

The effects of vitamin E supplementation on autoimmune-prone New Zealand black × New Zealand white F₁ mice fed an oxidised oil diet

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The purpose of the present study was to investigate the effect of vitamin E supplementation on autoimmune disease in New Zealand black × New Zealand white F₁ (NZB/W F₁) female mice fed an oxidised oil diet. First, 5-month-old mice were fed an AIN-76 diet containing either 150 g fresh soyabean oil/kg (15S), 50 g fresh soyabean oil/kg + 100 g oxidised frying oil/kg (5S10F) or 5S10F supplemented with all-rac- α -tocopheryl acetate at 275 mg/kg diet level (5S10F5E) or 550 mg/kg (5S10F10E), respectively, in experiment 1. The results showed that mice fed the 5S10F10E diet had a lower anti-double-stranded DNA IgG antibody level and a longer lifespan than those fed the 15S and 5S10F diets. Therefore, the 5S10F and 5S10F10E treatments were repeated in experiment 2 for further analysis. The results showed that vitamin E supplementation in the oxidised oil significantly decreased thiobarbituric acid-reactive substance values in the kidney and spleen of NZB/W F₁ mice. Interferon- γ and IL-6 production by mitogen-stimulated splenocytes decreased in mice fed the 5S10F10E diet, whereas the secretion of IL-2 and IL-10 was not affected. The percentage of T-cells was significantly higher and that of MHC class II-bearing cells was lower in the spleens of the 5S10F10E group. The 5S10F10E group had a significantly higher linoleic acid (18:2n-6) composition than the 5S10F diet group. Therefore, vitamin E supplementation in oxidised oil might decrease oxidative stress, anti-double-stranded DNA IgG antibody, regulate cytokines and lymphocyte subsets, and subsequently alleviate the severity of autoimmune disease such as systemic lupus erythematosus under oxidative stress.

Vitamin E: Oxidised oil: NZB/W F₁ mice: Systemic lupus erythematosus

New Zealand black × New Zealand white F₁ (NZB/W F₁) mice are commonly used as a murine lupus model to provide much insight into the pathogenic mechanisms of systemic lupus erythematosus (SLE)-like autoimmune disease (Theofilopoulos *et al.* 1989). NZB/W F₁ mice have been characterised with features including polyclonal B-cell activation, the production of autoantibodies and rheumatoid factor, circulating immune complex-mediated glomerulonephritis, vasculitis and arthritis, dying of uraemia in early life (Drake *et al.* 1994; Kotzin, 1996). Although the mechanism of pathogenesis of the autoimmunity remains unclear, many studies have suggested a genetic contribution to the development of autoimmunity in New Zealand black mice. One study showed that an increased number of MHC molecules on the surface of immune effector cells might contribute to the abnormal expansion of autoreactive cells and production of autoantibodies in SLE (Santiago-Raber *et al.* 2004), suggesting that lupus susceptibility alleles within or closely linked to the MHC locus play an important role in the development of or protection against SLE.

Cytokine imbalances also play a significant role in the acceleration of lupus-like autoimmune disease. The development of SLE is mediated to an equal extent by either T helper (Th) 1 cytokines or Th2 cytokines (O'Shea *et al.* 2002). In patients with SLE, serum levels of Th2 cytokines such as IL-4, IL-6 and IL-10 are

elevated, whereas there is a decrease in the production of Th1 cytokines, including IL-2 and interferon- γ (IFN- γ). The shifting of Th1 to Th2 immune responses leads to B-cell hyperactivity and the production of pathogenic autoantibodies (Akahoshi *et al.* 1999). Dietary factors, such as a 20% high-fat diet composed of equal amounts of lard and soyabean oil, have been shown to increase MHC class II expression, increase IL-6, TNF- α and prostaglandin E₂ (PGE₂) production, lower the IL-2:IL-4 ratio and shorten the lifespan of NZB/W F₁ mice, although IFN- γ was not significantly affected (Lin *et al.* 1996b). IFN- γ has recently been demonstrated to be a prerequisite for lupus development in autoimmune-prone NZB/W F₁ and MRL/lpr mice (Balomenos *et al.* 1998; Haas *et al.* 1998). NZB/W F₁ mice treated intraperitoneally with IFN- γ -encoding plasmid had a shorter lifespan (Hasegawa *et al.* 2002), whereas IFN- γ receptor deletion prevented autoantibody production and glomerulonephritis, and thus prolonged lifespan (Haas *et al.* 1998). IFN- γ enhances production of the proinflammatory cytokines IL-1, IL-6 and TNF- α . IFN- γ hyperproduction may increase MHC expression and autoantigen presentation, and promote local immune and inflammatory processes. Therefore, two stages of T-cell activation and cytokine secretion, i.e. the initial expression of Th1-type cytokines, followed by the induction of Th2-type cytokines, have been proposed in mice with experimental SLE (Akahoshi *et al.*

Abbreviations: ds, double-stranded; IFN, interferon; NZB/W F₁, New Zealand black × New Zealand white F₁; PGE₂, prostaglandin E₂; SLE, systemic lupus erythematosus; ss, single-stranded; Th, T helper.

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1999). Whether dietary factors may affect these two stages is worthy of investigation.

