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The effect of low doses of glyphosate on reactive oxygen species production by human granulocytes

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ABSTRACT: Glyphosate is the base of numerous herbicides used widely all over the world. Strong hepato- and nephrotoxicity of high doses of this reagent was reported in laboratory animal studies. In European Union countries the acceptable daily intake for humans is set at 0.5 mg/kg body weight. We investigated the effects of glyphosate on peripheral blood polymorphonuclear cells (PMNs) at relatively low concentrations of the reagent, from 0.01 mg/L to 10 mg/L (from ~0.06 μM to 59 μM). As the biological half-life of this compound in the human body is estimated to be 3 to 10 hours, we decided to incubate blood samples with glyphosate for a period of one hour. Such incubation caused a statistically significant increase of reactive oxygen species production in granulocytes stimulated with N-formylmethionine-leucyl-phenylalanine and *Escherichia coli* cells. This increase was not associated with the toxic effects of glyphosate or with increased phagocytic activity of granulocytes. The reagent, when applied at specified concentrations, did not induce a respiratory burst in granulocytes or affect the amount of production of reactive oxygen species in blood samples stimulated with 12-myristate phorbol 13-acetate. On the basis of the results obtained, it may be suggested that glyphosate affects signaling pathways leading to NADPH oxidase activation, independent of protein kinase C activation. Thus, it can be concluded that although low doses of glyphosate are not harmful to humans, synergistic effects of this compound with other environmental pollutants may be an important part of pathogenic mechanisms.

Keywords: Glyphosate; Reactive oxygen species; Human peripheral blood polymorphonuclear cells.

1. INTRODUCTION

Glyphosate (N-phosphonomethylglycine) is an inhibitor of the shikimate pathway in weeds, preventing aromatic amino acid production. Is widely used worldwide as the base of numerous herbicides, although applicable mainly to genetically modified glyphosate-resistant crops. The persistence of glyphosate in soil varies greatly, with a half-life ranging from 1 to 200 days, depending on i.a. on temperature, humidity and type of soil, microbial composition and pH of the environment, content of phosphates, aluminum and iron oxides/hydroxides [1,2]. As the reagent water soluble without affinity to fat tissue is believed to be safe to humans, because its half-life estimated to be 3 to 10 hours allows to quick elimination with urine. However, in experiments on cultured cell lines or laboratory animals, the harmful effects were observed. It included inflammation, immune, endocrine and neurological disorders, adverse effects on reproduction and development, induction of cancerogenic changes. Most of the studies concerning human exposure to glyphosate have thus far

included peoples exposed occupationally to the pesticide. Some publications suggest that glyphosate may be an endocrine disruptor. Environmental exposure to glyphosate was associated with an increased risk of spontaneous abortion or preterm delivery [3-6]. Sometimes, increased glyphosate exposure is associated with the spread of thyroid disease [7,8]. It should be noted, however, that despite the research undertaken, glyphosate-endocrine system interactions are still speculative [9,10]. An exposure to preparations containing this substance maybe associated with the occurrence of non-Hodgkin's lymphomas [11-13] or acute myeloid leukemia [14], although there are publications denying such a carcinogenic effect [15]. European Food Safety Authority (EFSA) disallows glyphosate as a carcinogen in opposition to IARC (International Agency for Research on Cancer) classifying this pesticide as probably carcinogenic to humans.

Studies on the effect of glyphosate on animal organisms were conducted in various experimental protocols. Both animals and cell lines of various origins, including human ones were used, as well as some model organisms [16-19]. However, the assessment of the potential harm of glyphosate to humans is based on studies using standard laboratory animals: mice and rats [e.g. 20-23]. Such studies, like studies on cell lines, however, concerned genetically homogeneous populations, while the individual response to glyphosate in the human population may be variable. In a few cases, normal human cells were used, most often isolated peripheral blood mononuclear cells [24-28]. Human whole blood has rarely been used in such studies [29-31], although it is assumed that such a model better reflects in vivo interactions, where higher cell diversity may affect the size and kinetics of response to the test substance [32]. In this paper, an attempt was made to examine the effect of glyphosate on human leukocytes in samples of whole peripheral blood taken from different people, as such a protocol seems to better reflect the possible effect of this agent on people in the natural living environment.

