

# Effects of Supplementation With Vitamins A, C, and E, Selenium, and Zinc on Immune Function in a Murine Sensitization Model

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**OBJECTIVE:** We compared the effects of supplementing with vitamins A, C, and E, selenium, and zinc on a range of innate and specific T-helper 1 (Th1) and Th2-driven adaptive immune responses.

**METHODS:** BALB/c mice were fed semi-purified AIN93 diets and randomly assigned to receive a diet supplemented with 120 mg/kg of vitamin A, 2500 mg/kg of vitamin C, 1000 mg/kg of vitamin E, 2 mg/kg of selenium, and 500 mg/kg of zinc ( $n = 15/\text{group}$ ). After 4 wk of supplementation, mice were sensitized by topical application of di-nitro-chlorobenzene (DNCB); 2 wk later mice were challenged; and 5 d later they were killed to assess the effect on a range of innate responses (phagocytic activity, oxidative burst and tumor necrosis factor- $\alpha$ ), adaptive Th1-driven responses (delayed-type hypersensitivity, DNCB-specific immunoglobulin [Ig] G2a and IgG2b, and interferon- $\gamma$  [IFN- $\gamma$ ]), and adaptive Th2-driven responses (DNCB-specific IgE and IgG1 and interleukin-4 [IL-4]).

**RESULTS:** Immune function was affected only in the vitamin A group. These mice gained less weight and were less capable of resolving the inflammatory response elicited during sensitization. The oxidative burst of blood cells was increased, but production of IFN- $\gamma$  and IL-4 and the ratio of IFN- $\gamma$  to IL-4 were markedly depressed. In concordance with the latter result, production of Th1-driven IgG2a antibodies was decreased, whereas Th2-driven isotypes were not affected (IgG1, IgE) and mucosal IgA was increased.

**CONCLUSIONS:** These findings confirmed that supplementary amounts of vitamin A above dietary requirements enhance inflammatory responses accompanied by decreased Th1 and increased mucosal responses. However, supplementation of these sufficiently fed, non-stressed, young adult mice with vitamins C and E, selenium, or zinc had no effect on immune function. We speculate that using this model in aged, physiologically, or nutritionally stressed mice may provide outcomes more similar to those in sensitive human populations. If so, this would improve the usefulness of the model to assess, characterize, and rank effects of foods or nutrients on a range of immune functions, including Th1/Th2 polarization. *Nutrition* 2003;19:940–946. ©Elsevier Inc. 2003

**KEY WORDS:** immunomodulation, animal model, vitamin A, vitamin C, vitamin E, selenium, zinc

## INTRODUCTION

The immune system has evolved as a defense mechanism against infections and contributes to the maintenance of good health. In recent years, the effect of diet on various aspects of immune function has become increasingly apparent.<sup>1</sup> Systematic research in this area, however, is hampered by the lack of generally accepted, validated animal models that are predictive for effects in humans.<sup>2</sup> Typically, different research groups have studied different nutrients, each using different animal models. Most of these models focus on selected aspects of immune function without assessing other functions. It is therefore difficult to compare the broader immunomodulating effects of diets or individual nutrients. A systematic approach to compare the impact of various nutrients and diets using an animal model to assess a range of innate and specific T-helper 1 (Th1)- and Th2-driven adaptive immune responses therefore would be valuable.

In a previous study we started to evaluate a murine epicutaneous sensitization model using the contact sensitizer di-nitro-chlorobenzene (DNCB) in BALB/c mice for application in nutrition studies.<sup>3</sup> Modulation of Th1- and Th2-driven responses can readily be detected in these mice, notwithstanding their tendency to develop Th2-biased responses to certain infections.<sup>4</sup> We therefore set out to determine whether this model can be used to compare the impact of nutrients on a range of immune functions, including Th1/Th2 polarization. Previously we compared the immunomodulatory effects of diets varying in fatty acid composition and vitamin E contents.<sup>3</sup> The aim of the present study was to further evaluate the usefulness of this murine sensitization model to assess, characterize, and rank effects of foods and nutrients on markers of innate responses (phagocytic activity, oxidative burst, and tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ]), of adaptive Th1-driven responses (delayed-type hypersensitivity [DTH], DNCB-specific immunoglobulin [Ig] G2a and IgG2b, and interferon- $\gamma$  [IFN- $\gamma$ ]), and of adaptive Th2-driven responses (DNCB-specific IgE and IgG1 and interleukin-4 [IL-4]). In addition, we assessed production of DNCB-specific IgA and lymphocyte proliferation.

In the current study we compared the immunomodulatory activity of supplementation with 120 mg/kg of vitamin A, 2500 mg/kg of vitamin C, 1000 mg/kg of vitamin E, 2 mg/kg of

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TABLE I.

