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Vitamin E and immune response

I. ENHANCEMENT OF HELPER T CELL ACTIVITY BY DIETARY SUPPLEMENTATION OF VITAMIN E IN MICE

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Summary. The effects of vitamin E on the humoral immune response to hamster erythrocytes (HRBC) and 2,4,6-trinitrophenyl (TNP) were studied in mice. Inbred SL mice were fed on a diet supplemented with 0, 20 or 200 mg of vitamin E per kg of food throughout the course of experiments. These mice were immunized primarily with HRBC 50 days after the beginning of treatment with vitamin E supplementation. Secondary immunization with TNP–HRBC, a hapten-carrier conjugate, was given 28 days after primary immunization with HRBC. Anti-HRBC and anti-TNP haemagglutinin titres were increased by supplementing mice with vitamin E. Moreover, the effect of previous priming of mice with HRBC on the hapten-specific antibody response to immunization with TNP-HRBC was also enhanced by vitamin E supplementation. These effects of vitamin E were dose-dependent, and vitamin E as tocopheryl acetate exerted more effect than vitamin E as tocopheryl nicotinate. In experiments with the mouse inbred strain DDD, vitamin E seemed to facilitate the shift of antibody production from IgM to IgG. Initial IgM response and late IgG response were not augmented by treating mice with vitamin E supplementation. These findings suggested that vitamin E stimulated the helper activity of T lymphocytes. This was confirmed using an adoptive transfer system involving stimulation of hapten-primed cells by a hapten-carrier conjugate in the presence of carrier-primed helper cells.

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INTRODUCTION

Vitamin E, which has its advocates in the treatment of peripheral vascular and thromboembolic disease, is now stated to have a powerful antioxidant effect and is known to have various biological activities such as fertility factor (Sure, 1924), inhibition of human platelet aggregation (Steiner & Anastasi, 1976), and stabilization of biological membranes (McCay, Poyer, Pheifer, May and Gilliam, 1971).

Recently, it has been reported that vitamin E enhances humoral immune response to antigenic stimulation and resistance to bacterial infection (Tengerdy, Heinzerling & Nockels, 1972; Tengerdy, Heinzerling, Brown & Mathias, 1973; Heinzerling, Nockels, Quarles & Tengerdy, 1974). The mode of action of vitamin E on humoral immune response is still unclear, particularly as to the function of T lymphocytes. To elucidate the effect of vitamin E on the

humoral immune response, it seems essential to determine whether vitamin E stimulates the functions of T cells or not.

In the present study, helper function was chosen, since it could better be estimated quantitatively than could other functions of T cells. We have tested the influence of vitamin E on antibody production in mice using a hapten-carrier conjugate as an antigen. It has been reported that a hapten-specific antibody response to immunization with a hapten-carrier conjugate is enhanced by previous priming with the same carrier (Rajewsky, Schirrmacher, Nase & Jerne, 1969). The effect of carrier priming on hapten-specific antibody response (carrier effect) has been attributed to co-operation between hapten-specific B cells and carrier-specific T cells (Mitchinson, 1971). Augmentation of this carrier effect was obtained by treating mice with increased dietary vitamin E, and hence it is suggested that vitamin E stimulated co-operation between T and B cells in antibody production.

MATERIALS AND METHODS

Mice

Four- to five-week-old female inbred SL mice and DDD mice were supplied from the Breeding Unit of Kyushu University.

Treatment with vitamin E

Two types of vitamin E were obtained from the Eisai Pharmaceutical Corp., Tokyo. One was α-tocopheryl acetate (vitamin EA) and the other, α-tocopheryl nicotinate (vitamin EN). Mice were maintained on a diet containing 0, 20, or 200 mg of vitamin EA, or 226 mg of vitamin EN per kg of food, throughout the course of experiments. Two hundred and twenty-six milligrams equivalent to 200 mg of vitamin EA. The normal diet contains 20 mg of vitamin EA per kg of food.

