PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, 146 465-469 (1974)

Vitamin E Enhances *in vitro* Immune Response by Normal and Nonadherent Spleen Cells[1] (38127)

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(Introduced by P. Kind)

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In vitro immune responses by murine spleen cells have been shown to require cells which adhere to plastic as well as nonadherent cells (1, 2). The adherent cell population is rich in macrophages, while nonadherent cells contain the thymus-derived and bone marrow-derived lymphocytes required for antibody production (1, 2). The adherent cell population can apparently be replaced by a supernatant prepared by incubating adherent cells in medium (3). Recent reports have demonstrated that the reducing agent, 2-mercaptothanol (2-ME), can stimulate nonadherent cells to respond to antigens in the absence of adherent cells (4, 5). Responses of unseparated spleen cells are also enhanced by 2-ME (4–6). Other sulfhydryl-containing reducing agents which have been reported to enhance *in vitro* immune responses include glutathione and dithiothreitol (5, 7). Alpha-tocopherol, or vitamin E, which may have biologic functions similar to those of a reducing agent, has also been shown to enhance the *in vivo* immune response of mice and chickens to sheep red blood cells and to tetanus toxoid (8, 9).

The experiments described below were designed to test whether alpha-tocopherol can: (1) enhance *in vitro* immune responses by normal spleen cells, (2) stimulate nonadherent cells to produce antibodies in the relative absence of adherent cells. The data indicate that alpha-tocopherol is a good *in vitro* adjuvant and that it stimulates nonadherent cells at least as well as does 2-ME.

Materials and Methods. Mice. Throughout these studies, 8–16-week-old male or female BDF_1 (C57BL/6 × DBA/2) mice, raised in our own colony, were the experimental animals. Mice of a single age and sex were used in each experiment. They were maintained on Wayne Lab Blocks, which contain 35 ppm alpha-tocopherol, and on water to which 16 ppm chlorine has been added.

Cultures. To obtain an immune response *in vitro,* normal mouse spleen cells were cultured according to the technique of Mishell and Dutton (10). The single cell suspensions were cultured in 1 ml Minimal Essential Eagle's medium (MEM), which contains no alpha-tocopherol, but which was supplemented with nonessential amino acids, glutamine, sodium pyruvate, and penicillin–streptomycin as described earlier (10). One further supplement was 5% fetal bovine serum (FBS) (Colorado Serum Co., Denver, Colorado) which contains naturally-occurring alpha-tocopherol. Medium supplemented with 5% FBS contains approximately 0.1 μg alpha-tocopherol per ml medium (11). After addition of 1 drop of a 2% sheep red blood cell (SRBC) suspension as antigen, the cultures were incubated at 37° in an atmosphere consisting of 12.5% CO_2, 8.5% O_2, and 80% N_2. The cultures were rocked on rocker platforms and fed daily with a nutritional cocktail consisting of 30% FBS in supplemented medium (10). The amount of cocktail used would provide approximately 0.1 μg alpha-tocopherol to the cultures daily. After 5 days of culture, the cells were harvested by scraping the dishes with a rubber policeman and were assayed for antibody-forming cells as described below.

Preparation of nonadherent cells. Adherent

[1] Supported by USPHS Research Grant AI-11240 and Allergy Disease Center Grant AI-10398.

and nonadherent cells can be separated by attachment to plastic or glass. In the experiments reported here, nonadherent cells were prepared as follows: 10 ml of washed Degalon V26 polymethylmetaacrylic beads (Degussa Wolfgang Au, Germany) were placed into a sterile 10 ml syringe which contained a small plug of nylon hosiery. After the beads were rinsed with about 20 ml sterile balanced salt solution (BSS) (10) containing 20% FBS, approximately 2×10^8 normal spleen cells were added to the bead column and washed slowly through with sterile BSS. This procedure, done at room temperature, quite probably removes some antibody-forming cell precursors as well (11). Approximately 50–80% of the cells which were put onto the columns were recovered and cultured. This population of nonadherent spleen cells was noticeably depleted of macrophages and developed low numbers of antibody-forming cells when stimulated with antigen *in vitro*.

Assay for antibody-forming cells. The number of plaque-forming cells (pfc) was determined by the slide modification of the Jerne plaque assay (10). Each group, containing 2–3 culture dishes, was harvested by scraping with a rubber policeman. The cells were pooled, coded, and several dilutions were plated in duplicate on microscope slides. After incubation at 37° for 2–3 hr with 1:10 normal guinea pig serum, the pfc were counted through a magnifying glass. Results are expressed as the number of pfc recovered per culture dish.