Micronutrient	COMPOSITION OF THE DIETS*					
	Dietary treatment groups					
	Control	Vitamin A	Vitamin C	Vitamin E	Selenium	Zinc
Vitamin A (mg/kg)	1.1	105.4				
Vitamin C (mg/kg)	0†		2500†			
Vitamin E (mg/kg)	47.8			1077.0		
Selenium (mg/kg)	0.2				1.8	
Zinc (mg/kg)	37.0					458.1

\* The following ingredients were added at identical levels to all diets (g/kg of diet): calcium caseinate, 163.3; maize starch, 609.9; cellulose, 50.0; mineral mixture, 35.0; vitamin mixture, 10.0; L-cysteine hydrochloride, 1.8; choline bitartrate, 2.5; fat blend, 127.5; Mineral and vitamin mixtures were made according to AIN93 recommendations. Experimental diets were supplemented with vitamins A, C, and E, selenium, and zinc given as vitamin A acetate, ascorbyl-2 polyphosphate, D- $\alpha$ -tocopheryl acetate, sodium selenite, and zinc carbonate, respectively. Indicated concentrations were measured in the diets except for vitamin C.

† Ascorbyl-2 polyphosphate could not be measured due to technical problems; therefore, indicated values for vitamin C are concentrations added to the diet.

selenium, and 500 mg/kg of zinc. These micronutrients were selected based on their claimed effects on immune function in humans. Although most studies have compared deficient states with sufficient states, some studies also have indicated beneficial effects of supplementation above recommended levels in humans and some animal models. Surprisingly, the immunomodulating activities of these micronutrients have never been compared side by side in one animal model.

## MATERIALS AND METHODS

### Animals and Maintenance

The Animal Experimentation Committee of the Faculty of Veterinary Sciences of Utrecht University approved the experimental protocol. Young adult, female BALB/c mice, ages 4 to 6 wk at the start of the experiment, were bred and housed at the animal facilities of the Utrecht University. They were kept on wood chips in filter-topped Macrolin cages under standard conditions (temperature  $23 \pm 2^\circ\text{C}$ , 50% to 60% relative humidity, 12-h dark and light cycle) with free access to food and acidified (pH 3) drinking water. They were randomly assigned to six experimental groups ( $n = 15/\text{group}$ ), and mice were individually identifiable through ink markings on their tails. Persons involved in handling the animals or sample analysis were blinded for the treatments.

### Diets and Feeding

All animals received the control diet for 2 wk before the intervention. During the 8-wk intervention, animals received an AIN93-based control diet or the same diet fortified with vitamin A, C, or E, selenium, or zinc. All diets were semi-purified and provided 16% energy as protein, 54% as carbohydrates, and 30% as fat (12% saturated fatty acids, 10% monounsaturated fatty acids, and 8% polyunsaturated fatty acids) to mimic the typical human high-fat diets of industrialized societies. The fat blend was prepared from 71.2% palm oil, 8.7% safflower oil, and 20.1% sunflower oil (all from Chempri BV). This resulted in a fatty acid composition of 0.3% C12:0, 0.8% C14:0, 34.0% C16:0, 0.2% C16:1, 4.3% C18:0, 32.8% C18:1, 26.2% C18:2, 0.2% C18:3, 0.4% C20:0, and 0.2% C22:0. In the control group, test compounds were given at recommended levels for rodents according to AIN93 guidelines.<sup>5</sup> Experimental diets were fortified with vitamin A acetate, ascorbyl-2 polyphosphate, D- $\alpha$ -tocopheryl acetate, sodium selenite, or zinc carbonate, aiming for concentrations of 120 mg/kg of

vitamin A, 2500 mg/kg of vitamin C, 1000 mg/kg of vitamin E, 2 mg/kg of selenium, and 500 mg/kg of zinc. These levels have been shown to affect immune function in various other animal models. The actual levels achieved and additional diet specifications are listed in Table I. To minimize oxidative degeneration, the diets were stored in portions for 2 to 3 d at  $-20^\circ\text{C}$ . Animals were fed by group, and a fresh diet replacing the old was given every 2 to 3 d. Vitamins A and E also were measured after 2 mo storage at  $-20^\circ\text{C}$  followed by 3 d at room temperature. Under these harsh conditions, vitamin A in the control diet was reduced by 12%; all other changes were reduced by less than 10%. Food and acidified water were provided ad libitum.

