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Effect of Vitamin E Supplementation on the Immune Response during Chemically Induced Hepatocarcinogenesis in the Rat

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Summary The effect of vitamin E (Vit E), both α-tocopherol (α-T) and γ-tocotrienol (γ-T3), supplementation on splenocyte proliferation and phagocytic activity of peritoneal macrophages in rats treated with diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF) was investigated. Splenocyte proliferation in response to concanavalin A or phytohemagglutinin and phagocytic activity of peritoneal macrophages in the DEN/AAF-treated rats were significantly reduced compared with the control levels. In contrast, mitogenesis and phagocytic activity of peritoneal macrophages were increased significantly in the DEN/AAF-treated rats supplemented with Vit E; and the vitamin effect was dose dependent. However, the increases were not so great as those observed in the controls. The highest doses of α-T and γ-T3 used effected the highest phagocytic activity, with α-T generally showing a higher activity than γ-T3. Although long-term Vit E supplementation at half the optimum dose significantly increased mitogenesis, phagocytic activity in the DEN/AAF-treated and control rats was only marginally increased.

Key Words: chemical hepatocarcinogenesis, immune response, α-

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tocopherol, palm oil γ-tocotrienol

Vitamin E (Vit E) has been shown to play an important role in the prevention of carcinogenesis by inhibiting the formation of free radicals and by scavenging them [1]. The antitumor activity of Vit E in a number of carcinomas and several transplantable murine tumors has been demonstrated [2–4], where γ-tocotrienol (γ-T3), a form of Vit E, possesses a higher anti-cancer property than α-tocopherol (α-T), the major form of Vit E [3]. In rat hepatocarcinogenesis chemically induced with diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF), γ-T3, purified from the oil of the palm *Elaies guineensis*, was shown to be capable of reducing the severity of the carcinogenesis as evaluated by determination of enzyme activities, morphology, ultrastructure and histology [5–7].

Vit E has been reported to enhance immune response [8–12]. Rats and mice deficient in Vit E had a reduced immune response as evaluated in terms of mitogenesis to concanavalin A (Con A), phytohemagglutinin (PHA), or lipopolysaccharide (LPS) [13, 14]. In contrast, rats supplemented with Vit E in their diet had increased immune responses as evaluated by determination of lymphocyte proliferation, T-cell activity, and efficiency of phagocytic functions [15, 16].

Carcinogens such as 2-AAF and DEN generate the formation of free radicals and also reduce the activity of the immune system [17]. This in turn facilitates the formation of cancer. Vit E has been reported to affect the process of carcinogenesis, probably by inhibiting the initiation and promotion stages [18, 19]. We would like to know if Vit E could also affect the activity of the immune system during hepatocarcinogenesis in the rat caused by the carcinogens DEN and AAF. We report the effect of dietary supplementation of α-T and palm oil γ-T3 on the immune response of rats during hepatocarcinogenesis induced by DEN and AAF. The immune response was evaluated in terms of phagocytic activity of peritoneal macrophages and splenocyte proliferation in response to the mitogens Con A, PHA and LPS.

MATERIALS AND METHODS

Materials. [^{3}H]Thymidine (specific activity, 80 mCi/mmol) was purchased from Amersham International, Buckinghamshire, England α-*dl*-tocopherol acetate, Con A, PHA, LPS, 2-mercaptoethanol, DEN, and AAF were obtained from Sigma Chemical Co., St. Louis, MO. The enriched γ-T3 fraction (80% γ-T3, 20% α- and β-tocotrienols) used was a gift from the Palm Oil Research Institute of Malaysia (PORIM), Bangi, Malaysia. The tissue culture products and other chemicals used were of the highest grade commercially available.

Animals and treatment. Male *Rattus norwegicus* rats, 6–7 weeks old and weighing 120–150 g, were divided into 12 groups of 6 rats. The three control groups were fed with a basal diet, a diet supplemented with 170 mg α-T/kg diet

and a diet supplemented with 150 mg γ-T3/kg diet, respectively. In the other nine groups, cancer was induced by a single intraperitoneal injection of DEN at 200 mg/kg body weight, followed, after a 2-week recovery period, by feeding a diet containing 0.02% (w/w) AAF according to the method described by Solt and Farber [20]. Eight of the induced-cancer groups were given a diet containing 0.02% (w/w) AAF and supplemented with 17, 34, 68, and 170 mg α-T/kg diet and 15, 30, 60, and 150 mg γ-T3/kg diet, respectively. The ninth group received no vitamin supplement. Food and water were given *ad libitum*. After 8 weeks of treatment, the rats were sacrificed by cervical dislocation.

