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Effect of multivitamins in an effervescent preparation on the respiratory burst of peritoneal macrophages in mice

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The effect of regular intake of low doses of an effervescent multivitamin preparation on the free-radical-producing activity of murine peritoneal macrophages under conditions resembling a possible infection was studied *in vitro*. Initially, several groups of mice were fed a basal diet and given, for 2 weeks, water without or with supplementation of either α -tocopherol, ascorbic acid, riboflavin or a multivitamin preparation. The supplementation period was followed by a 2-week wash-out time interval during which control and multivitamin groups received deionized water. Macrophage stimulation tests using chemiluminescent spectroscopy were performed at the end of the supplementation and wash-out periods to determine cell counts and their capacity to produce free radicals. Multivitamin supplementation increased the number, and the reactive oxygen species-producing activity, of macrophages. This effect persisted for 2 weeks after higher doses of supplementation were stopped. Multivitamin supplementation lowered the steady-state free radical concentrations of liver and spleen as measured by electron paramagnetic resonance spectroscopy. It also increased the antioxidant reactivity of the same organs, while there was no effect on the free radical concentration and antioxidant capacity of the kidney and brain. When taken regularly, low doses of multivitamin supplementation may have a beneficial effect on the defence mechanisms of the organism.

Vitamins: Reactive oxygen species: Chemiluminescence: Respiratory burst

Nutrition has a great importance in the maintenance of immunity and health of both man and animals (for reviews see: Brown, 1977; Sharmanov, 1982; Burkholder & Swecker, 1990; Chandra & Kumari, 1994; Hannigan, 1994; Keusch, 1998; Chandra, 1999). Nutritional deficiencies impair immune responsiveness and, thereby, increase morbidity and mortality. On the other hand, nutritional supplementation of vitamins and minerals often enhances certain aspects of immune function. There is a great number of different vitamin and mineral preparations available in the form of caplets or effervescent tablets recommended for daily consumption in addition to an everyday diet.

A group of vitamins with potent antioxidant activities have recently received a great deal of attention because of their action on immunity and disease aetiology (Bendich, 1996; Meydani *et al.* 1998; Hughes, 1999). Superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are continuously produced in the course of normal aerobic cellular metabolism and are capable of injurious interactions with cell macromolecules (de Zwart *et al.* 1999). On the other hand, phagocytic immune cells like neutrophils, monocytes and macrophages, when stimulated undergo respiratory burst to produce high levels of these reactive oxygen species (ROS) to kill invading pathogens (Babior, 1978). Thus, respiratory burst and the consequent high production of ROS play a crucial role in host defence against micro-organisms and represents part of the defence mechanisms of the organism.

There are many studies in the literature dealing with the effect of different vitamins on the activity and respiratory burst of phagocytic immune cells. High dietary intakes of vitamin A (retinyl palmitate) increased *in vitro* phagocytic ability and tumouricidal activity of peritoneal macrophages, as well as mitogenic activity of isolated murine splenocytes (Moriguchi *et al.* 1985). Additional, but non-toxic, amounts of retinyl palmitate have been shown to lower the susceptibility of rats to salmonella infection *in vivo* by enhancing phagocytic functions of isolated Kupffer cells, peritoneal and splenic macrophages (Hatchigian *et al.* 1989). Dietary deprivation of B vitamins, and especially

Abbreviations: EPR, electron paramagnetic resonance; ROS, reactive oxygen species.

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that of riboflavin, significantly lowers the macrophage counts in murine spleen (Matthews *et al.* 1994). Treatment of monocytic human cell lines with cholecalciferol *in vitro* increases their phagocytic activity and enhances their oxidative burst capacity (Hauck *et al.* 1997).