### Sensitization Model

In the fifth week after start of dietary intervention, animals were sensitized by ear painting with 25  $\mu\text{L}/\text{ear}$  0.5% (w/v) DNCB on 3 consecutive days. Fourteen days after the first sensitization (in week 7), animals were challenged with 25  $\mu\text{L}$  of 0.25% (w/v) DNCB on their ears to elicit a secondary immune response. Just before this challenge and 24 and 48 h later, ear thickness was assessed with an electronic micrometer (Mitoyota) to determine DTH responses. Blood samples were obtained by orbital puncture before sensitization in week 5. Five days after challenge (week 8 of dietary intervention), mice were anesthetized by ether inhalation and blood was obtained. Ten drops of blood heparinized with 20  $\mu\text{L}$  of tromboliquine were used in the phagocytosis and burst test. The remainder of the blood was used to prepare serum samples. Animals were subsequently killed by cervical dislocation. Spleens and auricular lymph nodes (ALNs) were used to prepare cell suspensions by mincing in RPMI medium under sterile conditions.

### Vitamins A and E in Serum

Serum samples were mixed with antioxidant (ascorbic acid and pyrogallol) and internal ( $\alpha$ -tocopheryl acetate and retinyl acetate) standards, deproteinized with ethyl alcohol, and extracted into *n*-heptane. Solvents were removed under  $\text{N}_2$ , and residues were re-dissolved in chloroform:methanol (2:1, v/v). Analytes were separated by high-performance liquid chromatography on an RP C18 (5  $\mu\text{m}$ ,  $250 \times 4$  mm) column by isocratic elution with methanol-2:propanol:water (50:50:8, v/v/v). Detection was by ultraviolet-vis absorption at 284, 292, and 325 nm. The coefficient

of variance of this analysis was smaller than 4%. Vitamin A and E in concentrations in the food samples were determined by a contract laboratory (TNO, Zeist, The Netherlands) according to good laboratory practice using standard procedures.

### **Proliferation and Cytokine Production**

Cells from spleen and ALN were resuspended in RPMI medium and counted on a Coulter counter (Coulter Electronics, Luton, UK). Cell suspensions were diluted to  $10^6$  cells/mL for cytokine production and  $5 \times 10^6$  cells/mL for proliferation. Autologous heat-inactivated serum was added to a final concentration of 0.75% (v/v). For proliferation, spleen cells were stimulated for 48 h with 0 or 3  $\mu\text{g/mL}$  concavalin A (ConA) and [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci/mL}$ ) was added for the last 8 h. DNA was harvested and counted on a scintillation counter (Beta Plate, Wallac). For cytokine production, ALN and spleen cells were cultured for 24 h with 5  $\mu\text{g/mL}$  of ConA (IL-4 and INF- $\gamma$ ) or 2  $\mu\text{g/mL}$  of lipopolysaccharide (TNF- $\alpha$ ). After this period, plates were centrifuged, and supernatants were collected and stored at  $-70^\circ\text{C}$ .

### **Enzyme-Linked Immunosorbent Assays**

DNCB-specific antibodies of distinct isotypes were measured by using in-house developed enzyme-linked immunosorbent assays, as described previously.<sup>6</sup> In brief, DNCB-specific IgG1, IgG2a, and IgG2b were captured on a coat of di-nitro-chlorophenol human serum albumin and were detected with alkaline phosphatase-conjugated isotype-specific antibodies. The lower levels of specific IgE and IgA were detected with reversed capture enzyme-linked immunosorbent assays by using isotype-specific antibodies for capture and biotinylated di-nitro-chlorophenol human serum albumin followed by streptavidin peroxidase for detection. Titers of DNCB-specific antibodies were calculated by means of sample dilution at which extinctions were higher than background, +2 times standard deviation. IL-4 and IFN- $\gamma$  were detected with in-house-developed sandwich enzyme-linked immunosorbent assays, as described previously.<sup>6</sup> Dilutions of mouse recombinant IFN- $\gamma$  and IL-4 were included on every plate for standard curve production (variation between plates was less than 10%).

### **Phagocytosis and Oxidative Burst**

Phagocytosis and oxidative burst were assessed with commercial kits (Orpegen Pharma, Heidelberg, Germany). In brief, blood samples were incubated (30 min,  $37^\circ\text{C}$ ) with fluorescently labeled (phagocytosis) or unlabeled *Escherichia coli* bacteria (oxidative burst). For oxidative burst, dihydro rhodamine 123 (fluorescent upon oxidation) was added for the last 10 min of the incubation. Extracellular fluorescence was quenched, and results were analyzed with flow cytometry to determine the number of granulocytes involved in phagocytosis and oxidative burst and the mean level of activity per granulocyte.

### **Commercial Kits**

According to the manufacturer's instructions, TNF- $\alpha$  was measured with a kit from Biosource (Nivelles, Belgium), and stable derivatives of prostaglandin  $\text{E}_2$  were assessed with the bicyclo prostaglandin  $\text{E}_2$  kit (Cayman, Ann Arbor, MI, USA).

