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# Vitamin E-Deficient Diets Enriched with Fish Oil Suppress Lethal *Plasmodium yoelii* Infections in Athymic and *scid*/*bg* Mice

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**Mice fed vitamin E-deficient diets containing omega-3 fatty acids survive infection with lethal *Plasmodium yoelii*. The current study sought to determine if antimalarial T- and B-cell responses were required for such dietary-mediated protection. In the first set of experiments, *nu*/*nu* mice (which lack αβ T-cell-receptor-positive T cells and do not produce antimalarial antibody) and *nu*/+ mice were fed casein-based diets containing 4% menhaden oil, with or without vitamin E supplementation, for 4 weeks prior to infection with lethal *P. yoelii*. All mice fed diets containing vitamin E developed fulminating parasitemias and quickly died, whereas both *nu*/*nu* and *nu*/+ mice fed diets deficient in vitamin E controlled their parasitemias for the first 18 days of infection. Thereafter, the *nu*/*nu* mice became anemic and died, whereas the *nu*/+ mice produced antimalarial antibodies and survived. In the second set of experiments, *scid*/*scid.bg*/*bg* mice (which lack B cells and αβ and γδ T cells and have reduced NK-cell activity) were fed the experimental diet for 6 weeks and then infected with the less virulent 17XNL strain of *P. yoelii*. Mice fed vitamin E-containing diets quickly died, whereas those fed the vitamin E-deficient diet survived without developing detectable parasitemias. Results from these experiments show that under prooxidant dietary conditions, mice were able to control and even survive malaria in the absence of malaria-primed T cells and antimalarial antibody. These results emphasize the importance of cellular oxidative processes in parasite elimination.**

Almost 40 years ago, Godfrey showed that an animal's diet could influence the course of malaria infection (9). He reported that when mice were fed a diet supplemented with cod-liver oil, they survived for a significantly longer period following infection with lethal *Plasmodium berghei* than mice fed a normal diet. The protective effect was reversed by adding vitamin E to the diet. In the last few years, several groups have extended this finding and have demonstrated that outbred mice routinely survive infection with lethal *P. berghei*, *Plasmodium vinckei*, and *Plasmodium yoelii* when fed a vitamin E-deficient diet containing menhaden fish oil (MO) or other sources of omega-3 polyunsaturated fatty acids, including ethyl linolenate, linseed oil, and ground flax (1, 4, 8, 15, 16, 18, 19). The mechanism responsible for the protective effect of vitamin E-deficient, fish oil-containing diets is unclear, but two major theories have been advanced (20).

First, it is well established that the nature of dietary fat alters the lipid composition of erythrocyte membranes (23, 29). Malarial parasites cannot biosynthesize their own fatty acids and thus must use those provided by the host (28). Therefore, in mice fed vitamin E-deficient, fish oil-containing diets, the membranes of both the parasite and the infected erythrocyte will contain large amounts of highly unsaturated omega-3 fatty acids. Such lipids have several double bonds that make them highly susceptible to peroxidation, especially in the absence of dietary antioxidants, such as vitamin E. Since reactive oxygen intermediates (ROI) are produced during normal physiological processes and malarial parasites are reported to be severely affected by ROI (12, 25), the diet could influence parasite growth by increasing their susceptibility to oxidative stress.

Alternately, acquired immunity may play an important role in diet-mediated parasite elimination. Recent studies have shown that mice develop a protective immune response during the course of primary malaria infection while fed prooxidant diets (1, 8). Once the diet-protected mice have recovered from primary infection and are then returned to a normal chow diet, they are protected from rechallenge with lethal malaria. These results suggest that an acquired immune response involving antimalarial T and B cells may be an important component in diet-induced protection.

