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Absorption rates and free radical scavenging values of vitamin C-lipid metabolites in human lymphoblastic cells

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: In this study we investigated the cellular absorption rates, antioxidant and free radical scavenging activity of vitamin C-lipid metabolites. The absorption was measured in a human lymphoblastic cell line using a spectrophotometric technique.

Material/Methods: Cellular vitamin C levels in the human lymphoblastic H9 cell line were measured using the 2,4-dinitrophenylhydrazine spectrophotometric technique. Free radical scavenging activity of vitamin C-lipid metabolites was measured by the reduction of 1,1-diphenyl-2-picryl hydrazyl (DPPH) to 1,1-diphenyl-2-picryl hydrazine. Vitamin C-lipid metabolite scavenging of peroxyl radical oxygen reactive species (ORAC) was determined by fluorescence spectrophotometry.

Results: Compared to ascorbic acid (AA), calcium ascorbate (CaA), and calcium ascorbate-calcium threonate-dehydroascorbate (Ester-C®), vitamin C-lipid metabolites (PureWay-C™) were more rapidly absorbed by the H9 human T-lymphocytes. The vitamin C-lipid metabolites (PureWay-C™) also reduced pesticide-induced T-lymphocyte aggregation by 84%, while calcium ascorbate-calcium threonate-dehydroascorbate (Ester-C®) reduced aggregation by only 34%. The vitamin C-lipid metabolites (PureWay-C™) demonstrated free radical scavenging activity of nearly 100% reduction of DPPH at 20 µg/ml and oxygen radical scavenging of over 1200 µ Trolox® equivalents per gram.

Conclusions: These data demonstrate that the vitamin C-lipid metabolites (PureWay-C™) are more rapidly taken-up and absorbed by cells than other forms of vitamin C, including Ester-C®. This increased rate of absorption correlates with an increased protection of the T-lymphocytes from pesticide toxicities. Further, vitamin C-lipid metabolites (PureWay-C™) are a potent antioxidant and have significant free radical scavenging capabilities.

key words: **absorption • T-lymphocyte • Vitamin C • DPPH • ORAC**

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Background

Vitamin C is an important dietary component which is required for physiological and metabolic activities including healthy neuronal development [1,2], prevention of neurodegenerative diseases [3,4], wound healing [1,5,6], and a healthy immune system [1,7,10]. Therefore, the bioavailability of vitamin C in the diet is the focus of intense research interest. A calcium ascorbate preparation with small amounts of dehydroascorbate, calcium threonate, xylonate and lyxonate, has been shown to lead to the increased cellular uptake of vitamin C [9,10], to provide increased protection from vitamin C deficiency in rats [11], and to improve uptake and circulating levels of vitamin C in humans [12]. More recently a formulation of vitamin C-lipid metabolites has been shown to more rapidly stimulate neurite outgrowth, fibroblast adhesion and protect cells of the immune system when compared to all other vitamin C formulations [2]. This increased rate of bioactivity suggests an increase in the bioavailability and rate of cellular uptake of vitamin C-lipid metabolites when compared to other vitamin C formulations.

Here, we measure the rate of cellular uptake of vitamin C-lipid metabolites (PureWay-C™) when compared to calcium ascorbate, ascorbic acid, and calcium ascorbate-calcium threonate-dehydroascorbate (Ester-C®). We also confirm that the vitamin C-lipid metabolites (PureWay-C™) maintain and deliver both high and effective antioxidant and free radical scavenging activity.

Material and Methods

Material

Dimethylsulfoxide was purchased from Sigma Chemical Co., St. Louis, Mo. and bifenthrin was purchased from Chem. Services, West Chester, PA. Bifenthrin was brought to a stock concentration of 10^{-2} M in DMSO Phytohemagglutinin was purchased from Sigma Chemical Co. St Louis, MO and suspended in RPMI 1640 and stored at –20°C. Formulations and certificates of analysis of ascorbic acid, calcium ascorbate, Ester-C® and PureWay-C™ were provided by Nature's Value, Coram, NY, from their respective suppliers, and were dissolved in sterile, serum-free RPMI-1640 at a 50 µM. 2,4-dinitrophenylhydrazine was purchased from Sigma-Aldrich (*www.sigmaaldrich.com*). 1,1-diphenyl-2-picryl hydrazine and Trolox® were provided by ChromDex Analytics (Boulder, CO) where the PDDH and ORAC assays were performed.