There are several studies showing a cooperative effect among multiple antioxidants (Palozza & Krynski, 1992; Gilligan *et al.* 1994; Nyyssonen *et al.* 1994; Chen & Tappell, 1995; Knudsen *et al.* 1996; Thomas *et al.* 1996; Mosca *et al.* 1997; Eberlein-König *et al.* 1998) focusing mainly on protection against oxidative damage of lipids and proteins, but there are not many studies reporting cell-mediated immune responses. Migration and phagocytosis increases when vitamins C or E are added to head-kidney leukocytes of gilthead seabream *in vitro*, while the respiratory burst is synergistically enhanced when both vitamins are used together (Mulero *et al.* 1998). This is explained by the ability of vitamin C to regenerate membrane-bound oxidized vitamin E (Tappell, 1962; Nikki, 1987; Chan, 1993). Although these vitamins are effective antioxidants *in vitro*, the results of *in vivo* and *ex vivo* studies are conflicting (McCall & Frei, 1999). An α-tocopherol acetate-enriched or -deficient diet failed to significantly activate Kupffer cell phagocytosis and to influence liver injury in rats (Rücker *et al.* 1997). Vitamin C when incubated *in vitro* neutralized the free radicals produced during respiratory burst of murine peritoneal phagocytic cells, but showed no effect on ROS production in cells harvested from mice pretreated with high doses of the vitamin *in vivo* (Arquette & Caren, 1992).

Current evidence is insufficient to draw conclusions on the effect of vitamins, and especially of vitamin combinations on the activity of phagocytic immune cells when taken as a supplement to an average diet. Therefore, we studied the effect of a low dose supplementation of a multivitamin preparation on the ROS-producing activity of murine peritoneal macrophages, and compared the results with those obtained in cells isolated from mice supplemented with α-tocopherol, ascorbic acid or riboflavin alone. When mentioning low doses of supplementation it has to be considered that animals were kept on a basal, not vitamin-deficient diet, meaning that they have received enough vitamins for the maintenance of normal cell functions on daily basis without any supplementation. We chose a typical effervescent multivitamin preparation readily accessible to consumers, which consists of ten vitamins, each at a dose corresponding to 100 % of the recommended dietary allowance for human adults (National Research Council, 2001).

Materials and methods

Experimental design

Five-week-old pathogen-free NMRI (Crl:NMRI BR SPF) male mice weighing 18–20 g were obtained from Charles River Co. (Budapest, Hungary). They were kept under standard conditions and fed a basal preformulated rat-mouse diet R/M-Z+H 15 mm (Ssniff Spezialdiaten GmbH, Soest, Germany). Animals were randomly divided into thirteen groups each containing twenty to twenty-five animals. Food and water were given *ad libitum*. On average, mice consumed 2 ml water and 2–3 g chow/d. Four control groups (Control 1, Control 2, Control 3 and Control 4) received basal diet and deionized water only, while two placebo groups (Placebo 1 and Placebo 2) received the basal diet for a 2-week period and an effervescent placebo in their drinking water, which contained the carrier components of the multivitamin supplement at two different concentrations without the vitamins. The other seven groups received basal diet and drinking water supplemented with an effervescent preparation containing either vitamin E in the form of α-tocopherol acetate (α-Tocopherol group), vitamin C or ascorbic acid (Ascorbic acid group), Vitamin B_2 or riboflavin (Riboflavin group), or a multivitamin complex (Plusssz Multivitamin formulated by Pharmavit Rt., a Bristol–Myers Squibb Company, Veresegyház Hungary) at two different concentrations (Multivitamin 1 and Multivitamin 2 groups at 2X dose, Multivitamin 3 and Multivitamin 4 groups at 5X dose (2X meaning a dose (calculated based on a 20 g mouse body weight) that is twofold, and 5X meaning a dose that is fivefold, the recommended dietary allowance for human adults)). Based on the manufacturer's formulation of the rat–mouse chow R/M-Z+H (Ssniff Spezialdiaten GmbH), the 2X dose did not exceed 10 % of an average daily vitamin intake of the mice). After 2 weeks, animals from Control 1 and 3, Placebo 1 and 2, Vitamin E, Vitamin C, Vitamin B_2, Multivitamin 1 and 3 groups were killed: elicited peritoneal macrophage cells were isolated from ten animals and organs from the remaining ten to fifteen animals extracted for free radical measurements. Control 2 and 4, Multivitamin 2 and 4 groups were given the basal diet and deionized water without supplementation for another 2 weeks (wash-out period), and killed 4 weeks after the beginning of the experiment. The vitamin content and doses of supplementation are given in Table 1. The protocol and animal care was approved by the local Committee for the Care and Use of Laboratory Animals in accordance with the European Community guidelines.