The study reported herein sought to determine if a prooxidant MO-enriched, vitamin E-deficient (MO−VE) diet could produce its beneficial effect against lethal *P. yoelii* infection in mice that lack the ability to produce an acquired antimalarial immune response. We initially studied the effect of the diet by using athymic (*nu*/*nu*) and euthymic (*nu*/+) BALB/c mice. Athymic mice lack αβ T-cell-receptor-positive T cells and do not produce antimalarial immunoglobulin M (IgM) or IgG antibodies (Ab) (26); however, they have γδ T-cell-receptor-positive T cells and normal NK-cell activity. Since the latter two cell types have been implicated in mediating protective immunity (27; reviewed in reference 13), we also studied severe combined immunodeficient (*scid*/*scid*) mice crossed with beige (*bg*/*bg*) mice (*scid*/*bg*). These mice lack αβ and γδ T cells, are deficient in B cells and antimalarial Ab, and have ~50% reduced NK-cell activity (5, 22).

## MATERIALS AND METHODS

**Mice.** Female *nu*/*nu* (athymic) and *nu*/+ (euthymic) BALB/c mice were obtained from Harlan-Sprague Dawley (Indianapolis, Ind.), and female BALB/c.ByJ (+/+) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). The *scid*/*scid* and *scid*/*scid.bg*/*bg* mice were kindly provided by Anne Croy, Department of Biomedical Sciences, University of Guelph. Approximately

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equal numbers of male and female *scid*/*bg* mice were assigned to each experimental group. The *nu*/*nu* and *nu*/+ mice were housed in plastic cages with bonnets in laminar flow hoods, while the *scid*/*bg* mice were maintained in cages equipped with individual HEPA-filtered air supplies. Following weaning, all mice were fed conventional lab chow (Formulab Chow 5008; Ralston Purina Co., St. Louis, Mo.) for a short period (0.5 to 3 weeks) prior to being fed the experimental diets.

**Diets.** The compositions of the diets have been detailed previously (16). In brief, the basal MO diet contains 20% casein, 4% MO, 1% tocopherol-stripped corn oil (source of essential fatty acids), 3.5% AIN-76 salt mix, 1% AIN-76A vitamin mix without vitamin E, 0.3% DL-methionine, 0.152% choline dihydrogen citrate, 20 mg of *para*-aminobenzoic acid per kg, and sucrose to 100%. This diet was used either without supplementation (MO−VE) or supplemented with 38.4 mg of *d*-alpha-tocopheryl acetate per kg (MO+VE), which is the recommended level of vitamin E for rodent diets. In experiment 2, the basal MO diet was not supplemented (MO−VE) or was supplemented with 100 mg of *d*-alpha-tocopheryl acetate per kg (MO+VE) or with 55.5 mg of another efficient antioxidant, *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD), per kg (the molar equivalent of vitamin E) (MO+DPPD) (6). Higher levels of these two antioxidants were used in this experiment to increase the likelihood of the DPPD effect. Diets were stored at 4°C, and fresh food was provided to the mice daily.

**Experimental infections. (i) Nude mice.** Two experiments were performed. In experiment 1, there were five groups of mice (five mice per group): (i) *nu*/+ mice fed the MO+VE diet, (ii) *nu*/*nu* mice fed the MO+VE diet; (iii) *nu*/+ mice fed the MO−VE diet, (iv) *nu*/*nu* mice fed the MO−VE diet, and (v) BALB/c (+/+) mice fed regular chow (controls). In experiment 2, the same five groups of mice (five mice per group) were used, along with two additional groups which included *nu*/+ and *nu*/*nu* mice fed the MO diet containing antioxidant DPPD (MO+DPPD diet). Mice were fed the experimental diets for 4 weeks prior to infection and then maintained on their respective diets throughout the infection.

The *nu*/*nu* and *nu*/+ mice were injected intraperitoneally with $10^4$ 17XL (lethal) *P. yoelii*-infected erythrocytes. Smears of tail blood were prepared three times a week and stained with Diff-quik (Baxter Healthcare Corp., Miami, Fla.). Parasitemias were determined by counting the number of parasitized erythrocytes per 500 erythrocytes. To determine if the parasites present in the blood of MO−VE-fed mice with persistent infections remained capable of inducing a lethal infection, blood samples (100 μl) were collected from surviving *nu*/*nu* and *nu*/+ mice on day 11 and from *nu*/*nu* mice on day 26 of infection and injected directly into naive BALB/c mice fed conventional laboratory chow. Parasitemias were monitored as described above.