The amount and degree of saturation of dietary fat affect disease course in autoimmune mice. A low-fat diet and fish oil decreased autoantibody production and prolonged the lifespan of NZB/W F₁ mice (Fernandes, 1994; Wu *et al.* 2000). Higher amounts of dietary fat or oxidised oil increased serum anti-cardiolipin antibody level in NZB/W F₁ mice (Lin & Jeng, 1997; Lin *et al.* 1997a), and promoted peritoneal cell PGE₂ production in mice (Lin *et al.* 1996b, 2000). Ovalbumin-sensitised BALB/c mice fed oxidised oil had higher serum ovalbumin-specific IgG₁ and IgE levels and Th2 responses (Lin *et al.* 2000). These studies indicate that the quantity and quality of dietary fat alter immune responses and antibody production, and affect disease severity in animal models.

Nowadays, fried foods are important contributors to a high-fat diet. The degree of lipid oxidation can affect lymphoid organs and affect immune responses in normal mice and rats (Oarada *et al.* 1989; Lin *et al.* 1997b). An increased dietary content of oxidised oil that causes oxidative stress and deleteriously changes lipoprotein, platelet and lipid metabolism might aggravate diseases such as autoimmune diseases, allergies and tumours (Kubow, 1992; Liu & Huang, 1995; Lin *et al.* 1996a, 2000). The oxidative stress, caused by reactive oxygen species, has been implicated as a cause of tissue damage and as a causative agent in the pathogenesis of several degenerative diseases (Blount *et al.* 1991; Ruiz-Torres *et al.* 1997). Reactive oxygen species-modified DNA has been found to be a better antigen for anti-DNA antibodies found in SLE sera (Blount *et al.* 1990). Furthermore, MRL/lpr mice had a lower mRNA expression of antioxidant enzymes and activities (Venkatraman *et al.* 1994). Serum from SLE patients had lower concentrations of α -tocopherol, superoxide dismutase and glutathione peroxidase, and a higher plasma malone dialdehyde level (Comstock *et al.* 1997; Bae *et al.* 2002). Lymphocytes isolated from patients suffering from rheumatoid arthritis and SLE contained higher oxidised DNA (Lunec *et al.* 1994), suggesting that a deficient repair system may result in the release of oxidised DNA and induce chronic inflammatory diseases (Ahsah *et al.* 2003).

Therefore, whether the supplementation with antioxidant vitamin E of autoimmune-prone mice fed with oxidised oil can retrieve the detrimental effect of oxidative stress is worthy of study. Vitamin E is not only the most effective free radical and peroxy radical scavenger (Packer, 1991), but may also enhance humoral and cellular immune reactions, Th cell function and anti-inflammatory reactions (Meydani, 1995; Meydani & Beharka, 1996; Lee & Wan, 2000). In addition, vitamin E has been shown to inhibit arachidonic acid metabolism and reduce the production of PGE₂, which indirectly regulates the immune response and proinflammatory process (Meydani & Beharka, 1996). In our previous experiments, a 200 g oxidised oil/kg diet supplemented with all-rac- α -tocopheryl acetate at the level of 220 mg/kg diet tended to retard the development of autoimmune disease in NZB/W F₁ mice (Lin & Chi, in press). In order to clarify the effect of vitamin E supplementation on autoimmune disease under oxidative stress, NZB/W F₁ mice fed the 100 g oxidised oil/kg supplemented with vitamin E were followed to determine their lifespan and autoantibody production; *in vivo* oxidative status, cytokine secretions, phenotype and fatty acid composition of the splenocytes, and urinary bicyclo-PGE₂ were investigated in this study.

Materials and methods

Experimental animals

Female NZB/W F₁ (7 weeks old) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the Institute of Microbiology and Biochemistry at the National Taiwan University. The animal room was maintained on a 12 h light-dark cycle with a constant temperature ($25 \pm 2^\circ\text{C}$) and humidity. Animal care and handling conformed to the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Experiment 1

The oxidised oil was prepared as follows: 4.5 kg soyabean oil (President Co., Tainan, Taiwan) was poured into a cast-iron wok (40 cm internal diameter, 10 cm central depth, 6.5 litre capacity) and heated on a gas stove that was adjusted to maintain the oil temperature at $205 \pm 5^\circ\text{C}$. The wheat-flour dough sheets ($12 \times 4.5 \times 0.15$ cm, about 11 g/weight) were fried in the oil, one at a time. The wheat-flour dough was made by mixing together 1500 g high-gluten wheat flour, 200 g sugar, 5 g baking powder and 600 g water. The frying proceeded for 6 h/d and was repeated successively for 4 d according to previously reported procedures (Lin *et al.* 1996a). The oxidised oil was stored at -20°C for preparation of the test diets.

In order to eliminate malnutrition caused by the high content of oxidised oil, but still show the effects of dietary fat, 10% oxidised oil and total 15% dietary fat were used in this study. Thirty-six 5-month-old mice were randomly assigned to four groups and fed one of four experimental diets containing the following: 150 g fresh soyabean oil/kg (15S), 50 g fresh soyabean oil/kg + 100 g oxidised frying oil/kg (5S10F) or 5S10F supplemented with all-rac- α -tocopheryl acetate 275 mg/kg (5S10F5E) or 550 mg/kg (5S10F10E). The composition of the four test diets is shown in Table 1. The mice were given free access to the test diets throughout the experiment and weighed twice weekly.

Table 1. Composition of test diets used in the feeding experiment (g/kg diet)*

Ingredient†	15S	5S10F	5S10F5E	5S10F10E
Fresh soyabean oil	150	50	50	50
Oxidised frying oil‡	0	100	100	100
Casein	224	224	224	224
Methionine	3	3	3	3
Corn starch	258	258	258	258
Sucrose	258	258	258	258
Cellulose	55	55	55	55
Mineral mixture	39	39	39	39
Vitamin mixture	11	11	11	11
Choline	2	2	2	2
Energy (kJ/kg)	18 102	18 102	18 102	18 102
% Energy from protein	20.8	20.8	20.8	20.8
% Energy from fat	31.3	31.3	31.3	31.3

* 15S, 150 g fresh soyabean oil/kg; 5S10F, 50 g fresh soyabean oil/kg + 100 oxidised frying oil/kg; 5S10F5E, 5S10F supplemented with 275 mg/kg all-rac- α -tocopheryl acetate; 5S10F10E, 5S10F supplemented with 550 mg/kg all-rac- α -tocopheryl acetate.