Simultaneously, in a long-term experiment, male *Rattus norwegicus* rats, 6–7 weeks old and weighing 120–150 g, were divided into 6 groups of 8 rats, consisting of a control fed a basal diet, two groups fed a basal diet supplemented with Vit E at 34 mg α-T/kg diet and 30 mg γ-T3/kg diet, respectively, and three groups treated with DEN/AAF as previously described, of which two groups were given a diet supplemented with Vit E at 34 mg α-T/kg diet and 30 mg γ-T3/kg diet, respectively. The rats were killed after 36 weeks of treatment.

Mitogenesis of splenocytes. The spleen of each rat was removed, immediately mashed with a sterile plunger against a stainless steel sieve, and suspended in RPMI 1640 culture medium containing 4 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 IU penicillin, and 50 μg/ml streptomycin. The viability of the splenocytes was greater than 95% as determined by trypan-blue exclusion. The splenocyte suspension was further diluted with RPMI 1640 culture medium containing 4 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 IU penicillin, 50 μg/ml streptomycin, and 10% (v/v) fetal bovine serum. The splenocytes were then plated at 2×10^6 cells/ml into 96-well Nunc plates and incubated at 37°C with 5% CO_2 and 95% air for 72 h. Con A, PHA, and LPS were added separately at their sub-optimal concentration of 1.0, 2.5, and 1.0 μg/ml, respectively into the wells. The cells were pulsed with 1.0 μCi [^{3}H]thymidine for 6 and 24 h for the 8 weeks- and 36 weeks-treated rats, respectively. Finally, the splenocytes were harvested with a cell harvester and placed onto a glass-fiber mat. The radioactivity of the incorporated [^{3}H]thymidine in the splenocytes was determined with a liquid scintillation counter.

Phagocytic activity of peritoneal macrophages. Resident peritoneal macrophages were obtained aseptically by washing the peritoneal cavity of each animal with 50 ml of Hanks' balanced salt solution. Viability of the peritoneal macrophages was greater than 98% by trypan-blue exclusion, and the yield obtained was greater than 5×10^8 cells per animal. The cells were pelleted by centrifugation at $300 \times g$ for 10 min, resuspended in RPMI 1640 culture medium containing 4 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 IU penicillin, and 50 μg/ml streptomycin to give a concentration of 5×10^6 cells/ml; and kept at 4°C for 1–2 h before being assayed. To 0.2 ml of the macrophage suspension containing 1×10^6 cells, 0.5 ml Hanks' balanced salt solution, 0.2 ml 0.01 mM luminol, and 0.1 ml 3 mg/ml zymosan opsonized with normal rat serum were added. The chemiluminescence produced was monitored with Luminometer 1250 (LKB Wallac, Finland) for 5

min, after which a final reading was taken.

Statistical analysis. The data obtained were analyzed by analysis of variance, Student's *t*-test, and linear regression analysis. A value of $p<0.05$ is considered significant.

RESULTS

Mitogenesis of splenocytes from rats fed short-term Vit E-supplemented diet

Splenocyte proliferation increased in response to the mitogens Con A and PHA in all the rats used in the study, but the increase in the DEN/AAF-treated rats was lower than that in the controls (Table 1). A significant dose-dependent effect ($p<0.05$) was observed when the DEN/AAF-treated rats were supplemented with increasing doses of α-T and γ-T3 at 17–170 mg/kg diet and 15–150 mg/kg diet, respectively (linear regression analysis; Con A: $r=0.95$, α-T; $r=0.96$, γ-T3; PHA: $r=0.94$, α-T; $r=0.87$, γ-T3). Vit E supplementation to the DEN/AAF-treated rats significantly ($p<0.05$) increased mitogenesis compared with that activity of the non-supplemented DEN/AAF-treated rats. In the control rats supplemented with Vit E at the highest dose used, no difference in splenocyte proliferation in response to Con A and PHA was observed compared with the controls. There was also no difference observed in the effect on splenocyte proliferation between supplementation with α-T and that with γ-T3.

Mitogenesis in the DEN/AAF-treated rats was significantly ($p<0.05$) reduced compared with that in the controls and the Vit E-supplemented rats. The B-lymphocyte cell response to LPS was generally not affected.

Table 1. Proliferation of splenic lymphocytes in short-term treated and control rats exposed to mitogens.

