Effects of Vitamin E-Deficiency and/or Nitrogen Dioxide Inhalation on Allergen-Sensitized Type IV and Type I Allergy Responses of Mice

Hongbin Mi,[a] Kazuyuki Hiramoto,[a] Kaori Kujirai,[a] Ken Ando,[a] Yoshiaki Ikarashi,[b] and Kiyomi Kikugawa*,[a]

[a]School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan and [b]Division of Medical Devices, National Institute of Health Sciences, Setagaya-ku, Tokyo 158–8501, Japan

(Received July 16, 2001; Accepted October 11, 2001)

Effect of oxidative stress induced by vitamin E-deficiency and/or nitrogen dioxide (NO_2) inhalation on Type IV and Type I allergy responses of mice was investigated. Mice were fed a vitamin E-adequate diet (control: C group) or a vitamin E-deficient diet (–E group). The vitamin E content in the blood of C and –E groups was 1.58 and 0.3 μg/ml, respectively. Mice of the C and –E groups were exposed to air or air containing NO_2 (5–6 ppm) (C + NO_2 and –E + NO_2 groups) by feeding them the corresponding diets for 1–2 week. In the sensitization with 2,4-dinitrochlorobenzene (DNCB), the degree of lymph node cell proliferation of mice of the C + NO_2 group, as assessed by lymph node assay, was similar to that of mice of the C group. While the degree of cell proliferation of mice of the –E group was lower than that of mice of the C group, the degree of cell proliferation of mice of the –E + NO_2 group was higher than that of mice of the C and C + NO_2 groups. While the DNCB-sensitized IgE levels of mice of the C + NO_2 group were similar to those of mice of the C group, the IgE levels of mice of the –E and –E + NO_2 groups were higher than those of the C and C + NO_2 groups. While the trimellitic anhydride (TMA)-sensitized IgE levels of mice of the –E group were similar to those of mice of the C group, the IgE levels of mice of the C + NO_2 and –E + NO_2 groups were much higher. These results suggest that allergen-sensitized type IV and I allergy responses of mice are enhanced by oxidative stress induced by vitamin E-deficiency and/or NO_2 inhalation.

Key words —— vitamin E-deficiency, nitrogen dioxide inhalation, type IV allergy, type I allergy

INTRODUCTION

Vitamin E is a major chain-breaking antioxidant in cell membranes that effectively scavenges lipid peroxyl radicals, and thus protects the cell membranes from oxidative damage.[1,2] In addition, vitamin E has another property unrelated to antioxidant activity. Vitamin E enhances immune responses, and stimulates mitogenic responses of lymphocytes and antibody production.[3–6] Our previous studies have shown that vitamin E-deficiency in mice brings about decreased contact sensitization responses to 2,4-dinitrochlorobenzene (DNCB).[7]

On the other hand, nitrogen dioxide (NO_2) is a free radical toxin usually present in polluted urban and room air (0.02–0.2 ppm), and in fresh smoke or cigarette smoke (upto 100 ppm). It has been shown that long-term inhalation of a low level of NO_2 causes oxidative damage, especially to lung tissue. Inhalation of air containing NO_2 causes pulmonary edema, pulmonary fibrosis, bronchitis[8–10] and cancer.[11] Inhalation of NO_2 into rats causes lung lipid peroxidation, as assessed by the increase in conjugated diene formation[12] and hydrocarbon exhalation.[13]

The aim of the present study was to determine the effect of oxidative stress induced by vitamin E-deficiency and/or NO_2 inhalation on allergen-sensitized type IV (contact sensitization) and type I (respiratory sensitization) allergy responses of mice. In this study, mice supplemented with a vitamin E-adequate diet or a vitamin E-deficient diet were exposed to purified air or to air containing NO_2. For investigation of the effect on type IV allergy response, contact sensitization responses induced by DNCB, a potent sensitizer used for many years to

*To whom correspondence should be addressed: School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan. Tel.: +81-426-76-4503; Fax: +81-426-76-4508; E-mail: kikugawa@ps.toyaku.ac.jp

induce contact sensitization,[14,15] were examined. For investigation of the effect on type I allergy responses, serum IgE levels were measured using mice sensitized on their flanks, with a subsequent challenge on the ears by DNCB or trimellitic anhydride (TMA).[16,17]