† Sources of ingredients: casein, ICN (Aurora, OH, USA); cornstarch (Samyang genex Seoul, Korea); sucrose, Taiwan Sugar Co. (Taipei, Taiwan), cellulose, J. Betten-Maier & Söhne, Rosenberg (Holzmühle, Germany); soyabean oil, President Co. (Tainan, Taiwan); methionine and choline, Sigma (St Louis, MO, USA); AIN-76 mineral mixture and AIN-76 vitamin mixture, ICN (Aurora).

‡ Prepared by frying dough sheet in soyabean oil at $205 \pm 5^\circ\text{C}$ for 24 h.

Determination of proteinuria and lifespan. Proteinuria was measured in a semi-quantitative manner, using tetrabromophenol paper (Yeongdong, Pharmaceutical Co. Seoul, Korea) on fresh urine samples. This colorimetric assay, which is relatively specific for albumin, was graded from 1+ to 4+, the approximate protein concentrations being as follows: 1+, 0.3 g/l; 2+, 1 g/l; 3+, 10 g/l; 4+, > 10 g/l. Proteinuria was defined as a reading higher than 2+ (1 g/l). Mice were followed up to determine their lifespan and for proteinuria, which is an index of lupus glomerulonephritis, every week to confirm pathological changes.

Determination of anti-DNA antibodies. Anti-double-stranded (ds) and anti-single-stranded (ss) DNA antibodies in the serum of NZB/W F₁ mice were determined by ELISA. Plates were coated with methylated bovine serum albumin (10 g/l; Sigma, St Louis, MO, USA). After incubation at 4°C overnight, the plates were washed, coated with 2.5 g/l mouse dsDNA antigen and incubated at 4°C overnight. Then 200 µl PBS containing 1% gelatin (Sigma) was added, and the plates were incubated at 25°C for 2 h and washed. Serum to be tested was diluted (1/150 v/v for IgG and IgM) in PBS-gelatin for 2 h at 25°C. The plates were washed with PBS containing 0.05% Tween 20 (wash buffer). Horseradish peroxidase-conjugated rabbit anti-mouse γ chain-specific antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was then added. After 2 h of incubation at 25°C, the plates were washed and incubated with 100 µl substrate solution ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid; Sigma). Absorbance was determined at 415 nm (Microplate; Bio-Tek Instrument, Winooski, VT, USA). The autoantibody results were calculated as the absorbance at 415 nm using a positive control of monoclonal antibody specific for IgG or IgM. The monoclonal antibodies 10F10A3 and 6H7B5 from the laboratory were prepared for use as positive controls for the anti-DNA IgG and IgM assays.

Experiment 2

Twenty 5-month-old mice were randomly assigned to two groups and fed one of two experimental diets, containing 5S10F and 5S10F10E, respectively. The mice were raised as described in experiment 1 and were maintained on the experimental diets for 2.5 months before being killed for *in vivo* analysis.

Determination of thiobarbituric acid-reactive substances. Oxidised tissue lipids were measured as tissue homogenate malondialdehyde production. A volume of 1 ml tissue homogenate and 1 ml 10% TCA were mixed and then centrifuged at 1500 rpm for 10 min. One millilitre of the upper layer was collected into a tube containing 1 ml 0.4% 2-thiobarbituric acid, and 0.1 ml 0.2% butylated hydroxy toluene was added and mixed in. The mixture was incubated at 50°C for 1 h. After cooling, 2 ml isobutanol was added, the mixture being mixed in a vortex until clear and then centrifuged. The sample supernatant was measured at excitation 515 nm and emission 550 nm. Yields of malondialdehyde were calculated using 1,1,3,3-tetra-methoxy propane as a standard.

Cell culture. The spleens were removed and single cells suspension were isolated by lysing erythrocytes with ammonia chloride-tris buffer and washed with Hank's balanced saline solution before use. Splenocytes at a concentration of 5×10^6 cells/ml were placed in twenty-four-well plates in RPMI-1640 medium supplemented with 10% TCM (mouse serum replacement; Celox Corp., Hopkins, MN, USA). Mitogens (phytohaemagglutinin

10 mg/l; lipopolysaccharide 10 mg/l; Sigma) were added as a stimulus for cell cultures. Suspensions were cultured at 37°C in a 5% CO₂-humidified incubator for 24 h.

Determination of cytokines. The collected supernatants were assayed for cytokines by sandwich ELISA. Predetermined concentrations of anti-IL-2, -IFN- γ , -IL-4, -IL-6 and -IL-10 antibodies (PharMingen, San Diego, CA, USA) were diluted in NaHCO₃ buffer at pH 9.6 and coated on plates. After incubation at 4°C overnight, the plates were washed and blocked with 200 µl blocking solution (PBS buffer containing 2% bovine serum albumin; Sigma). The plates were washed with PBS containing 0.5 ml Tween 20/l, the supernatant then being added and the plates incubated at 25°C for 2 h. The plates were then washed before adding biotin-conjugated anti-IL-2, -IFN- γ , -IL-4, -IL-6 and -IL-10 antibodies (PharMingen) at 25°C for 2 h. After washing, horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch) was added at 25°C for 30 min. After washing, the plates were incubated with ABTS and measured for absorbance at 415 nm. The data were calculated according to the cytokine standard curve (PharMingen).