2. MATERIALS AND METHODS

For experiments performed on the peripheral blood samples, the stock solution of glyphosate (CAS# 1071-83-6) (98.9% w/w, Institute of Organic Industry, Warsaw, Poland) in the concentration of 1 mg/mL in non-pyrogenic water was prepared. This solution was filtered through a 0.22 μm PES syringe filter (Merck Millipore), aliquoted into sterile deep freeze tubes (1.8 mL) and stored in a freezer (-18°C) until use.

Blood from healthy donors purchased at the Provincial Blood Donation Station in Poznań was used in the study, so the consent of the Bioethics Committee was not required. Blood in the volume of 7.5 mL was collected into tubes with lithium heparin (Sarstedt, Germany) and delivered to the laboratory in the shortest possible time (maximum 1 hour).

The results of all carried reactions were measured in the Sysmex Partec-CyFlow®Space flow cytometer at excitation wavelength 488-492 nm and emission at 515-535 nm (green) or >610 nm (red).

2.1. Methods

The effects of glyphosate on cell viability, production of reactive oxygen species (ROS), and rates of phagocytosis were analyzed using standard cytometric assays. All tests were carried out according to the manufacturer's instructions.

The FLIVO® In vivo Poly Caspase Assay (ICT) kit from ImmunoChemistry Technologies, LLC (USA) was used to assess granulocytes viability *in vitro*. The caspase substrate (FLICA - Fluorochrome Inhibitor of Caspases) contains the binding sequence for most caspase, which is labeled with a carboxyfluorescein (FAM) dye (green fluorescence). The assay, based on the use of Fluorescent Labeled Inhibitors of CAspases and propidium iodide (PI, red fluorescence) staining, allows to discriminate between living (non-stained) apoptotic (FAM+) and dead cells (PI+).

Polymorphonuclear leukocytes ROS production was determined by applying PHAGOBURST™ kit (Glycotope Biotechnology GmbH, Heidelberg, Germany). The oxidative burst was stimulated by unlabeled opsonized E. coli bacteria and the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP) (particulate and low physiological stimulus), or protein kinase C ligand phorbol 12-myristate 13-acetate (PMA) (high stimulus). The production of ROS was measured by the percentage of leukocytes which oxidize the fluorogenic substrate dihydrorhodamine (DHR) 123 to rhodamine 123 and the mean fluorescence intensity (MFI) of rhodamine 123 positive neutrophils in flow cytometry.

The phagocytic function of leukocytes was estimated using the PhagotestTM kit (Glycotope, Biotechnology GmbH, Heidelberg, Germany). Briefly, blood samples were incubated with fluorescein isothiocyanate (FITC)-labelled *Escherichia coli* and the percentage of neutrophils which had ingested bacteria was quantified by means of FITC-positive cells. Their corresponding phagocytic activity was measured through mean fluorescence intensity (MFI).

2.2. Statistical analysis

Analyses were performed using MedCalc® Statistical Software version 19.5.3 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020). In all calculations, p-value <0.05 was considered statistically significant.

For each series of experiments, mean, standard deviation, median, as well as minimum and maximum values were calculated. Next, normality was checked in the Shapiro-Wilk test. Statistical analysis was carried out with non-parametric Friedman's test for related variables because assumptions for parametric tests were not met. In the next step, a post hoc Conover's test was applied to determine which groups of variables were statistically different (p<0,05). Where applicable, ANOVA analysis was used.

3. RESULTS

3.1. Evaluation of the potentially toxic effect of glyphosate on peripheral blood leukocytes

To assess the toxicity of glyphosate, the flow cytometry method using the FLICA probe and propidium iodide (PI)was used. The glyphosate was added to blood samples in 0.01 mg/L, 0.1 mg/L, 1 mg/L or 10 mg/L concentrations. Apoptotic cells, stained by FAM, showed fluorescence in the green range of the spectrum, dead cells (PI stained) in the red range (Figure 1).