### **Antibodies and Chemicals**

Alkaline phosphatase-conjugated anti-IgG1, anti-IgG2a, and anti-IgG2b were from Southern Biotechnology Association (Birmingham, AL, USA); unconjugated anti-IgA, anti-IgE, anti-IFN- $\gamma$ , anti-IL-4, biotinylated anti-IFN- $\gamma$ , and anti-IL-4 were from Pharmingen (San Diego, CA, USA). ConA and [ $^3\text{H}$ ]thymidine were from ICN Bio-

medicals (Costa Mesa, CA, USA); diethanolamine, Tween-20, and casein were from BDH (Poole, Dorset, UK); and streptavidin and horse radish peroxidase were from the Central Laboratory of Blood Transfusion (CLB, Amsterdam, The Netherlands). DNCB, bovine serum albumin, 3,3',5,5'-tetramethylbenzidine, *p*-nitrophenol phosphate, *N*-hydroxysuccinimide biotin, and di-nitro-chlorophenol human serum albumin were obtained from Sigma (St. Louis, MO, USA). RPMI-1640 was supplemented with bicarbonate, penicillin plus streptomycin,  $\beta$ -mercapto-ethanol, and L-glutamine were from Gibco BRL (Life Technology, Breda, The Netherlands). All other chemicals were obtained from Merck (Darmstadt, Germany).

### **Statistics**

The study was run in five cohorts, each with three mice per treatment group. On section days, samples were taken from these animals, coded, and tested in random order in the various assays by people blinded to the experimental treatments. Data were analyzed by analysis of variance, with dietary group as factor and cohort as a block. Differences from the control group were determined with Dunnett's test. Where required, data were log transformed to normalize distribution before analysis. Data are reported as means  $\pm$  standard error of the mean.

## **RESULTS**

### **Animal Growth**

The mean weight of the animals at the start of the intervention did not differ significantly between groups (range, 17.8–18.9 g). However, animals fed the vitamin A-supplemented diet gained less weight ( $4.6 \pm 0.4$  g,  $P < 0.05$ ) than did animals fed the control diet ( $6.5 \pm 0.4$  g). Weight gains of the animals fed diets fortified with vitamin C ( $5.4 \pm 0.4$  g), vitamin E ( $5.9 \pm 0.5$  g), selenium ( $5.4 \pm 0.4$  g), or zinc ( $5.6 \pm 0.5$  g) did not differ from those of animals fed the control diet.

### **Concentrations of Experimental Micronutrients**

Fortification of the experimental diets with vitamins A, C, and E and zinc was reflected in the elevated concentrations of these micronutrients in sera isolated at the end of the intervention as measured in three to five animals (except the vitamin C group, which was measured in all animals) per treatment group (Table II). Observed increases reached statistical significance only for vitamins C and E ( $P < 0.05$ ). Supplementation with selenium or zinc did not result in appreciable increases of selenium in serum or zinc in the liver.

### **Parameters of Innate Immunity**

**PHAGOCYTOSIS AND OXIDATIVE BURST.** The percentage of phagocytosing granulocytes (range, 80–84%), the mean level of phagocytotic activity per granulocyte (range, 145–165), mean fluorescence intensity, and the percentage of granulocytes responding with an oxidative burst (range, 52–53%) was not influenced by the supplement. However, the mean level of oxidative burst per involved granulocyte was higher in the vitamin A group ( $213 \pm 8$  mean fluorescence intensity) than in the control group ( $181 \pm 9$  mean fluorescence intensity,  $P < 0.01$ ).

**PRODUCTION OF TNF- $\alpha$ .** The concentration of TNF- $\alpha$  was assessed in supernatants of lipopolysaccharide-stimulated spleen cells. Production of this proinflammatory cytokine in the control group ( $175 \pm 13$  pg/mL) did not differ significantly from those of any of the supplementary intervention groups ( $147 \pm 14$ ,  $201 \pm$

TABLE II.

Micronutrient	Dietary treatment groups					
	Control	Vitamin A	Vitamin C	Vitamin E	Selenium	Zinc
Vitamin A (nMol/mL)	0.75 ± 0.03	1.01 ± 0.01				
Vitamin C (μMol/L)	58.7 ± 3.5	63.6 ± 4.3	92.4 ± 2.7†	70.0 ± 2.8	50.5 ± 2.4	49.2 ± 4.1
Vitamin E (nMol/mL)	13.6 ± 0.2			33.4 ± 2.8†		
Selenium (μg/L)	0.34 ± 0.01				0.35 ± 0.01	
Zinc (serum) (μMol/L)	16.0 ± 0.4					23.2 ± 3.3
Zinc (liver) (mg/kg)	25.8 ± 0.9					27.2 ± 1.5

\* Serum concentrations (and liver concentration for zinc) of fortified micronutrients. Values are means ± standard error of the mean of three to five animals, in principle one per group per cohort, except for vitamin C, which was measured in all animals ( $n = 15$ ).

