

Organophosphorous Pesticides Metabolite Reduces Human T CD8 Homeostasis and Proliferation by Inducing Cellular Death

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Abstract

Background: We have previously shown that Diethyldithiophosphate (DEDTP), a metabolite of Organophosphorous (OP) compounds biotransformation with longer half life than its parental compound, can modulate T CD4 lymphocyte functions. To explore if DEDTP can also alter T CD8 homeostasis and proliferation we evaluated cellular viability and proliferation by propidium iodide (PI) incorporation and carboxyfluorescein succinimidyl ester (CFSE) assay by flow cytometry, respectively, in peripheral blood mononuclear cells (PBMCs) and T CD8 cells from healthy male donors.

Results: *In vitro* exposure to 1-50 μ M DEDTP decreased T CD4 vs. T CD8 proportion on resting T CD3 lymphocytes. DEDTP decreased T CD8 viability in a dose-dependent manner after 24 h without affecting the rest of T CD3 lymphocytes. DEDTP also decreases CFSE dilution in T CD8 cells stimulated with anti-CD3/CD28 by arresting cells at the first round of division (M1). Decrease in cell proliferation was not only due to cellular arrest, but also to a consequence of cell death. Although cell death and cell cycle arrest were observed in the majority of the T CD8 cells, some particular T CD8 subset clones presented a high proliferative rate in the presence of DEDTP.

Conclusion: DEDTP showed higher toxicity and cytostaticity in T CD8 cells than in T CD4 lymphocytes. This is relevant in exposed individuals as their ability to deal with viral infections and cancer cells could be limited by exposure to OP pesticides.

Keywords: Cell death; Cell viability; Immunotoxicology; Organophosphorous compounds; T CD8 proliferation

Abbreviations: APC: Apophycocyanine; CD: Cluster Differentiation; CFSE: Carboxy Fluorescein Succinimidyl Ester; CTL: Cytotoxic T Lymphocyte; DAPs: Dialkylphosphates; DEDTP: Diethyldithiophosphate; DETP: Diethylthiophosphate; DEP: Diethylphosphate; DMDTP: Dimethyldithiophosphate; DMTP: Dimethylthiophosphate; DMP: Dimethylphosphate; DMSO: Dimethyl Sulfoxide; ELISA: Enzyme-linked immune-adsorbent Sandwich Assay; FITC: Fluorescein Isothiocyanate; FBS: Foetal Bovine Serum; IL: Interleukin; OP: Organophosphorous; PBMCc: Peripheral Blood Mononuclear Cells; PBS: Phosphate Buffered Saline Solution; PE: Phycoeritrin; PI: Propidium Iodide; SD: Standard Deviation; SOCS: Suppressor of Cytokine Signaling; STAT: Signal Transduction Activation Transcription Factor; TCM: Central Memory T Cells; TCR: T Cell Receptor; TEM: Effector Memory T cells; TSCM: T Memory Stem Cells

Introduction

Today, organophosphorous (OP) compounds are widely used as pesticides due to their low residual power into the environment and have replaced chlorinated compounds as the famous DDT. Some commercial uses of OP compounds are as control of farm plagues and transmissible diseases like malaria, yellow fever, dengue and chagas [1], disinfection and safety of private industry, hospitals and schools, or as fumigants for flower production and maintenance of golf or football stadiums gardens [2]. In fact, it is recently proposed that all beings are exposed directly or indirectly to OP [3]. Although the neurotoxic effects of OP compound have been widely studied and have been attributed to the Oxon formed by metabolic cleavage of OP compounds by esterases, little is known regarding the immunotoxic effects of OP pesticides or their metabolites, called dialkylphosphates (DAPs); Diethyldithiophosphate (DEDTP), Diethylthiophosphate (DETP), Diethylphosphate (DEDP), Dimethyldithiophosphate

(DMDTP), Dimethylthiophosphate (DMTP) and Dimethylphosphate (DMP). Many questions have emerged about the immunotoxic potential of OP compound metabolites mainly due to their long persistence in the body and long-lasting effects.