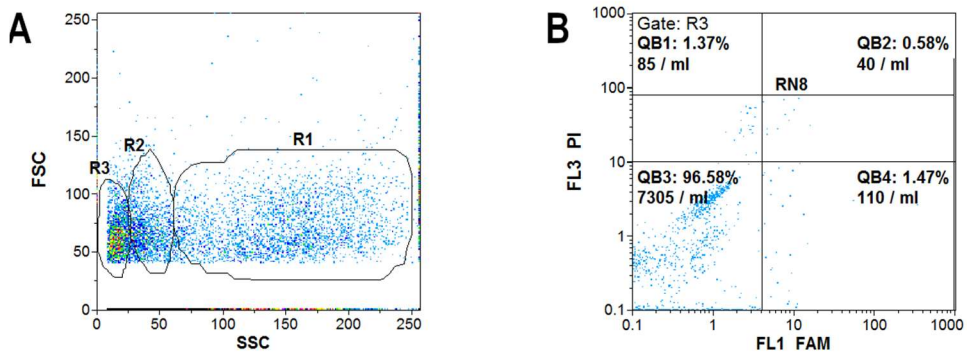

Figure 1. Flow cytometry analysis of peripheral blood white cells.

A. Forward side scatter (FSC) vs side scatter (SSC) cytogram in dot plots showing regions gating lymphocytes (R3), monocytes (R2) and granulocytes (R1). B. Flow cytometry analysis of viability of granulocytes. A two-dimensional density plot, indicating the correlation of green and red fluorescence for the cells in a data file. QB3. unstained live cells - FAM(-)PI -); QB4. cells in early apoptosis - FAM(+)PI(-); QB2. cells in late apoptosis - FAM(+)PI(+); QB1. dead cells - FAM(-)PI(+).

Statistical analysis confirmed that a one-hour incubation of blood cell samples with the tested concentrations of glyphosate did not affect nor the granulocytes viability (Table 1) neither lymphocytes (results not shown).

Table 1. The effect of incubation of blood samples with different concentrations of glyphosate on the fluorescence channel, as a rate of living cells in a granulocyte population. Data from 7 experiments. p - Friedman's test result (p>0.05 means statistically insignificant differences between the compared groups).

Glyphosate concentration [mg/L]	Percentage of living cells [%]			*p*-value
	mean ± SD	median	min-max	
0 (control)	95.85±2.03	95.88	93.04-98.50	0.2729
0.01	96.50±1.75	96.88	93.28-98.60	
0.1	96.68±0.86	97.13	95.31-97.46	
1	94.43±2.07	93.76	92.47-97.17	
10	95.55±3.14	96.84	89.29-98.09	

SD - standard deviation, min-max - minimum-maximum.

3.2. Determination of polymorphonuclear leukocytes reactive oxygen species (ROS) production

Granulocytes (as well as monocytes) produce reactive oxygen metabolites (superoxide anion, hydrogen peroxide, hypochlorous acid) when activated by different stimuli. The formation of ROS can be estimated by measuring the green fluorescence intensity of rhodamine 123, the oxidized derivative of dihydrorhodamine 123 (DHR 123) (Figure 2). To exclude artifacts, the DHR fluorescence was analyzed in the gate containing DNA-stained neutrophils (red fluorescence).