Cells culture and cell pellet preparation

The H9 human lymphoblastic cell line was obtained from the NIH AIDS reagent program and cultured in a medium of RPMI-1640 containing 10% fetal bovine serum and 0.01% gentamycin and incubated in a CO_2 incubator at 37°C. Cell viability and cell numbers for plating during experimentation was determined using trypan blue and a hemocytometer counting slide.

Briefly, 3.0×10^7 H9 cells were collected in into one pellet by repeated centrifugation at 500 × g and the supernatant was removed. In order to completely remove the serum component from the culture medium and starve the cells for vitamin C and nutrients that alter cellular vitamin C levels, the pellet was washed three times by repeated resuspension in 50 ml of serum free RPMI and centrifuged at 500 × g. After three washes with serum-free RPMI-1640, the pellet was resuspended in a final 20 ml of serum-free RPMI, seeded in a 150 cm^3 tissue culture flask and incubated in a CO_2 water-jacketed incubator at 37.5°C. After 18 hours of starvation the cells were collected into one pellet as described above and counted for viability using trypan blue.

In order to test the uptake of the four various forms of vitamin C, each vitamin C preparation was dissolved at 50 µM in RPMI-1640. After determining cell viability as described above, sets of eight 75 cm^3 tissue culture flask were seeded with 6×10^5 cells in 4.0 ml of RPMI-1640 containing each type of vitamin C preparation and these flasks were placed horizontally for incubation in a 37.5°C water-jacketed CO_2 incubator seeded in a and placed in the CO_2 water jacket incubator at 37.5°C. Every 15 minutes, one flask containing each of the different vitamin C preparations was removed from the incubator and agitated to assure equal cell suspension which was divided into four 1.0 ml aliquots in microcentrifuge tubes and centrifuged at 500 × g. The supernatant was discarded and the cell pellets (1.5×10^5 cells/pellet) were frozen at –20°C for further analysis. Three of the pellets were used to determine Vitamin C levels and one was used to determine protein content. This design permitted triplicate vitamin C readings at times ranging 15 through 120 minutes at 15 minute intervals for each of the four Vitamin C formulations and the determination of protein content. Therefore the cellular vitamin C concentration could be determined as nmol/mg cellular protein.

Vitamin C assay

The cellular content of vitamin C was measured using the method described by Lowry et al., 1947 [13]. Briefly, to the cell pellets, 40 µl of 5% trichloroacetic acid was added. For adequate extraction of ascorbic acid the samples were mixed well by vortex. Next, 13 µl of freshly prepared dinitrophenylhydrazine reagent (2% dinitrophenylhydrazine, 0.25% thiourea, 0.03% $CuS0_4\cdot5H_20$ prepared in 9 N H_2S0_4) was added to each tube. The samples were again mixed well and incubated at 37.5°C for six hours. The samples were then placed on ice and 50 µl of chilled (4°C) 65% H_2S0_4 were added and mixed well by vortex. The optical density of the samples was then read at 520nm in a Benchmark plus Bio-Rad microplate reader and compared to a standard curve (described below). Blanks were prepared by treating 400 µl of 5% trichloroacetic acid in the same manner as the cell pellets. The standard curve was generated by dissolving various amounts of ascorbic acid (0.05–1.5 mg/ml) in 5% trichloroacetic acid and treated in the same manner as the cell extracts.

Protein assay

The protein content of the cell pellets was determined using a BioRad Bradford Assay Kit. Briefly, the cell pellets were lysed and homogenized in 100 µl of RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% Deoxycholate and 5 mM EDTA). Next, 400 µl of the the BioRad Bradford Kit dye reagent concentrate was added directly to the samples. For the standard curve, five dilutions

of a bovine serum albumin ranging from 0.1 to 0.5 mg/ml were prepared and 400 µl of the the BioRad Bradford Kit dye reagent concentrate was added directly to 100 µl of each standard. Samples and standards were then vortexed and allowed to sit on the bench-top for 10 minutes and the absorbance of 200 µl was then read at 595 nm in a Benchmark plus Bio-Rad microplate reader.