Samples of heparinized blood (approximately 100 μl) were collected from surviving mice at 18 to 33 days postinfection and tested for the presence of antimalaria Ab by enzyme-linked immunosorbent assay (see below).

**(ii) *scid*/*bg* mice.** In experiment 3, *scid*/*bg* mice were fed the MO+VE (five mice per group) and MO−VE (three mice per group) diets for 6 weeks prior to injection of $10^4$ 17XNL (nonlethal) *P. yoelii*-infected erythrocytes. The nonlethal strain was selected for this study because it grows preferentially in reticulocytes and thus has a lower growth rate and longer course of infection. This strain, however, produced a lethal infection in *scid*/*bg* mice (see results). A fourth experiment was conducted, in which *scid*/*scid* mice were fed the MO+VE diet for 14 days. During this time, the *scid*/*bg* mice surviving from experiment 3 were maintained on the MO−VE diet. Both groups of mice were injected intraperitoneally with $10^4$ 17XNL *P. yoelii*-infected erythrocytes, and parasitemias were monitored by preparing tail blood smears three times a week. *scid*/*scid* mice were used as controls in this experiment, because age-matched *scid*/*bg* mice were not available.

**Determination of antimalarial Ab titers.** The enzyme-linked immunosorbent assay used was similar to one reported previously (31). In brief, *P. yoelii*-parasitized erythrocytes were purified by cellulose chromatography and Percoll density gradient centrifugation. The cells were extracted in 1% Tween 20 in 0.1 M phosphate-buffered saline (PBS) (pH 7.2) and diluted in carbonate buffer (pH 9.6) to $5 \times 10^5$ parasites per ml. Microtiter wells (NUNC Maxisorp; Thomas Scientific, Swedesboro, N.J.) were coated with 100 μl of extract overnight at 4°C. The plates were washed, blocked with 10% milk in PBS containing 0.05% Tween 20 for 1 h, and washed, and 100 μl of plasma, serially diluted in 1.0% milk in PBS–0.05% Tween 20, was added to duplicate wells. Plasma from normal, uninfected BALB/c mice and mice immune to *P. yoelii* (i.e., infected with 17XNL *P. yoelii* followed by two challenges of 17XL *P. yoelii*) were used as the negative and positive controls, respectively. After incubation at room temperature for 1 h, the plates were washed, and either a 1:5,000 dilution of alkaline phosphatase-labeled goat anti-mouse IgG (γ chain specific) (Sigma Immunochemicals, St. Louis, Mo.) or a 1:1,000 dilution of alkaline phosphatase-labeled goat anti-mouse IgM (μ chain specific) (Southern Biotech Associates Inc., Birmingham, Ala.) was added. After 1 h, the plates were washed, 100 μl of Sigma 104 substrate was added, and color development was measured at an optical density of 405 nm. Titers were determined by using an endpoint of 25% maximum specific binding.

## RESULTS

**Course of infection in *nu*/*nu* and *nu*/+ mice.** In experiment 1, *nu*/*nu* and *nu*/+ mice were fed either the MO+VE or MO−VE diet for 4 weeks prior to infection with 17XL *P. yoelii*. Since most studies on *P. yoelii* have been conducted with BALB/c mice fed normal laboratory chow, this group was also included for comparison. The courses of infection are compared in Fig. 1. Mice fed the MO+VE diet developed fulminating parasitemias and quickly died (mean days of survival ± standard deviation [SD]: *nu*/*nu*, 8 ± 0; *nu*/+, 7.6 ± 2.0; and +/+, 7.8 ± 1.2) (Fig. 1). The course of infection in *nu*/*nu* and *nu*/+ mice fed the MO−VE diet was significantly different. Between days 4 and 10, both groups developed slowly ascending parasitemias which reached ~12% by day 10. Over the next 8 to 18 days, mice in the *nu*/*nu* and *nu*/+ groups modulated the infection, with parasitemias ranging between 5 and 25%. Thereafter, the *nu*/+ mice eliminated their parasites and survived, whereas the athymic mice became anemic, developed high parasitemias of >50%, and were euthanized on day 29 of infection.