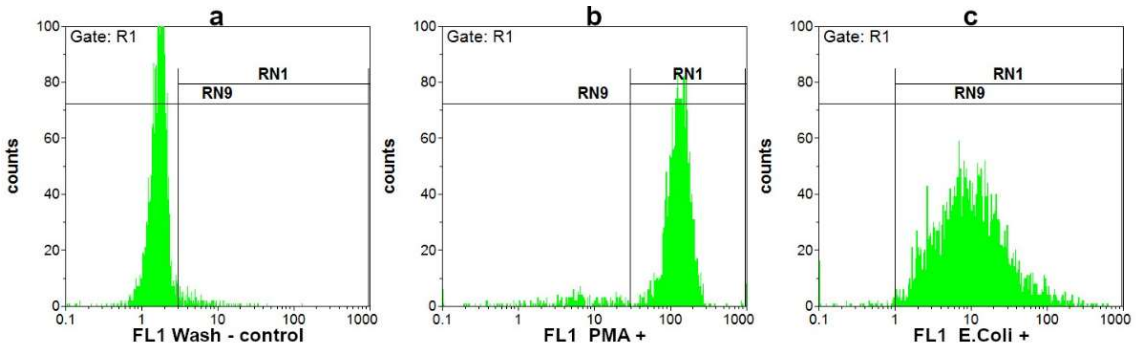

Figure 2. A histogram showing the rhodamine 123 fluorescence intensity pattern in (a) non stimulated (control), (b) PMA or (c) E.coli+ fMLP stimulated peripheral blood granulocytes.

Statistical analysis confirmed that a one-hour incubation of blood cell samples with the tested concentrations of glyphosate did not induce ROS production in non-stimulated granulocytes (Table 2) nor affect ROS production in PMA-simulated cells (Table 3). However, an increase of rhodamine 123 fluorescence intensity was observed in *E. coli* and fMLP-stimulated granulocytes (Table 4).

In experiments when granulocytes were stimulated by fMLP and *E.coli*, the preincubation of blood samples with glyphosate caused an increase of oxidative burst. All tested concentrations increased the production of free radicals, but no linear relationship was observed dose-dependent.

Table 2. The effect of incubation of blood samples with different concentrations of glyphosate on the rhodamine 123 fluorescence intensity (channel) in a non-stimulated granulocytes population. Data from 32 experiments. p - Friedman's test result (p>0.05 means statistically insignificant differences between the compared groups).

Glyphosate concentration [mg/L]	Rhodamine 123 fluorescence (channel value)			*p*-value
	mean ± SD	median	min-max	
0	1.39±0.40	1.37	0.76-2.40	0.4124
0.01	1.46±0.38	1.43	0.62-2.26	
0.1	1.44±0.51	1.31	0.67-2.55	
1	1.39±0.47	1.29	0.75-3.16	

SD - standard deviation, min-max - minimum-maximum.

Table 3. The effect of incubation of blood samples with different concentrations of glyphosate on the rhodamine 123 fluorescence intensity (channel) in a PMA stimulated granulocytes. Data from 16 experiments. p - Friedman's test result (p>0.05 means statistically insignificant differences between the compared groups).

Glyphosate concentration [mg/L]	Rhodamine 123 fluorescence (channel value)			*p*-value
	mean ± SD	median	min-max	
0	49.77±13.79	49.90	23.70-67.20	0.1409
0.01	60.99±11.38	60.70	45.20-81.80	
0.1	60.20±12.90	61.90	34.00-81.80	
0.5	61.16±10.68	60.95	42.80-83.30	
1	62.42±8.64	60.95	49.70-84.90	
5	61.19±10.77	60.65	41.30-90.40	

SD - standard deviation, min-max - minimum-maximum.

Table 4. The effect of incubation of blood samples with different concentrations of glyphosate on the rhodamine 123 fluorescence intensity (channel) in granulocytes stimulated by fMLP and *E.coli*. Data from 32 experiments. p - Friedman's test result (p>0.05 means statistically insignificant differences between the compared groups). The post hoc test results for the Friedman test mean that the concentration 0 (control) is definitely significantly different from the others, and the concentration of 0.1 mg/L is probably different from 1 mg/L.

Glyphosate concentration [mg/L]	Rhodamine 123 fluorescence (channel value)			*p*-value
	mean ± SD	median	min-max	
0	12.06±7.63	10.60	2.20-36.2	0.0000
0.01	16.74±9.97	14.60	5.20-48.9	
0.1	16.21±8.70	14.45	4.50-35.00	
1	17.67±8.32	17.65	2.60-35.30	

SD - standard deviation, min-max - minimum-maximum. The post-hoc test results: p<0.05 when 0 vs. 0.01, 0 vs.0.1 and 0 vs 1 were compared.

Table 5. Glyphosate-induced changes in DHR123 mean fluorescence channel value in the granulocytes stimulated by fMLP and *E.coli* as a percentage of the control.