† Significant difference from control group ( $P < 0.001$ ).

22, 152 ± 15, 181 ± 13, and 164 ± 15 pg/mL for vitamins A, C, and E, selenium, and zinc, respectively).

### Parameters of Adaptive Immunity

**DELAYED-TYPE HYPERSENSITIVITY.** As represented in Figure 1, all experimental groups showed a clear increase in ear thickness at 24 h after challenge. It is noteworthy that, whereas ears of mice in all other groups had fully recovered from the sensitization 10 d earlier, ears in the vitamin A-supplemented group were still swollen before challenge with DNCB. The DTH response expressed as an increase in ear thickness due to challenge ( $\Delta$  ear thickness) also appeared to be increased in this group, but this did not reach statistical significance (12.1 ± 0.8 for vitamin A versus 9.9 ± 1.0 for control, 9.8 ± 0.8 for vitamin C, 8.8 ± 1.0 for vitamin E, 8.5 ± 1.0 for selenium, and 10.6 ± 1.0 for zinc × 10<sup>-2</sup> mm).

**PROLIFERATION IN ALN AND SPLEEN.** The number of cells in the ALN draining the site of DNCB challenge reflects mainly in vivo activation of lymphocytes. The number of cells in both ALN combined was highest in the vitamin A group but did not differ significantly between groups (18.1 ± 2.0, 23.7 ± 2.8, 19.5 ± 2.8, 18.2 ± 2.0, 17.0 ± 1.6, and 17.6 ± 1.7 × 10<sup>6</sup> cells/2 ALN for control, vitamins A, C, and E, selenium, and zinc,

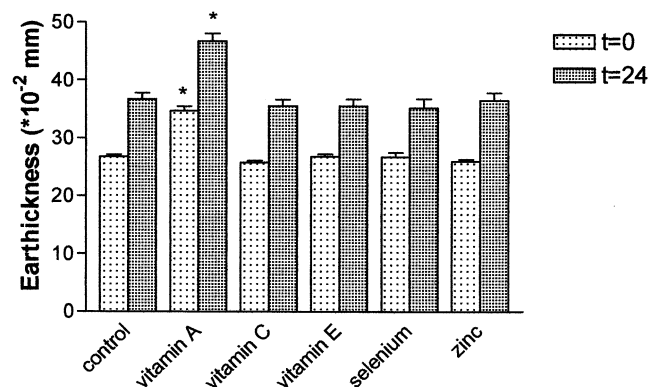


FIG. 1. Delayed-type hypersensitivity response. Ear thickness of the sensitized mice was measured before ( $t = 0$ ) and 24 h ( $t = 24$ ) after challenge with di-nitro-chlorobenzene. \*Significant differences in ear thickness. The increase in ear thickness due to challenge is not significantly different between groups.

respectively). All these numbers were clearly increased as compared with non-sensitized BALB/c mice (typically 5–8 × 10<sup>6</sup> cells/2 ALN; M. Bol, unpublished data). The ex vivo polyclonal proliferation of splenocytes after stimulation by ConA was highest in the vitamin C group, but this was not significantly different between diets (stimulation indices: 28.7 ± 3.3, 30.7 ± 4.5, 31.4 ± 3.4, 28.9 ± 3.9, 30.1 ± 3.8, and 28.1 ± 3.0 for control, vitamins A, C, and E, selenium, and zinc, respectively).

**LEVELS OF DNCB-SPECIFIC ANTIBODIES IN SERUM.** After sensitization, a clear time-dependent increase of serum titers of DNCB-specific Ig was observed in all groups, indicating initiation of a specific response to DNCB (data not shown). Figure 2 shows the titers of DNCB-specific IgG1, IgG2a, IgG2b, IgE, and IgA in sera collected at the end of the study, 5 d after challenge. Feeding the vitamin A-supplemented diet affected the isotype of the DNCB-specific antibodies formed after sensitization and subsequent challenge. In particular, concentration of IgG2a ( $P < 0.001$ ) was lower as compared with the control group, whereas IgA was increased ( $P < 0.05$ ). The other isotypes, i.e., IgG2b, IgG1, and IgE, were not significantly different from the control group. The other diets did not affect the DNCB-specific antibody concentrations.

**PRODUCTION OF IFN- $\gamma$  AND IL-4.** IFN- $\gamma$  and IL-4 were measured in the supernatants of ConA-stimulated cultures of ALN and spleen cells (Table III). ALN cells produced more of both cytokines than did spleen cells. This was particularly evident for IFN- $\gamma$ . Production of IFN- $\gamma$  by ALN cells was dramatically lower in the vitamin A group ( $P < 0.001$ ). The same trend was seen for IL-4 production and for production of IFN- $\gamma$  by spleen cells (not significant). The other micronutrients, except vitamin C, also tended to decrease production of IFN- $\gamma$  by ALN cells (not significant). The ratio of IFN- $\gamma$  to IL-4 was calculated because this ratio can indicate immunomodulation with respect to Th1 versus Th2 activation dominance.<sup>6</sup> This ratio was lower in the vitamin A group, but this was significant only in the ALN ( $P < 0.001$ ), not in the spleen cells.

