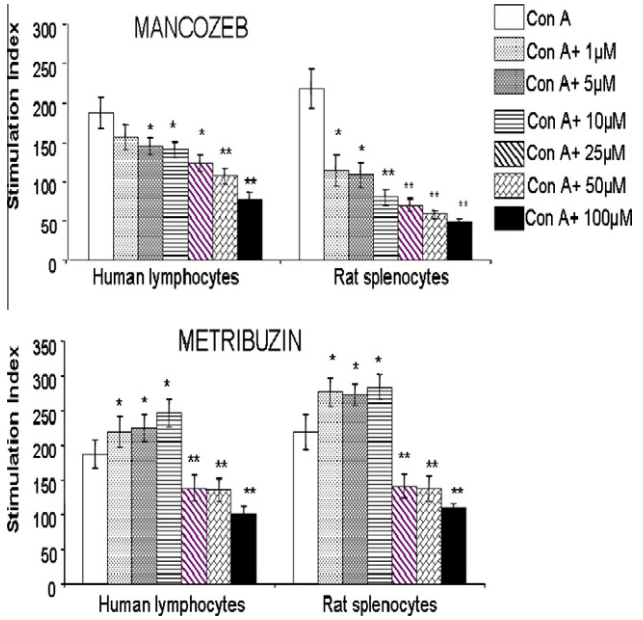

Fig. 1. In vitro influence of different concentrations of Mancozeb and Metribuzin on the proliferative response (stimulation index) of human lymphocytes and rat splenocytes stimulated by mitogen ConA. Cells were incubated in the presence of the following agents: concanavalin A (ConA, 5 μg/ml), Mancozeb (1–100 μM) or Metribuzin (1–100 μM) for 48 h. Stimulation index was expressed as pourcentage of the control. The values are means ± SD of triplicate assays from 8 healthy subjects and from spleens of 8 rats. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test, with $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. cells treated with ConA alone.

to Mancozeb; the reduction of stimulation index was apparent and occurred at 1 μM and continued to fall as the Mancozeb concentrations were increased, reaching a significant maximal inhibition at 100 μM.

The effects of Metribuzin on lymphocyte proliferation were different from those of Mancozeb; human and rat lymphocytes were affected to the same degree. Metribuzin at concentration between 1 and 10 μM resulted in an activation of ConA-stimulated lymphocyte proliferation as shown by the increase in the stimulation index. However, Metribuzin at concentration between 25 and 100 μM significantly reduced lymphocyte proliferations in a dose-dependent manner.

3.2. Effects of pesticides on cytokine production

To determine the Th1 and Th2 phenotype, the secretion of cytokines (IL-2, IL-4 and INFγ) was examined at 48 h of culture (Figs. 2 and 3). Human and rat splenic T-lymphocyte Th-1 (IL-2 and IFNγ) and Th-2 (IL-4) cytokine secretions were altered in the presence of Mancozeb and Metribuzin. IL-2, IFNγ and IL-4 productions were significantly decreased by Mancozeb in a progressive dose-related manner (Fig. 2). In addition, the Th1/Th2 ratio measured as the ratio IFNγ/IL-4 was significantly increased as the Mancozeb concentrations increased; the highest values were observed at 100 μM (Table 1).

A dichotomic effect on cytokine production was observed with Metribuzin depending on the concentration used (Fig. 3). In fact, human and rat lymphocyte IL-2, IFNγ and IL-4 secretions were significantly enhanced by Metribuzin at 1, 5 and 10 μM. However, at 25, 50 and 100 μM, Metribuzin induced a significant and progressive reduction in lymphocyte cytokine secretions. The IFNγ/IL-4 ratio was unaffected by Metribuzin at 1–50 μM but it was significantly increased by Metribuzin at 100 μM.