Blood samples from MO−VE-fed mice collected at day 11 (*nu*/*nu* and *nu*/+ mice) and day 26 (*nu*/*nu* mice) of infection were subinoculated into naive recipient BALB/c mice fed conventional lab chow. The recipient mice died of malaria 8 to 9 days later, demonstrating that the parasites had remained fully virulent in the MO−VE-fed donor animals.

In the second experiment, groups of mice were fed either the MO+VE, MO−VE, or MO+DPPD diet. As shown in Fig. 2, the results were similar to those of experiment 1, in that all mice receiving diets containing an antioxidant (vitamin E or DPPD) died of lethal malaria within 2 weeks of infection (mean days of survival ± SD for MO+VE: *nu*/*nu*, 12.8 ± 3.2, and *nu*/+, 9.2 ± 0.8; mean days of survival ± SD for MO+DPPD: *nu*/*nu*, 14.6 ± 2.6, and *nu*/+, 12.8 ± 3.4) (Fig. 2A and B). Again, all mice fed the MO−VE diet initially controlled their parasitemias for about 17 days (Fig. 2C). Thereafter, *nu*/+ mice fed the MO−VE diet cleared their parasites and survived. Athymic mice developed high parasitemias after day 16, became anemic, and were euthanized on day 33.

Plasma samples were collected from MO−VE-fed *nu*/+ and *nu*/*nu* mice on days 18 and 28 (experiment 1) and days 22 and 33 (experiment 2) of infection and tested for the presence of antimalarial IgM and IgG Ab (Table 1). Antimalarial IgM and IgG Ab were detected in the plasma samples of *nu*/+ mice in both experiments, but plasma samples from *nu*/*nu* mice were negative (titer of <1:50). Thus, the *nu*/+ mice mounted a successful antimalarial Ab response, whereas the athymic mice did not.

**Studies with *scid*/*bg* mice.** In experiment 3, two groups of *scid*/*bg* mice were fed two experimental diets for 6 weeks prior to infection with 17XNL *P. yoelii*. As can be seen in Fig. 3A, mice fed the MO+VE diet quickly developed malaria. After one mouse died on day 9, the remainder were euthanized. Mice fed the MO−VE diet were monitored for 31 days, during which time they did not develop any detectable parasitemias. To ensure that the results were not a methodological artifact, the MO−VE mice were again infected with $10^4$ parasitized erythrocytes per mouse, as were three naive *scid*/*scid* mice fed the MO+VE diet. As before, the mice fed the MO+VE diet quickly developed malaria and died (Fig. 3B), whereas the *scid*/*bg* mice fed the MO−VE diet again did not develop any detectable parasitemias. Thus, *scid*/*bg* mice, which lack T and B cells and have reduced NK-cell activity, were able to eliminate an otherwise lethal malaria infection when fed the prooxidant MO−VE diet.

## DISCUSSION

Previous studies have shown that outbred mice are able to control lethal *P. yoelii* parasites if fed a vitamin E-deficient diet