## DISCUSSION

In the present study, we compared the immunomodulatory activity of supplementing mice with 120 mg/kg of vitamin A, 2500 mg/kg of vitamin C, 1000 mg/kg of vitamin E, 2 mg/kg of selenium, and 500 mg/kg of zinc. Supplementation at these dose levels was shown previously to affect immune function in various other

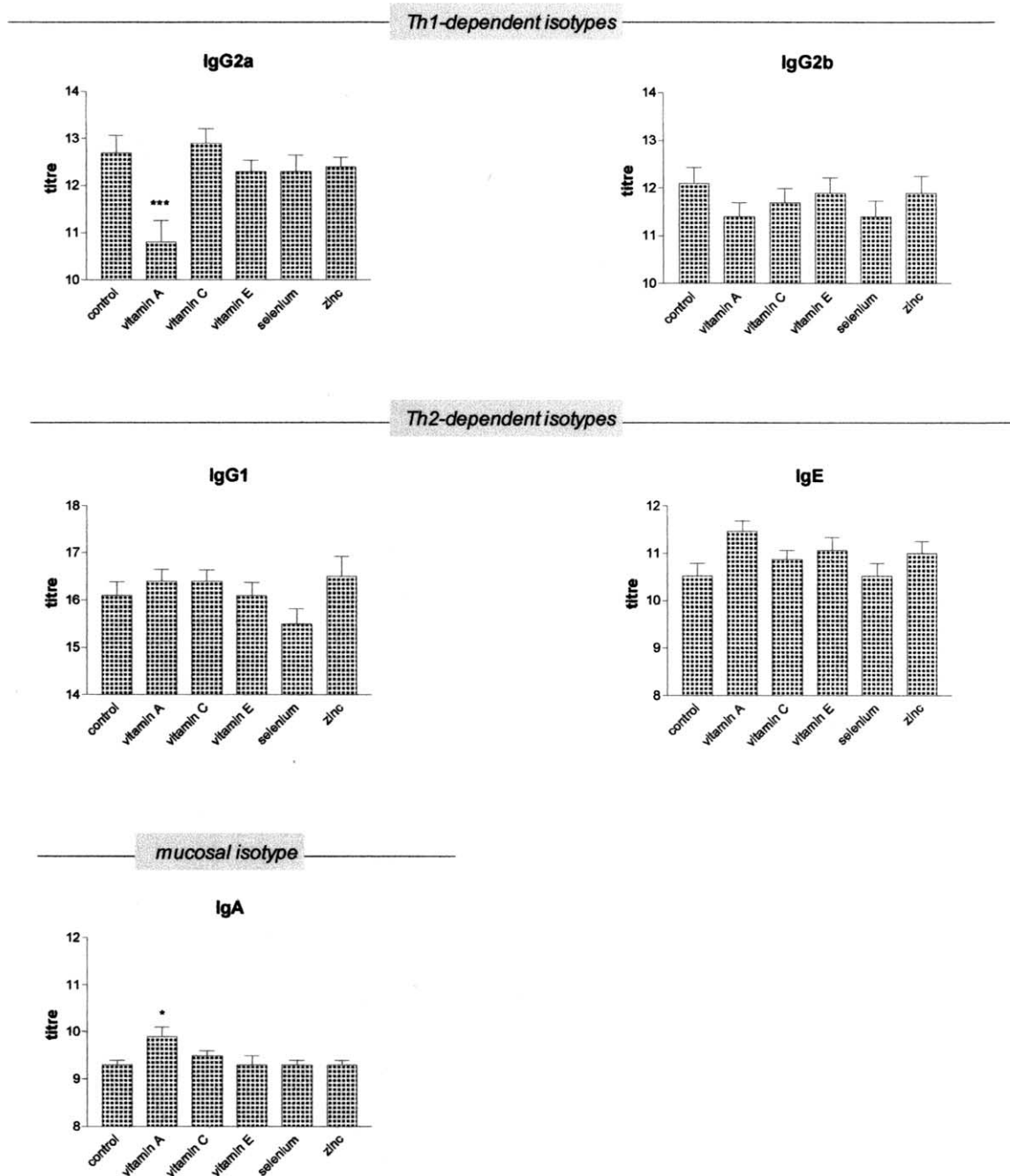


FIG. 2. Serum titers of di-nitro-chlorobenzene-specific antibodies of different isotypes were measured in sera obtained 5 d after challenge. Indicated values are means  $\pm$  standard error of the mean ( $n = 15$ ). \*\*\*Significantly different from control ( $P < 0.001$ ). Ig, immunoglobulin; Th, T helper.