Antigens

Hamster erythrocytes (HRBC) were obtained by cardiac puncture from outbred golden hamsters. These erythroyctes were washed three times with normal saline and used as immunogen and test antigen. Trinitrophenylated HRBC (TNP-HRBC) were prepared by heavy coupling of 2,4,6-trinitrobenzene sulphonic acid (TNBS) to HRBC as described by Kettman & Dutton (1971). For titration of the antibody, trinitrophenylated human erythrocytes of type O (TNP–ORBC) were prepared by light coupling of TNBS to ORBC, according to Rittenberg & Pratt (1969). TNP–ORBC were used as test antigen because ORBC do not show immunological cross-reactivity with HRBC.

Immunization

0·2 ml of a 25% (v/v) suspension of HRBC in saline were injected intravenously 50 days after the beginning of treatment with vitamin E supplementation. In order to estimate the carrier effect on antibody production to a hapten, 0·2 ml of 25% TNP–HRBC suspension were injected intravenously 28 days after primary immunization with HRBC.

System of adoptive transfer secondary response

The procedures for an adoptive immunization were carried out according to Hamaoka, Kitagawa, Matsuoka & Yamamura (1969). Donors of carrier-primed spleen cells were maintained on a diet supplemented with 0 or 200 mg of vitamin EA and injected intravenously with 0·2 ml of 25% HRBC suspension, 50 days after the beginning of treatment with vitamin E supplementation. Donors of hapten-primed spleen cells were maintained on the normal diet and immunized with bovine serum albumin (BSA) conjugated with TNP, according to Rittenberg & Amkraut (1966). There was no cross-reactivity between HRBC and BSA. Each mouse of this group was injected subcutaneously with 0·5 mg of trinitrophenylated BSA (TNP–BSA) in Freund's complete adjuvant. Mice received subsequent injections subcutaneously with the same dose of TNP–BSA in saline 14 and 21 days after primary immunization. Spleens were removed 7 days after priming with HRBC and 28 days after priming with TNP-BSA. Spleen cell suspensions were prepared by squeezing between two glass slides in chilled RPMI 1640 culture medium (GIBCO) and washed three times with the same medium. One ml of the suspension containing $2{\cdot}0 \times 10^7$ HRBC-primed cells, $1{\cdot}3 \times 10^8$ TNP-BSA-primed cells and $1{\cdot}0 \times 10^8$ TNP–HRBC were injected intravenously into a syngeneic recipient which had been exposed to 600 rad from a ^{60}Co. beam therapy unit 4 h before the cell transfer.

Assay of antibody titre

Blood specimens were obtained by capillary tubes from the retro-orbital venous plexus at various times after immunization. Haemagglutinin (HA) titres

against HRBC and TNP were measured by a microtitration method as described previously (Kitamura, Nomoto, Torisu & Takeya, 1976). Haemagglutination titres were expressed as $\log_2$. For examination of 2-mercaptoethanol (2-ME)-resistant antibody, sera were diluted with saline containing 2-ME so as to obtain 0·1 M of a final concentration, and incubated for 30 min at 37° before the addition of test antigen.

Statistics

The mean and standard error were calculated in each experimental group and observations were compared using the Student's *t* test.

RESULTS

Influence of treatment with vitamin E on antibody response to HRBC in SL mice

Vitamin E-supplemented mice were immunized primarily with HRBC, and haemagglutinin titres to HRBC were measured on days 0, 4, 8 and 14. The results are shown on the left side of Fig. 1. Anti-HRBC antibody titres of mice treated with vitamin EA (200 mg) were significantly augmented, as compared with those treated with vitamin E-deficient diet ($P < 0·05$ on Day 4, $P < 0·01$ on Days 8 and 14). Anti-HRBC antibody titres of mice treated with vitamin EN (226 mg) were also significantly augmented on Day 4 ($P < 0·05$), but those on Days 8 and 14 were not significant ($P > 0·1$). Treatment with vitamin EA (20 mg) seemed to augment antibody production, but the results failed to reach statistical significance ($P > 0·1$ on Day 4, $P > 0·8$ on Days 8 and 14). From these results, it was confirmed that vitamin E could augment antibody production *in vivo* and that the effect of vitamin E was dose-dependent. Vitamin EA showed a greater effect than vitamin EN.