MATERIALS AND METHODS

Materials —— Purified air and air containing 10 ppm NO_2 were prepared by Nihonsanso, Ltd. (Tochigi, Japan). Hanks' balanced salt solution (HBSS) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were obtained from Dojindo Laboratories (Kumamoto, Japan). RPMI-1640 medium and penicillin-streptomycin solution were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Fetal calf serum (FCS) was obtained from Bio Whittaker (Walkersvile, MD, U.S.A.). Thiobarbituric acid (TBA) was obtained from Merck KGaA (Darmstadt, Germany). 2,4-DNCB, TMA, olive oil and normal goat serum (NGS) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Standard DL-α-tocopherol was obtained from Tokyo Chemical Company (Tokyo, Japan). Standard bovine serum albumin (BSA) (γ-globulin-free and fatty acid-free), mouse monoclonal IgE anti-dinitrophenol (DNP) antibody (clone SPE-7) and *o*-phenylenediamine dihydrochloride tablet kits were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). [^{3}H]Methyl thymidine ([^{3}H]TdR) was obtained from Amersham Pharmacia Biotech UK, Ltd. (Buckingham, U.K.). Rat monoclonal anti-mouse IgE antibody was from Southern Biotechnology Associates, Inc. (Birmingham, AL, U.S.A.), and goat anti-mouse IgE conjugated with horseradish peroxidase was from Nordic Immunology (Tilberg, The Netherlands).

Animals and Diets —— The protocol of animal preparations for the present experiment was approved by the Ethics Committee of our institute. Three-week-old male BALB/c mice weighing 10–15 g, obtained from Japan Laboratory Animals Incorporation (Tokyo, Japan), were used. Six mice were housed together in a plastic cage in a room of controlled temperature at 23 ± 1°C, humidity at 55 ± 5% and lighting with a 12 hr dark-daylight cycle. The animals were allowed free access to food. The animals were fed a vitamin E adequate-powdered diet MF containing protein at 254 g/kg, calories at 3422 kcal/kg, fat at 44 g/kg, fiber at 41 g/kg and vitamin E at 70×10^{-3} g/kg (Oriental Yeast Company, Tokyo, Japan) solidified by mixing 450 g food and 230 g water (C group), or, a powdered vitamin E-deficient-diet, Oriental Composition Type A containing protein at 226 g/kg, calories at 3600 kcal/kg, fat at 62 g/kg, fiber at 49 g/kg and vitamin E at 0×10^{-3} g/kg prepared by Oriental Yeast Company (Tokyo, Japan), solidified by mixing 450 g diet and 230 g water (–E group), for 3 week.

Each mouse was then moved to a special gastight glass cage, Metabolica MM-AP type, prepared by Sugiyama-Gen Iriki Company (Tokyo, Japan) of controlled temperature at 23 ± 1°C, and a 12 hr dark-daylight cycle. Air (C and –E groups) or air containing 10 ppm NO_2 (C + NO_2 and –E + NO_2 groups) was introduced into the glass cage at 100 ml/min or 200 ml/min, respectively. Under these conditions, NO_2 concentration in the cage, as assessed by the Saltzman method,[18] was maintained at 5–6 ppm when the animal and food were placed in the cage. Mice of the C and C + NO_2 groups were continuously supplemented with a vitamin E-adequate diet, and mice of the –E and –E + NO_2 groups were continuously supplemented with a vitamin E-deficient diet for 1–2 weeks. Weights of the mice after 2-week housing were 19–21 g.

Determination of Vitamin E Levels in Blood —— The vitamin E level of mouse blood was determined according to a method described elsewhere.[19] To 1.0 ml of heparinized blood from a mouse, 1.0 ml of a 60 mg/ml pyrogallol solution in ethanol and 1.0 ml of ethanol were added. After heating the mixture at 70°C for 2 min, 0.2 ml of a 600 mg/ml KOH solution was added, and the mixture was heated at 70°C for 30 min. Vitamin E was extracted by the addition of 2.5 ml of water and 5.0 ml of *n*-hexane, followed by centrifugation at 3000 rpm for 5 min.

The upper *n*-hexane phase (4.0 ml) was removed and evaporated to dryness to be redissolved into 0.2 ml of methanol. High performance liquid chromatography (HPLC) was carried out using a Hitachi 655 liquid chromatograph (Tokyo, Japan) equipped with an Inertsil ODS-2 column (4.6 mm i.d. × 250 mm) (GL Sciences Incorporation, Tokyo, Japan) by injection of 10 μl of the sample solution in methanol, and the column was eluted with a mobile phase composed of methanol/water (98 : 2, v/v) at a flow rate of 1.4 ml/min. A fluorescent peak was detected by excitation at 292 nm/emission at 335 nm with a Shimadzu RF-535 fluorescence spectromonitor (Osaka, Japan). The peak due to vitamin E appeared at a retention time of 17–19 min.

The amount of vitamin E in the sample was estimated by comparing the peak area with that of the calibration curve of the standard DL-α-tocopherol. Vitamin E levels in blood were expressed per ml blood.