We have previously reported that exposure to some DAPs alters the proliferation induced by interleukin (IL)-2 in human peripheral blood mononuclear cells (PBMCs) [4] and especially, DEDTP modifies T CD4 lymphocyte IL-2 receptor-dependent proliferation by modifying the phosphorylation status of MAPKs and STAT5 proteins [5]. Indicating that the transduction signal pathway of activation of IL-2 is the main pathway altered by OP compounds in lymphocytes [6]. Besides T CD4 lymphocytes, T CD8 cells have an essential role in some cellular processes, such as the cytotoxic response known as CTL to kill virus and cancer cells. Therefore, this subset of CD3 lymphocytes are very important as part of the immune response inducing clonal expansion of effectors and memory clones, which are determinant to control pathogens or cancer development [6].

Actually, it is known that many external insults can affect the immune system and, in fact, it is the target of many xenobiotic agents [7]. Some investigators suggest that the high incidence of certain diseases

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Received December 01, 2011; Accepted March 07, 2012; Published March 09, 2012

Citation: Esquivel-Sentíes MS, Vega L (2012) Organophosphorous Pesticides Metabolite Reduces Human T CD8 Homeostasis and Proliferation by Inducing Cellular Death. J Environment Analytic Toxicol S4:004. doi:10.4172/2161-0525.S4-004

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is associated with high contact with xenobiotics [8], in particular the incidence of viral infections or even cancer development [9].

Materials and Methods

Materials

The OP metabolite diethyldithiophosphate (DEDTP) was obtained from Sigma, 99.9% purity (St. Louis, MO, USA). The antibodies and recombinant proteins for ELISA and immunophenotyping assays were obtained from BD Biosciences (San Jose, CA, USA), Cell signaling (Danvers, MA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), Invitrogen (Carlsbad, CA, USA) and Miltenyi Biotech (Auburn, CA, USA). The rest of the reagents were purchased from Sigma or Gibco (Grand Island, NY, USA), as indicated.

Cell isolation

Peripheral blood mononuclear cells (PBMCs) from 14 different young, disease-free male donors were separated from a buffy coat fraction obtained from the blood bank of the “La Raza” Hospital (Mexico, DF) by density gradient centrifugation for 30 min at 2000 rpm using Ficoll-Hypaque (Sigma). PBMCs were resuspended in culture medium (RPMI 1640, Gibco), supplemented with penicillin/streptomycin (10 U/ml and 10 µg/ml, respectively, Sigma), 2 mM L-glutamine (Gibco), 1 mM non-essential amino acids (Gibco) and 10% heat inactivated fetal bovine serum (FBS, Gibco) and incubated at 37°C in a humidified atmosphere with 5% CO₂. Cells were allowed to adhere to 75 ml Falcon flasks for 2 h. PBMCs were recovered from non-adherent cells and washed with phosphate buffered saline solution (PBS) and centrifuged at 1500 rpm for 5 min. Cells were resuspended

in RPMI and seeded in culture plates as needed in 24 or 96 wells/plate at 1x10⁶ cells/ml.

Treatments

Cultures were treated with either dimethyl sulfoxide (DMSO), at a final concentration of 0.5%, or different doses of DEDTP from 1 to 50 µM dissolved in DMSO. Cells were further incubated, harvested and stained as described below.

T cell stimulation

Cells were deprived of FBS for 16 h before treatment and stimulation in order to synchronize cells. Experiments were carried out by triplicate. Stimulation of T cells was performed in 24 or 96-well plates containing 10⁶ cells/ml (1 or 0.2 ml, respectively, Costar, NY, USA) with or without plate-bound human anti-CD3 and anti-CD28 (1.5 µg/ml each, BD Biosciences) to induce activation and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24-96 h.

Cell proliferation

The cell replication index was determined by carboxyfluorescein succinimidyl ester (CFSE) dilution of labeled cells with 0.5 µM final concentration of the fluorochrome and following the manufacturer’s recommendation (CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes). Briefly; CFSE solution was added to 10⁷ cells in a final concentration of 0.5 µM, then cells were incubated at 37°C for 10 min and quenching the staining by adding 5 volumes of ice-cold culture media to the cells, incubated 5 min on ice and pelleted by centrifugation for 5 min at 1500 rpm and washed two times with