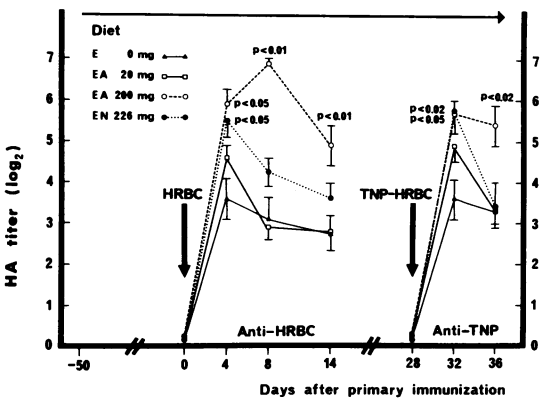

Figure 1. Influence of treatment with dietary supplementation with vitamin E on antibody production to HRBC and TNP-HRBC. SL mice were maintained on diet supplemented with various amounts of vitamin EA or vitamin EN for 50 days and following experimental period. Primary immunogen, HRBC, secondary immunogen, TNP-HRBC. Each value represents the mean ± SEM. of six mice.

Influence of treatment with vitamin E on antibody response to TNP in SL mice

Mice which were immunized primarily with HRBC received secondary immunization with TNP–HRBC 28 days after primary immunization. Haemagglutinin titres to TNP were measured on Days 28, 32 and 36. The right side of Fig. 1 shows the results obtained. Treatment with vitamin EA (200 mg) augmented antibody response to TNP in comparison with vitamin E-deficient mice ($P < 0·02$ on Days 32 and 36). Treatment with vitamin EN (226 mg) also augmented it on Day 32 ($P < 0·05$), but the effect was short-lasting ($P > 0·8$ on Day 36). Treatment with vitamin EA (20 mg) gave a slight augmentation, although the difference was not significant ($P > 0·1$ on Day 32, $P > 0·7$ on Day 36).

These results indicated that vitamin E had a dose-dependent enhancing effect on antibody response to immunization with a hapten-carrier conjugate, when mice had received previous priming with the same carrier. Also in this experiment, vitamin EA increased and prolonged antibody production more than vitamin EN.

Influence of treatment with vitamin E on the carrier effect in SL mice

In an attempt to determine whether or not vitamin E can enhance the effect of carrier priming on antibody response to a hapten, antibody production against TNP–HRBC was studied in HRBC-primed and in HRBC-non-primed mice with and without treatment with vitamin E supplementation. In HRBC-primed mice, TNP-HRBC were injected 28 days after primary immunization with HRBC in the same way as in the above mentioned experiment. Simultaneously, TNP–HRBC were injected into HRBC-non-primed mice. Antibody titres to TNP were measured on Days

Table 1. Influence of dietary supplementation with vitamin E on the carrier effect on anti-TNP antibody production in SL mice

	Treatment* (mg/kg of feed)	Primary immunization	Anti-HRBC† HA titre	Secondary immunization	Anti-TNP HA titre† (log_2)	
Expt days		0	7	28	32	36
Group I	None	None	0	None	0	0
II	E deficient	None	0	TNP–HRBC	2·7±0·3	2·5±0·2
III	EA 20 mg	None	0	TNP–HRBC	2·5±0·2	2·5±0·2
IV	EA 200 mg	None	0	TNP–HRBC	2·8±0·4	2·7±0·3
V	En 226 mg	None	0	TNP–HRBC	2·2±0·3	1·9±0·1
VI	E deficient	HRBC	3·4±0·3	TNP-HRBC	3·9±0·7	3·1±0·5
VII	EA 20 mg	HRBC	4·6±0·4	TNP–HRBC	5·0±0·5	3·5±0·2
VIII	EA 200 mg	HRBC	6·2±0·2	TNP–HRBC	6·1±0·4	5·3±0·5
IX	En 226 mg	HRBC	5·5±0·3	TNP–HRBC	5·7±0·4	3·7±0·6

* Treatment with diet supplemented with various amounts of vitamin E acetate or vitamin E nicotinate for 50 days and following experimental period.

† Haemagglutinin titres in mice after immunization with HRBC or TNP–HRBC. Each value represents the mean ± SEM of eight mice.