Alpha-tocopherol. Although many different forms of alpha-tocopherol were tested, only 2 produced consistently significant enhancement. These were both kindly provided by Hoffmann-La Roche, Inc., and were solubilized in a commercial preparation containing 0.1 ml Emulphor EL 620, 0.1 ml anhydrous alcohol, 0.1 ml propylene glycol, 0.01 ml benzyl alcohol, 0.3 mg sodium acetate, 2.5 mg glacial acetic acid, 9 mg sodium chloride, 0.1 mg disodium edetate, and 50 mg *dl*-alpha-tocopherol or *dl*-alpha-tocopheryl acetate. Both of these materials were readily soluble in our culture medium. A placebo Emulphor containing all the above materials, but lacking the *dl*-alpha-tocopherol, was also tested.

2-mercaptoethanol. 2-mercaptoethanol (2-ME), purchased from Eastman Kodak Co., New York, was diluted in sterile BSS and added to the cultures. The final concentration was 10^{-5}–10^{-4} *M*.

Results. Effect of alpha-tocopherol on normal spleen cells. Vitamin E administered as a dietary supplement enhances the immune response of mice to SRBC (8). Experiments were designed to confirm this enhancement *in vitro*. Normal mouse spleen cells were cultured

TABLE I. Effect of Alpha-Tocopherol on Immune Responses by Normal Spleen Cells.[a]

μg alpha-tocopherol added per culture	Pfc per culture		
	Experiment 1	Experiment 2	
	(alcohol)[b]	(alcohol)	(acetate)[b]
none	8,606	18,360	18,360
0.6	—[c]	19,002	36,990
1.8	—	27,853	34,471
5.0	33,314	31,214	25,786
50.0	31,533	—	—
500.0	0	—	—

[a] 10^7 normal spleen cells were cultured with alpha-tocopherol for 5 days, and the number of pfc was then determined. Pfc = plaque-forming cells.

[b] Alcohol = *dl*-alpha-tocopherol solubilized in Emulphor and acetate = *dl*-alpha-tocopheryl acetate solubilized in Emulphor.

[c] — = not done.

TABLE II. Effect of Alpha-Tocopherol on Immune Responses by Nonadherent Spleen Cells.[a]

μg alpha-tocopherol added per culture	Pfc per culture			
	Experiment 1		Experiment 2	
	(alcohol)[b]	(acetate)[b]	(alcohol)	(acetate)
none	91	91	471	471
0.05[d]	—[c]	—	687	1387
0.5	—	—	2825	3109
0.6	3263	1470	—	—
1.8	3723	1057	—	—
5.0	2097	1731	2786	2875
50.0	—	—	1663	209
none + 10^{-5} *M* 2-ME	1395			
none + 10^{-4} *M* 2-ME	1854			

[a] 10^7 column-separated spleen cells were cultured for 5 days, and the number of pfc was then determined. Pfc = plaque-forming cells.

[b] Alcohol = *dl*-alpha-tocopherol solubilized in Emulphor, and acetate = *dl*-alpha-tocopheryl acetate solubilized in Emulphor.

[c] — = not done.

[d] Approximately 10^{-4} *M*.

at a concentration of 10^7 cells per dish. Various doses of alpha-tocopherol or its acetate were added to the cultures at the time of setup and the culture dishes were assayed for pfc 5 days later. Table I demonstrates that alpha-tocopherol enhanced slightly the *in vitro* response of normal mouse spleen cells to SRBC. In these experiments there was no increase in the number of cells recovered from cultures to which vitamin E was added.

Effect of alpha-tocopherol on nonadherent spleen cells. Nonadherent spleen cells separated from adherent spleen cells are unable to respond normally to SRBC *in vitro* (1, 2). A reducing agent, 2-mercaptoethanol, stimulates nonadherent cells to respond in cultures depleted of adherent cells (4, 13). Since alpha-tocopherol is an antioxidant and may also be a reducing agent, its ability to stimulate nonadherent cells depleted of adherent cells was tested. Nonadherent cells were prepared by passage through a plastic bead column, and 10^7 cells were cultured with various doses of alpha-tocopherol or alpha-tocopheryl acetate. Five days later, the cultures were harvested and the number of pfc determined. Table II depicts the results of 2 typical experiments. These data clearly show that alpha-tocopherol can stimulate nonadherent spleen cells to respond to SRBC when adherent cells are depleted. Furthermore, alpha-tocopherol is at least as effective as 2-ME. The failure of these nonadherent cells to respond as well as do adherent cells is probably due to the removal of some pfc precursors by the columns (11). In some experiments there was an increase in cell recovery when alpha-tocopherol was added to the cultures. This could be due either to enhanced cell growth, decreased cell death, or both, and will be discussed in a subsequent publication.