Cell culture

Mice were injected intraperitoneally with 1 ml sterile sodium caseinate (100 g/l; Sigma, St Louis, MO, USA) prepared in NaCl (9 g/l). Elicited macrophages were harvested by peritoneal lavage 48 h after injection of caseinate: 5 ml RPMI 1640 medium (Sigma) containing fetal calf serum (100 ml/l; Gibco BRL™, Life Technologies Ltd., Paisley, UK) and heparin (5 u/ml) was injected into the peritoneal cavity, which was then massaged, and the fluid withdrawn. An additional 5 ml portion of the medium was injected into the cavity and the procedure repeated. The peritoneal exudate cells (primarily macrophages) were counted, viability tests performed using trypan blue staining, and viable cell concentration was adjusted in the same medium to 2×10^6 cells in each glass cuvette of the luminometer. To obtain a homogeneous macrophage monolayer, incubations were performed for 2 h at 37°C in the glass cuvettes, after which the non-adhered cells (macrophages and lymphocytes) were washed off with Hank's balanced salt solution (Sigma; pH 7·2).

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Table 1. Content and doses of effervescent vitamin supplementation in mice

	Daily dose			
	2X†		5X†	
Vitamins*	mg/kg body weight per d	μg/ml drinking water	mg/kg body weight per d	μg/ml drinking water
α-Tocopherol	0·3	3·0		
Ascorbic acid	2·0	20·0		
Riboflavin	0·05	0·5		
Multivitamin:				
α-Tocopherol	0·3	3·0	0·75	7·5
Ascorbic acid	2·0	20·0	5·0	50·0
Thiamin	0·04	0·4	0·1	1·0
Riboflavin	0·05	0·5	0·125	1·25
Pyridoxine	0·06	0·6	0·15	1·5
Cyanocobalamin	$0{\cdot}03 \times 10^{-3}$	$3{\cdot}0 \times 10^{-4}$	$0{\cdot}075 \times 10^{-3}$	$7{\cdot}5 \times 10^{-4}$
Folic acid	0·006	0·06	0·015	0·15
Biotin	0·004	0·04	0·01	0·1
Nicotinamide	0·5	5·0	1·25	12·5
Pantothenic acid	0·17	1·7	0·425	4·25

* Vitamins were formulated by Pharmavit Rt., a Bristol–Myers Squibb Co., Hungary.
† 2X, 5X, doses corresponding to two- and fivefold the recommended dietary allowance for adult humans (National Research Council, 2001) based on body weight.

Luminol-dependent chemiluminescence measurements

Details of chemiluminescence measurements have been described by us earlier (Gál *et al.* 1997; Kriska *et al.* 1999). In summary, measurements were performed by a VG-05-type computer-controlled chemiluminometer (Central Research Institute for Chemistry, Hungarian Academy of Sciences, Budapest, Hungary) with a 9658 EMI (S-20) type photomultiplier. Macrophages were stimulated with 0·5 mg zymosan/ml obtained from Sigma (Castranova *et al.* 1987). The reaction mixture (2 ml) contained 100 μM-luminol, bovine serum albumin (2 g/l; Sigma), and glucose (2 g/l; Sigma). Luminol, 3-aminophthalhydrazide, was purchased from Sigma and prepared as a stock solution in dimethylsulfoxide at a concentration of 100 mM.