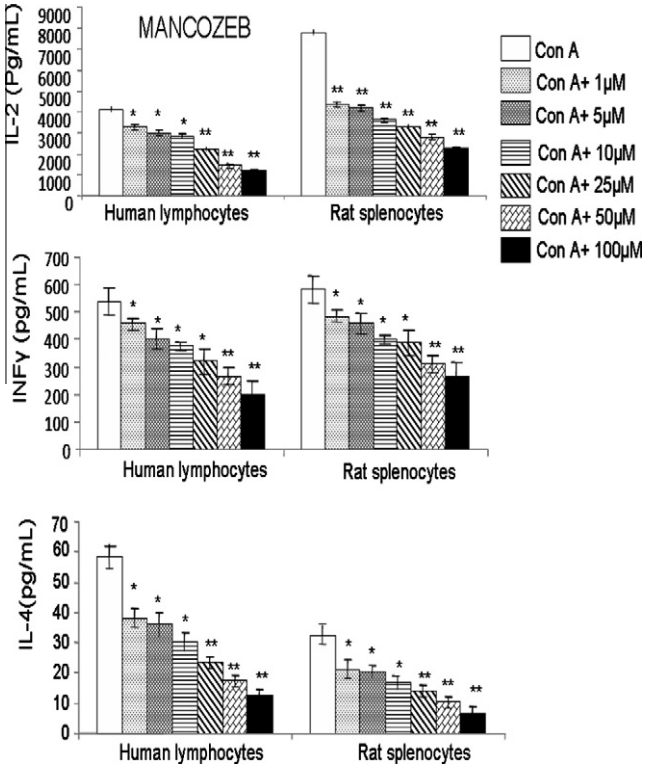

Fig. 2. Th1 (IL-2, INFγ) and Th2 (IL-4) cytokine secretion by ConA-stimulated T lymphocytes in the presence of Mancozeb. Cells were incubated in the presence of the following agents: concanavalin A (ConA, 5 μg/ml), Mancozeb (1–100 μM) or Metribuzine (1–100 μM) for 48 h. The values are means ± SD of triplicate assays from 8 healthy subjects and from spleens of 8 rats. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test, with $^{*}P < 0.05$ and $^{**}P < 0.001$ vs. cells treated with ConA alone.

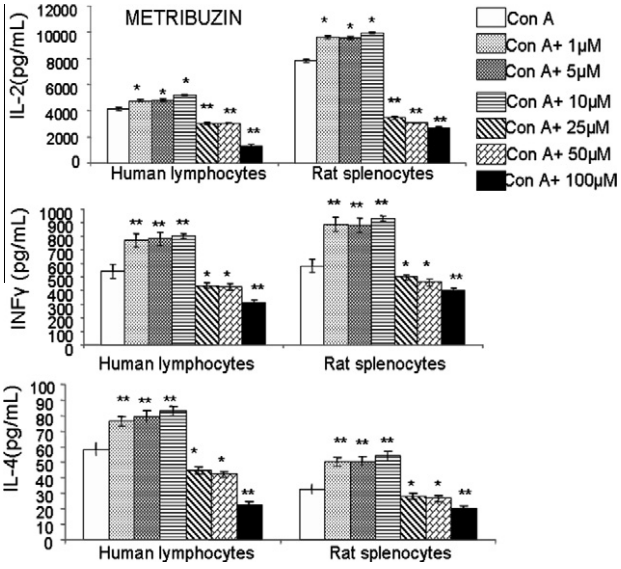

Fig. 3. Th1 (IL-2, INFγ) and Th2 (IL-4) cytokine secretion by ConA-stimulated T lymphocytes in the presence of Metribuzin. Cells were incubated in the presence of the following agents: concanavalin A (ConA, 5 μg/ml), Mancozeb (1–100 μM) or Metribuzin (1–100 μM) for 48 h. The values are means ± SD of triplicate assays from 8 healthy subjects and from spleens of 8 rats. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test, with $^{*}P < 0.05$ and $^{**}P < 0.001$ vs. cells treated with ConA alone.

3.3. Effects of pesticides on T lymphocyte oxidant/antioxidant status

3.3.1. Cellular GSH, hydroperoxide and carbonyl protein contents of stimulated lymphocytes

As shown in Table 2, human and rat lymphocyte intracellular glutathione (GSH) levels were not sensitive to Mancozeb adding in the medium at the concentrations of 1, 5 and 10 μM. However, at higher concentrations (25–100 μM), Mancozeb induced a significant reduction in lymphocyte GSH contents in a dose-dependent manner. Addition of Mancozeb in the culture medium (1–100 μM) produced significant increases in human and rat lymphocyte hydroperoxide (markers of lipid peroxidation) and carbonyl protein (markers of protein oxidation) levels in a dose-dependent fashion.

Metribuzin has no effects on human and rat lymphocyte intracellular GSH, hydroperoxide and carbonyl protein levels at low concentrations (1–10 μM) while it induced a significant decrease of GSH and an increase in hydroperoxide and carbonyl protein contents at high concentrations (25–100 μM).