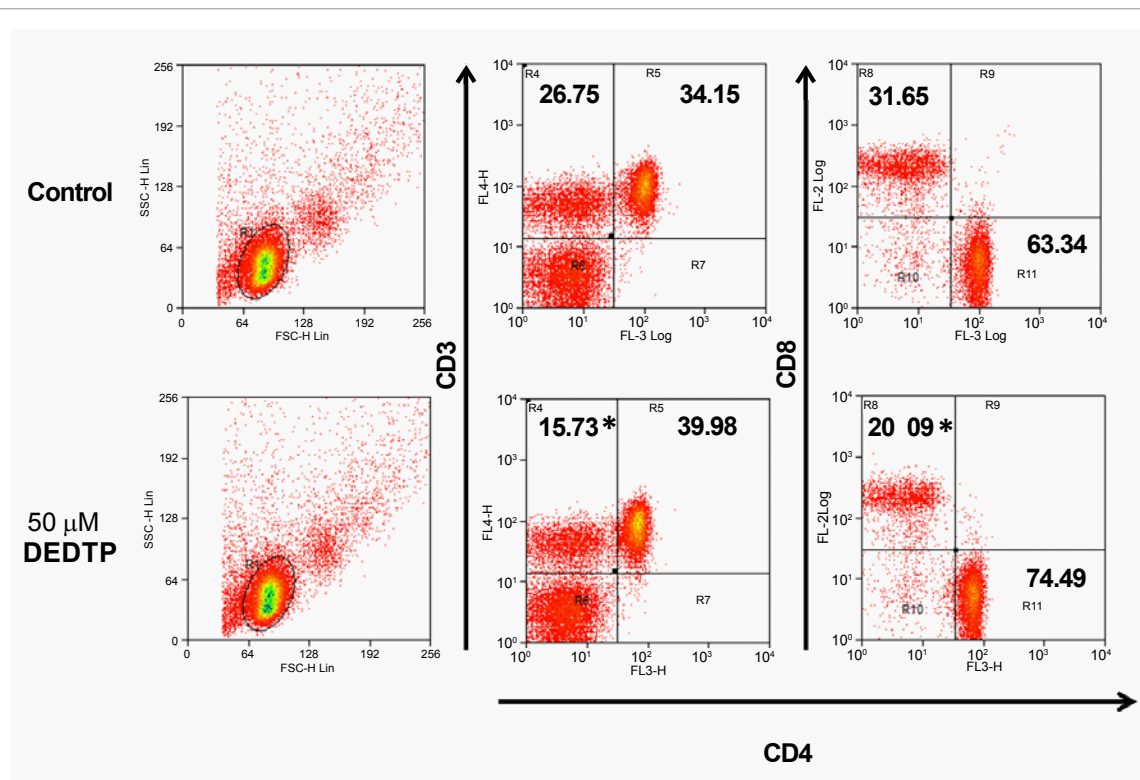


Figure 1: DEDTP exposure modifies the proportion of T-CD8-positive lymphocytes in resting PBMCs. The PBMCs were treated with 50 µM DEDTP for 24 h. Cells were stained with anti-CD3-APC, anti-CD4-PerCP and anti-CD8-PE; their expression was assessed by flow cytometry in 10000 events. One representative experiment of four. Mean ± SD (n=8), *p<0.05, Student’s t-test.