animal models. At these levels, we observed that serum concentrations of the supplemented micronutrients were generally elevated, although not always statistically significantly due to the limited number of samples analyzed. Only selenium concentrations were not increased despite a 10-fold higher intake, which underlines the known tight regulation of the serum concentration of selenium.<sup>8</sup> Selenium and zinc tended to decrease serum vitamin C concentrations, whereas the antioxidant vitamin E tended to do the reverse. As at high levels of selenium intake<sup>9</sup> (1 part/million in the diet, which is in the same range as the levels used in the present study, 1.8 parts/million) and at high zinc-concentrations,<sup>10</sup> these trace elements may have prooxidant activity rather than antioxidant activity, and lower vitamin C levels in these experimental groups may be due to this prooxidant activity.

Statistically significant effects on immune function were seen only in the vitamin A group. It appears that feeding mice vitamin A at approximately 100-fold the recommended level affects their overall condition. These mice gained less weight and seemed less capable of resolving the inflammatory response elicited during sensitization with DNCB. Unlike the other mice, they had markedly swollen ears 10 d after sensitization. The oxidative burst was increased in these animals, which likely resulted in the observed release of more reactive oxygen species during the local inflammation elicited by sensitization with DNCB. Previously, it has been shown that increased vitamin A intake increases numbers of Langerhans cells in the skin.<sup>11</sup> Similar mechanisms involving sustained inflammation contribute to the increased chemically induced liver injury in vitamin A-supplemented rodents<sup>12</sup> and may

TABLE III.

## CYTOKINE PRODUCTION BY CELLS FROM THE ALN AND THE SPLEEN\*

	Dietary treatment groups					
	Control	Vitamin A	Vitamin C	Vitamin E	Selenium	Zinc
ALN						
IFN- $\gamma$ (pg/mL)	816 $\pm$ 213	99 $\pm$ 26†	870 $\pm$ 237	484 $\pm$ 159	578 $\pm$ 177	505 $\pm$ 211
IL-4 (pg/mL)	24.0 $\pm$ 4.5	13.2 $\pm$ 2.3	33.5 $\pm$ 10.0	20.6 $\pm$ 3.7	15.5 $\pm$ 3.2	21.1 $\pm$ 4.2
IFN- $\gamma$ /IL-4 (-)	31.7 $\pm$ 6.9	7.6 $\pm$ 1.7†	30.1 $\pm$ 8.6	20.3 $\pm$ 4.3	35.6 $\pm$ 9.1	20.8 $\pm$ 7.1
Spleen						
IFN- $\gamma$ (pg/mL)	122 $\pm$ 42	79 $\pm$ 19	108 $\pm$ 23	89 $\pm$ 28	119 $\pm$ 29	128 $\pm$ 34
IL-4 (pg/mL)	9.2 $\pm$ 1.9	8.6 $\pm$ 0.7	9.7 $\pm$ 1.0	7.4 $\pm$ 1.0	8.8 $\pm$ 1.3	9.5 $\pm$ 1.6
IFN- $\gamma$ /IL-4 (-)	10.5 $\pm$ 1.8	8.9 $\pm$ 1.7	11.5 $\pm$ 2.6	10.1 $\pm$ 2.6	11.9 $\pm$ 2.1	12.6 $\pm$ 2.1

\* Production of IFN- $\gamma$  and IL-4 after stimulation of ALN and spleen cells with concavalin A. Values are means  $\pm$  standard error of the mean ( $n = 12$ ).

† significantly different from control ( $P < 0.001$ ).

ALN, auricular lymph node; IFN- $\gamma$ , interferon- $\gamma$ ; IL-4, interleukin-4

underlie the augmented ear swelling and local lymph node tests in vitamin A-supplemented mice.<sup>13</sup> Here we assessed cell number in the draining ALN, which is similar to the outcome measured in the local lymph node assay. Although the number of cells was highest in vitamin A-supplemented mice, this did not reach statistical significance. However, production of IFN- $\gamma$  and IL-4 by these ALN cells was markedly depressed. The ratio of IFN- $\gamma$  to IL-4 also was decreased because production of IFN- $\gamma$  was more affected than that of IL-4. In line with this result, the production of DNCB-specific Th1-driven IgG2a ( $P < 0.001$ ) and IgG2b (not significant) antibodies was decreased, whereas Th2-driven isotypes were not affected (IgG1 and IgE) and mucosa-associated IgA was increased ( $P < 0.05$ ). These findings are in agreement with the general notion that supplementation with vitamin A (to a serum level of  $\sim 1$  nM/ml, similar to serum levels in the vitamin A group of this study) depresses IFN- $\gamma$  production<sup>14</sup> and favors Th2 responses and production of IgA.<sup>15</sup> In contrast, deficiency of vitamin A results in overexpression of IFN- $\gamma$  and dominating Th1 responses.<sup>14</sup>