Thiobarbituric Acid-Reactive Substances (TBARS) in Mouse Lung —— After being anesthetized with an air/diethylether mixture, the mouse was sacrificed, and the lung quickly isolated and washed with physiological saline. To the lung preparation, 10 equivalent amounts (v/w) of phosphate buffered saline (pH 7.4) (PBS) were added, and the mixture was homogenized using a Potter-type teflon homogenizer on an icebath. The protein content of the homogenate was determined by the Lowry method[20] using BSA as a reference standard.

The levels of TBARS in the homogenate were determined according to the –EDTA and +EDTA assay of Kosugi *et al*.[21–23] in the presence of butylated hydroxytoluene (BHT) as an antioxidant. To 0.2 ml of the homogenate were added 0.2 ml of a solution of 5.2% sodium dodecylsulfate in water, 50 μl of a solution of 0.8% BHT in glacial acetic acid, 1.50 ml of 0.8% TBA solution in water, 1.70 ml of water, 0.65 ml of water with or without EDTA (final concentration: 2 mM), and 0.15 ml of 20% sodium acetate buffer (pH 3.5) in this order. After being kept at 5°C for 60 min, the mixture was heated at 100°C for 60 min. Red pigment formed in the solution was extracted by the addition of 1.0 ml of a mixture of 1-butanol/pyridine (15 : 1, v/v). The absorbance at 532 nm of the extract was measured. The levels of TBARS (nmol/mg protein) were calculated using a molecular extinction coefficient of red pigment of 156000, and the protein content of the homogenate.

Local Lymph Node Assay —— Local lymph node assay was performed according to the method described elsewhere.[14,15] A set of three mice was treated on both ears by painting 25 μl of a 1% (w/v) DNCB solution in acetone-olive oil (4 : 1, v/v), or the vehicle alone daily for 3 consecutive days from the fourth day of the exposure of purified air or air containing NO_2. On the seventh day, the mice were anesthetized with an air/diethylether mixture, then the draining auricular lymph nodes were excised, combined for each experimental set and weighed.

LNC suspensions were prepared by mechanical disaggregation through a sterile 200-mesh gauze. The cells were transferred through nylon mesh into a 15 ml centrifuge tube, and the tube was centrifuged at 1200 rpm for 5 min. The LNC suspensions were washed once with HBSS, and total LNC were resuspended in 3.0 ml of an RPMI-1640 culture medium supplemented with 2.5 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% FCS. The total LNC number was counted. The LNC suspension was made at a cell concentration of 5×10^6 cells/ml. The LNC suspensions (200 μl) were seeded into 96-well culture plates (4 wells per set) and cultured with 18.5 kBq [^{3}H]TdR at 37°C for 24 hr in a humidified atmosphere of 5% CO_2 in air. Culturing was terminated by a semiautomatic cell harvester, and [^{3}H]TdR incorporation was determined using a liquid scintillation counter. The mean value of 4 culture wells was obtained.

Four sets of experiments were done for mice of C and C + NO_2 groups with vehicle alone, 8 sets of experiments for mice of C and C + NO_2 groups with DNCB-sensitization, and 4 sets of experiments for mice of –E and –E + NO_2 groups with DNCB-sensitization were performed.

Mouse IgE Test —— The mouse IgE test was performed as described elsewhere.[16,17] At the beginning of exposure to air or air containing NO_2, mice of the C, –E, C + NO_2 and –E + NO_2 groups, three mice for each group, received 50 μl of 1% (w/v) DNCB solution in acetone-olive oil, 25% (w/v) TMA in the vehicle or the vehicle alone on both shaved flanks, and seven days later, received 25 μl of the same solution on both ears. On the seventh day after the challenge, blood was collected by cardiac puncture after anesthetization with an air/diethylether mixture. After standing at room temperature for 12 hr, serum was prepared by centrifugation at 4°C and 2000 rpm for 20 min, then stored at –20°C.

Serum IgE was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). In a Costar E.I.A/R.I.A 96-well strip (Corning Incorporated, Corning, NY, U.S.A.) 120 μl of rat monoclonal anti-mouse IgE antibody (2.5 μg/ml in 0.1 M carbonate buffer pH 9.6) was placed, and the plate was kept at 4°C overnight. The plate was blocked with 120 μl of 5% NGS in PBS at 37°C for 30 min. One hundred microliters of the test sample or mouse monoclonal IgE anti-DNP antibody solution (standard) diluted to various extents in 0.5% NGS-PBS were added to the wells of the plate. The plate was incubated at 37°C for 2 hr. Subsequently, the plates were incubated at 37°C for 2 hr with 100 μl of a 1 : 2000 diluted solution of goat anti-mouse IgE conjugated with horseradish peroxidase in 0.5% NGS-PBS. The plate was washed with 0.05% Tween 20 in PBS three times. *o*-Phenylenediamine and urea

hydrogen peroxide solution (100 μl) was placed in the wells, and the plate was incubated at 37°C for 10 min. The enzyme reaction was terminated by the addition of 0.5 M citric acid. The absorbance at 450 nm of the product of the enzyme reaction was determined using a microplate reader, Benchmark (Bio Rad Laboratories, Tokyo, Japan). The concentration of serum IgE was obtained from the standard curve for the mouse monoclonal IgE.