Flow cytometry. Single-cell suspensions were prepared from the spleen of mice. Direct staining was performed by incubating viable cells (2.5 – 5.0×10^5 cells) from the splenocytes at 4°C in the dark for 30 min with fluorescein isothiocyanate-, phycoerythrin- or cyochrome-conjugated monoclonal antibodies against CD3, CD4, CD8, CD5, B220, NK1.1, IA^k and IA^d (PharMingen). The cells were washed and resuspended in 0.5 ml PBS with 2% fetal bovine serum (GIBCO, Grand Island, NY, USA) plus 0.1% sodium azide and subjected to fluorescence-activated cell sorting (FACScan) analysis. A total of 10^4 cells were counted, the frequency of each cell surface marker being determined using appropriate software (FACScan; Becton Dickinson, Mountain View, CA, USA).

Splenocytes fatty acid composition. To investigate further the effect of vitamin E supplementation on the fatty acid composition of immune cells, splenocyte fatty acid compositions were analysed. Pooled splenocytes from two mice were extracted with chloroform-methanol (2:1, v/v), and the fatty acid compositions were analysed according to the method of Lin *et al.* (1997a).

Bicyclo-prostaglandin E₂ level in urine. The urine samples were collected for 24 h, and bicyclo-PGE₂ was measured by enzyme immunoassay kit (Correlate-EIA bicyclo PGE₂ kit; Cayman Chemical Co., Ann Arbor, MI, USA). The bicyclo-PGE₂ assay was developed as a method of converting all the PGE₂ plasma metabolites to a single, stable derivative that could be easily quantified according to an operation manual. The amount of the creatinine in the urine was also determined with colorimetric assay by creatinine kit (Randox, Crumlin, Co. Antrim, UK).

Statistical analysis and mouse survival analysis. Data were expressed as means with their standard errors for each group. Significance was determined by analysis of Duncan's multiple range test or Student's *t* test using the SAS software program (SAS/STAT version 8; SAS Institute, Cary, NC, USA). Survival analysis was compared between two different curves using Cox's proportional hazards regression test (STATA version 8.0; Stata Corp., College Station, TX, USA). The difference was considered significant at a *P*-value of less than or equal to 0.05.

Results

Experiment 1

Growth, proteinuria and lifespan. There was no significant difference in body weight gain and food efficiency in mice

consuming the four diets containing different dietary fat and vitamin E supplementation (data not shown). The growth curves were similar in the four groups by 8 months of age. At the late stages of disease, the mice had serious proteinuria and their body weight decreased. The mice in 5S10F10E group had a delayed drop in body weight ($P < 0.05$ v. 5S10F from 11 months old). The mice began to show proteinuria at 6 months old (Fig. 1(a)). Half of the mice in the 15S group developed proteinuria at 9 months old, and all had proteinuria at 12 months old, whereas only 60% of the mice in 5S10F10E group had proteinuria. Only half of the 15S group survived at 13 months of age, while all the 5S10F10E mice were still alive at that age (Fig. 1(b)). The lifespan of the 5S10F10E group (453 (SEM 17) d) was significantly longer than that of the 15S (378 (SEM 22) d; $P = 0.029$) and 5S10F (377 (SEM 24) d; $P = 0.020$) groups. The lifespan of the 5S10F5E group was 382 (SEM 31) d. The data demonstrated that the oxidised frying oil diet supplemented with a high dose of vitamin E could delay the onset of proteinuria and improve the lifespan of NZB/W F₁ mice.

Anti-double-stranded DNA and anti-single-stranded DNA antibody levels. The levels of IgG and IgM anti-DNA antibodies were followed every month. The levels of IgG anti-dsDNA and

IgM anti-ssDNA antibodies in the vitamin E supplementation groups were significantly lower than those of the 5S10F group ($P < 0.05$) at 8 and 6 months of age, respectively (Table 2). IgG anti-ssDNA and IgM anti-dsDNA antibodies showed the same tendency, although this was not statistically significant (data not shown). The amount of 10% oxidised oil used in this experiment did not inhibit anti-DNA antibody production caused by malnutrition as shown in a previous study in which 200 g oxidised oil/kg was used (Lin & Chi, in press). The effect of vitamin E supplementation could therefore be shown to decrease the levels of anti-DNA autoantibodies, especially IgG anti-dsDNA antibody, which is the most important pathological index in autoimmune disease.

Experiment 2

Thiobarbituric acid reactive substances levels. To further investigate the mechanisms by which vitamin E delayed the onset of autoimmune disease of NZB/W F₁ mice fed with oxidised oil, the oxidised oil group (5S10F) and the oxidised oil supplemented with vitamin E group (5S10F10E) treatments were repeated for *in vivo* analysis. As shown in Table 3, tissue weight was not significantly affected by vitamin E supplementation. The thiobarbituric acid reactive substance value representing oxidative status was significantly lower in the kidneys and spleens of NZB/W F₁ mice fed with oxidised oil plus vitamin E. The data showed that vitamin E supplementation could ameliorate oxidative stress in the kidney and spleen of NZB/W F₁ mice fed the oxidised oil.