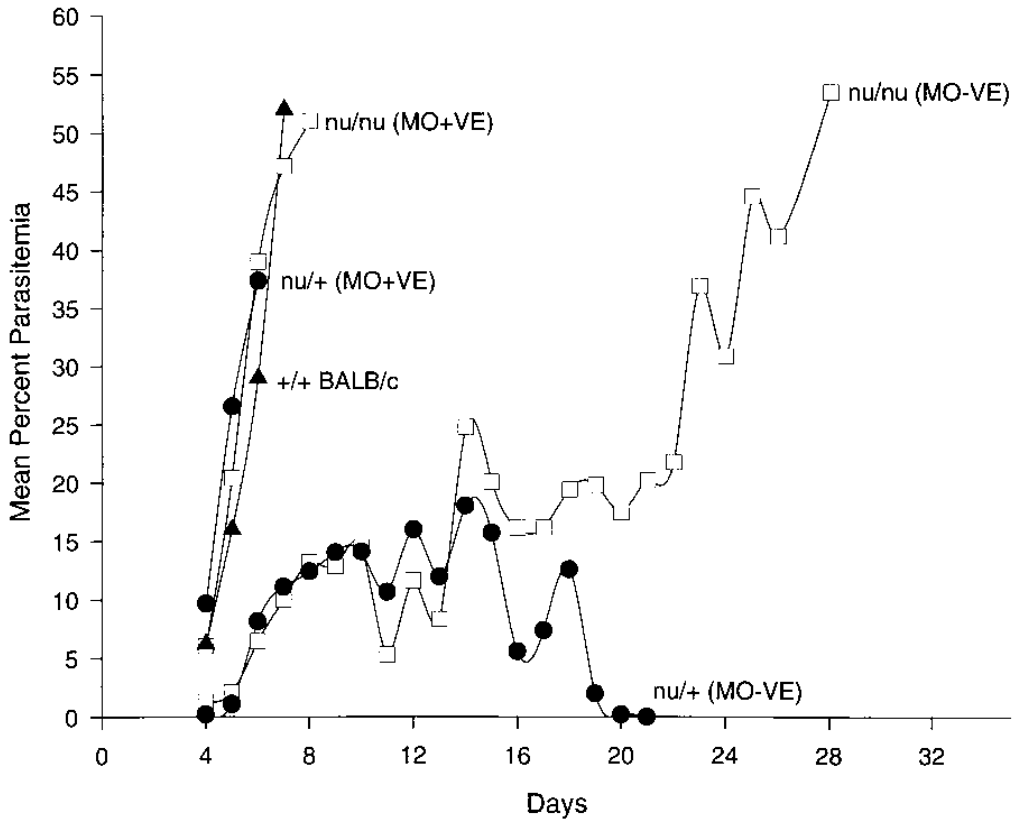


FIG. 1. Course of lethal *P. yoelii* infection in *nu/nu* and *nu/+* mice fed MO−VE and MO+VE diets (experiment 1). Each data point represents the mean percent parasitemia for five mice.

containing omega-3 polyunsaturated fatty acids (20). The mice not only survive but also develop a protective immune response, as demonstrated by the fact that they rapidly eliminate secondary 17XL *P. yoelii* challenges after being returned to normal laboratory chow (1, 8). The current study sought to define the role of acquired immunity in diet-mediated protection. Our results show that mice fed a prooxidant diet were successful in controlling their parasitemias in the absence of T cells, T-cell-derived cytokines, and antimalarial Ab.

Naive mice require T and B cells for the acquisition of immunity to *P. yoelii* (24, 30). T cells provide cognate help for Ab production, as well as secrete cytokines that enhance macrophage functions, including ROI production. Ab are required for the elimination of asexual-stage parasites (24). Thus, it was logical to assume that acquired immunity would contribute significantly to protection in intact mice fed the prooxidant diet. Accordingly, the results obtained in this study were not entirely anticipated.

The data show that during the first 17 to 18 days of infection, *nu/nu* mice fed the MO−VE diet were able to control lethal *P. yoelii* as well as intact *nu/+* animals were (Fig. 1 and 2). They had similar ascending parasitemias and successfully modulated the parasites thereafter. Thus, during this time, non-T-cell-mediated effector functions were sufficient in the *nu/nu* mice to allow them to control the infection. Clearly, the effector mechanisms operating during this time were not determined, but one can speculate that the incorporation of MO into membranes made the parasites more susceptible to peroxidation. It is well established that macrophages and neutrophils secrete ROI during malaria (3, 12). Although *nu/nu* mice lack high levels of T-cell-derived cytokines, they have other cell types that secrete immunomodulating cytokines. For example, NK cells secrete gamma interferon, and B cells produce transforming growth factor β, both of which enhance ROI production. Thus, it is likely that innate immune cells and non-T-cell-derived cytokines contributed significantly to the initial control of malaria in diet-protected mice. In addition, recent studies with *Plasmodium chabaudi* have demonstrated a profound role for γδ T cells in malarial immunity (27). This constitutes an additional potential effector mechanism that could be operative at this time.