Statistical analysis: Data were analyzed by Student's *t*-test.

RESULTS

Male BALB/c mice were fed a vitamin E-adequate or a vitamin E-deficient diet for 3 weeks. The vitamin E-adequate diet contained vitamin E at 70×10^{-3} g/kg dried solid, and the vitamin E-deficient diet contained vitamin E at 0×10^{-3} g/kg dried solid. Vitamin E levels in the blood of mice fed the vitamin E-adequate diet (C group) were 1.58 ± 0.2 μg/ml blood, and those of mice with the vitamin E-deficient diet (–E group) were 0.3 ± 0.04 μg/ml blood. Vitamin E levels of mice of the –E group were significantly lower than those of mice of the C group. Vitamin E levels in blood were similar to those of male ddY mice fed the same diets.[24] Mice of the C and –E groups were used in the following experiments.

Mice of the C and –E groups, six mice each, were continuously housed in an atmosphere of purified air and air containing NO_2 (C + NO_2 and –E + NO_2 groups), and fed the corresponding diets for 1 week. In order to know the effect of vitamin E-deficiency and NO_2 inhalation on the degree of lipid peroxidation of the lung, a TBA assay was performed. The TBA assay was conducted by a method that could distinguish between TBARS derived from malonaldehyde derivatives + alkadienal/alkenal derivatives (–EDTA assay) and TBARS derived from malonaldehyde derivatives alone (+EDTA assay) in the presence of BHT as an antioxidant.[21–23] As shown in Fig. 1, there were no significant differences in the levels of TBARS from malonaldehyde derivatives or from malonaldehyde derivatives + alkadienal/alkenal derivatives among the lung tissues from mice of the C, –E, C + NO_2 and –E + NO_2 groups. The results indicate that vitamin E-deficiency and/or NO_2 inhalation did not cause a severe increase in the degree of lipid peroxidation of lung tissues of mice under the experimental conditions.

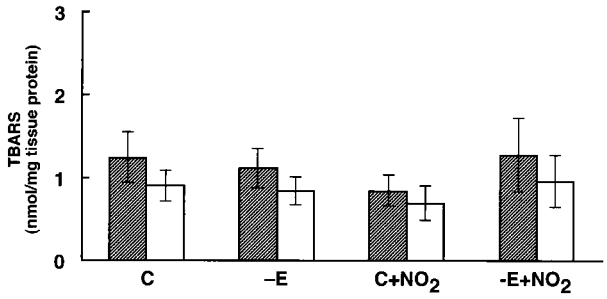

Fig. 1. Levels of TBARS in Lung of Mice of C and –E Groups Housed in an Atmosphere of Purified Air, and Mice of C + NO_2 and –E + NO_2 Groups Housed in an Atmosphere of NO_2 for 1 week

TBARS were obtained in the +EDTA assay (white bars) and –EDTA assay (slashed bars). Results are expressed as TBARS (nmol/mg lung tissue protein) (mean value ± S.D.) of six mice for each group.

Mice of the C, –E, C + NO_2, and –E + NO_2 groups, three mice for each group, were housed for 1 week. Mice from each group were treated on both ears by DNCB solution or the vehicle alone, daily for 3 consecutive days from the fourth day of exposure. On the seventh day of exposure, draining auricular lymph nodes were excised. Lymph nodes from three mice were combined for each experimental set. The weight of the lymph nodes and total LNC number were counted. A local lymph node assay of DNCB-sensitized and non-sensitized cells of each set was performed.