Measurement of total antioxidant reactivity of tissue homogenates

The capacity of biological samples to scavenge free radicals was determined based on the method described by Lissi *et al.* (1995), which monitors the intensity of luminol-induced chemiluminescence by radicals derived from the thermolysis of 2,2′-azo-bis(2-amidinopropane). The luminescence intensity is quenched by the addition of biological fluids or samples like tissue homogenates. Briefly, approximately 100 mg liver or spleen tissue were homogenized in PBS solution on an ice bath using an Elvehjem (Wheaton Science Products, Millville, NJ, USA) potter to a final homogenate concentration of 100 g/l. The homogenates were centrifuged at 500 ***g*** for 10 min at 4°C and supernatant fractions were used for measurements of antioxidant reactivity. Homogenates (25 μl) were added to 2·7 ml 100 mM-glycine buffer, pH 8·6 containing 10 μM-luminol at 40°C in the cuvette of the chemiluminometer. 100 mM-2,2′-azo-bis(2-amidinopropane) (Fluka, Buchs, Switzerland) (−0·3 ml, final concentration 10 mM), were added to the reaction mixture and measurements started right away. Trolox, ((±)-6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid), a soluble vitamin E derivative purchased from Fluka, was used as standard to quantify the reductive capacity of tissue samples.

Electron paramagnetic resonance measurements of frozen tissue samples

Electron paramagnetic resonance (EPR) spectroscopy is a direct method for detection of free radicals and was used to measure the *in vivo* generated free-radical concentration of tissue samples. Tissue sampling was accomplished within 5 min of killing the animals (when the organs are still alive) by freezing an approximately 100 mg rod-shaped piece of brain, kidney, spleen and liver in liquid N_2. The spectra were obtained using an X-band computer-controlled spectrometer with a 100 kHz modulation frequency constructed by Magnettech GmbH (Berlin, Germany). Measurements were performed in a quartz finger-dewar filled up with liquid N_2 in a similar way to that described earlier (Shulyakovskaya *et al.* 1993). The concentrations of free radicals were determined using a MnO–Mn internal standard and an EPR simulation program developed by Dr Antal Rockenbauer (Rockenbauer & Korecz, 1996) at the Technical University of Budapest, Hungary.

Statistical analyses

Unpaired Student's *t* test was used to determine the significance of the differences between mean values of the groups using Statistica™ program package (Statsoft, Tulsa, OK, USA). Differences associated with P values $<0{\cdot}05$ or $<0{\cdot}01$ were regarded as statistically significant.

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Results

Compared with control groups, the placebo induced no significant changes in any of the measured variables.

The number of elicited peritoneal macrophages increased in caseinate-injected animals that were pretreated with α-tocopherol, riboflavin or the multivitamin complex, but had no effect in animals pretreated with ascorbic acid in a dose that was twofold the recommended dietary allowance for human adults (National Research Council, 2001) (Table 2). The cell counts in mice given the multivitamin preparation remained high for 2 weeks after supplementation was terminated and animals were given basic diet and deionized water again.

We have shown previously (Gál *et al.* 1997) that luminol-dependent chemiluminescence during respiratory burst of zymosan-stimulated macrophage cell cultures gives an asymmetrical bell-shaped curve, while resting cells give no detectable signal. Under controlled conditions, this is a useful model for quantitative evaluation of the amount of ROS produced by cells *in vitro* (Németh *et al.* 1999). Figure 1 shows a typical luminol-dependent chemiluminescence response of zymosan-stimulated murine peritoneal macrophages elicited from control and multivitamin-supplemented mice for 2 weeks. Addition of 1 mM-N^G-methyl-L-arginine, an NO synthase inhibitor to the reaction mixture had no effect on the intensity of the signal (results not shown), suggesting no significant contribution of NO radicals to luminol-dependent chemiluminescence.

The ability of stimulated macrophages to produce ROS was elevated only in cells elicited from animals supplemented with riboflavin and the multivitamin preparation compared with control and placebo groups (Table 2). Values obtained with cells isolated from multivitamin-supplemented animals were significantly higher than those obtained in cells isolated from animals pretreated with α-tocopherol, ascorbic acid or riboflavin alone, showing an additive effect. This difference in ROS-producing capacity of cells isolated from the multivitamin group disappeared after the 2-week wash-out period. Results were similar when the experiment was repeated with a higher dose of the multivitamin mixture, fivefold the recommended dose for adult humans (National Research Council, 2001) (Table 3). In this case, however, macrophages retained their high ROS-producing ability even after the 2-week wash-out period, suggesting that the effect of some of the vitamins on the activity of immune cells persists longer when taken at higher doses.