Similar experiments were conducted using water-insoluble alpha-tocopheryl acetate which had been emulsified in sterile BSS. Although this material enhanced the response of nonadherent cells to SRBC, it did so at much higher concentrations than did the solubilized forms, and the stimulation was never more than three-fold higher than in the unstimulated cultures. This insoluble vitamin E is much less effective than solubilized vitamin E in enhancing immune responses.

TABLE III. Effect of Emulphor Placebo on Immune Responses by Normal and Nonadherent Spleen Cells.[a]

	Pfc. per culture					
	Normal spleen cells			Nonadherent spleen cells		
	(alcohol)[b]	(acetate)[b]	(placebo)[b]	(alcohol)	(acetate)	(placebo)
none	1800	1800	1800	107	107	107
10 μg	5054	5037	1322	853	1061	18
50 μg	5888	5260	733	936	889	15

[a] 10^7 normal or column-separated nonadherent spleen cells were cultured with alpha-tocopherol or a placebo of the Emulphor solution in which it was solubilized. After 4 days in culture, the number of pfc was determined. Pfc = plaque-forming cells.

[b] Alcohol = *dl*-alpha-tocopherol solubilized in Emulphor, acetate = *dl*-alpha-tocopheryl acetate solubilized in Emulphor, and placebo = Emulphor solubilizing vehicle.

Effect of Emulphor placebo on response of normal and nonadherent spleen cells. To confirm that the enhancement was due to the alpha-tocopherol and not to the Emulphor solubilizing vehicle, the effect of Emulphor placebo on normal and nonadherent spleen cells was tested. Alpha-tocopherol in Emulphor, alpha-tocopheryl acetate in Emulphor or Emulphor alone was added to 10^7 normal or nonadherent spleen cells. The cells were cultured in the presence of antigen for 4 days, and the number of pfc per culture was determined. Table III shows that Emulphor alone, cultured at the same concentrations as in the alpha-tocopherol-containing solutions (diluted 1:1000–1:5000), did not enhance the immune response of either normal or nonadherent cells. In fact, the data suggest an inhibitory effect of Emulphor. This could be considered evidence that one mode of action of alpha-tocopherols may be to decrease the fragility of cells in culture. Cell recovery in this experiment ranged from 33% to 57% with no consistent trend.

Discussion. We have demonstrated that alpha-tocopherol, or Vitamin E, stimulates nonadherent spleen cells to respond to antigens when the number of adherent spleen cells is depleted, thus replacing the requirement for normal numbers of adherent spleen cells. It is unlikely that these nonadherent cell populations are completely void of residual adherent cells. Consequently, one cannot determine whether alpha-tocopherol and other materials which appear to replace the requirement for adherent cells (1) actually act directly on the few remaining adherent cells to enhance their effectiveness, or (2) act directly on nonadherent cells, thus bypassing the adherent cells entirely. Nevertheless, alpha-tocopherol clearly enhances the immune responses of nonadherent spleen cells when the number of adherent spleen cells is markedly depleted, apparently by providing some function normally provided by normal numbers of adherent cells. In addition, soluble alpha-tocopherols enhance the *in vitro* immune response of normal mouse spleen cells to SRBC, confirming earlier findings that a dietary supplement of alpha-tocopherol enhances *in vivo* immune responses (8, 9). Since alpha-tocopherol is highly insoluble in water, it was necessary to find a soluble form of the vitamin which was effective in a culture system. Commercial preparations of *dl*-alpha-tocopherol and *dl*-alpha-tocopheryl acetate solubilized in emulsifying agents were found to be very effective *in vitro*. Control experiments showed that the emulsifying agents alone did not stimulate spleen cells or nonadherent cells.

The stimulation of cultures of normal spleen cells and of nonadherent spleen cells by alpha-tocopherol is similar to that previously reported for 2-mercaptoethanol (4–7). Alpha-tocopherol and 2-ME, which are both antioxidants, may enhance *in vitro* immune responses by a common mode of action. One possibility is that these agents may enhance respiratory

and metabolic activities of cells by keeping sulfhydryl groups on cell membranes in the reduced state (6, 7, 14, 15). Another common mode of action of these agents is their protective effect against O_2 toxicity. The protective effect of alpha-tocopherol in hyperoxia has been described by Mengel (16). Similar protective effects of 2-ME against high oxygen concentrations in spleen cell cultures have been reported (17). Other recent studies report that both sulfhydryl compounds and disulfides promote the growth of normal lymphoid cells *in vitro* (18) and enhance the action of mitogens on these cells (14). The mechanisms by which both 2-ME and alpha-tocopherol enhance immune responses may simply be by creating more favorable conditions for cell growth or for cell interactions.