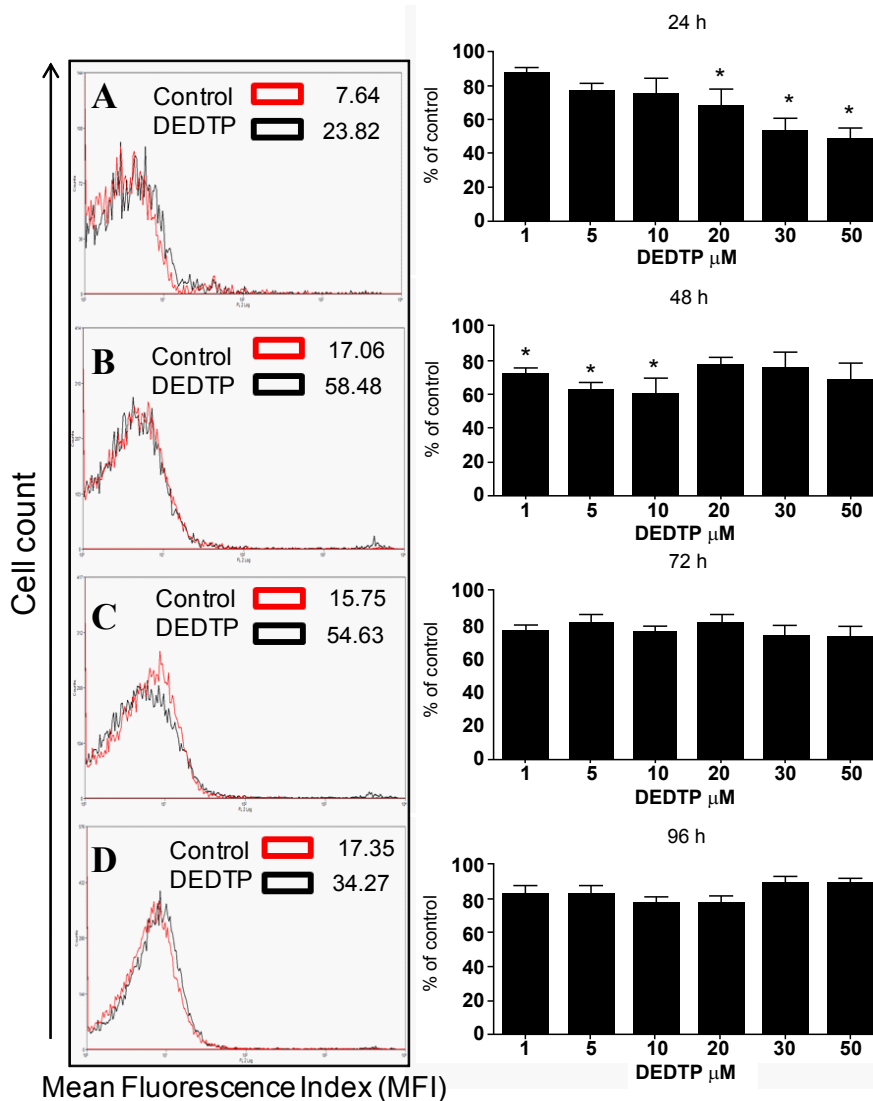


Figure 2: DEDTP induces cell toxicity on T-CD8-positive resting lymphocytes. PBMCs were treated with DEDTP in a 96-well plate for 24 (A), 48 (B), 72 (C), or 96 h (D), PI incorporation was quantified in T-CD8-positive lymphocytes by flow cytometry in 10000 events. One representative experiment of four is presented in dot plots. Mean \pm SD (n=8), *p<0.05, ANOVA test.

fresh media. Cells were resuspended in 10^6 cells/ml of supplemented medium and resuspended in an appropriate volume to be analyzed by flow cytometry counting 10000 events per sample. Flow cytometry was performed on a fluorescence activated cell sorter FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were evaluated using the Summit 4.3 research software (Dako, CO, USA).

Viability assay

The cytotoxic effect of DEDTP on T cells was determined by quantifying necrosis with the Detection Kit II (BD Pharmingen™), following the manufacturer's recommendations. Briefly, cells were washed twice with cold PBS and then resuspended in 1X binding buffer at 10^6 cell/ml, 100 μ l of the solution were transferred to a 5 ml culture tube adding 5 μ l of propidium iodide (PI) and incubated for 15 min at 25°C in the dark, 400 μ l of 1X binding buffer was added to each tube and cells were analyzed by flow cytometry counting 10000 events per sample.

Phenotyping of T CD8 lymphocytes

For extracellular membrane staining, 10^6 cells were incubated for 30 min at 4°C with anti-human CD3-APC or anti-human CD3-FITC, anti-human CD8 PE and anti-human CD4-PerCP. Cells were washed twice with PBS with 5% FBS and 0.02% sodium azide and 20000 events were evaluated by flow cytometry.

Statistical analysis

Numeric data are presented as mean \pm standard deviation (SD). Statistical significance of the data was obtained using a two way ANOVA and Student's t-test, differences were considered significant when $p < 0.05$.