Cytokine production by mitogen-stimulated splenocytes. Cytokines such as IL-2, IFN- γ , IL-4, IL-10 and IL-6 produced by mitogen-stimulated splenocytes were determined, the results being summarised in Table 4. Mice fed the vitamin E supplementation diet had significantly lower phytohaemagglutinin-stimulated IFN- γ secretion ($P = 0.0351$) and slightly, although not significantly, lower IL-4 secretion ($P = 0.1099$). The level of IL-6 produced by lipopolysaccharide-stimulated splenocytes was

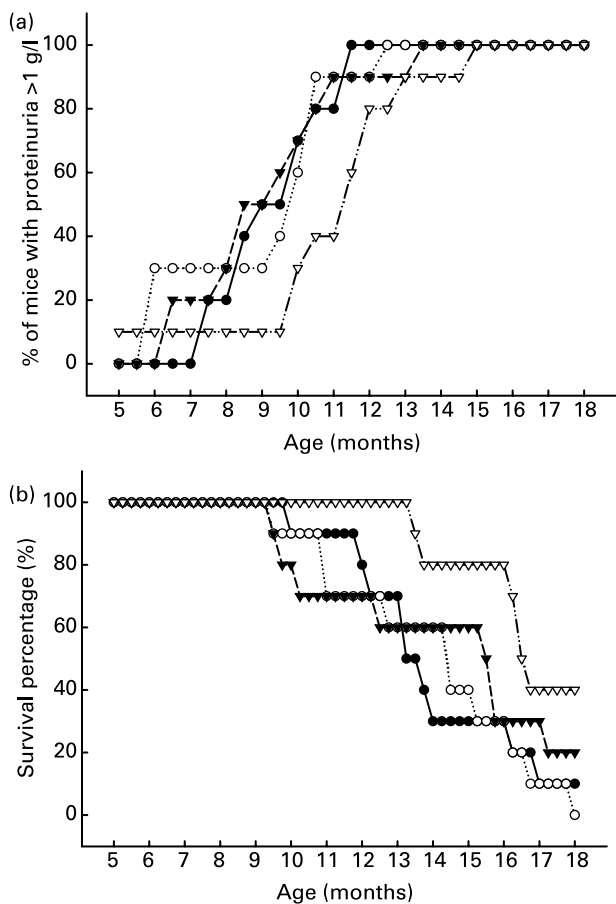


Fig. 1. (a) Proteinuria and (b) survival curve of New Zealand black x New Zealand white F₁ mice fed on diets containing 15% fresh soyabean oil (●, 15S), 5% fresh soyabean oil + 10% oxidising frying oil (○, 5S10F) or 5S10F supplemented with all-rac- α -tocopheryl acetate 275 (▼, 5S10F5E) or 550 mg/kg (▽, 5S10F10E). Mice with proteinuria were defined as those with more than 1g/l protein in the urine, nine for each group. Lifespan data were analysed by Cox's proportion hazards regression test; the lifespan of the 5S10F10E group was longer than that of the 5S10F group, $P = 0.020$.

Table 2. Serum anti-double-stranded (ds) DNA IgG and anti-single-stranded (ss) DNA IgM antibodies content in female New Zealand black x New Zealand white F₁ mice fed diets containing different dietary fats and vitamin E supplementation*

(Mean values with their standard errors for nine mice per dietary group)

	15S		5S10F		5S10F5E		5S10F10E	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Anti-dsDNA autoantibodies IgG (ELISA unit)†								
5 mo	0.05	0.01	0.05	0.02	0.03	0.01	0.04	0.02
6 mo	0.06	0.02	0.10	0.03	0.05	0.02	0.05	0.03
7 mo	0.47 ^a	0.14	0.38 ^{ab}	0.10	0.10 ^b	0.04	0.20 ^{ab}	0.10
8 mo	0.37 ^{ab}	0.07	0.52 ^a	0.14	0.20 ^b	0.07	0.15 ^b	0.06
Anti-ssDNA autoantibodies IgM (ELISA unit)								
5 mo	0.26	0.02	0.28	0.02	0.27	0.04	0.26	0.02
6 mo	0.23 ^b	0.03	0.31 ^a	0.03	0.23 ^b	0.02	0.20 ^b	0.02
7 mo	0.76 ^a	0.05	0.84 ^a	0.02	0.61 ^{ab}	0.05	0.58 ^b	0.03
8 mo	0.63	0.08	0.64	0.08	0.77	0.09	0.54	0.05

^{a,b}Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$) as determined by Duncan's multiple range test.

*Mice were fed a diet containing either 150 g fresh soyabean oil/kg (15S), 50 g fresh soyabean oil/kg + 100 g oxidised frying oil/kg (5S10F) or 5S10F supplemented with all-rac- α -tocopheryl acetate 275 mg/kg (5S10F5E) or 550 mg/kg (5S10F10E). For details of diets and procedure, see p. 656.

†ELISA unit: data are expressed as the ratio of serum antibody titre to positive control antibody titre.

Table 3. Tissue weight and thiobarbituric acid reactive substance (TBARS) levels in the liver, kidney and spleen homogenate of New Zealand black x New Zealand white F₁ mice fed on oxidised oil diets with or without vitamin E supplementation†

(Mean values with their standard errors for ten mice per dietary group)

	5S10F		5S10F10E	
	Mean	SE	Mean	SE
Tissue weight (g)				
Liver	2.45	0.07	2.38	0.07
Kidney	0.44	0.01	0.46	0.02
Spleen	0.12	0.01	0.13	0.01
TBARS content (nmol/g tissue)				
Liver	1.78	0.20	1.52	0.10
Kidney	6.34	0.85	3.39*	0.24
Spleen	35.0	1.36	23.4*	1.63

Mean values were significantly different as determined by the Student's *t* test, **P*<0.05.
† Mice were fed a diet containing either 50 g fresh soyabean oil/kg + 100 g oxidised frying oil/kg; (5S10F) or 5S10F supplemented with all-*rac*- α -tocopheryl acetate 550 mg/kg (5S10F10E) for 2.5 months. For details of diets and procedure, see p. 656.

also significantly lower in mice fed the vitamin E supplementation diet (*P*=0.0181).