3.3.2. Lymphocyte cellular antioxidant enzyme activities

As shown in Table 3, catalase activity was significantly enhanced in T lymphocytes from humans and rats in the presence of Mancozeb at 1–100 μM concentrations. Lymphocyte superoxide dismutase (SOD) activity was less sensitive to Mancozeb which induced a significant increase only at 100 μM.

Metribuzin had no effect on human and rat lymphocyte catalase activity at 1–10 μM. However, it stimulated catalase activity at 25–100 μM. SOD activity was also less sensitive to Metribuzin with a significant rise in its activity only at 100 μM.

3.4. Micronucleus formation

Only concentrations of 50 and 100 μM of Mancozeb and of Metribuzin induced a significant increase in the micronucleus (MN) frequency in both human and rat lymphocytes (Table 4). In contrast, no differences in the MN frequency were found between 1 and 25 μM of Mancozeb or of Metribuzin and control.

4. Discussion

In the present study, the effects of in vitro exposure to different concentrations of Mancozeb (fungicide) and of Metribuzin (herbicide) were evaluated in human and rat spleen lymphocytes. In vitro lymphocyte proliferation and cytokine production are a useful technique to evaluate lymphocyte function. ConA represents the most powerful mitogen for lymphocyte stimulation. Immunomodulation may result in suppression or stimulation of lymphocyte proliferation, cytokine secretion and/or in the homeostasis between Th1 (cell mediated) and Th2 (humoral) lymphocytes. Our results showed that Mancozeb and Metribuzin, at the concentrations used in our experiment (1–100 μM), have in vitro an important modulatory effects on cell mediated and humoral immune responses, with similar toxicokinetic profiles in human and rat spleen lymphocytes; the latter being more sensitive. The relationships between these effects and the ability of these pesticides to induce an intracellular oxidative stress are evident at high concentrations (50 and 100 μM). To the best of our knowledge, the immunomodulating activities of these two pesticides and their effects on lymphocyte antioxidant/oxidant status have not been documented previously. We observed that in vitro Mancozeb significantly reduced lymphocyte proliferation in both human and rat spleen lymphocytes in a dose-dependent manner. The immuno-suppressive action was more pronounced at 100 μM. The sensitivity of rat splenocytes to the fungicide used was higher than that of human lymphocytes. Similar changes were also reported in cell culture following exposure to others pesticides [33]. A decrease in lymphocyte activation with mitogens was observed after pesticide exposure [5]. The proliferative response of other cells such as astrocytes was significantly slower after

Table 1
Th1/Th2 ratio (INFγ/IL-4) in ConA-stimulated T lymphocytes in the presence of pesticides.

	Mancozeb		Metribuzin	
	Human lymphocytes	Rat splenocytes	Human lymphocytes	Rat splenocytes
INFγ/IL-4				
ConA	9.25 ± 0.86	17.80 ± 1.33	9.25 ± 0.86	17.80 ± 1.33
ConA + 1 μM	11.95 ± 0.62*	22.79 ± 1.25*	10.11 ± 0.93	17.72 ± 1.42
ConA + 5 μM	11.20 ± 0.64*	22.45 ± 1.31*	9.86 ± 0.63	17.50 ± 1.31
ConA + 10 μM	12.39 ± 1.02*	23.90 ± 1.27*	9.70 ± 0.72	17.25 ± 1.27
ConA + 25 μM	13.73 ± 1.17**	27.50 ± 1.08**	9.75 ± 0.68	17.67 ± 1.34
ConA + 50 μM	15.30 ± 1.06**	29.70 ± 1.45**	10.08 ± 0.91	17.19 ± 1.05
ConA + 100 μM	16.05 ± 1.14**	39.81 ± 1.17***	13.80 ± 1.04**	20.03 ± 1.26*

Cells were incubated in the presence of the following agents: concanavalin A (ConA, 5 μg/ml), Mancozeb (1–100 μM) or Metribuzin (1–100 μM) for 48 h. The values are means ± SD of triplicate assays from 8 healthy subjects and from spleens of 8 rats. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test, with $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. cells treated with ConA alone.

Table 2
Cellular GSH, hydroperoxide and carbonyl protein contents of stimulated T lymphocytes in the presence of pesticides.