T-cell homotypic aggregation assays

Cell were collected and 3.0 ml of 2×10^5 cells/ml were seeded in wells of a six-well tissue cluster and then treated with 10 µg/ml of phytohemagglutinin and or 10^{-5} M bifenthrin. The stock bifenthrin was 10^{-2} M in DMSO therefore a vehicle control of 0.1% DMSO was used. Therefore the control untreated cells for this experiment contained 1% DMSO. The PHA was diluted in RPMI 1640 and so did not require a vehicle control. Immediately after seeding the cells, triplicate wells were treated with the 0.5 µM ascorbic acid from the various formulations and the cells were incubated in a in a CO_2 incubator at 37°C for 30 minutes. After treatment for 30 minutes the ability of vitamin C to inhibit homotypic aggregation was measured by counting aggregate size at 400× magnification. The center field of each well was assessed by visual inspection and the number of cells in each aggregate was counted and divided by the number of aggregates to derive the number of cells per aggregate. Since the treatments were done in triplicate, three fields were counted for each treatment. While the number of aggregates was noted, these data are not presented. The number of cells per aggregates is. The treatment with few cells per aggregate also had very few aggregates to count. The aggregate size was counted

DPPH and ORAC assays

The DPPH assay was carried out as described by Vani et al., 1997 [14] with some modifications. Briefly, 200 ml of a 1mg/ml ascorbic acid solution (1.0 mg PWC in 1.0 ml distilled water) was mixed with 50 µl of 0.659 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) solution and incubated at 25°C for 20 minutes. The absorbance was then read at 510 nm.

The ORAC assay was performed as described by Cao et al., 1993 [15]. Briefly, 2,2'-Asobix (2-amidinopropane) dihydrochloride (AAPH) (0.414 g) was dissolved in 10 ml of 75 mM phosphate buffer to final concentration of 153 mM and kept on ice. The fluorescein stock solution was prepared at 4.19×10^{-3} mM in 75 mM phosphate buffer and kept at 4°C in the dark. For the (±)-6-Hydroxy-2,5,7,8- tetramethylchromane-2-carboxylic acid, (Trolox®) standard preparation, 0.25 g of Trolox® dissolved was in 50 ml of phosphate buffer to yield 0.02 M stock. Next, PureWay-C™ (PWC) was dissolved in acetone/water mixture (50–50) and subsequently diluted with 75 mM phosphate buffer (pH 7.4) to a varying extent to yield 200, 100, 50, 25, 12.5, and 6.25 µM for the test reactions. The ORAC assay detects free radical damage to the fluorescein and a loss of fluorescence. Antioxidants inhibit the free radical range damage to the fluorescent compound and prevent the reduction in fluorescence. Reactions containing the PureWay-C™ (PWC) and blanks (solvent) were run in parallel using equivalent amounts of a generator of a radical oxygen species and the fluoroscein and the area under the curve from the experimental sample was calculated. After subtracting the area under the curve for the for the blank, the resultant difference is expressed as antioxidant activity of the PureWay-C™ (PWC). Results from different concentrations are compared with Trolox® and the ORAC results are expressed as Trolox® equivalents (TE) per gram of sample.