32 and 36 after primary immunization with HRBC. The results are shown in Table 1. Anti-TNP antibody after immunization with TNP-HRBC in HRBC-non-primed mice (Group II–V) was detectable on days 4 and 8 after the injection with TNP-HRBC. There was, however, no significant difference between the anti-TNP antibody titres of mice whose diet was supplied with vitamin EA (Group III, IV) and those of vitamin E-deficient mice (Group II). In particular, mice whose diet was supplemented with 226 mg of vitamin EN (Group V) made less antibody than vitamin E-deficient mice (Group II). These results suggested that vitamin E could not stimulate, or might even suppress, co-operation between T and B cells in the primary response to a hapten-carrier conjugate.

In contrast, mice which received previous priming with HRBC (Group VI–IX) showed significantly greater hapten-specific antibody response to immunization with TNP–HRBC than HRBC-non-primed mice (Group II–V). In addition, a dose-dependent augmentation by vitamin E was noticed in hapten-specific antibody response to TNP–HRBC injection in HRBC-primed mice (Group VI–IX). Again, the effect of vitamin EA was superior to vitamin EN, and prolonged augmentation was achieved only by supplementing mice with 200 mg of vitamin EA. These results indicated that vitamin E could increase cooperation between T and B cells in an antibody response to a hapten-carrier conjugate under circumstances where memory cells responding to the carrier had already been well developed. Therefore, it is suggested that vitamin E stimulates the effect of previous priming with the carrier, i.e. the carrier effect.

Influence of treatment with vitamin E on antibody response to HRBC in DDD mice

In other experiments DDD mice were immunized with HRBC, and anti-HRBC antibody titres were measured on days 4, 7, 14 and 21. The results are shown in Table 2. The time-course of antibody production in DDD mice was different from that in SL mice: the peak of antibody was on Day 7, whereas that in SL mice was on Day 4, except for animals treated with vitamin EA (200 mg) (Fig. 1). In DDD mice, the difference of anti-HRBC antibody titres between vitamin E supplemented mice and vitamin E-deficient mice on Days 4, 14 and 21 failed to reach significance. The titres on Day 7 only in vitamin E-supplemented mice showed significant elevation, as compared with those of vitamin E-deficient mice [$P < 0{\cdot}005$ in mice supplied with vitamin EA (200 mg), $P < 0{\cdot}02$ in mice supplied with vitamin EA (20 mg)]. 2-ME resistant antibody titres on Day 7 were also higher in mice supplied with vitamin E than those of vitamin E-deficient mice [$P < 0{\cdot}001$ in mice supplied with vitamin EA (200 mg), $P < 0{\cdot}05$ in mice supplied with vitamin EA (20 mg)]. Primary IgM and later IgG antibody seemed to be augmented by vitamin E supplementation, but the difference from vitamin E-deficient mice was not significant. These results suggested that vitamin E facilitated the shift of antibody from IgM to IgG.

Table 2. Influence of dietary supplementation with vitamin E on the immune response against HRBC in DDD mice

Group	Vit. E acetate Supplementation* (mg/kg)	Anti-HRBC HA titre† (log_2)		(Net / 2ME-resistant)	
		Day 4	Day 7	Day 14	Day 21
I	200	5·31 ± 0·21	6·50 ± 0·20¶	5·38 ± 0·18	5·15 ± 0·15
	0	0	4·80 ± 0·38**	5·12 ± 0·22	5·08 ± 0·26
II	20	5·28 ± 0·18	6·00 ± 0·20§	5·25 ± 0·14	5·10 ± 0·12
	0	0	4·46 ± 0·33‡	5·20 ± 0·24	5·05 ± 0·13
III	0	5·25 ± 0·17	5·46 ± 0·18	5·23 ± 0·13	5·08 ± 0·14
		0	3·15 ± 0·33	5·01 ± 0·18	5·00 ± 0·16

* Mice were maintained on diet supplemented with various amounts of vitamin E acetate for 50 days and following experimental period.

† Haemagglutinin titres in mice after primary immunization with HRBC. Each value represents the mean ± SEM of twelve mice.

‡ $P < 0·05$ § $P < 0·02$ ¶ $P < 0·005$ ** $P < 0·001$.