	Mancozeb		Metribuzin	
	Human lymphocytes	Rat splenocytes	Human lymphocytes	Rat splenocytes
GSH(nM/10^{6}cells)				
ConA	14.50 ± 2.11	16.75 ± 1.02	14.50 ± 2.11	16.75 ± 1.02
ConA + 1 μM	14.11 ± 2.02	16.33 ± 1.66	13.26 ± 1.54	16.02 ± 1.22
ConA + 5 μM	14.15 ± 1.65	15.73 ± 1.54	13.54 ± 1.32	15.78 ± 1.31
ConA + 10 μM	14.24 ± 1.28	15.42 ± 1.43	13.18 ± 1.26	15.62 ± 1.30
ConA + 25 μM	12.05 ± 1.01*	13.41 ± 1.27*	10.02 ± 1.03*	13.11 ± 1.11*
ConA + 50 μM	10.73 ± 1.03**	13.22 ± 1.31*	9.45 ± 1.02*	12.35 ± 1.27*
ConA + 100 μM	9.36 ± 1.10**	13.07 ± 1.02*	7.73 ± 0.76**	10.45 ± 1.05**
HYDP (nM/10^6 cells)				
ConA	1.72 ± 0.13	2.08 ± 0.21	1.72 ± 0.13	2.08 ± 0.21
ConA + 1 μM	2.67 ± 0.22*	3.42 ± 0.25*	1.78 ± 0.22	2.22 ± 0.23
ConA + 5 μM	3.44 ± 0.30*	3.68 ± 0.32*	1.80 ± 0.25	2.31 ± 0.15
ConA + 10 μM	3.83 ± 0.25*	4.56 ± 0.34**	1.86 ± 0.16	2.27 ± 0.27
ConA + 25 μM	4.52 ± 0.26**	5.22 ± 0.28**	3.11 ± 0.32*	3.55 ± 0.31*
ConA + 50 μM	5.22 ± 0.18**	5.61 ± 0.22**	3.42 ± 0.28*	3.67 ± 0.34*
ConA + 100 μM	6.78 ± 0.24***	7.03 ± 0.30***	4.47 ± 0.24**	4.31 ± 0.28**
PCAR (nM/10^6 cells)				
ConA	2.36 ± 0.45	1.44 ± 0.25	2.36 ± 0.45	1.44 ± 0.25
ConA + 1 μM	3.40 ± 0.33*	2.75 ± 0.31*	2.48 ± 0.33	1.56 ± 0.26
ConA + 5 μM	4.33 ± 0.41*	3.22 ± 0.27*	2.56 ± 0.35	1.58 ± 0.21
ConA + 10 μM	5.66 ± 0.28**	3.59 ± 0.32*	2.68 ± 0.27	1.76 ± 0.31
ConA + 25 μM	6.83 ± 0.36**	4.56 ± 0.24**	4.07 ± 0.48*	3.56 ± 0.29*
ConA + 50 μM	8.45 ± 0.35***	5.82 ± 0.35**	4.63 ± 0.43*	3.81 ± 0.30*
ConA + 100 μM	9.56 ± 0.40***	6.75 ± 0.37**	5.42 ± 0.37**	4.84 ± 0.33**

Legends: see Table 1.

Mancozeb treatment [34]. Indeed, the relative sensitivity of rodent splenocytes to different poisons has been found higher or equal to that of human lymphocytes [35]. Our results showed that the addition of Metribuzin to the culture medium at low concentrations (1–10 μM) resulted in a significant activation of ConA lymphocyte proliferation in both human and rat spleen cells. However, at high concentrations (25–100 μM), Metribuzin induced a dose-dependent inhibition of lymphocyte proliferation. It was clear that this herbicide exerted either stimulatory or inhibitory activity to the immune system. This study provided evidence that the same pesticide can exert both effects, depending on the applied concentration. It could be speculated that the observed stimulatory and inhibitory effects of Metribuzin towards rat and human lymphocytes is related to hormesis, a phenomenon observed with dose–response curve. Hormesis was defined as the response of cells to an effector, with stimulatory results at low doses and inhibitory at high doses, thus explaining a molecule showing hormesis has the opposite effect in small doses than in large doses [36].

