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Vitamin E Protects Mice Against *Diplococcus pneumoniae* Type I Infection

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Vitamin E protects nonimmunized and immunized mice against fatal *Diplococcus pneumoniae* type I (DpI) infection. A dietary supplementation of 180 mg of DL-α-tocopheryl acetate per kg of diet increased survival of nonimmunized mice from 20 to 80% when challenged with 20 organisms, and of mice immunized with 0.5 ng of DpI polysaccharide from 15 to 70% when challenged with 20,000 organisms. The phagocytic index of immunized mice was four times higher in the 180-mg vitamin E group than in the control group. Both the survival and phagocytic index revealed a biphasic dose response, indicating a cause-effect relationship between phagocytosis and survival. Vitamin E also significantly increased the rate of carbon clearance from blood, indicating a general increase in phagocytic activity. The data indicated that increased macrophage activity probably aided by increased antibody production was the principal reason for increased protection.

We have reported earlier that dietary supplementation of pharmacological doses of vitamin E significantly increased the protection of young chicks against *Escherichia coli* infection (9). The increased protection was associated with a 2 to 3 $\log_2$ titer increase in hemagglutinating antibody against *E. coli* lipopolysaccharide. We also demonstrated earlier that vitamin E enhances humoral immune responses against sheep erythrocytes and tetanus toxoid both in vivo (11, 12, 13) and in vitro (5).

The mechanism of increased humoral immunity and protection by vitamin E is not yet clear, although previous experiments suggested that antioxidant action (5, 13) and enhancing electron transport via increased ubiquinone synthesis (unpublished data) may play significant roles. Ubiquinones, especially coenzyme Q, have been shown to increase phagocytic activity (4, 6).

The main purpose of the present study was to correlate disease protection with phagocytic activity in mice immunized or not immunized with *Diplococcus pneumoniae* type I (DpI) polysaccharide (SI). Injection of SI may induce protective immunity in mice by low doses, and immune paralysis by very low or high doses. A few DpI cells are enough to cause high lethality in nonimmunized mice and the cells are apparently refractory to phagocytosis in the absence of opsonizing antibody (7). We expected that in such a precariously balanced host-parasite system a regulatory substance, such as vitamin E, may shift the balance toward increased protection by stimulating immune phagocytosis.

MATERIALS AND METHODS

DpI suspension and DpI SI. DpI cells from a third serial mouse passage were maintained in a skim milk lyophilized culture and used as the stock culture for all experiments. For challenge, 17-h blood agar cultures were resuspended in cold tryptose broth medium with agitation to break up chains, and adjusted to an optical density approximately giving 2.0×10^8 cells; this suspension was diluted to approximately 20 or 20,000 cells per 0.1 ml for injection. Actual numbers of viable organisms were confirmed by plate count after challenge. These cell numbers gave an optimal challenge dose for nonimmunized or immunized mice in previous experiments (10). SI was prepared by ethanol precipitation of whole DpI cells, followed by chloroform-butanol (5:1) extraction of proteins. The polysaccharide was precipitated from the aqueous layer of the extract with isopropanol and purified by repeated extraction and precipitation. This is a procedure used by Heidelberger (8) except that he used the supernate of phenol-precipitated cells for the ethanol precipitation.

Mice and diets. Four-week-old Swiss Webster female mice, in groups of 35, were fed a standard Purina mouse chow diet supplemented with 0 to 360 mg of vitamin E (as DL-α-tocopheryl acetate) per kg of the Purina mouse chow diet, in 60-mg increments for separate groups (seven dietary groups). Food and water were given ad libitum, and the mice were maintained on the diet for 4 weeks before challenge with DpI.

Challenge, mortality, and phagocytic index. Ten nonimmunized mice in each group were challenged

with approximately 20 DpI cells (approximately an 80% lethal dose) intraperitoneally, and mortality was recorded 5 days later. The other 25 mice in each group were immunized intraperitoneally with 0.5 ng of SI. Ten mice were challenged a week later intraperitoneally with 20,000 DpI cells. This combination of immunization and challenge also produced around 80 to 85% mortality in control mice. Mortality was recorded 5 days later. The remaining 15 immunized mice were used in the phagocytic study.

Phagocytic index (PI) was expressed as the mean number of DpI cells found in 100 peritoneal macrophages in a treatment group over the same in the control group. The PI was determined by a modification of the original method of Wood (14). Fifteen mice per group received intraperitoneally 100,000 DpI cells 1 week after immunization, and three mice per group were sacrificed at 0, 2, 4, 6, and 8 h after DpI injection. Four smears of the peritoneal wall scrapings per mouse were prepared, fixed, stained by Wright stain, and counted for PI. The peritoneal wall scrapings contain predominantly macrophages. The counts were evaluated statistically. In nonimmunized mice the PI could not be determined because the macrophages did not take up the DpI cells.