Influence of treatment with vitamin E on the helper cell activity in adoptively immunized DDD mice

Spleen cells were transferred from DDD mice primed with HRBC (heterologous carrier-primed) together with those primed with TNP-BSA (hapten-primed) into 600 rad irradiated syngeneic mice. Recipients were immunized with TNP-HRBC (hapten-heterologous carrier conjugate) simultaneously with the cell transfer. Antibody titres to TNP were assessed 4, 7, 14 and 21 days after secondary immunization with TNP-HRBC. The results are shown in Table 3. When irradiated mice did not receive the cell transfer, im-

Table 3. Influence of dietary supplementation with vitamin E on helper cell activity in adoptively immunized DDD mice

Group	Carrier-primed cell donors		Hapten-primed cell donors	^{60}Co irradiation of recipients	HA titre† (log_2)		(Anti-TNP / Anti-HRBC)	
	Diet*	Carrier	Hapten-carrier		Day 4	Day 7	Day 14	Day 21
I	Vit. E 200 mg/kg	HRBC	TNP–BSA	Yes	0	1·83 ± 0·11¶	1·92 ± 0·66	2·67 ± 0·59
					0	0·33 ± 0·21	2·92 ± 0·77§	4·57 ± 0·34§
II	Vit. E deficient	HRBC	TNP–BSA	Yes	0	0·33 ± 0·24	0·50 ± 0·22	1·83 ± 0·49
					0	0·17 ± 0·16	0·66 ± 0·49	3·33 ± 0·49
III	Normal	None	TNP–BSA	Yes	0	0	0	1·58 ± 0·78
					0	0	0·16 ± 0·14	1·58 ± 0·55
IV‡	—	—	—	Yes	0	0	0	1·50 ± 0·51
					0	0	0	1·56 ± 0·80

* Treatment with diet supplemented with various amounts of vitamin E acetate for 57 days.

† Haemagglutinin titres in mice after secondary immunization with TNP–HRBC.
Each value represents the mean ± SEM of eight recipients.

‡ Mice received only ^{60}Co irradiation before immunization with TNP–HRBC.

§ $P < 0·05$, ¶ $P < 0·01$, significance between group I and group II.

munization with TNP-HRBC did not give rise to appreciable antibody production against TNP up to Day 14 (Group IV). When HRBC-non-primed normal spleen cells were transferred together with TNP–BSA-primed cells, anti-TNP antibodies were also not detectable up to Day 14 (Group III). In contrast, Group I and Group II mice, which received the cell transfer both from HRBC-primed donors and from TNP–BSA-primed donors, demonstrated appreciable anti-TNP antibody production on Day 7. Moreover, anti-TNP antibody titres of Group I mice which received HRBC-primed cells from vitamin E-supplied donors, were significantly higher than those of Group II mice which received HRBC-primed cells from vitamin E-deficient donors ($P < 0{\cdot}01$ on Days 7 and 14). These results indicate that the activity of helper cells (T cells) was enhanced by treatment with vitamin E supplementation.

DISCUSSION

These results show that the antibody response in mice is augmented by treatment with dietary supplementation of vitamin E. In addition, it is shown that vitamin E can increase the effect of carrier priming on the antibody response to immunization with a hapten-carrier conjugate, possibly by the stimulation of carrier-specific helper T cells.

Since Tengerdy *et al*, (1972) reported that vitamin E increased antibody responses in chickens to sheep red blood cells, vitamin E has been mentioned as an agent protecting animals from bacterial infection. In fact, Heinzerling *et al*, (1974) have reported that dietary supplementation of 150 mg or 300 mg of vitamin E per kg of food gave chickens an increased protection to *Escherichia coli* and a correlative two- to three-fold increase in antibody titres against the bacteria. It is well known that vitamin E shows powerful antioxidant activity, and the primary function of vitamin E *in vivo* is thought to be the prevention of destructive peroxidation of polyunsaturated lipids according to the antioxidant hypothesis (Tappel, 1972). It has been demonstrated that 2-ME, a reducing agent, can enhance antibody-forming capacity of lymphocytes *in vitro* (Chen & Hirsch, 1972). Recently, 2-ME has been found to facilitate induction of helper T cells in antibody response *in vitro* (Erb & Feldmann, 1975). These facts suggest that vitamin E, which has biological functions of a reducing agent, can also affect the function of T cells. This hypothesis prompted us to study the effect of vitamin E on the helper functions of T cells *in vivo*.