In our study, the effects of the two pesticides on in vitro cytokine production were concomitant to those on lymphocyte proliferation. In fact, we have measured different cytokine produced by lymphocytes to assess Th1/Th2 functional properties of lymphocytes. Measurement of cytokine production has been used previously in different studies and has been demonstrated to be an efficient method to detect Th1/Th2 differences. Th1 cells are mainly responsible for phagocyte-mediated host defence and these cells are the principal effectors of cell-mediated immunity, delayed-type hypersensitivity reactions and chronic inflammation via Th1 cytokines (IL-2, INFγ). Th2 lymphocytes are responsible for the immune defence not mediated by phagocytes, the recruitment of eosinophils and allergic reactions promoting humoral immunity via Th2 cytokines (IL-4). There are several possible mechanisms as to how Mancozeb may exert its inhibitory effects. Firstly, it reduced IL-2 secretion by ConA-stimulated lymphocytes in a dose-dependent manner, suggesting that the suppressed lymphoproliferation is due in part to a reduction in IL-2 production, knowing that IL-2 is a potent T lymphocyte growth factor. IL-4 and INFγ production was also diminished by Mancozeb. The significant decrease in IFNγ levels could enhance infection risk in pesticide exposure. There is a change in the profile of Th1/Th2 lymphocytes after Mancozeb exposure. We noted that while Mancozeb induced a decrease in the synthesis of both IL-2, IFNγ and IL-4 synthesis within lymphocytes, a Th1-like response was nevertheless dominant reflecting probably the inflammatory effect of Mancozeb. Previous studies

Table 3
Cellular antioxidant enzyme activities of stimulated T lymphocytes in the presence of pesticides.

	Mancozeb		Metribuzin	
	Human lymphocytes	Rat splenocytes	Human lymphocytes	Rat splenocytes
Catalase (U/mg)				
ConA	20.50 ± 2.26	30.53 ± 3.66	20.50 ± 2.26	30.53 ± 3.66
ConA + 1 μM	25.66 ± 1.64*	35.83 ± 3.25	20.34 ± 2.24	31.02 ± 2.08
ConA + 5 μM	32.16 ± 2.11*	43.02 ± 2.77*	20.05 ± 1.88	30.84 ± 2.53
ConA + 10 μM	35.74 ± 2.46**	45.92 ± 2.06*	19.87 ± 2.17	30.22 ± 2.44
ConA + 25 μM	42.31 ± 3.05**	56.11 ± 3.20**	38.66 ± 2.46*	42.50 ± 1.75*
ConA + 50 μM	46.37 ± 3.17**	58.05 ± 3.05**	40.11 ± 1.53*	50.03 ± 2.82*
ConA + 100 μM	48.22 ± 3.08**	60.33 ± 3.66**	49.53 ± 2.61**	60.62 ± 2.21**
SOD (U/mg)				
ConA	89.32 ± 6.63	122.27 ± 8.46	89.32 ± 6.63	122.27 ± 8.46
ConA + 1 μM	88.56 ± 5.44	120.34 ± 6.72	91.11 ± 7.02	121.50 ± 7.77
ConA + 5 μM	88.63 ± 5.68	125.72 ± 7.23	90.34 ± 6.88	120.06 ± 8.25
ConA + 10 μM	87.46 ± 6.33	122.05 ± 8.05	91.50 ± 8.45	122.42 ± 8.50
ConA + 25 μM	90.50 ± 6.02	124.26 ± 7.26	102.74 ± 8.28	125.63 ± 8.02
ConA + 50 μM	92.25 ± 4.26	130.11 ± 6.81	100.32 ± 8.55	130.84 ± 7.64
ConA + 100 μM	132.44 ± 5.38*	168.28 ± 6.50*	155.57 ± 6.43*	173.11 ± 6.33*

Cells were incubated in the presence of the following agents: concanavalin A (ConA, 5 μg/ml), Mancozeb (1–100 μM) or Metribuzin (1–100 μM) for 48 h. The values are means ± SD of triplicate assays from 8 healthy subjects and from spleens of 8 rats. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test, with $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. cells treated with ConA alone.

Table 4
Induction of micronuclei by pesticides in stimulated T lymphocytes.

MN (%)	Human lymphocytes	Rat splenocytes
Mancozeb		
ConA	4 ± 2	5 ± 2
ConA + 1 μM	5 ± 2	6 ± 1
ConA + 5 μM	6 ± 1	5 ± 1
ConA + 10 μM	7 ± 1	7 ± 1
ConA + 25 μM	7 ± 2	7 ± 1
ConA + 50 μM	15 ± 2*	20 ± 2*
ConA + 100 μM	26 ± 3**	32 ± 4**
Metribuzin		
ConA	4 ± 2	5 ± 2
ConA + 1 μM	6 ± 2	7 ± 3
ConA + 5 μM	6 ± 1	7 ± 2
ConA + 10 μM	7 ± 2	7 ± 1
ConA + 25 μM	7 ± 1	8 ± 1
ConA + 50 μM	12 ± 1*	18 ± 2*
ConA + 100 μM	18 ± 2**	26 ± 3**