Phenotypic analysis of lymphocyte subsets (surface marker). The results of the phenotypic analysis of the splenocytes are summarised in Table 5. There were no significant differences in the percentages of CD4⁺ T-cells, CD8⁺ T-cells, B-cells, CD5⁺ B-cells, natural killer cells and MHC class I-bearing cells in the spleen between the two groups. However, the level of MHC class II-bearing cells significantly decreased in mice fed the vitamin E supplementation diet (*P*=0.0490). The percentage of

Table 4. IL-2, interferon- γ (IFN- γ), IL-4, IL-10 and IL-6 production by splenocytes in New Zealand black x New Zealand white F₁ mice fed oxidised oil diets with or without vitamin E supplementation†

(Mean values with their standard errors for ten mice per dietary group)

	5S10F		5S10F10E	
	Mean	SE	Mean	SE
IL-2 (pg/10 ⁶ cell)				
Spontaneous‡	3.47	0.96	5.83	0.75
Phytohaemagglutinin‡	43.6	4.31	43.8	5.92
IFN- γ (pg/10 ⁶ cell)				
Spontaneous	ND	ND	ND	ND
Phytohaemagglutinin	0.52	0.16	0.12*	0.04
IL-4 (pg/10 ⁶ cell)				
Spontaneous	7.22	0.69	7.40	0.67
Phytohaemagglutinin	15.2	1.44	12.0	1.22
IL-10 (pg/10 ⁶ cell)				
Spontaneous§	1.86	1.63	2.97	1.30
Phytohaemagglutinin	31.7	5.87	32.4	5.07
IL-6 (ng/10 ⁶ cell)				
Spontaneous	1.59	0.40	2.27	0.36
Lipopolysaccharide‡	10.7	0.55	7.92*	0.89

ND, not detectable.
Mean values were significantly different as determined by the Student's *t* test, **P*<0.05.
† Mice were fed a diet containing either 50 g fresh soyabean oil/kg + 100 g oxidised frying oil/kg (5S10F) or 5S10F supplemented with all-*rac*- α -tocopheryl acetate 550 mg/kg (5S10F10E) for 2.5 months. For details of diets and procedure, see p. 656.
‡ Splenocytes at a concentration of 5 × 10⁶ cells/ml in RPMI-1640 medium supplemented with 10% TCM were incubated for 24 h.
§ Cells were incubated without mitogen (10 mg/l phytohaemagglutinin or 10 mg/l lipopolysaccharide) stimulation.

Table 5. Flow cytometric analysis of immune cell populations in the splenocytes of New Zealand black x New Zealand white F₁ mice fed oxidised oil diets with or without vitamin E supplementation†

(Mean values with their standard errors for ten mice per dietary group)

	5S10F		5S10F10E	
	Mean	SE	Mean	SE
T-cells	39.0	1.0	41.2*	1.3
B-cells	38.3	2.6	37.4	3.7
T helper cells (CD4 ⁺)	32.0	1.0	34.9	1.4
T suppressor cells (CD8 ⁺)	13.8	1.4	13.0	1.1
CD4 ⁺ /CD8 ⁺	2.4	0.1	2.9	0.3
CD5 ⁺ B-cells	5.3	0.3	4.8	0.5
Natural killer cells	3.0	0.3	3.7	0.4
MHC class I	98.6	0.3	98.6	0.2
MHC class II	37.2	2.9	28.4*	2.6

Mean values were significantly different as determined by the Student's *t* test, **P*<0.05.
† Mice were fed a diet containing either 50 g fresh soyabean oil/kg + 100 g oxidised frying oil/kg (5S10F) or 5S10F supplemented with all-*rac*- α -tocopheryl acetate 550 mg/kg (5S10F10E) for 2.5 months. For details of diets and procedure, see p. 656.

T-cells in the spleen was higher in mice fed the vitamin E supplementation diet (*P*=0.0201).

Splenocyte fatty acid composition. To investigate whether the fatty acid composition of the splenocytes could be affected by vitamin E supplementation, splenocytes from two mice of the same group were, owing to the small size of the spleen, pooled for this measurement. As shown in Table 6, mice fed the 5S10F10E diet had a significantly greater linoleic acid (18:2*n*-6) composition than the 5S10F diet group. The arachidonic acid (20:4*n*-6) content tended to be lower in the vitamin E supplementation group (*P*=0.1068), although not significantly so due to limited samples.

Bicyclo-prostaglandin E₂ level in urine. The whole-body production of PGE₂ was evaluated in terms of the urinary level of bicyclo-PGE₂, which is a stable PGE₂ derivative (Table 7). Although there was no significant difference between the two groups, mice fed the vitamin E supplementation diet tended to have lower levels of PGE₂ metabolites. The hepatic bicyclo-PGE₂ production also showed a similar trend (data not shown).