The 'carrier effect' has been attributed to cooperation between two types of lymphoid cells, T cells and B cells (Mitchison, 1971). We selected TNP-HRBC as a hapten-carrier conjugate and studied the effect of vitamin E on the immune response to HRBC and TNP–HRBC in SL mice. SL mice react strongly in antibody response to HRBC (Nomoto, Mashiba & Takeya, 1972) The strain difference in immune response to HRBC is attributed to the difference in the functions of HRBC-specific T cells, which are under genetic control.

The antibody response of SL mice to immunization with HRBC was augmented by dietary supplementation with vitamin E. Moreover, we found that vitamin E enhanced the carrier effect, because a dose-dependent increase in anti-hapten antibody production against the hapten-carrier conjugate by treatment with vitamin E supplementation was observed in the case of HRBC-primed mice (Table 1, Group VI–IX). This suggested that vitamin E could increase cooperation between T and B cells. Vitamin EA acts more than vitamin EN. Gallo-Torres, Miller, Hamilton & Trantnyek (1971) have reported that these agents show different tissue uptake and turnover ratios after oral administration.

In contrast to the effect of vitamin E in HRBC-primed mice, there was no enhancing effect in the case of HRBC-non-primed mice (Table 1, Group II–V). Although the reason why vitamin E, particularly vitamin EN, failed to stimulate, or even suppressed, hapten-specific antibody production in this condition is uncertain, two explanations are possible. First, BCG (*Bacillus Calmette-Guérin*), a potent adjuvant, can increase helper T cell activity in the same experimental system as ours (Kitamura *et al*, 1976). BCG can stimulate hapten-specific antibody response to primary immunization with a hapten-carrier conjugate even in the absence of previous priming with the carrier. OK-432, an immunopotentiator prepared from a strain of *Streptococcus hemolyticus*, did not stimulate such a response, although the agent also exhibited an enhancing effect on helper T cells (Kai, Tanaka, Nomoto & Torisu, 1979). Immunopotentiation by vitamin E is not so potent as that effected by the above immunostimulants and this may determine the difference observed.

Secondly, anti-carrier antibody can suppress hapten-specific antibody response to a hapten-carrier conjugate at some stage in primary immunization

(Takatsu, Hamaoka & Kitagawa, 1974). Vitamin E might augment anti-carrier antibody production. Neither of these explanations, however, are satisfactory for the understanding of the data presented, and studies are currently in progress in an attempt to clarify the events of the primary response.

We confirmed the enhancing effect of vitamin E on humoral immunity using another inbred mouse strain, DDD. DDD mice belong to a high responder strain against antigenic stimulation with HRBC (Nomoto *et al*, 1972), and treatment with vitamin E supplementation gave mice dose-dependent augmentation of antibody production exclusively on day 7 after immunization with HRBC (Table 2). Also 2-ME resistant antibody production was augmented by treatment with vitamin E only on day 7. Day 7 was just at the point of transition of antibody production from IgM class to IgG, as shown in the time-course of 2-ME resistant antibody response against HRBC in Table2. IgG responses require the cooperation of helper T cells to a greater extent than do IgM responses (Miller, Dukor, Grant, Sinclair & Sacquet, 1967; Britton & Möller, 1968). Activated T cells help the shift of antibody production from IgM class to IgG (Hurme, Kontiainen, Seppälä & Mäkelä, 1973; Doria, Agarossi, Borashi & Amendolea, 1977). Our results with HRBC in DDD indicated that vitamin E stimulates the helper function of T cells and are consistent with the report by Tengerdy, Heinzerling, Brown & Mathias (1973) which shows that vitamin E influenced IgG production more than IgM production.

The cellular specificity of the effect of vitamin E, however, remains to be determined. That is, it can not be decided whether the enhancement of hapten-specific antibody production by vitamin E results from the specific stimulation of a T-cell population responding to the carrier immunization, or from the general stimulation of cell proliferation, including the stimulus to the hapten-specific B cells which indeed produce the anti-hapten antibody. Nevertheless, it has been clarified that anti-carrier helper T cells are essential for the development of anti-hapten B cells (Takatsu *et al.* 1974). We believe that the stimulated helper T cell activity plays an important role in the effect of vitamin E on humoral immune response.

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