The mean values of lymph node weight and LNC number obtained from more than 4 sets of experiments are listed in Table 1. The mean values of local lymph node assay obtained from more than 4 sets of experiments are shown in Fig. 2. As has been shown in the previous papers,[7,14] the lymph node weight and total LNC number of the C group was increased by DNCB-sensitization (Table 1). There were no differences in weight or LNC number between unsensitized mice of C and C + NO_2 groups, or among DNCB-sensitized mice of C, –E, C + NO_2 and –E + NO_2 groups (Table 1). As has been shown previously,[7,14] the degree of cell proliferation of mice of the C group was increased by DNCB-sensitization. The DNCB-sensitized increase in cell proliferation was not affected by NO_2 inhalation (C + NO_2 group) (Fig. 2). It is interesting to note that although the degree of DNCB-sensitized cell proliferation of mice of the –E group was lower than that of mice of the C group,[7] that of mice of the –E + NO_2 group was higher than those of mice of the –E group (Fig. 2). The results indicate that while NO_2 inhalation alone does not affect the DNCB-sensitized in-

Table 1. Averaged Effect of Vitamin E-Deficiency and/or NO_2 Inhalation on Draining Auricular Lymph Node Weight and LNC Number Induced by Painting DNCB on Ears

Group	Set number	DNCB	Lymph node weight (mg ± S.D./group)	Total LNC number [(number ± S.D.) $\times 10^{-6}$/group)]
C	4	−	28.50 ± 17.80	14.40 ± 5.70
C	8	+	60.05 ± 25.10	62.61 ± 11.90
−E	4	+	49.53 ± 26.00	77.43 ± 7.10
C+NO_2	4	−	24.70 ± 10.90	16.50 ± 1.90
C + NO_2	8	+	65.11 ± 18.10	65.23 ± 16.90
−E + NO_2	4	+	43.90 ± 22.30	61.50 ± 26.70

Mice of the C and −E groups were housed in an atmosphere of purified air, and mice of the C + NO_2 and −E + NO_2 groups were housed in an atmosphere of NO_2 for 1 week. A set of three mice for each group was treated on both ears by painting DNCB solution or the vehicle alone daily for 3 consecutive days from the fourth day of exposure. On the seventh day of exposure, the draining auricular lymph nodes were excised, combined for each experimental set, and weighed, and the total LNC number was counted. The LNC suspensions were cultured with [^{3}H]TdR, and [^{3}H]TdR incorporation was determined. Mean values ± S.D. of 4 or 8 sets of the experiments.

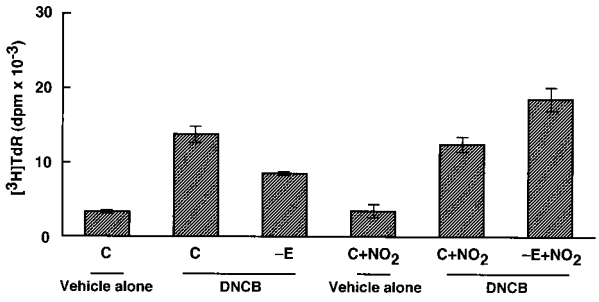

Fig. 2. Averaged Effect of Vitamin E-Deficiency and/or NO_2 Inhalation on Draining Auricular LNC Proliferation Induced by Painting DNCB on Ears

Mice of the C and –E groups housed in an atmosphere of purified air, and mice of C + NO_2 and –E + NO_2 groups housed in an atmosphere of NO_2 for 1 week, as shown in Table 1. The LNC suspensions were cultured with [^{3}H]TdR, then [^{3}H]TdR incorporation was determined. Mean values ± S.D. of 4 or 8 sets of the experiments of [^{3}H]TdR incorporation (dpm) in the local lymph node assay are shown.

crease in the degree of cell proliferation, the combination of vitamin E-deficiency and NO_2 inhalation did cause such an increase.

Mice of the C, –E, C+ NO_2, and –E+ NO_2 groups were housed for 2 weeks. Three mice from each group were treated on both flanks by either the DNCB solution, the TMA solution or the vehicle alone, at the beginning of the exposure. Seven days later, both ears were challenged with the same solution. On the seventh day after the challenge, blood serum was obtained, and the serum IgE levels were determined by sandwich ELISA. The mean IgE levels of each group are shown in Fig. 3. There were no differences in the unsensitized IgE levels among mice of the C, –E, C + NO_2, and –E + NO_2 groups, indicating that vitamin E-deficiency alone or NO_2 inhalation alone did not affect the serum IgE levels. When sensitized with DNCB, significantly increased levels of serum IgE were observed in mice of the –E and –E + NO_2 groups, as compared to the levels in mice of C and C + NO_2 groups, indicating that vitamin E-deficiency caused an increase in DNCB-sensitized serum IgE levels. When sensitized with TMA, the serum IgE levels of mice of the C and –E groups increased, and those in mice of the C + NO_2 and –E + NO_2 groups increased to a much greater extent, indicating that NO_2 inhalation caused an increase in TMA-sensitized serum IgE levels.