Treatment	No mitogen	PHA (2.5 μg/ml)	Con A (1.0 μg/ml)	LPS (1.0 μg/ml)
Control	431.2±39.8	973.2±122.2	1,354.0±187.0	658.2±180.2
Control (+170 α-T)	445.5±64.8	604.2±172.5	2,409.5±1,102.6*	444.8±160.7
Control (+150 γ-T3)	248.7±14.0	1,303.9±310.3*	2,468.3±520.2*,+	259.5±38.3+
DEN/AAF	492.3±62.1	631.9±70.3+	822.5±28.1+	484.7±202.8
DEN/AAF				
+ 17 α-T	421.6±10.2	1,196.3±118.2*	2,131.3±351.5*,+	350.7±107.6
+ 34 α-T	284.3±99.2	2,440.0±589.7*,+	2,457.8±70.9*,+	474.8±146.2
+ 68 α-T	290.0±36.0	2,843.8±219.7*,+	8,767.8±810.3*,+	922.6±186.6
+170 α-T	175.7±24.2	1,641.4±249.4*,+	5,413.9±688.7*,+	225.8±39.6*
DEN/AAF				
+ 15 γ-T3	452.2±95.9	1,092.3±117.0*	2,060.1±246.2*,+	520.6±99.6
+ 30 γ-T3	452.0±46.0	538.2±27.3+	2,379.7±330.6*,+	1,334.8±240.8*,+
+ 60 γ-T3	211.0±72.7	801.4±196.2	7,655.2±1,491.2*,+	1,337.8±707.6*,+
+150 γ-T3	302.6±78.8	1,982.5±291.9*,+	3,574.6±1,581.7*,+	998.8±575.3

Values shown are the mean±SD, cpm of [^{3}H]thymidine incorporation ($n=6$). Significance values, $^*p<0.05$ compared to DEN/AAF treatment, $^+p<0.05$ compared to control (-Vit E). Unit of Vit E-supplemented, mg/kg diet.

Long-term Vit E supplementation

Long-term Vit E supplementation at 34 mg α-T/kg diet and 30 mg γ-T3/kg diet in the control and DEN/AAF-treated rats significantly ($p<0.05$) increased mitogenesis in response to Con A and PHA compared with the control values

Table 2. Proliferation of splenic lymphocytes in long-term treated and control rats exposed to mitogens.

Treatment	No mitogen	PHA (2.5 μg/ml)	Con A (1.0 μg/ml)	LPS (1.0 μg/ml)
Control	520.6±24.0	9,110.1±3,560.8	9,070.2±2,060.8	2,641.3±1,411.7
Control (+34 α-T)	4,011.4±394.3	32,373.4±6,262.7^{+}	28,634.5±3,605.8^{+}	9,398.7±4,362.3^{+}
Control (+30 γ-T3)	2,400.8±534.6	34,763.6±8,984.6^{+}	32,645.6±2,805.2^{+}	6,338.6±1,613.0^{+}
DEN/AAF	493.5±389.5	307.7±176.0^{+}	477.7±70.0^{+}	3,680.4±1,800.5
DEN/AAF				
+34 α-T	4,890.9±2,655.9	15,576.0±3,866.2*	17,705.4±4,950.6*	5,910.2±5,659.2
+30 γ-T3	4,139.4±2,414.4	25,198.2±10,279.2*	26,237.6±10,528.8*	8,815.7±2,736.8*

Values shown are the mean±SD, cpm of [^{3}H]thymidine incorporation ($n=8$). Significance values, *$p<0.05$ compared to DEN/AAF treatment, $^{+}p<0.05$ compared to control (-Vit E). Unit of Vit E-supplemented, mg/kg diet.

Table 3. Phagocytic activity of peritoneal macrophages isolated from control rats; Vit E-treated control rats; DEN/AAF-treated rats; and Vit E-supplemented, DEN/AAF-treated rats in short-term experiment.

Treatment	Chemiluminescence (Arbitrary unit)
Control	2.46±0.60
Control	
+170 α-T	34.79±2.15*,$^{+}$
+150 γ-T3	25.22±0.59*,$^{+}$
DEN/AAF	1.99±0.61
DEN/AAF	
+17 α-T	8.41±1.41*,$^{+}$
+15 γ-T3	5.78±0.65*,$^{+}$
+34 α-T	10.20±0.43*,$^{+}$
+30 γ-T3	7.24±1.16*,$^{+}$
+68 α-T	24.49±2.06*,$^{+}$
+60 γ-T3	18.75±0.96*,$^{+}$
+170 α-T	68.96±3.46*,$^{+}$
+150 γ-T3	45.09±3.09*,$^{+}$

Values shown are the mean±SD ($n=6$). The rats were killed after 8 weeks of treatment. Unit of Vit E supplemented, mg/kg diet. Significance values, *$p<0.05$ compared to DEN/AAF treatment, $^{+}p<0.05$ compared to the control.

Table 4. Phagocytic activity of peritoneal macrophages isolated from control rats, Vit E-treated control rats; DEN/AAF-treated rats; and Vit E-supplemented, DEN/AAF-treated rats in a long-term experiment.

Treatment	Chemiluminescence (Arbitrary unit)
Control	7.31±4.89
Control	
+34 α-T	13.18±1.02
+30 γ-T3	11.09±3.91
DEN/AAF	5.42±1.12
DEN/AAF	
+34 α-T	8.71±1.49
+30 γ-T3	10.00±2.30

Values shown are the mean±SD ($n=8$). The rats were killed after 36 weeks of treatment. Unit of Vit E-supplemented, mg/kg diet.