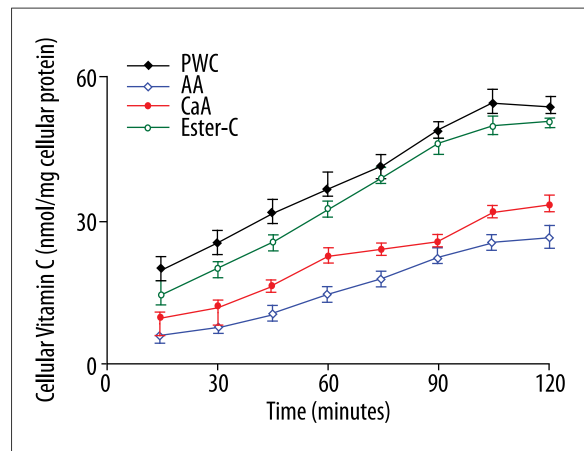

Figure 1. Vitamin C absorption rates in the H9 human T-cell line. Cells were starved of vitamin C for 18 hours in serum-free RPMI-1640 and subsequently suspended in 50 µM of vitamin C in various formulations: ascorbic acid (AA), calcium ascorbate (CaA), calcium ascorbate-calcium threonate-dehydroascorbate- Ester-C® (Ester-C), and the vitamin C lipid metabolites-PureWay-C™ (PWC). At the indicated times, cells were harvested and measured for vitamin C and protein content as described in the Materials and Methods section. An analysis of variance was performed and the asterisks indicate a significant difference at 95% confidence (Scheffe F test) between the vitamin C-lipid metabolites (PWC) and calcium ascorbate-calcium threonate-dehydroascorbate (Ester-C).

Results

The rate of vitamin C absorption in human T-cells was compared between several different vitamin C formulations. We found significantly different rates of uptake depending on the formulation of vitamin C. Over a two hour period, the level of vitamin C-lipid metabolite uptake was consistently higher than that observed with ascorbic acid, calcium ascorbate, and calcium ascorbate-calcium threonate-dehydroascorbate (Figure 1). At fifteen minutes, cellular vitamin C levels ranged from 7±1.4 nmols/mg cellular protein with ascorbic acid, to over double that amount (15±2.4nmol/mg) for the vitamin C-lipid metabolites (Figure 1). The absorbed vitamin C levels rose significantly with time, peaking at approximately two hours with cellular levels ranging from 31 nmol/mg for ascorbic acid and 50 nmol/mg for vitamin C-lipid metabolites (Figure 1).

Since ascorbic acid showed the least uptake at all time points (Figure 1), the values for ascorbic acid were considered to be 100% for all time point thus allowing a percent increase comparison for the other formulations in Table 1. When compared to ascorbic acid, calcium ascorbate-calcium threonate-dehydroascorbate (Ester-C®), showed a 189% increase in up-

Table 1. Percent differences in Vitamin C absorption rates in the H9 human T-lymphocyte line.

	Percent	Increase	Compared to ascorbic acid
	CaA	Ester-C®	PureWay-C™
Time after addition			
15 min	128	171	214
30 min	122	189	233
45 min	133	192	233
	Percent	**Increase**	**Compared to Ester-C®**
	CaA	**Ester-C®**	**PureWay-C™**
Time after addition			
15 min	–	0	125
30 min	–	0	124
45 min	–	0	122

Calcium ascorbate (CaA), ascorbate-calcium threonate-dehydroascorbate (Ester-C®), and vitamin C-lipid metabolites (PureWay-C™) all showed better cellular absorption rates compared to ascorbic acid (AA). In the top portion of the table, the levels of cellular vitamin C are presented as the percent of that observed with ascorbic acid (AA) at the indicated time points. In the lower portion of the table, the cellular levels of PureWay-C™ are shown as the percent of that observed with Ester-C®.

Table 2. Vitamin C-lipid metabolites (PureWay-C™) inhibits xenobiotic induced homotypic aggregation in human T-lymphocytes more effectively than calcium ascorbate-calcium threonate-dehydroascorbate Ester-C®.

	Activators of T-cell Aggregation		
	None	PHA	Bifenthrin
Vit. C Added			
None	10±5	170±15	300±13
AA	9±4	75±12	120±5
CaA	12±4	110±10	137±8
EsterC	8±2	130±17	200±8
*PWC	11±6	20±9	50±10

Human H9 T-lymphocytes were either untreated (None) or treated with activators of T-lymphocyte aggregation phytohemagglutinin (PHA) or bifenthrin and then either given no further vitamin C treatments (None) (note, bifenthrin was dissolved in 0.1% DMSO, so the no treatments contained 0.1% DMSO as a vehicle control) or treated further with 50 µM ascorbic acid (AA), calcium ascorbate (CaA), Ester-C® (EsterC) or PureWay-C™ (PWC). The cells were then incubated for 30 minutes and the number of cells per aggregate was determined as described in the Materials and Methods section. The error is represented as ± the standard error of the mean with an n of 6.
* While all vitamin C preparations stimulated a statistically significant reduction in aggregation, PWC (PureWay-C™), showed a statistically significant greater reduction when compared to Ester-C®. PHA was used as a positive control for the activation of aggregation.

take, which is similar to earlier reports of 177% at 30 minutes. The vitamin C-lipid metabolites (PureWay-C™) showed a 233% increase at both 30 and 45 minutes compared to ascorbic acid (Table 1) which is over a 120% improvement in absorption in the first 45 when compared to calcium ascorbate-calcium threonate-dehydroascorbate (Ester-C®).