Cells were incubated in the presence of the following agents: concanavalin A (ConA, 5 μg/ml), Mancozeb (1–100 μM) or Metribuzin (1–100 μM) for 48 h. The values are means ± SEM of triplicate assays from 8 healthy subjects and from spleens of 8 rats. Micronuclei (MN) frequency was calculated for 200 binucleated cells scored for each donor and for each case. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test, with $^{*}P < 0.05$ and $^{**}P < 0.001$ vs. cells treated with ConA alone.

have obtained a Th1 or a Th2 response using other pesticides [37]. One possible explanation for the predominance of Th1 over Th2 in Mancozeb cultures could be an increase in the rate of Th2 lymphocyte apoptosis relative to that of Th1. Since Mancozeb induced a reduction in T-lymphocyte proliferation, potential differences in the rate of proliferation between Th1 and Th2 lymphocytes may also explain an imbalance of Th1/Th2 in Mancozeb exposure.

Metribuzin induced an increase in cytokine secretion (IL-2, INFγ, IL-4) by ConA stimulated lymphocytes at low concentrations, and a reduction in these cytokines at high concentrations. The proportion of lymphocytes Th1 in relation to Th2 was unaffected by Metribuzin at 1–50 μM. However, at 100 μM, the proportion of Th1 lymphocytes is higher than that of Th2 lymphocytes, showing that human or spleen rat lymphocytes exposed to high concentration of Metribuzin mainly exhibit a Th1 response. An imbalance in cytokine production was reported by several authors after herbicide exposure [38]. It is possible that Metribuzin may act directly on Th lymphocytes, inducing activation and also inhibition of cell proliferation. Once they have been activated, Th die by apoptosis rather than proliferating. The variation in cytokine levels in the culture supernatants may be due to variation in cytokine secretion by the same number of cells, variation in the number of secreting cells or by different cell subpopulations.

Several studies showed that chronic exposure to pesticides suppresses humoral immune response, which is associated with increased excess of free radical generation [12–15,17]. Therefore, pesticides induced oxidative stress which may contribute to its immunotoxicity as previously reported [39]. Mancozeb was able to alter the antioxidant system of astrocytes in culture [17].

The result of redox markers in this work revealed that there was a oxidative stress reflected by an increase in hydroperoxides (lipid peroxidation marker) and carbonyl proteins (protein oxidation marker) and a significant decrease in GSH levels in human and rat lymphocytes exposed to Mancozeb in a dose-dependent manner. Oxidative stress markers were increased in Mancozeb-treated rats [17,18]. However, a significant increase in antioxidant enzyme activities was observed, catalase activity at 5–100 μM of Mancozeb exposure and superoxide dismutase activity at 100 μM. Metribuzin induced intracellular oxidative stress only at concentrations higher than 25 μM. The variation in catalase and SOD activities after Metribuzin exposure were similar to that found with Mancozeb. It could be suggested that antioxidant enzyme activities are modulated by the presence and the magnitude of oxidative stress following pesticide exposure. Thus, the increase in catalase and SOD activities could be adaptative responses to oxidative stress following pesticide exposure. It was clear that the oxidant/antioxidant status of lymphocytes from humans and spleen rats was equally sensitive to pesticides.

Several reports suggested DNA damage in cells exposed to reactive oxygen species [5,17]. The induction of MN formation (a marker of cytogenetic damage) by different pesticides has been reported by different authors [5]. In our study, Mancozeb and of Metribuzin induced a significant increase in the micronucleus (MN) frequency in both human and rat lymphocytes at 50 and 100 μM. Previous studies on other pesticides at concentrations similar to that used in our study found a significant increase in MN frequency only for the cultures treated with the higher concentration [5,40].

In conclusion, Mancozeb and Metribuzin displayed immunomodulatory properties depending on the concentration used.

Mancozeb was immunosuppressive while Metribuzin was immunostimulant at low concentrations and immunosuppressive at high concentrations. These two pesticides modulated the production of both Th1-type and Th2-type cytokines with a shift away to Th1 phenotype specially at high concentrations. These alterations were linked to several effects of Mancozeb and Metribuzin on behaviour of oxidative stress biomarkers of both human and rat spleen lymphocytes.

5. Conflict of interest

The authors have no conflicting financial interests.

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