In order to see how multivitamin supplementation affects the overall free-radical concentration of some of the organs, we have measured the steady-state free-radical concentration of the spleen, liver, kidney and brain tissues using EPR spectroscopy. Samples submerged in liquid N_2 give rise to a broad and intense singlet, which is the envelope of EPR signals derived from many organic radicals (Reynolds & Moslen, 1980). Figure 2 represents a typical EPR spectrum of the kidney, where points indicate the measured values and the line shows the simulated spectrum.

Results in Table 4 have been derived from the measured EPR signals and show that the overall free radical concentration of the liver and spleen was affected by the 2-week supplementation with α-tocopherol, riboflavin and the multivitamin preparation, whereas the free-radical content of the kidney and brain remained unchanged. The differences

Table 2. Number of elicited peritoneal macrophages and their reactive oxygen species (ROS)-producing capacity in mice drinking different solutions of vitamins at a 2X dose§ for 2 weeks and after a wash-out period of another 2 weeks||

(Mean values and standard deviations for nine or ten mice per group)

	No. of macrophages ($n \times 10^6$ cells)		ROS-producing activity (2×10^6 cells)			
			% Control		% Placebo	
Vitamins¶	Mean	SD	Mean	SD	Mean	SD
After 2-week supplementation at a 2X dose§						
Control 1	6·49	1·84	100			
Placebo 1	5·76	0·98	112·1	16·6	100	
α-Tocopherol	9·79*††	1·02	132·9	49·5	118·5	43·4
Ascorbic acid	7·08	0·75	163·4	52·4	145·9	46·3
Riboflavin	12·5*††	2·96	177·9*	54·1	158·8†	38·3
Multivitamin 1	11·06*††	2·26	253·2**‡	72·4	226·1††‡	64·2
After 2-week wash-out period						
Control 2	5·95	1·23	100			
Multivitamin 2	10·34*	1·67	134·5	28·2		

Mean values were significantly different from those of the control groups: *$P<0·05$, **$P<0·01$.
Mean values were significantly different from those of the placebo group: †$P<0·05$, ††$P<0·01$.
Mean values were significantly different from those of individual vitamin treatments: ‡$P<0·05$.
§ 2X, dose corresponding to twofold the recommended dietary allowance for adult humans (National Research Council, 2001) based on body weight.
|| For details of diets, supplements and procedures, see Table 1 and p. 502. Peritoneal exudate cells (primarily macrophages) were counted and, viability tests performed using trypan blue staining. ROS-producing ability was measured based on luminol-dependent chemiluminescence during respiratory burst of zymosan-stimulated macrophage cell cultures *in vitro*.
¶ Vitamins were formulated by Pharmavit Rt., a Bristol–Myers Squibb Co., Hungary.

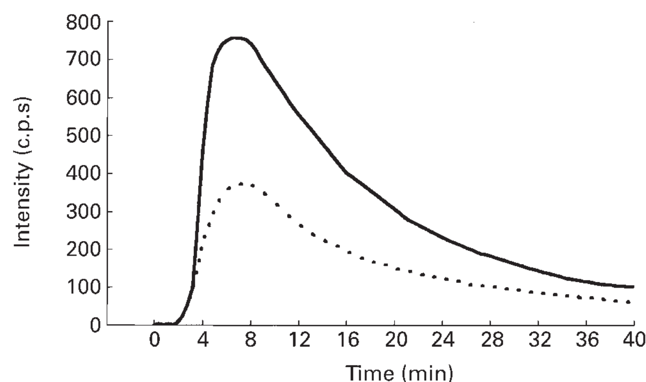

Fig. 1. Time course of luminol-dependent chemiluminescence of stimulated murine peritoneal macrophages elicited from control mice and mice supplemented with multivitamin preparation for 2 weeks. —, Multivitamin; ----, control. For details of diets, supplement and procedures, see Table 1 and p. 502. To obtain a monolayer, 2×10^6 cells were incubated for 2 h at 37°C in a glass cuvette of the luminometer. After several washes, macrophages were stimulated with 0·5 mg zymosan/ml in a medium containing 100 μM-luminol (time 0). The emitted light was measured using a chemiluminescence spectrometer.