Results

DEDTP induces cell death of CD8 on resting PBMCs

As a first step to determine the effects of DEDTP exposure on

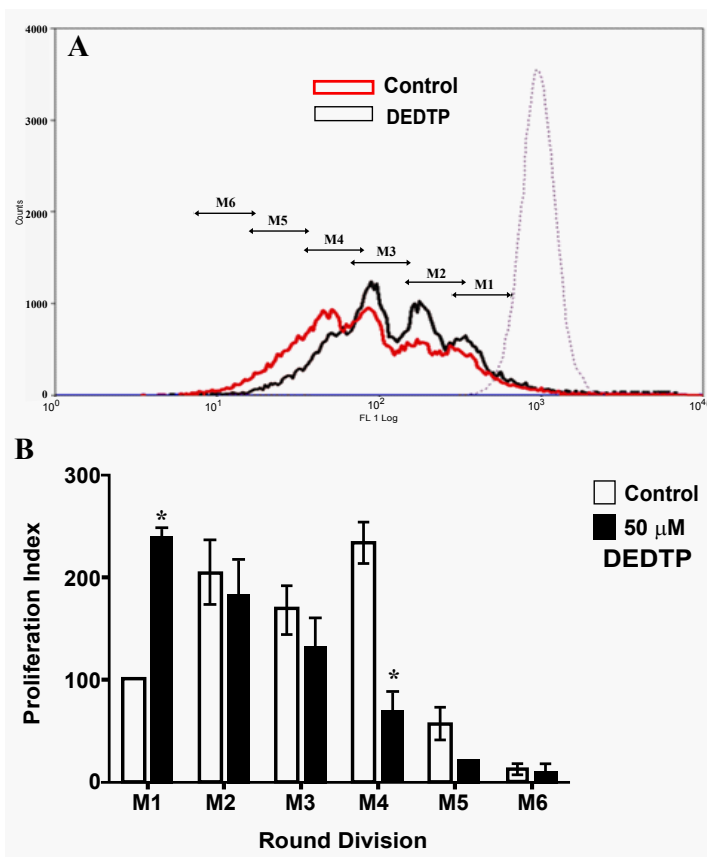


Figure 3: Exposure to DEDTP decreased proliferation in T-CD8-positive cells. PBMCs were labeled with 1 μ M CFSE, and stimulated with plate-bound anti-CD3/CD28 antibodies [1.5 μ M/ml, each] in 24-well plates and treated with 50 μ M DEDTP for 96 h. PBMCs were stained with anti-CD3-APC, and anti-CD8-PE (A). Histogram shows the main fluorescence index of CFSE of one representative experiment of four. The round division of T-CD8-positive cells was calculated by mitotic percentage and each division is represented as M (B). Mean \pm SD (n=8), *p<0.05 by, ANOVA test.

PBMCs we treated resting cells with different concentrations of DEDTP within the range of levels found on human plasma from intoxicated people [10]. We evaluated the cytotoxic effect over T cells by determining T CD3-lymphocytes subsets as CD8- or CD4-positive cells. The PBMCs were treated with 1-50 μ M DEDTP for 24-96 h and we observed that the proportion of T-CD3/CD8-positive lymphocytes was reduced in more than 10% by DEDTP treatment when compared with untreated cells (Figure 1A). However, DEDTP had no effect on T-CD4 lymphocytes as previously reported by our group [5]. In order to evaluate if this change in the proportion of CD4- vs. CD8-positive lymphocytes was due to cell toxicity, we determined PI incorporation on T-CD8-positive lymphocytes (Figure 2). Incorporation of PI in non-treated cells was considered as 100% of cell viability evaluated by flow cytometry. We observed that exposure to DEDTP reduced, up to 40%, in a dose-dependent manner, the cellular viability of T-CD8-positive cells after 24 h of exposure (Figure 2A). Nevertheless, viability was recovered in T-CD8-positive cells treated with the highest doses of DEDTP after 48 h of treatment (Figure 2B) and completely recovered after 72 and 96 h of exposure (Figure 2C and 2D).

DEDTP treatment alters CD8 proliferation when stimulated with anti-CD3 and anti-CD28

To determine if the changes produced by exposure to DEDTP in T-CD8-positive resting PBMCs could affect cellular response to an activation and proliferative signal, we determined the effects of

DEDTP on T-CD8-positive proliferation induced by stimulation of the T cell receptor (TCR) and the CD28 co-stimulatory molecule in CFSE marked cells. We observed that exposure to 50 μ M DEDTP for 96 h decreased T cell proliferation of CD3 lymphocytes and particularly reduced proliferation of T-CD8-positive cells (Figure 3A). Most of the T-CD8-positive lymphocytes were arrested at the first metaphase (M1) as calculated by the proliferative index (Figure 3B). Additionally, the reduction on T-CD8-positive cells proliferation was also due to cell toxicity as evaluated by PI incorporation at 24 (Figure 4A), 48 (Figure 4B), 72 (Figure 4C), or 96 h (Figure 4D), with the main peak of cell death induction at 48 h of exposure (Figure 4E).