During the first 2 weeks of infection, the *nu/+* mice developed an acquired immune response against the 17XL strain of *P. yoelii*, as evidenced by the production of antimalarial IgG Ab, and were able to eliminate their parasites, whereas the *nu/nu* mice failed to produce antimalarial Ab (Table 1), became anemic, and died (Fig. 1 and 2). Since antimalarial Ab are required for elimination of *P. yoelii* infections, the absence of this effector mechanism in *nu/nu* mice resulted in lethal consequences.

Results from the *scid/bg* mouse study showed that these mice could also control an otherwise lethal infection under certain dietary conditions (Fig. 3). When *scid/bg* mice were fed the MO−VE diet, they failed to develop patent 17XNL *P. yoelii* infections. The 17XNL strain, however, produced a lethal infection in *scid/bg* mice fed the MO+VE diet. The major differences between the nude and *scid/bg* mouse studies were that the *scid/bg* mice were fed the diet for 6 instead of 4 weeks prior to infection and were infected with a less virulent strain of *P. yoelii*. Previous studies have shown that longer prefeeding pe-

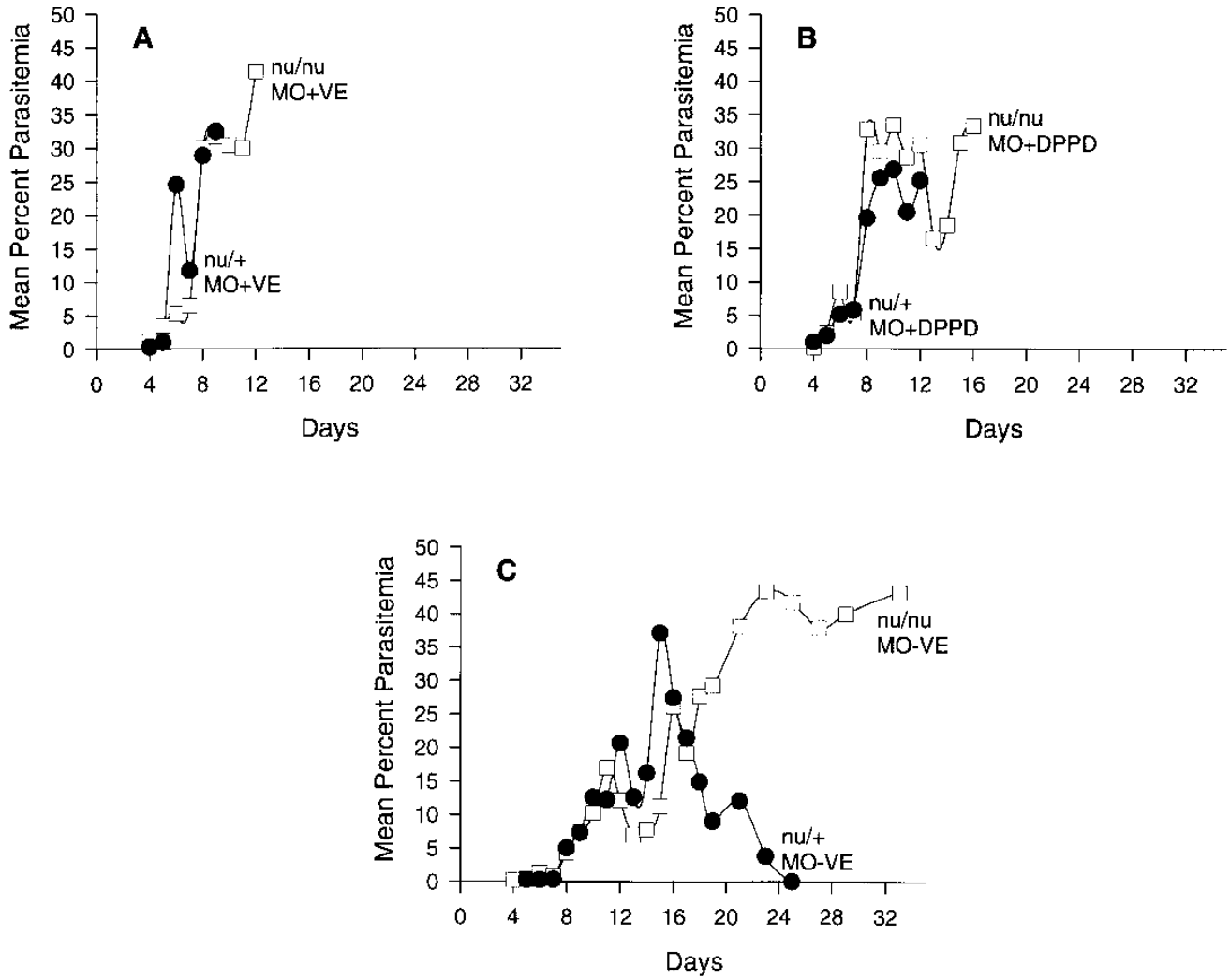


FIG. 2. Course of lethal *P. yoelii* infection in mice fed MO diets with or without an antioxidant (experiment 2). *nu*/*nu* and *nu*/+ mice were fed one of three diets (see Materials and Methods) for 4 weeks and then infected with 17XL *P. yoelii*. Each data point represents the mean parasitemia for five animals.

riods with vitamin E-deficient diets often result in enhanced survival, presumably because of greater depletion of tissue tocopherol stores (16, 17). It is likely that the level of prooxidant stress immediately following the injection of 17XNL *P. yoelii* was sufficient to eliminate the parasites before patency developed.

TABLE 1. Antimalarial Ab titers in diet-protected mice