disappeared 2 weeks after the termination of supplementation. We looked at the total antioxidant reactivity of liver and spleen (Table 5) and found that their reductive capacity has been significantly increased in cases of α-tocopherol-, multivitamin-, and/or ascorbic acid-, riboflavin-pretreatment.

Discussion

Macrophages stimulated *in vitro* with zymosan (phagocytizable yeast-cell-wall particles) give a respiratory burst response similar to that of resident cells during the phagocytosis, which is a key process in combating infections in man and animals. The effect on the respiratory burst capacity of macrophages upon stimulation was additive in animals pretreated with multivitamins compared with those given only additional α-tocopherol, ascorbic acid or riboflavin (Tables 2(A) and 3(A)). These results indicate that even low doses of multivitamins taken as a supplement to the basal diet are able to modulate the functions of macrophages. There was no concentration-dependent difference in the magnitude but in the duration of the ROS-producing response, suggesting that lower doses of multivitamins are enough to obtain the maximum effect.

Among non-enzymatic antioxidant α-tocopherol, ascorbic acid and riboflavin are of major importance (Abrams *et al.* 1993; Böhles, 1997). α-Tocopherol is the most relevant radical scavenger in lipid phases, while ascorbic acid is a potent antioxidant in aqueous fluids and cell compartments. Riboflavin acts mainly as cofactor of glutathione reductase keeping glutathione in reduced state. It can therefore be considered as an indirect water-soluble antioxidant vitamin. Our findings show that not only high (Moriguchi *et al.* 1990; Gore & Qureshi, 1997), but also low doses of α-tocopherol supplementation are able to increase the number of elicited macrophages *in vivo*. This could be due to a vitamin-induced enhancement of macrophage proliferation or to the migration of these cells in higher proportions to the peritoneum of α-tocopherol-supplemented animals. Carotenoids and α-tocopherol have been reported to increase the proliferative capacity of phagocytic cells (Moriguchi *et al.* 1990; Chew, 1995), while ascorbic acid and α-tocopherol were able to increase their migration (Mulero *et al.* 1998; Del Rio *et al.* 1998). In our present case, the cause of higher cell numbers has yet to be established. Interestingly, supplementation with low doses of riboflavin increased not only the number, but also the ROS-producing activity of

Table 3. Number of elicited peritoneal macrophages and their reactive oxygen species (ROS)-producing capacity in mice drinking different solutions of vitamins at a 5X dose‡ for 2 weeks and after a wash-out period of another 2 weeks§

(Mean values and standard deviations for ten mice per group)

	No. of macrophages ($n\times10^6$ cells)		ROS-producing activity (2×10^6 cells)			
			% Control		% Placebo	
Vitamins‖	Mean	SD	Mean	SD	Mean	SD
After 2-week supplementation at a 5X dose‡						
Control 3	6·23	1·70	100			
Placebo 2	5·22	1·18	119·3	15·1	100	
Multivitamin 3	12·56††	1·89	229·5**	36·3	192·4††	24·1
After 2-week wash-out period						
Control 4	5·92	1·16	100			
Multivitamin 4	9·98**	1·02	172·8**	15·3		

Mean values were significantly different from those of the control groups: $^{**}P<0{\cdot}01$.
Mean values were significantly different from those of the placebo group: $^{††}P<0{\cdot}01$.
‡ 5X, dose corresponding to fivefold the recommended dietary allowance for adult humans (National Research Council, 2001) based on body weight.
§ For details of diets, supplements and procedures, see Table 1 and p. 502. Peritoneal exudate cells (primarily macrophages) were counted and viability tests performed using trypan blue staining. Ros-producing ability was measured based on luminol-dependent chemiluminescence during respiratory burst of zymosan-stimulated macrophage cell cultures *in vitro*.
‖ Vitamins were formulated by Pharmavit Rt., a Bristol–Myers Squibb Co., Hungary.