Discussion

A group of xenobiotics that had shown immunotoxic potential are the OP pesticides [11]. The relationship between OP pesticides and the increase of recurrent diseases have clearly been described in many epidemiologic studies [12]. In humans, there are some reports indicating that OP pesticides can alter the complement function, subpopulation levels, cell proliferation, cytokine secretion, surface markers, chemotactic migration, phagocytosis, antigen presentation and apoptosis induction [13].

Although human PBMCs are not known to biotransform OP pesticides, a significant amount of OP metabolites have been detected in the serum of exposed individuals [14], indicating that PBMCs are

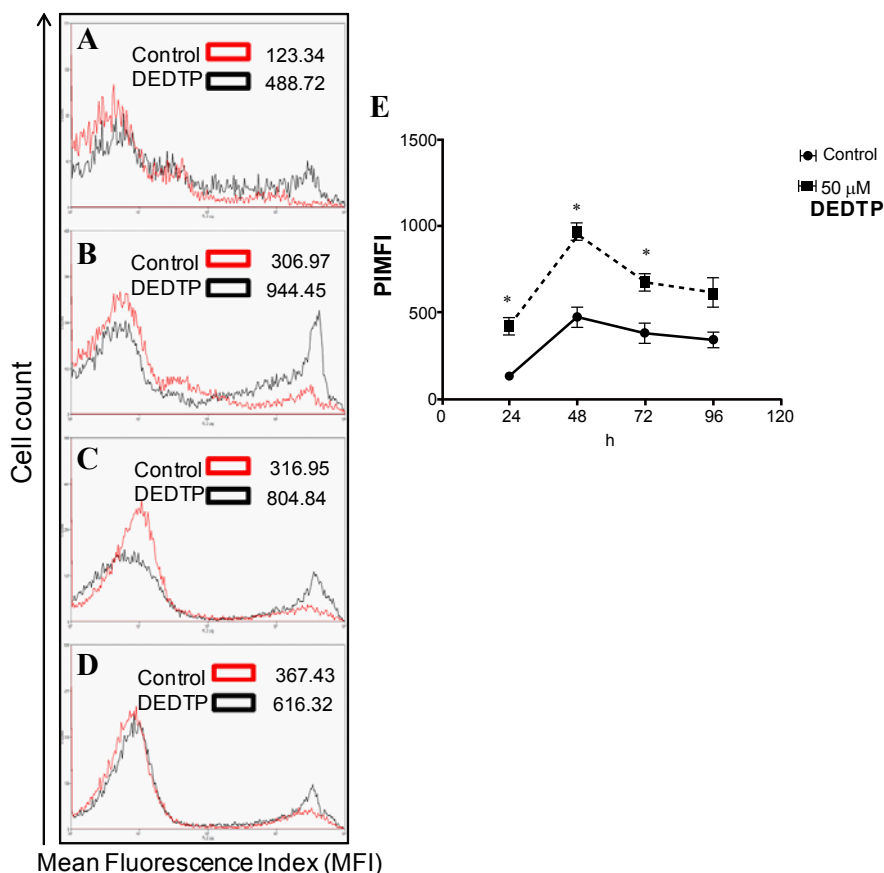


Figure 4: DEDTP inhibits TCR/CD28-induced proliferation in T-CD8-positive cells by inducing cellular death. T-CD8-positive cells were stimulated with anti-CD3/CD28 antibodies [1.5 μM/ml, each] in 24-well plates and treated with 50 μM DEDTP for 24 (A), 48 (B), 72 (C), or 96 h (D). T-CD8-positive lymphocytes were stained with PI. Histograms show PI mean fluorescence index of one representative experiment of four. Mean ± SD (n=8), *p<0.05 by ANOVA test.

exposed to OP pesticide metabolites. Despite the known and clear information related to the molecular mechanism of the toxic effects of OP compounds over the central nervous system, there is not information regarding the mechanism by which pesticides, or their metabolites, may modulate the immune system. One possible explanation is that these effects are produced by the interaction of DAPs with PBMCs. However, until today, these kinds of metabolites are considered to be unreactive and are commonly used only as exposure makers.