| Expt or serum | Day of infection | Ab titer[a] | | | |
|---|---|---|---|---|---|
| | | IgM | | IgG | |
| | | *nu*/+ mice | *nu*/*nu* mice | *nu*/+ mice | *nu*/*nu* mice |
| 1 | 18 | 1:1,120 | <1:50 | 1:1,250 | <1:50 |
| | 28 | 1:480 | <1:50 | 1:250 | <1:50 |
| 2 | 22 | 1:100 | <1:50 | 1:250 | <1:50 |
| | 33 | 1:70 | <1:50 | 1:450 | <1:50 |
| Hyperimmune | | 1:316 | | 1:5,010 | |
| Normal mouse | | <1:50 | | <1:50 | |

[a] Mean titer in plasma samples pooled from three to five mice; 1:50 was the lowest dilution tested.

Previous studies have suggested that diets that increase oxidative stress can be beneficial not only against rodent malaria but also against human malaria and possibly other parasitic diseases as well. For example, when Chinese patients with *Plasmodium falciparum* malaria were treated with either qinghaosu (artemisinin) or qinghaosu plus a vitamin E-free fish oil concentrate, they became clinically cured but developed recrudescent malaria. The period of recrudescence, however, was significantly extended in those individuals receiving the dietary supplement (21). In addition, the addition of omega-3 fatty acids derived from fish oil to culture medium inhibits *P. falciparum* growth *in vitro* (7, 14). Research by others has indicated that fish oil-containing diets may have value against other parasites. For instance, the early work of Godfrey (10) demonstrated that a diet low in vitamin E and containing fish oil protected mice against *Babesia rodhaini*. He later showed that a similar diet suppressed the growth of *Trypanosoma congolenses* and *Trypanosoma vivax* but did not appear to be effective against *Trypanosoma cruzi* or *Trypanosoma brucei* (11). More recently, Allen et al. (2) found an anticoccidial effect of omega-3 fatty acids in that diets containing fish oil reduced cecal