DISCUSSION

The degree of lipid peroxidaion, as assessed by TBARS of the lung of mice, was not affected by feeding them a vitamin E-deficient diet for a long period, nor was it affected by NO_2 inhalation. It has been shown that long-term inhalation of NO_2 causes oxidative damage to the lung. NO_2 inhalation causes pulmonary edema, pulmonary fibrosis, bronchitis[8–10] and cancer,[11] and inhalation of NO_2 into rats causes lung lipid peroxidation, as assessed by the increase in conjugated diene formation[12] and hydrocarbon exhalation.[13] In the present study, inhalation of NO_2 to mice, even in the vitamin E-deficient state, caused no remarkable increase in the degree of lipid peroxidation in the lung. Nevertheless, previous studies[7] have shown that the levels of TBARS in the spleen and lymph nodes of mice fed a vitamin E-deficient diet showed a remarkable increase in comparison to those of mice fed a vitamin E-adequate diet.

In the lymph node assay for type IV allergy (contact sensitization) responses, the degree of DNCB-

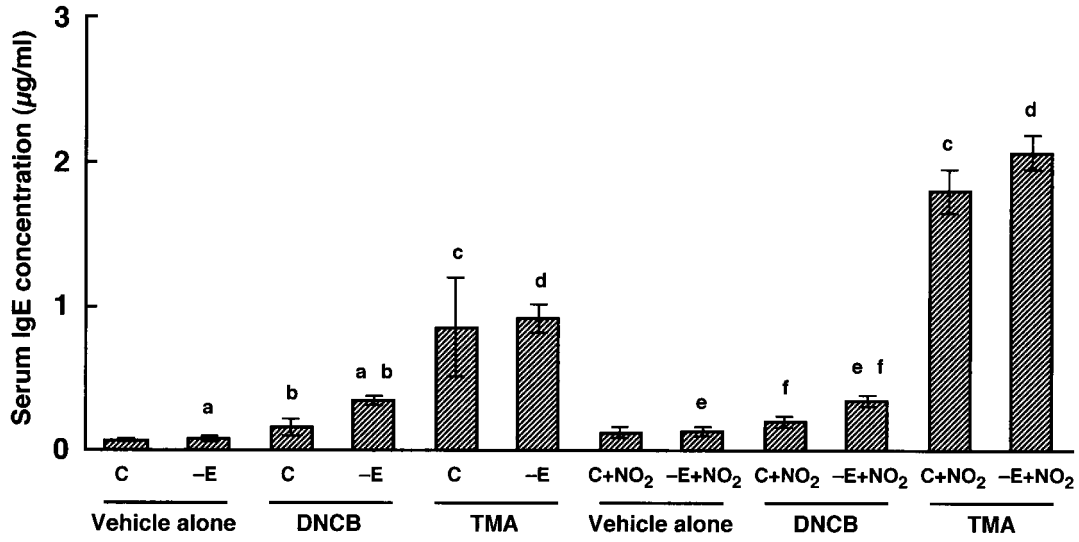

Fig. 3. Averaged Effect of Vitamin E-Deficiency and NO_2 Inhalation on Serum IgE Levels of Mice Treated with DNCB or TMA

Mice of the C and –E groups were housed in an atmosphere of purified air, and mice of C + NO_2 and –E + NO_2 groups were housed in an atmosphere of NO_2 for 2 weeks. Three mice for each group were treated on both shaved flanks by painting on a DNCB solution, TMA solution or the vehicle alone at the biginning of exposure, and 7 days later the same solution was painted to both ears. Seven days after the challenge, blood was collected, and the serum IgE level was determined by sandwich ELISA. Mean values of serum IgE levels ± S.D. of three serum samples are shown. a: $p < 0.002$, b: $p < 0.03$, c: $p < 0.02$, d: $p < 0.01$, e: $p < 0.03$ and f: $p < 0.07$.

sensitized LNC proliferation was lowered by vitamin E-deficiency. This result was consistent with a previous observation.[7] In the present study, DNCB-sensitization did not increase serum IgE levels in vitamin E-adequate mice in type I allergy (respiratory sensitization) responses, which is consistent with the earlier observation by Hilton *et al.*[17] However, serum IgE levels in type I allergy responses by DNCB-sensitization were increased by vitamin E-deficiency.