(Table 2). In contrast, mitogenesis in the DEN/AAF-treated rats was significantly ($p<0.05$) reduced compared with that of the control and the Vit E-supplemented rats. Splenocyte proliferation in response to LPS was not affected.

Phagocytic activity of peritoneal macrophages in Vit E-supplemented rats

The phagocytic activity of peritoneal macrophages from short-term, Vit E-supplemented DEN/AAF-treated rats showed a dose-dependent effect of the supplementation (linear regression analysis; $r=0.96$, α-T; $r=0.97$, γ-T3; Table 3). The increase in phagocytic activity was significant ($p<0.05$) compared with the activity in the control and DEN/AAF-treated rats. Generally, supplementation with α-T resulted in a significantly higher corresponding phagocytic activity than that with γ-T3 in the DEN/AAF-treated rats ($p<0.05$). There was no difference in phagocytic activity of peritoneal macrophages between the control and DEN/AAF-treated rats.

The phagocytic activity of peritoneal macrophages in the long-term Vit E (34 mg α-T/kg diet and 30 mg γ-T3/kg diet)-supplemented control and DEN/AAF-treated rats was marginally increased compared with that of the control and the DEN/AAF-treated rats (Table 4).

DISCUSSION

In the present study, Vit E supplementation of DEN/AAF-treated rats was observed to enhance T-lymphocyte proliferation and phagocytic activity of peritoneal macrophages in a dose-dependent manner. The data obtained is in accordance with previous studies showing the enhancement of immune response by Vit E supplementation, also in a dose-dependent manner [10, 11, 13, 14].

α-T (17–170 mg/kg diet) and γ-T3 (15–150 mg/kg diet) supplementation increased mitogenesis and phagocytic activity in rats under carcinogen administration, with a dose of 60 mg γ-T3/kg diet and 68 mg α-T/kg diet achieving the maximum response. There was no difference observed in the effect on mitogenesis between α-T and γ-T3 supplementation. However, phagocytic activity of peritoneal macrophages in rats with cancer induced with DEN and AAF was shown to be affected more by α-T than by γ-T3, indicating that α-T enhances the immune response better than γ-T3. Our data is substantiated by the previous finding that showed α-T has a higher bioactivity than γ-T3 in the biosynthesis of immunoglobulins and prostacyclin in rats [21].

Macrophages from Vit E-deficient mice have been found to be defective in their ability to present antigens to T-lymphocytes [22]. Since the rats used in the present study were not deficient in Vit E, the peritoneal macrophages in the DEN/AAF-treated rats were presumably not defective since there was no reduction in phagocytic activity in the treated rats compared with that in the controls. In addition, Vit E could activate production of lymphokines, leading to the maintenance of normal phagocytic activity in the DEN/AAF-treated rats. Vit E supplementation in the diet has been shown to influence immune response through the production of lymphokines, as substantiated by the report of the appearance of macrophage-activating factor in mice supplemented with Vit E [11].

In the present study, long-term supplementation with Vit E (34 mg α-T and 30 mg γ-T3/kg diet) increased mitogenesis but not the phagocytic activity of peritoneal macrophages in rats treated with DEN/AAF. Long-term Vit E supplementation has been reported to attenuate the increase in tumor-marker enzyme activities in DEN/AAF-treated rats [7]. A higher dose of Vit E than that used presently probably would be required to increase the phagocytic activity of peritoneal macrophages. It would be advantageous to use a higher dose of Vit E since both lymphocyte proliferation and phagocytic activity would be increased, hence an overall increase in the immune response.

During carcinogenesis, immune functions of the host are often suppressed, resulting in a diminished resistance to infections and a higher susceptibility to cancer metastasis [4, 17]. Free radicals generated by carcinogens also suppress the activity of the immune system. In the present study, the marked reduction in mitogenesis in the DEN/AAF-treated rats over the long term could probably indicate suppression of immune functions. Vit E supplementation, both in the short- and long-term experiments, moderated the reduction of mitogenesis, indicating that Vit E supplementation inhibits suppression of immune functions. As an antioxidant, Vit E probably acts as a scavenger of the free radicals that are generated during carcinogenesis.

In conclusion, Vit E supplementation in the diet enhanced immune response in the rat in a dose-dependent manner. During chemically induced hepatocarcinogenesis, dietary Vit E supplementation possibly inhibits suppression of immune functions by scavenging the free radicals generated by the carcinogens.

α-T had a higher bioactivity than γ-T3 in increasing phagocytic activity of peritoneal macrophages in the DEN/AAF-treated rats. Long-term Vit E supplementation at a low dose enhanced splenocyte proliferation but not phagocytic activity of peritoneal macrophages.

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