Supplementation with the other micronutrients did not significantly affect immune function. This is somewhat surprising because these micronutrients were selected on the basis of their claimed efficacy. In particular, vitamin C supplementation has been shown to increase lymphocyte proliferation in mice.<sup>16</sup> However, there are only few studies with vitamin C in mice, presumably because rodents, unlike humans, can provide their own vitamin C. Here we observed no significant effects on immune function, although lymphocyte proliferation tended to be highest in the vitamin C-supplemented group. The effects of (high dose) vitamin C supplementation on common cold symptoms and immune responsiveness in humans are controversial. It has been suggested that this may be due to selective effects only in populations that are deficient or marginal in their vitamin C status.<sup>17</sup> Vitamin E has been shown to increase phagocytic activity of alveolar macrophages<sup>18</sup> and Th cell functions<sup>18,19,20</sup> in particular of Th1.<sup>21</sup> These effects appeared most pronounced in aged mice.<sup>22,23</sup> Despite a approximate 2.5-fold increase in serum concentration, we found no effect on immune function after supplementation with similar levels of vitamin E.<sup>24,25</sup> Trials in elderly humans have demonstrated beneficial effects of vitamin E supplementation on aspects of immune function, but whether similar effects can be obtained in younger people is unclear. Selenium at levels similar to those used in this study has been shown to increase lymphocyte proliferation and natural killer cell activity in mice.<sup>26,27</sup> We could not confirm these findings because we found only a slight decrease

in DNCB-specific IgG2b, IgG1, and IgE serum levels (not significant). These decreased antibody responses are in line with findings in rats, in which supplementation with selenium at 2.5-fold higher levels was found to result in lower IgG responses.<sup>28</sup> Supplementation with 300 to 500 mg/kg of zinc has been found to enhance natural killer cell function, phagocytosis, and lymphocyte proliferation, whereas effects on antibody production have been variable.<sup>30,31</sup> In the present study, supplementation with 450 mg/kg of zinc moderately increased serum zinc concentration but did not affect immune function.

There are several possible explanations for the lack of immune effects of vitamin C, E, selenium, and zinc. In principle, this model may not be sufficiently sensitive to allow detection of the immunomodulatory effects of these nutrients. However, with the same model we previously demonstrated effects of fatty acids on various aspects of immune responsiveness, and in this study we demonstrated immunomodulatory effects of vitamin A. These effects are in line with the existing literature, which has been collated with a range of different models, each assessing selected aspects of immune responsiveness. These findings therefore support our previous conclusion that this sensitization model provides a valuable tool to assess, characterize, and rank effects of foods and nutrients on a broad range of immune functions. Possibly the lack of efficacy of vitamins C and E, selenium, and zinc is (in part) attributable to the feeding regimen used. Whereas our supplemented levels were in the same range as concentrations previously shown efficacious, the level in our control group was higher than that used in many other studies. This is the consequence of our choice to determine the effect of supplementation beyond AIN93 recommendations. For instance, most previous studies on vitamin E have used deficient control levels or the older AIN76 guidelines recommending 30 mg/kg as opposed to 48 mg/kg used in this study.<sup>23,30,31</sup> The age and physiologic condition of the mice may be other important factors. Similar to humans, aged mice seem to be most susceptible to the immunomodulatory effects of vitamin E. In younger mice (as used in this study), effects tend to become apparent only in the presence of a stressor such as an infection, exposure to oxygen radicals, or exposure to ultraviolet light.

In conclusion, supplementation with vitamins A, C, and E and zinc, but not with selenium, increased their serum concentrations. However, only vitamin A exerted immunomodulatory effects similar to those previously described. In humans, it is now becoming increasingly clear that beneficial effects of supplementation differ among subgroups. In a recent study,<sup>31</sup> no favorable effects of vitamin E supplementation were noted on the incidence of respi-

ratory tract infection in a group of 60-y-old individuals. However, it appears that in particular elderly or physiologically stressed individuals, who have a marginal nutrition status, may benefit. In this study, the absence of predicted effects on immune function after supplementation of sufficiently fed, non-stressed, young adult mice suggests that the same may be true in this murine sensitization model. If so, we predict that using this model in aged, physiologically, or nutritionally stressed mice will provide outcomes more similar to those in the putative human target populations. Future studies should compare the effects of supplementation in normal and aged or physiologically or nutritionally stressed mice.

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