The increase rate of absorption of the vitamin C-lipid metabolites correlated with a greater protective effect at after 30 minutes of exposure to pesticide (Table 2). When cells were treated with the pesticide bifenthrin and various formulations of vitamin C at time zero, the resultant pesticide-mediated aggregation of the T-lymphocytes was reduced by 84% with bifenthrin and only by 34% with calcium ascorbate-calcium threonate-dehydroascorbate treatment (Table 2).

In order to confirm that the vitamin C-lipid metabolite formulation has potent antioxidant and have free radical scavenging capabilities, ORAC and PDDH evaluations were conducted. Figure 2 shows that the vitamin C-lipid metabolites were able to scavenge 93% of the PDDH free radicals at 20 µg/ml (Figure 2). Gallic acid, a known scavenger was used as a positive control and also showed a maximal 93% scavenging. The vitamin C-lipid metabolites showed a classic dose dependency scavenging of DPPH free radicals (Figure 2) and by reaching 93% scavenging capability is indicative of an excellent free radical scavenger [14].

In addition to free radical scavenging, the antioxidant capabilities of the vitamin C lipid metabolites were measured by the ORAC method and the values obtained were compared to other sources of antioxidants also measured by the ORAC method (Table 3). The vitamin C lipid metabolites showed the stronger antioxidant activity on a gram basis than common natural sources of antioxidants. For example, PureWay-C™ has over 1300 units of antioxidant activity per gram, in stark contract to freeze-dried Acai, teas, some berries, and broccoli (Table 3). While these comparisons are limited in value, it can be concluded that PureWay-C™ is an excellent dietary supplement for it antioxidant capabilities.

Discussion

Vitamin C is an important component of the diet to ensure healthy physiological and metabolic activities such as the development of a healthy nervous system [1,2]; prevention of neurodegenerative diseases [3,4]; wound healing *in vitro* [5], and *in vivo* [6]; and protection of the immune system from xenobiotics [1,7,8]. For example, vitamin C has been shown to enhance NGF-mediated neurite outgrowth [1,2]. Further, fibroblast interaction with extracellular matrix proteins and subsequent migration are indicators of wound healing events [22–24], and, indeed, vitamin C has been shown to enhance fibroblast adhesion to and interaction with the extracellular matrix [1,25,26]. Further, leukocyte cell-cell adhesion is associated with xenobiotic induced hyperactivation and inflammatory damage [1,7,8,27], and

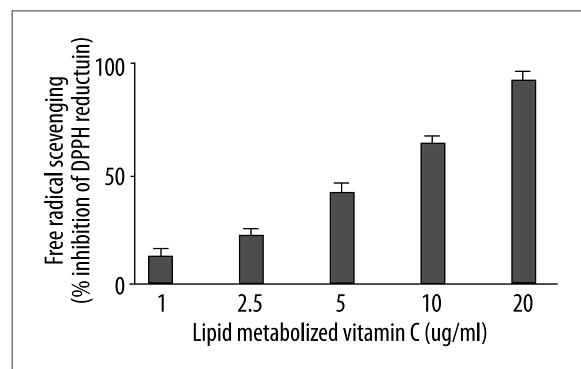

Figure 2. Vitamin C lipid metabolite scavenging of free radicals. The lipid metabolized vitamin C, at concentrations ranging from 1–20 µg/ml was mixed with 2,2-diphenyl-1-picryl hydrazyl (PDDH) solution and incubated at 25°C for 20 minutes. The absorbance was then read at 510 nm as described in the Materials and Methods section.

vitamin C has been shown to prevent cigarette smoke-induced leukocyte aggregation and attachment to vascular endothelium [7,8]. Vitamin C has also been shown to reduce pesticide mediated T-cell hyperactivation [1]. For each of the above beneficial activities, vitamin C-lipid metabolites (PureWay-C™) have been shown to be more active and act more quickly than calcium ascorbate-calcium threonate-dehydroascorbate (Ester-C®) [1].