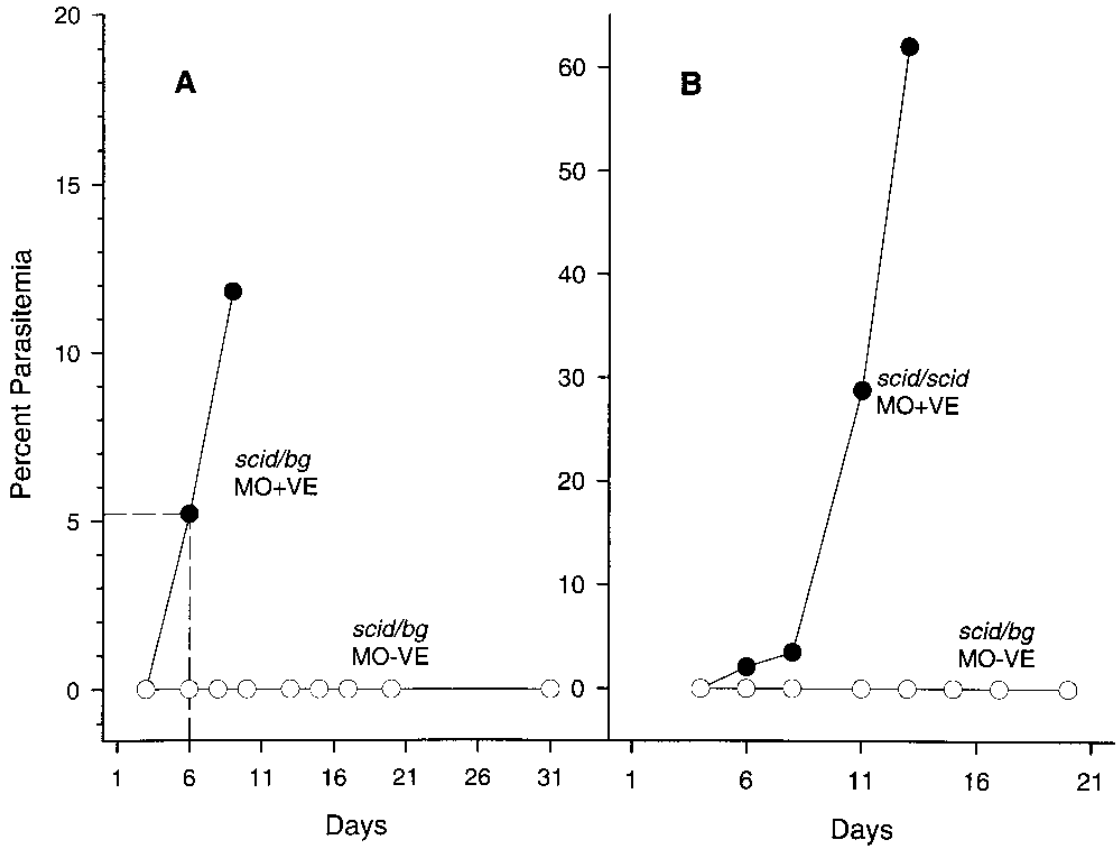


FIG. 3. Course of *P. yoelii* infection in *scid/bg* mice. (A) *scid/bg* mice were fed either the MO+VE or MO−VE diet before and after challenge with *P. yoelii*. Mice given the MO−VE diet did not develop detectable parasitemias (experiment 3). (B) The *scid/bg* mice were rechallenged along with three naive *scid* mice. Again, the *scid/bg* mice did not develop patent infections (experiment 4). Each data point represents the mean parasitemia for three to five mice.

lesion scores in chickens infected with *Eimeria tenella*. Thus, dietary fish oil may be a promising approach to the control of several parasites.

Clearly, there is much to be learned about the mechanism(s) by which prooxidant diets influence the course of malaria infections. Likewise, there remains much to be learned about immunity to asexual-stage malarial parasites. The mouse model with the prooxidant diet and lethal malaria described herein may prove useful for gaining additional basic information about malarial immunity, especially the role of immunologically induced oxidative stress in parasite elimination. In addition, athymic mice fed the MO−VE diet control the parasite but ultimately die due to lack of acquired immunity. Thus, with this model, one should be able to transfer various factors such as Ab to specific antigens, T-cell clones, or immunomodulatory cytokines to identify those immunologic components necessary for the elimination of lethal malarial parasites.

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