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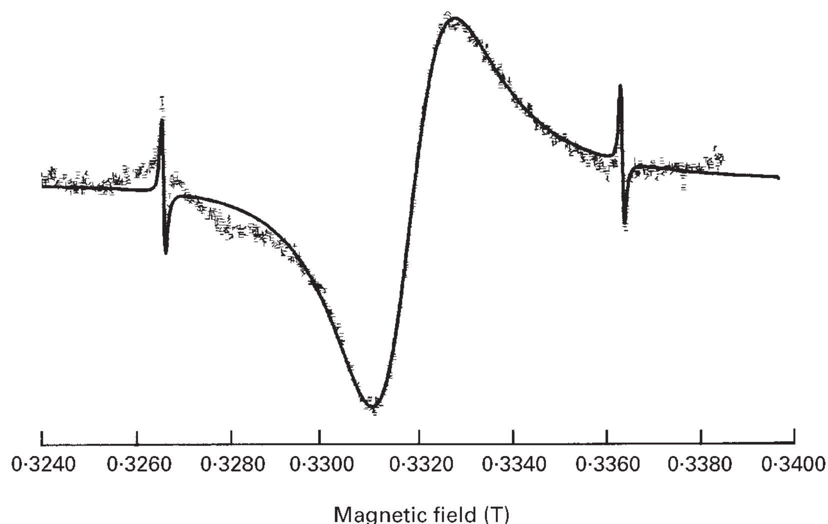

Fig. 2. Typical electron paramagnetic resonance spectrum of the kidney between two manganese peaks. Measurements were performed using an X-band electron paramagnetic resonance spectrometer in a quartz finger-dewar filled up with liquid nitrogen. The concentrations of free radicals were determined using a MnO–Mn internal standard. For details of procedures see p. 502. Points indicate the measured values and the line shows the simulated spectrum.

peritoneal macrophages, a phenomenon never reported before. α-Tocopherol has been shown to suppress Kupffer cell activation at points beyond the initiation of activation and free-radical release *in vitro* (Fox *et al.* 1997). This may explain why α-tocopherol was not effective in increasing ROS-producing activity of peritoneal macrophages.

It should be mentioned that in our present study we used cells that were elicited into the peritoneal cavity by prior injection of caseinate and stimulated to produce ROS by addition of zymosan *in vitro*. Under normal circumstances, macrophages become activated after an infection with parasites. Elicited or activated peritoneal macrophages respond to stimulation with particles or other agents much more vigorously than do resident cells (for review see Badwey & Karnovsky, 1980). Therefore, one must take account of the state of their 'activation'. Our present results refer to an elicited macrophage population and show that multivitamin supplementation of the diet can modify their ROS-producing ability after being stimulated *in vitro*, as it could possibly happen in the case of an *in vivo* infection.

Free-radical mechanisms of liver injury have been proposed for a number of xenobiotic compounds. Only α-tocopherol and multivitamin supplementations were able

Table 4. Concentration of free radicals in the organs of mice drinking different solutions of vitamins at a 2X dose† for 2 weeks and after a wash-out period of another 2 weeks‡

(Mean values and standard deviations for ten to fifteen mice per group)

	Concentration of free radicals (AU)							
	Liver		Kidney		Spleen		Brain	
Vitamins§	Mean	SD	Mean	SD	Mean	SD	Mean	SD
After 2-week supplementation at 2X dose†								
Control 1	1028	194	1154	177	412	105	93	13
Placebo 1	986	158	1305	158	410	79	92	17
α-Tocopherol	827**	125	1042	153	318**	53	99	15
Ascorbic acid	949	121	1063	201	389	97	114	20
Riboflavin	1030	184	1084	125	310**	52	111	23
Multivitamin 1	889*	174	1152	174	337*	54	97	9
After 2-week wash-out period								
Control 2	1124	170	1020	119	445	146	91	13
Multivitamin 2	1177	162	1268	215	381	131	100	14

Mean values were significantly different from those of the control groups: $^{*}P<0·05$ $^{**}P<0·010$.