In order for vitamin C to exert these benefits; it must be taken into the cell. Therefore, the cellular uptake and retention of vitamin C have been an area of significant study and formulations of vitamin C have been examined for rates of cellular uptake. To date, the most rapidly adsorbed vitamin C formulation, calcium ascorbate-calcium threonate-dehydroascorbate (Ester-C®) was shown to have a 177% increased absorption in the H9 human T-cell line [7]. The level of vitamin C in human T-lymphocytes in fasting has been measured at approximately 12 nmol vitamin C per mg of cellular protein (12 nmol/mg) [28] and as high as 88 µg vitamin C per 10^8 T-cells in people eating a healthy diet [29]. Here, after starving human T-cell *in vitro*, we find that the ascorbic acid uptake in these cells peaks at 31 nmol/mg cellular protein or 54 µg/10^8 cells while the vitamin C-lipid metabolites peak at 51 nmol/mg cellular protein or 90 µg/10^8 cells. The more rapid cellular localization of vitamin C-lipid metabolites (PureWay-C™) compared to ascorbic acid and calcium ascorbate-calcium threonate-dehydroascorbate (Ester-C®) explains the ability of this formulation to better enhance neurite formation and fibroblast adhesion. Here we confirm that the increase cellular absorption of the vitamin C-lipid metabolites (PureWay-C™) is directly correlated to an improved protection of human T-lymphocytes from xenobiotic-induced hyperactivation.

Vitamin C is a chemical reducing agent (antioxidant) in many intracellular and extracellular reactions such as oxidative DNA and or protein damage, low-density lipoprotein oxidation, lipid peroxidation, oxidants and nitrosamines in gastric juice, extracellular oxidants from neutrophils and endothelium-dependent vasodilation. Vitamin C-lipid metabolites (PureWay-C™), which exhibited potent antioxidant and free radical scavenging effect *in vitro*, can serve as a good dietary for further evaluation of their bio-efficacies and molecular and biological mechanism *in vitro* as well as *in vivo* on antioxidation effects, and may provide efficient antioxidant protection to humans and animals from all these oxidation products or processes of oxidation that contribute to the pathogenesis of cancer, cardiovascular diseases, and other age-related diseases by cytotoxic, genotoxic and proinflammatory mechanism and atherosclerosis.

Table 3. ORAC valuses comparing the antioxidant activity of vitamin C lipid metabolites (PureWay-C™) with known dietary antioxidants.

Nutrient source	ORAC (µM TE/g)	Reference	[Ref#]
PureWay-C™	1343	Present study	
	trial #1 1062		
	trial #2 1394		
	trial #3 1402		
	trial #4 1440		
Cinnamon	1243	Lan et al., 2007	[16]
Freeze-Dried Acai	1027	Schauss et al., 2006	[17]
Green and black teas	761.1 (235–1526)	Prior and Cao, 1999	[18]
Chokeberry	161	Wu et al., 2004	[19]
Broccoli	65.8 to 121.6	Kurilich et al., 2002	[20]
Soft wheat	32–48	Moore et al., 2005	[21]
Careless gooseberry	21	Wu et al., 2004	[19]

The ORAC values of the vitamin C-lipid metabolites was measured four times (trial #1–#4) and the average is presented. Here the ORAC values are presented in µM Trolox® Equivalents/gram of substance tested. ORAC values can often be express as per serving, or per volume, etc. Here a review of the literature for reports showing ORAC values per gram was prepared so that the comparison with the vitamin C-lipid metabolites could be made in the same units.

Conclusions

Vitamin C-lipid metabolites (PureWay-C™) are excellent antioxidants and free radical scavengers and are more rapidly absorbed and lead to higher cellular vitamin C than do calcium ascorbate-calcium threonate-dehydroascorbate or any other tested vitamin C formulation. This rapid uptake explains the ability of the vitamin C-lipid metabolites (PureWay-C™) to better enhance neurite outgrowth and wound healing [1] and to provide protection to the cells of the immune system from pesticide exposure as shown here.

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