Carbon clearance test. A method similar to that used by Biozzi et al. (3) was followed. One group of 20 4-week-old Swiss Webster mice was maintained on the control diet for 3 weeks, and the other group was maintained on a diet supplemented with 180 mg of vitamin E per kg of the diet. After 3 weeks all mice were injected via the tail vein with a carbon particle (India ink) suspension passed through a 0.4-μm membrane filter (Millipore Corp.). The dose of injection was 0.01 ml of a 8-mg/ml carbon suspension in saline per g of body weight. At 5-min intervals after injection two mice per group were bled from the retro-orbital plexus, taking 0.025 ml of blood. The blood was diluted to 2.0 ml with distilled water, and optical density was read at 710 nm in a spectrophotometer to record the decrease in absorbance due to the clearing of carbon from the blood.

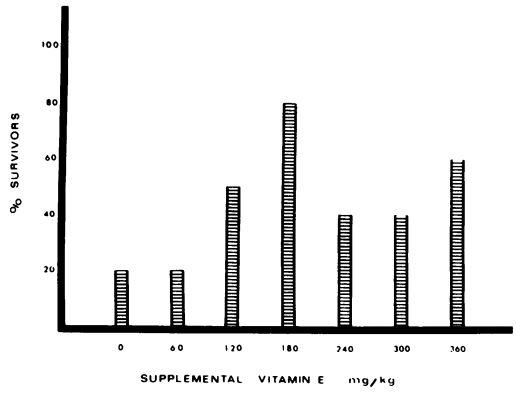

FIG. 1. *Protection of nonimmunized mice against DpI infection by vitamin E. The challenge dose was 20 organisms and survivors of each group of 10 were counted 5 days later.*

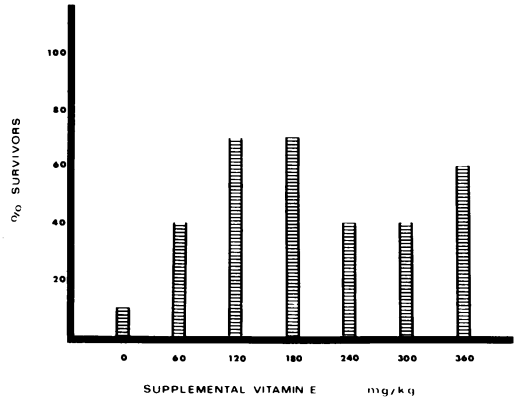

FIG. 2. *Protection of immunized mice against DpI infection by vitamin E. Mice were immunized with 0.5 ng of SI, and 1 week later challenged by 20,000 organisms. Survivors of each group of 10 were counted 5 days later.*

RESULTS

Vitamin E protects nonimmunized and immunized mice against DpI infection. Figures 1 and 2 are representative examples of one of five similar trials. The results of the five separate trials could not be combined, averaged, and statistically evaluated, because the low number of DpI cells (20 or 20,000) used for challenge caused an inevitable 10 to 15% variation in the number of viable organisms injected in each trial, and thus the survival patterns of all groups shifted up or down ± 20 to 30%. The relative survival patterns, however, remained in the same order in all five trials, the optimal level of vitamin E being always 180 mg/kg of the diet, resulting in a very significant, approximately four times, increase in survival over the controls.

It is interesting to note the biphasic dose effect; 360 mg of vitamin E gave protection comparable to 180 mg, but doses in between were less effective. This finding is meaningful in the light of similar findings in the following phagocytic study.

It is also interesting to note that the relative increase in protection attributable to vitamin E was about the same in nonimmunized as in immunized mice, although the challenge doses of DpI differed by a factor of 1,000.

Vitamin E enhances phagocytosis of DpI in mice immunized against SI. The result of this experiment is presented in Fig. 3. Maximal PI was always found 4 h after challenge with 100,000 DpI. At that time the PI of the 120-mg and 300-mg vitamin E groups was four times higher than the control, a highly significant ($P < 0.01$) difference. The decrease in the PI after

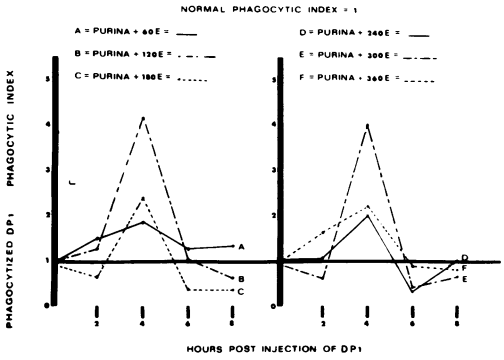

FIG. 3. *PI of immunized mice. Baseline is the control diet.*

4 h is caused by the rapid disappearance (lysis) of the DpI cells from the macrophages. The more active the macrophages are, as in the vitamin E groups, the faster the DpI cells are lysed, and the more rapidly the PI drops. Noticeable again is the biphasic nature of the phagocytic response. Numerically the optimal responses shifted lower (120 and 300 mg) compaired to the optima of the overall protection (180 and 360 mg), but the proportions were similar, suggesting that the effect of vitamin E on both phagocytosis and overall protection were strongly related.