Glyphosate concentration [mg/L]	0	0.01	0.1	0.5	1	5	10
DHR123 mean fluorescence channel value	12.0	16.7	16.2	19.7	17.7	18.7	14.8
Percentage of the control	100	137	135	164	147	156	123

3.3. Estimation of granulocyte phagocytic activity

The quantitative determination of leukocyte phagocytosis was done by the use of contains fluorescein (FITC)-labeled opsonized *Escherichia coli* bacteria. The cytometric analysis allowed both to measure the phagocytic activity of neutrophils (number of bacteria per cell) and the percentage of phagocytic cells.

Incubation of whole blood samples with various concentrations of glyphosate did not increase the phagocytic activity of granulocytes (Table 6). The number of phagocytic cells also did not change (results not shown).

Table 6. The effect of incubation of blood samples with different concentrations of glyphosate on the FITC fluorescence intensity (channel) in granulocytes phagocytized *E. coli*. Data from 10 experiments. p - ANOVA test result (p>0.05 means statistically insignificant differences between the compared groups).

Glyphosate concentration [mg/L]	FITC fluorescence (channel value)			*p*-value
	mean ± SD	median	min-max	
0	18.91±4.44	19.34	12.66-28.93	0.0687
0.01	16.36±4.05	16.64	9.11-22.77	
0.1	16.89±4.94	16.67	10.44-26.24	
1	18.30±5.38	18.02	10.63-29.97	
10	17.07±4.44	17.19	11.16-23.99	

SD - standard deviation, min-max - minimum-maximum.

4. DISCUSSION

Glyphosate, a strong herbicide is regarded as safe for animals. However, the field observations and laboratory experiments indicated harmful effects of this agent on different invertebrate and vertebrate species [33-37]. Among the mechanisms of such toxic action, the induction of oxidative stress should be considered. The biomarkers of such glyphosate-induced stress were found in human blood or urine [38-41] as well in plasma and tissues of various laboratories [19, 42-45] and domesticated animals [46-49].

One of the markers of the oxidoreductive balance in animal organisms is the production of reactive oxygen derivatives [ROS]. However, the results of experiments on glyphosate effect on redox status in laboratory animals were ambiguous, possibly due to the variety of analytical methods used. Oral administration of glyphosate in daily doses of 0.5 to 10 mg/kg of body weight for 28 days did not change ROS concentrations in the blood plasma of rats compared to the control group [50], while daily doses of 50 and 500 mg/kg administered for 5 weeks caused an increase in the concentration of hydrogen peroxide (H_2O_2) in the serum or liver cell homogenates [51]. Applying intraperitoneally this herbicide to animals at a dose of 50 mg/kg increased the peroxide content in hepatocytes by 50% [52]. So, it can be concluded that glyphosate can induce the production of ROS in animal tissues.

In the results of experiments with human cell lines and isolated blood cells a variation was observed. Incubation of SH-SY5Y neuroblastoma cells in medium with the addition of 5 mM glyphosate resulted in an approx. 25% increase in the level of ROS after 48 hours [53]. Production of ROS by human bronchial epithelial (BEAS-2B) cells, treated with 50 or 100 μM Roundup for 24 h, increased 1.24- and 1.47-fold as compared to control, however decreased significantly at 200 μM of this agent [54]. In 24-hour cultures of the human epidermal cell line HaCaT, the presence of this herbicide in the medium in concentrations up to 0.1mM stimulated the production of ROS almost twice [55]. The percentage of these cells showing the presence of

H_2O_2 increased from 10% to about 80% after 6 and 18 h incubation with 15, 20, 30 and 45 mM glyphosate solutions [56].

Incubation of blood samples for 1 hour with glyphosate at concentrations of 0.01 mg/L, 0.1 mg/L, 1 mg/L, 10 mg/L, i.e. from ~0.06 μM to 59 μM, did not increase production of ROS in granulocytes (Table 2). This is consistent with the studies of other authors who observed an increase in ROS content in mononuclear cells after 4 or 24 hours of incubation with glyphosate at a concentration above 0.25 mM [27,57]. When human cell lines were used, short-term incubation (0.5 h) of HaCaT human epidermal cells with 30 mM glyphosate solution did not change intracellular ROS concentrations [56]. Similarly, no increase in the concentration of ROS was noticed in the human hepG2 hepatoma cell line after 4 hours of incubation with this herbicide at concentrations from 0.5 μg/mL (0.5 mg/L) to 3.5 μg/mL (3.5 mg/L) [58].