Alpha-tocopherol noticeably differs from these previously studied reducing agents in that it does not contain sulfhydryl groups. Because Vitamin E apparently stimulates *in vitro* responses as well as does 2-ME, this stimulation clearly is not dependent on the presence of sulfhydryl groups. This conclusion is in apparent conflict with that of Browne and Jeng (18) who feel that the presence of a thiol or disulfide group is required for the growth promotion of lymphocytes by mitogens. However, these investigators assayed stimulation of immune lymphocytes by mitogens, while we studied stimulation of antibody-formation by nonadherent cells in the relative absence of a second population which is normally required. These two parameters may well require different culture conditions.

If alpha-tocopherol enhances immune responses by virtue of its antioxidant effects, then it is somewhat surprising that dietary *N, N*-diphenyl-*p*-phenylene diamine (DPPD), which is also a water-insoluble antioxidant, did not enhance *in vivo* responses in experiments in which alpha-tocopheryl acetate was effective (8). However, DPPD was presented to the mice in powdered form in their foods and may not have reached the cells in a metabolizable form or in sufficient concentrations. Preliminary *in vitro* experiments with DPPD indicate that it, too, can stimulate nonadherent cells to respond *in vitro*.

Summary. Antibody production by normal mouse spleen cells *in vitro* requires the presence of both adherent and nonadherent cell populations. The data presented here demonstrate that by the addition of alpha-tocopherol (vitamin E) to the medium nonadherent spleen cells can be stimulated to respond to sheep red blood cells in the relative absence of adherent cells. In addition, alpha-tocopherol will enhance *in vitro* immune responses by populations of spleen cells containing both adherent and nonadherent cells.

The authors would like to thank Dr. M. Brin and Dr. L. Machlin for their help in the preparation of the manuscript and for providing the alpha-tocopherol and placebo preparations.

1. Mosier, D. E., Science **158**, 1573 (1967).
2. Hartmann, K., Dutton, R. W., McCarthy, M. M., and Mishell, R. I., Cell. Immunol. **1**, 182 (1970).
3. Hoffmann, M. and Dutton, R. W., Science **172**, 1048 (1971).
4. Chen, C. and Hirsch, J. G., Science **176**, 60 (1972).
5. Click, R. E., Benck, L. and Alter, B. J., Cell. Immunol. **3**, 264 (1972).
6. Click, R. E., Benck, L. and Alter, B. J., Cell. Immunol. **3**, 156 (1972).
7. Heber-Katz, E. and Click, R. E., Cell. Immunol. **5**, 410 (1972).
8. Tengerdy, R. P., Heinzerling, R. H., Brown, G. L., and Mathias, M. M., Int. Arch. Allergy **44**, 221 (1973).
9. Tengerdy, R. P., Heinzerling, R. H. and Nockels, C. F., Inf. and Imm. **5**, 987 (1972).
10. Mishell, R. I. and Dutton, R. W., J. Exp. Med. **126**, 423 (1967).
11. Shortman, K., Williams, N., Jackson, H., Russell, P., Byrt. P. and Diener, E., J. Cell. Biol. **48**, 566 (1971).
12. *Blood and Other Body Fluids,* Ed. D. S. Dittmer, Fed.Amer.Soc.Exper.Biol., Washington, D. C., 1961, p. 91.
13. Chen, C. and Hirsch, J. G., J. Exp. Med. **136**, 604 (1972).
14. Fanger, M. W., Hart, D. A., Wells, J. V. and Nisonoff, A., J. Immunol. **105**, 1043 (1970).
15. Schwartz, K., Ann. N. Y. Acad. Sci. **203**, 45 (1972).
16. Mengel, C. E., Ann. N. Y. Acad. Sci. **203**, 163 (1972).
17. Dutton, R. W. and Jacobs, D., Fed. Proc. **32**, 4266 (1973).
18. Broome, J. D. and Jeng, M. W., J. Exp. Med. **138**, 574 (1973).

Received Nov. 29, 1973. P.S.E.B.M. 1974, Vol. 146.