It has been shown that chemical allergens of different types provoke divergent immune responses by selective activation of discrete CD^{4+} Th cell populations[25–28] of two main populations, Th1 and Th2, which differ with respect to cytokine secretion patterns.[29,30] In type IV allergy responses, contact allergens induce immune responses characterized by Th1 T cell activation,[31] which releases cytokine IFN-γ to antagonize the production of IgE.[32] In contrast, in type I allergy responses, respiratory allergens induce immune responses characterized by Th2 cell activation, which produce cytokine IL-4 to initiate and maintain IgE production.[33] The decreased LNC proliferation by vitamin E-deficiency in DNCB-sensitized type IV allergy responses observed here may be derived from vitamin E activity unrelated to its antioxidant activity on the immune responses. Vitamin E is known to enhance immune responses, and to stimulate mitogenic responses of lymphocytes.[3–6] The increased IgE levels caused by vitamin E-deficiency in type I allergy responses with DNCB-sensitization may be derived from increased Th2 cell activation and the production of IL-4.

In the lymph node assay, although the degree of DNCB-sensitized LNC proliferation was not affected by NO_2 inhalation alone, the combination of NO_2 inhalation and vitamin E-deficiency caused a remarkable increase in DNCB-sensitized LNC proliferation. Moreover, a TMA-sensitized increase in serum IgE levels was remarkably enhanced by NO_2 inhalation alone.

Allergen-sensitized type IV and type I allergy responses observed here might be mediated by the oxidative stress induced by vitamin E-deficiency and NO_2 inhalation. With respect to this point, we clearly demonstrated that oxidative stress induced by food reductones, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone and hydroxyhydroquinone, enhanced lung lipid peroxidation, DNCB-sensitized type IV allergy responses and DNCB- and TMA-sensitized type I allergy responses of mice.[34] The present results are consistent with those of these separate experiments, and it is likely that the increases in allergen-sensitized type IV and type I allergy responses induced by vitamin E-deficient and/or NO_2 inhalation are due to oxidative stress.

In conclusion, vitamin E-deficiency and/or NO_2 inhalation in mice enhanced the increase in DNCB-

sensitized LNC proliferation, and enhanced DNCB- or TMA-sensitized serum IgE increase. The results may postulate that vitamin E-deficiency and/or NO_2 inhalation causes undesirable effects on both allergen-sensitized type IV and type I allergy responses.

Acknowledgement This study was performed through Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1) Burton, G. W., Joyce, A. and Ingold, K. U. (1982) First proof that vitamin E is a major lipid-soluble, chain-breaking antioxidant in human blood plasma. *Lancet*, **8292**ii, 327.
2) Tappel, A. L. (1972) Vitamin E and free radical peroxidation of lipids. *Ann. N.Y. Acad. Sci*., **203**, 12–28.
3) Bendich, A., Gabriel, E. and Machlin, L. J. (1986) Dietary vitamin E requirement for optimum immune response in the rat. *J. Nutr*., **116**, 675–681.
4) Corwin, L. M. and Shloss, J. (1980) Influence of vitamin E on the mitogenic response of lymphoid cells. *J. Nutr.*, **110**, 916–923.
5) Meydani, M., Meydani, S. N., Leka, L., Gong, J. and Blumberg, J. B. (1993) Effect of long-term vitamin E supplementation on lipid peroxidation and the immune response of young and old subjects. *FASEB J*., **7**, A415.
6) Sheffy, B. E. and Schultz, R. D. (1979) Influence of vitamin E and selenium on immune response mechanisms. *Fed. Proc*., **38**, 2139–2143.
7) Ikarashi, Y., Tsuchiya, T., Nakamura, A., Beppu, M. and Kikugawa, K. (1998) Effect of vitamin E on contact sensitization response induced by 2,4-dinitro chlorobenzene in mice. *J. Nutr. Sci. Vitaminol*. (Tokyo), **44**, 225–236.
8) Dawson, S. V. and Schenker, M. B. (1979) Health effects of inhalation of ambient concentrations of nitrogen dioxide. *Am. Rev. Respir. Dis*., **120**, 281–292.
9) Guidotti, T. L. (1972) The higher oxides of nitrogen: Inhalation toxicology. *Environ. Res*., **15**, 443–472.
10) Stephen, R. J., Freeman, G. and Evans, M. (1972) Early response of lungs to low levels of nitrogen dioxide. *Arch. Environ. Health*, **24**, 160–179.
11) Richter, A. (1988) Effects of nitrogen dioxide and ozone on blood-borne cancer cell colonization of the lungs. *J. Toxicol. Environ. Health*, **25**, 383–390.
12) Thomas, H. V., Mueller, P. K. and Lyman, R. L. (1968) Lipoperoxidation of lung lipids in rats exposed to nitrogen dioxide. *Science*, **159**, 532–534.
13) Sagai, M., Ichinose, T., Oda, H. and Kubota, K. (1981) Studies on biochemical effects of nitrogen dioxide: I. Lipid peroxidation as measured by ethane exhalation of rats exposed to nitrogen dioxide. *Lipids*, **16**, 64–67.
14) Ikarashi, Y., Tsuchiya, T. and Nakamura, A. (1993) Evaluation of contact sensitivity to rubber chemicals using the murine local lymph node assay. *Contact Dermatitis*, **28**, 77–80.
15) Kimber, I., Dearman, R. J., Sholes, E. W. and Basketter, D. A. (1994) The local lymph node assay: Developments and applications. *Toxicology*, **93**, 13–31.
16) Dearman, R. J., Basketterr, D. A. and Kimber, I. (1992) Variable effects of chemical allergens on serum IgE concentration in mice. Preliminary evaluation of a novel approach to the identification of respiratory sensitizers. *J. Appl. Toxicol*., **12**, 317–323.
17) Hilton, J., Dearman, R. J., Basketter, D. A., Scholes, E. W. and Kimber, I. (1996) Experimental assessment of the sensitizing properties of formaldehyde. *Food Chem. Toxicol*., **34**, 571–578.
18) Saltzman, B. E. (1960) Colorimetric microdetermination of nitrogen dioxide in the atmosphere. *Anal. Chem*., **3**, 135–136.
19) Lehmann, J. and Martin, H. L. (1982) Improved direct determination of α- and γ-tocopherols in plasma and platelets by liquid chromatography, with fluorescence detection. *Clin. Chem*., **28**, 1784–1787.
20) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., **193**, 265–275.
21) Kosugi, H., Kato, T. and Kikugawa, K. (1988) Formation of red pigment by a two-step 2-thiobarbituric acid reaction of alka-2,4-dienals. Potential products of lipid peroxidation. *Lipids*, **23**, 1024–1031.
22) Kosugi, H., Kojima, T. and Kikugawa, K. (1991) Characteristics of the thiobarbituric acid reactivity of oxidized fats and oils. *J. Am. Oil Chem. Soc*., **68**, 51–55.
23) Kosugi, H., Kojima, T., Yamaki, S. and Kikugawa, K. (1992) Interpretation of the thiobarbituric acid reactivity of rat liver and brain homogenates in the presence of ferric ion and ethylenediaminetetraacetic acid. *Anal. Biochem*., **202**, 249–255.
24) Ando, K., Nagata, K., Kazama, O., Furusawa, T. and Kikugawa, K. (2002) Biphasic adverse effects of vitamin E on mouse macrophage binding and uptake of oxidized low density lipoprotein. *J. Oleo Sci.,* submitted for publication.
25) Dearman, R. J. and Kimber, I. (1991) Differential stimulation of immune function by respiratory and contact chemical allergens. *J. Immunol*., **72**, 563–