The effect of low concentrations of glyphosate on fMLP or PMA-induced granulocyte ROS production was analyzed by flow cytometry using dihydrorhodamine 123 (DHR123). Compared to conventional quantitative techniques estimating ROS production by entire cell populations, flow cytometry measures the ROS content in individual cells, what allows the determination of functional changes within specific subpopulations.

The presence of glyphosate did not affect the respiratory burst in PMA-stimulated granulocytes but increased the production of ROS in fMLP-stimulated granulocytes (Tables 3-5). Differences in ROS production by granulocytes stimulated by fMLP or PMA was already reported in patients with chronic renal failure. Although the oxidative burst stimulated by direct activation of protein kinase C by PMA remained unchanged, *Staphyloccus aureus* and fMLP-induced burst was significantly increased [59].

The observed changes in ROS production could not be associated with cells viability or increased phagocytic activity of granulocytes. One-hour incubation of blood samples with the tested solutions of glyphosate did not cause statistically significant changes in the percentage of apoptotic or necrotic leukocytes. This is in agreement with data from other authors on the effects of low doses of glyphosate on isolated peripheral blood mononuclear cells [24,26,57,60]. Such treatment of blood samples caused also a decrease in the number of phagocytosed *E. coli*, especially at the glyphosate concentration of 0.01 mg/L (Table 6). Although observations on the effect of glyphosate on vertebrate phagocytes are few, a similar decrease in phagocytic activity was described in fishes [34].

The different effects of glyphosate on ROS production by granulocytes stimulated with PMA or fMLP and *E. coli* can reflect its action on different way of NADPH activation. One of the key processes in the activation of NADPH oxidase is the phosphorylation of its subunits by appropriate kinases. In vitro, PMA crosses the plasma membrane and directly activates protein kinases C (PKC), which in turn activates available NADPH molecules. The stimulation of granulocytes with fMLP and *E.coli* seems to reflect what happens in vivo. The binding of the bacterial fMLP to a specific receptor induces signal transduction across the plasma membrane via a family of small heterotrimeric G proteins that are activated by the exchange of bound guanosine diphosphate (GDP) to guanosine triphosphate (GTP). G proteins activate a number of enzymes: phospholipase C (PLC), phospholipase A2 (PLA2), phospholipase D (PLD) and protein tyrosine kinases, which trigger signaling cascades inside the cell leading to NADPH activation [61,62]. The glyphosate targets in this pathway are not yet identified, they may be enzymes or other regulatory proteins.

The ways of entry of glyphosate into animal cells have not yet been described. In the case of *Bacillus subtilis*, the main route of glyphosate entry is via the glutamate/aspartate symporter [62]. A cystine/glutamate and aspartate transmembrane transport system (XAG system) was described in animal cells also [63]. To some

extent, observations that incubation of cultures of HeLa or Hep G2 lines with this herbicide at concentrations of 100 µg/L (0.1 mg/L) and higher interferes with the transport of cysteine into cells, could support the hypothesis of the role of XAG in the glyphosate transmembrane transport. The similar range of the changes in the respiratory burst of granulocytes incubated with different concentrations of glyphosate (from 0.06 µM to 59 µM) allows also to suppose that this compound may cross cell membranes by active transport.

The low concentrations of glyphosate tested in this study were neither cytotoxic nor did stimulate the production of ROS in resting cells but increased the production of ROS during the phagocytosis. This may be important in some inflammatory processes, as stimulation of ROS production in such cases may result in increased tissue damage. Detectable amounts of glyphosate were found in human urine, eg. in 99% of samples from French people [64] or in 70-100% of examined samples from different Mexico regions [65]. As the glyphosate biological elimination half-life in human is estimated to be 3 to 10 hours [66-68], the presence of this herbicide in the urine of a significant part of the population indicate a general contamination, what's may cause of several problems in public health.

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