Table 6. The fatty acid composition of splenocytes in New Zealand black x New Zealand white F₁ mice fed oxidised oil diets with or without vitamin E supplementation†

(Mean values with their standard errors for three mice per dietary group)

	5S10F		5S10F10E	
	Mean	SE	Mean	SE
Fatty acid (g/100 g total fatty acids)				
16:0	30.1	0.54	28.1	0.56
16:1 <i>n</i> -9	1.66	0.23	2.41	1.17
18:0	20.4	0.57	20.6	3.2
18:1 <i>n</i> -9	15.2	0.55	11.6	2.66
18:2 <i>n</i> -6	14.4	0.26	21.2*	1.28
18:3 <i>n</i> -3	0.12	0.06	1.11	0.72
20:4 <i>n</i> -6	18.1	0.66	14.4	1.69

Mean values were obtained from a pooled spleen sample from two mice.
Mean values were significantly different as determined by the Student's *t* test, **P*<0.05.
† Mice were fed a diet containing either 50 g fresh soyabean oil/kg + 100 g oxidised frying oil/kg (5S10F) or 5S10F supplemented with all-*rac*- α -tocopheryl acetate 550 mg/kg (5S10F10E) for 2.5 months. For details of diets and procedure, see p. 656.

Table 7. Urine bicyclo-prostaglandin E₂ (PGE₂) levels in New Zealand black x New Zealand white F₁ mice fed oxidised oil diets with or without vitamin E supplementation*

(Mean values with their standard errors for three mice per dietary group)

	5S10F		5S10F10E	
	Mean	SE	Mean	SE
Bicyclo-PGE ₂ (ng/24 h urine)	1.26	0.29	0.73	0.04
Bicyclo-PGE ₂ (ng/mg 24 h creatinine)	1.61	0.53	1.43	0.07
Creatinine (mg/24 h urine)	0.62	0.17	0.49	0.07

Mean values were obtained from a pooled urine sample from two mice.

*Mice were fed a diet containing either 50 g fresh soyabean oil/kg + 100 g oxidised frying oil/kg (5S10F) or 5S10F supplemented with all-rac- α -tocopheryl acetate 550 mg/kg (5S10F10E) for 2.5 months. For details of diets and procedure, see p. 656

Discussion

Many age-associated diseases, particularly autoimmune diseases, appear to be exacerbated by an adverse change of food habits such as high energy intake, a high-fat but low *n*-3 lipid diet, and especially a high intake of fast, fried food over the past two decades (Fernandes, 1994; Lin *et al.* 1996a, 1997a). Oxidative stress may be formed by dietary oxidised frying oil intake, can cause tissue damage and may play a role in the inflammatory process of autoimmune disease (Blount *et al.* 1990, 1991; Ruiz-Torres *et al.* 1997). Antioxidants may therefore play a protective role in autoimmune disease under oxidative stress. In the present study, vitamin E, not only an antioxidant but also an immune modulator (Meydani, 1995; Meydani & Beharka, 1996; Lee & Wan, 2000), was added as a supplement to the oxidised frying oil diet in order to evaluate the role of vitamin E on autoimmune disease under oxidative stress. However, NZB/W F₁ mice fed a high-fat diet containing 200 g oxidised oil/kg had a suppressed serum anti-DNA antibody as a result of malnutrition (Lin *et al.* 1996a). Thus, a 150 g high-fat/kg diet containing 100 g oxidised frying oil/kg was applied in the present study. The growth curves of mice from the four groups were similar, and autoantibody production did not differ between the 15S and 5S10F groups by 8 months of age, indicating that the 100 g oxidised oil/kg plus 50 g fresh oil/kg diet was more feasible for evaluating the antioxidative effect by first avoiding the suppression of autoantibody production caused by malnutrition.

Supplemented with vitamin E, the oxidised oil diet apparently decreased thiobarbituric acid reactive substance values in the kidney and spleen in this study. The hydroxyl radical-modified nucleic acids, such as RNA and DNA, might be agents for the induction of circulating SLE anti-DNA autoantibodies (Tasneem & Ali, 2001). Supplementation with vitamin C, vitamin E and β -carotene in human lymphocyte significantly decreased the DNA endogenous oxidative base and increased resistance to oxidative damage when challenged *in vitro* with H₂O₂ (Duthie *et al.* 1996; Brennan *et al.* 2000). Vitamin E may prevent DNA damage from oxidative stress and thus decrease autoantibody production, as shown in this study.

Th1 and Th2 responses play a prominent role in the pathogenesis of lupus-associated tissue injury (Akahoshi *et al.* 1999; O'Shea *et al.* 2002). Both Th1 cytokines such as IL-2 and IFN- γ , and Th2 cytokines such as IL-4, IL-10 and IL-6, secreted from splenocytes were measured in this study. The secretion of IL-2 and IL-10 was not affected by vitamin E supplementation

in the oxidised oil diet. IL-4 is a key cytokine directing the differentiation and development of naïve Th cells into Th2 cells. Vitamin E was shown to inhibit IL-4 gene expression in human peripheral blood T-cells and T-cell lines through blocking the DNA binding of NF- κ B and activator protein-1 and thus interfering with promoter activity upon T-cell activation (Li-Weber *et al.* 2002). However, the inhibitory effect of vitamin E supplementation on IL-4 secretion did not reach significance ($P=0.1099$) in this study.

Significantly, IFN- γ secretion decreased in the vitamin E-supplemented group. The lowering effect of vitamin E on IFN- γ had also been found in healthy elderly subjects supplemented with 50 and 100 mg vitamin E for 6 months (Pallast *et al.* 1999). IFN- γ is a proinflammatory promoter and aids the production of IL-1, IL-6 and TNF- α . IFN- γ might contribute to glomerulonephritis by up-regulating the expression of MHC gene products and adhesion molecules (Grimm *et al.* 2002). Deletion of the IFN- γ receptor prevented autoantibody production and thus glomerulonephritis in the NZB/W F₁ mice (Haas *et al.* 1998). The NZB/W F₁ mice treated with IFN- γ -encoding plasmid showed higher urea N values and a reduced survival rate (Hasegawa *et al.* 2002). These studies demonstrated that IFN- γ promoted the development of lupus and is possibly required in the development of lupus through the induction of MHC class II expression on the antigen-presenting cells, which are responsible for autoantigen presentation (Balomenos *et al.* 1998).