570.
26) Dearman, R. J. and Kimber, I. (1992) Divergent immune responses to respiratory and contact chemical allergens: antibody elicited by phthalic anhydride and oxazolone. *Clin. Exp. Allergy*, **22**, 241–250.
27) Dearman, R. J., Spence, L. M. and Kimber, I. (1992) Characterization of murine immune responses to allergenic diisocyanates. *Toxicol. Appl. Pharmacol.*, **112**, 190–197.
28) Dearman, R. J., Ramdin, L. S. P., Basketter, D. A. and Kimber, I. (1994) Inducible interleukin-4-secreting cells provoked in mice during chemical sensitization. *Immunol.*, **81**, 551–557.
29) Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986) Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.*, **136**, 2348–2357.
30) Mosmann, T. R. and Coffman, R. L. (1989) Heterogeneity of cytokine secretion patterns and functions of T helper cells. *Adv. Immunol.*, **46**, 111–147.
31) Cher, D. J. and Mosmann, T. R. (1987) Two types of murine helper T cell clones. II. Delayed type hypersensitivity is mediated by Th 1 clones. *J. Immunol.*, **138**, 3688–3694.
32) Finkelman, F. D., Katona, I. M., Mosmann, T. R. and Coffman, R. L. (1988) IFN-γ regulates the isotypes of Ig secreted during *in vivo* humoral immune responses. *J. Immunol.*, **140**, 1022–1027.
33) Finkelman, F. D., Katona, I. M., Urban, J. F., Jr., Holmes, J., Ohara, J., Tung, A. S., Sample, J. G. and Paul, W. E. (1988) IL-4 is required to generate and sustain *in vivo* IgE responses. *J. Immunol.*, **141**, 2335–2341.
34) Mi, H., Hiramoto, K., Kujirai, K., Ikarashi, Y., Ando, K. and Kikugawa, K. (2001) Effect of food reductones, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF) and hydroxyhdroquinone (HHQ), on lipid peroxidation, type IV and type I allergy responses of mouse. *J. Agric. Food Chem.*, **49**, 4950–4955.