As previously reported, our data on cell proliferation and activation parameters on PBMCs of T-CD4-positive cells exposed to DEDTP suggest an uncoupling of the IL-2R transduction signal pathway without relationship to cell toxicity [5]. T-CD8-positive lymphocytes are known to be more reactive than T-CD4-positive cells as effector cells and part of the main component of immune cellular response to viruses, intracellular pathogens and cancer cells. Naïve T-CD8-positive cells migrate mainly through lymphoid tissues, where they meet pathogen-derived antigen together with appropriate co-stimulation, both delivered by specialized and activated antigen-presenting cells. This encounter can lead to an enormous clonal expansion that is subsequently followed by substantial cell death, leaving behind long-lived memory cells comprising only 5–10% of the initial burst size. During the proliferation of antigen-reactive cells, these cells undergo variable phenotypical and functional changes, which have been identified as distinct T cell subsets with very specific

functions in immunity and the quality of memory responses [15]. Limited clonal expansion of activated T cells is necessary to maintain immune homeostasis and is achieved by growth arrest or apoptosis in dependence of the IL-2 signal transduction pathway. For example, in resting T cells, which were previously stimulated via TCR and then stimulated with IL-2, no proliferation was recovered due to an inhibition on STAT5 phosphorylation and an increase on SOCS3 expression [16]. T-CD8-positive cells activation and proliferation can be dangerous if it is not under the correct control and context causing cellular and tissular damage to the host.

Although there was inhibition on cell proliferation by DEDTP treatment in most T-CD8-positive cells, a small portion of these cells continued proliferating after 72 h in some donors. We consider that this proliferation correspond to CD25-, CD122-positive memory cells. Effector memory T (TEM) cells are terminally differentiated and acquire effector function immediately after re-stimulation, whereas central memory T (TCM) cells have a longer lifespan and can differentiate into TEM cells following antigenic challenge. A recent study published in *Nature Medicine* [17] reports that an additional memory T cell subset, T memory stem (TSCM) cells, which was previously identified in mice, exists in humans and exhibits stem cell properties. Our results suggest that this TSCM subset could be more resistant to DEDTP effects than naïve T-CD8-positive cells and more experiments are needed to further confirm this hypothesis.

Regarding the fact that most effects in proliferative PBMCs seem to be reversible, we can consider that the degradation of DEDTP, as proposed by Vega et al. [18], also allows the cell to return to a basal level of protein phosphorylation and the cells can be released from cellular arrest. IL-2R signaling in T cells is regulated by phosphorylation events, so unusual changes in the phosphorylation profile will drive cells to uncoupling behavior. Monocytes and lymphocytes, STAT5 downregulation and SOCS3 upregulation have been reported to produce an inactivation profile, or negative feedback signals [19]. On the other hand, T-CD8-positive memory cells can continue proliferating in response to IL-15 signaling, maintaining memory cells alive to respond to further specific challenges. Also, carboxylesterases in monocytes are inhibited by OP [20], so it is possible that DEDTP can inhibit enzymes such as PKA and then block the movement of CD28 to the synaptic union site [21] and therefore, block the initial anti-CD3/CD28 activation contact with the cells.

The effects observed in this report indicate that DAPs, such as DEDTP, can alter the function of certain elements of the immune system response. Thus, it is possible that diseases related to immunosuppression, hypersensitivity and autoimmunity can be produced by the exposure to OP compounds, as already reported with some OP pesticides [22]. Cancer is another immune-related disorder associated to OP compounds exposure as NHL in occupationally exposed individuals [23].

Finally, DAPs have been reported as widely detected compounds in cultures, waste water, flowers, fruit, etc. In fact, one report in the United States shows that all children tested in one elementary school excreted all six DAPs without referring previous exposure to OP compounds [2], so it is not surprising that in the future, this kind of metabolites would be on the agenda of immunotoxicologist.

Acknowledgments

We thank M. Sc. Victor Hugo Rosales-Garcia and M. Sc. Elizabeth Estrada-Muñiz from CINVESTAV for their technical assistance. This work was supported by CONACyT's Research Grand numbers 51359, 127946 and 153468. Esquivel-Sentíes M. S. received a scholarship number 172660.

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This article was originally published in a special issue, **Toxicology of Pesticides** handled by Editor(s). Dr. Francisco Sanchez Bayo, Australia; Dr. Richard ORTEGA, France