† 2X, dose corresponding to twofold the recommended dietary allowance for adult humans (National Research Council, 2001) based on body weight.

‡ For details of diets, supplements and procedures, see Table 1 and p. 502. The concentration of free radicals was determined using electron paramagnetic spectroscopy of tissue samples frozen in liquid nitrogen.

§ Vitamins were formulated by Pharmavit Rt., a Bristol–Myers Squibb Co., Hungary.

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Table 5. Total antioxidant reactivity of liver and spleen homogenates prepared from mice drinking different solutions of vitamins at a 2X dose† for 2 weeks and after a wash-out period of another 2 weeks‡

(Mean values and standard deviations for ten mice per group)

	Total antioxidant reactivity (μmol free radicals/g tissue)			
	Liver		Spleen	
Vitamins§	Mean	SD	Mean	SD
After 2-week supplementation at 2X dose†				
Control 1	936	207	248	181
Placebo 1	864	192	332	108
α-Tocopherol	1524**	380	1161**	276
Ascorbic acid	1205	362	994**	202
Riboflavin	987	196	757**	96
Multivitamin 1	1318**	263	1634**	453
After 2-week wash-out period				
Control 2	835	205	246	159
Multivitamin 2	962	183	462	193

Mean values were significantly different from those of the control groups: $^{**}P<0{\cdot}01$.

† 2X, dose corresponding to twofold the recommended dietary allowance for adult humans (National Research Council, 2001) based on body weight.

‡ For details of diets, supplements and procedures, see Table 1 and p. 502.

§ Vitamins were formulated by Pharmavit Rt., a Bristol–Myers Squibb Co., Hungary.

to lower the overall concentration of free radicals in the liver (Table 4). This seems to be related to the fact that liver is one of the best antioxidant-supplied organs (Fehér *et al.* 1992) and it has the largest storage capacity for α-tocopherol (Bjørneboe *et al.* 1991; Surai *et al.* 1996). In our present experiments we have not measured the concentration of vitamins in any of the organs, but the antioxidant reactivity of liver homogenates prepared from animals supplemented with α-tocopherol (Table 5) suggests that it was able to lower the steady-state level of free radicals by virtue of its ability to enhance the reductive capacity of the liver. Results obtained in spleen homogenates showed a similar correlation between the lower free-radical concentrations and higher antioxidant capacity of the organ.

In conclusion, low-dose supplementation of a basal mouse diet with a multivitamin mixture for 2 weeks was enough to enhance the ROS-producing activity of elicited peritoneal macrophages when stimulated with a pathogen-like substance. This increase may imply a higher phagocyte microbial killing power of these important immune cells. Del Rio *et al.* (1998) showed that several antioxidants when added to cells at low concentrations *in vitro* not only induced the production of superoxide anion, but also stimulated the phagocytic process in peritoneal macrophages. Supplementation of the diet with the multivitamin preparation did not increase the overall free-radical concentration of any organs studied, showing no potential danger of cell injury. In contrast, intake of some vitamins improved the endogenous defence system of the liver and spleen in the protection of cell components against attack by reactive species. The results presented here are not sufficient to decide which component or components of the multivitamin preparation are responsible for the observed effect. Moreover, ascorbic acid is synthesized by mice, hence the effects of supplementation with this vitamin are difficult to extrapolate to man, who lacks endogenous ascorbic acid.

Nonetheless, it seems that supplementation of the diet with low doses of the multivitamin preparation modulates the immune functions of macrophages by enhancing their ROS-producing activity, and contributes to the overall defence mechanism of some organs against damaging free radicals. More detailed evaluation of different vitamin combinations would provide insight into the *in vivo* contribution of each component to the beneficial effect of multivitamins on immune cells.

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