Vitamin E increases phagocytic activity in mice. Since increased phagocytosis may well have been due to an increased protective antibody production alone, independent evidence was needed to test the effect of vitamin E on macrophage activity in the absence of immunological phenomena. The rate of carbon clearance of vitamin E-fed mice (180 mg/kg of the diet) and control mice are shown in Fig. 4. The vitamin E-fed mice cleared the carbon from the blood about twice as fast as the controls. The level of carbon remaining in the blood 25 min postinfection was significantly different ($P < 0.01$) between the two groups of 20 mice each.

DISCUSSION

The results of these experiments indicated that the observed increased protection of mice against DpI infection by vitamin E was associated with an increased phagocytic activity. Vitamin E increased phagocytic activity even in noninfected, nonimmunized mice as demonstrated by the carbon clearance test, but effective phagocytosis of DpI was found only in mice immunized against SI, suggesting the indispensable role of opsonizing or cytophilic antibody.

It was shown in our earlier work that a very low level of antibody, resulting from immunization with only 0.5 ng of SI, was adequate to give a good protection against DpI infection, and this protection was transferable by serum to nonimmunized mice (10). This low level of antibody, however, is not detectable by conventional serological techniques. There is no sensitive test for the detection of SI antibodies in contrast to SIII antibodies (2). Even with the normally sensitive passive hemagglutination test we could not demonstrate titerable SI antibody in our immunized mice. Nevertheless, the protection study clearly demonstrates that effective phagocytosis requires the immune state of the mice. Since we know from earlier work that vitamin E enhances immune responses (5, 11, 12, 13) and that enhanced immunity is correlated with increased protection (9), it is safe to assume that the increased protection in this experiment was due to both an increased phagocytic and an increased immunological activity.

The observed protection of nonimmunized mice against DpI infection by vitamin E may also be explained by the generally increased activity (both phagocytic and immunological) of the reticuloendothelial system, which at this very low level of infection may have been able to phagocytize the infective cells with or without the help of a very low level of antibody. Such events are, however, below the level of experimental detection. There is evidence that as early as 5 to 24 h after an intravenous injection of SI, antibody-producing cells were already present (1). An alternative explanation may be a direct or indirect bactericidal or bacteriostatic action of vitamin E itself that may have pre-

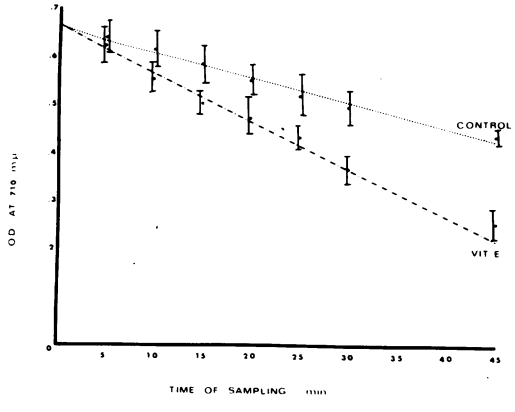

FIG. 4. *Rate of carbon clearance. Mice on control diet and on vitamin E supplemented diet (180 mg/kg) were used. The means of four determinations are shown, the vertical bars representing the standard deviation.*

vented the multiplication of DpI.

The biphasic dose response curves of the phagocytic index and survival corroborated a cause-effect relationship between phagocytosis and survival. A similar biphasic phagocytic effect was observed by Bliznakov and Adler in studying the effect of coenzyme Q_{10} on phagocytosis (4). In another study Casey and Bliznakov found that ubiquinones and vitamin K increased phagocytic activity in rats but vitamin E did not (6). Their failure to demonstrate increased phagocytosis stimulated by vitamin E was most likely due to the very low dose of vitamin E used (50 to 400 μg per rat, i.e., 10 to 80 times lower than in our study). The similarity of action of ubiquinones in the Casey and Bliznakov experiments and of vitamin E in ours suggests a possible common mode of action. In some very recent experiments (unpublished data) we have observed an increased ubiquinone synthesis stimulated by vitamin E. One possible mode of action of vitamin E could be an increased ubiquinone synthesis leading to a more efficient electron transport and metabolism in phagocytic and immunocompetent cells. This mode of action would also explain why vitamin E acts, or purported to act, on so many different cells or cellular functions.

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