It is therefore noteworthy that vitamin E supplementation affected the splenocyte phenotypes of NZB/W F₁ mice in the present study. The percentages of T-cells in the spleens were apparently higher, and those of MHC class II-bearing cells lower, in mice fed the vitamin E supplementation diet in this study. Increased MHC molecules on the surface of immune effector cells might contribute to an abnormal expansion of autoreactive cells and production of autoantibodies in SLE (Santiago-Raber *et al.* 2004). MRL/lpr mice deficient in MHC class II expression did not develop autoimmune nephritis or autoantibodies. The disease was prevented by a lack of thymic class II expression, which prevented autoaggressive T-cell development (Jevnikar *et al.* 1994). One study demonstrated the inhibition of IFN- γ -induced MHC class II gene transcription by NO and antioxidants, which might attenuate autoreactive T-cell development and the abnormal B-cell production of autoantibodies (Grimm *et al.* 2002). In this study, vitamin E supplementation decreased MHC class II expression in the spleens of mice fed on oxidised oil and might thus play a protective role in SLE. Furthermore, the age-associated decline in T-lymphocyte proportions had been shown to be unfavourable and could be restored by reducing energy intake. The combined therapy of fish oil and energy restriction completely abolished the age-dependent reduction in T-lymphocyte proportions and delayed the onset of autoimmune disease (Jolly & Fernandes, 1999), suggesting that the maintenance of an appropriate T-lymphocyte population was important in autoimmune development. Higher percentages of T-cells found in the spleens of the 5S10F10E group might be an additional consideration in explaining the improvement in disease course of vitamin E supplement-fed NZB/W F₁ mice.

The IL-6 production induced by chronic inflammation is intimately related to polyclonal B-cell activation and autoantibody production (Ishihara & Hirano, 2002). NZB/W F₁ mice treated with an anti-IL-6 receptor monoclonal antibody prevented the production of IgG anti-DNA antibody, significantly reduced

proteinuria and prolonged lifespan (Mihara *et al.* 1998). The incubation of rat glomerular mesangial cells with anti-dsDNA antibody from MRL/lpr spleen cells increased IL-6 expression (Yu *et al.* 2001). However, the higher IL-6 and IL-10 production by the murine splenocytes could be significantly normalised by vitamin E supplementation (Wang *et al.* 1995). In the present study, IL-6 secretion was also lower in the mice fed the vitamin E supplemented diet, which might contribute to attenuating inflammation and B-cell differentiation.

Further, vitamin E was also reported to exert the effect of the arachidonic acid metabolism, cell proliferation, anti-inflammation, signal transduction and gene expression (Azzi & Stocker, 2000; Rimbach *et al.* 2002). Arachidonic acid can be metabolised to a variety of eicosanoids, such as PGE₂, which inhibit IL-2 production and lymphocyte proliferation, activate autoantibody production and alter the balance of Th cells (Harris *et al.* 2002). Studies demonstrated that *n*-3 fatty acid induced suppression in the production of arachidonic acid-derived inflammatory eicosanoids in immune cells and successfully managed several inflammatory and autoimmune diseases (Fernandes, 1994; Calder *et al.* 2002). An essential fatty acid-deficient diet had been shown to prevent glomerulonephritis and prolong survival in NZB/W F₁ mice, suggesting that the essential fatty acid deficiency diminished inflammation by reducing the available prostaglandins (Hurd *et al.* 1981). Although arachidonic acid content stayed constant in the tissue, its content and metabolism could be affected by γ -linolenic acid supplementation, linoleic acid, fish oil and Δ -6 desaturase activity (Despret *et al.* 1992; Johnson *et al.* 1997; Chang *et al.* 1999). Vitamin E was reported to decrease the Δ -6 desaturation of PUFA in rat liver (Despret *et al.* 1992). Our data showed that the vitamin E supplemented diet was associated with a higher splenocytic linoleic acid (18:2*n*-6) level, suggesting a possible effect of vitamin E on fatty acid composition through the suppression of Δ -6 desaturation.

In addition, there was evidence that vitamin E decreased the formation of PGE₂ due to decreased cyclooxygenase activity, transcription and protein synthesis (Wu *et al.* 2001; Egger *et al.* 2003). Thus, vitamin E supplementation could decrease PGE₂ production in ageing and inflammation with higher PGE₂ production. The present study attempted to investigate overall PGE₂ production by determining the urinary bicyclo-PGE₂ level. However, the 24 h urinary bicyclo-PGE₂ level was not significantly lower in mice fed the vitamin E-supplemented diet.

Therefore, the effect of vitamin E supplementation in NZB/W F₁ mice fed the oxidised oil diet acted to decrease oxidative stress, IFN- γ and IL-6 secretion and MHC class II expression, and alter the fatty acid composition of the splenocytes. These might indirectly reduce autoantigen production and B-cell differentiation in autoimmune-prone mice. All the data indicated that vitamin E supplementation for mice under oxidative stress could effectively diminish autoantibody production and inflammation, and thus delay the development of autoimmune disease and prolong the lifespan in NZB/W F₁ mice. Therefore, vitamin E exerted not only an antioxidant, but also an immunomodulatory effect on autoimmune-prone mice under oxidative stress.

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