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Immunological memory is compromised by food restriction in deer mice *Peromyscus maniculatus*

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Martin, Lynn B., 2nd, Kristen J. Navara, Zachary M. Weil, and Randy J. Nelson. Immunological memory is compromised by food restriction in deer mice *Peromyscus maniculatus. Am J Physiol Regul Integr Comp Physiol* 292: R316–R320, 2007. First published August 10, 2006; doi:10.1152/ajpregu.00386.2006.—The immune system protects organisms against infection, but this protection presumably comes at a cost. Here, we asked whether food restriction would compromise the ability of an organism to generate an immune response on reexposure to an antigen, which would represent a functional cost of immunological memory. Immunological memory is generated when B and T lymphocytes sensitive to components of pathogens (i.e., antigens) proliferate after exposure and persist in circulation to hinder reinfection. To test the possibility that B cell memory, the component of the immune system responsible for antibody production, is expensive to maintain, secondary antibody production against a novel protein [keyhole limpet hemocyanin (KLH)] was compared in food-restricted and ad libitum-fed male deer mice (*Peromyscus maniculatus*). To determine whether compromised secondary antibody production was solely due to elevated corticosterone independent of resource availability, some food-restricted and ad libitum-fed mice were subjected to unpredictable, chronic (2 h/day) restraint. Mice fed 70% of their ad libitum diet 2 wk after primary antigen challenge produced ~95% less IgG against KLH after a second antigen challenge than mice fed ad libitum, even though all mice were fed ad libitum during the secondary antibody response period. Restraint had no effect on secondary IgG production in response to KLH, and corticosterone concentrations 1 day after food restriction did not differ between food-restricted and ad libitum-fed mice. Together, these data imply that secondary antibody responses and the benefits of immunological memory are energetically costly in this species.

humoral; stress; immunocompetence; rodent

THE IMMUNE SYSTEM EXISTS PREDOMINANTLY to protect organisms against infection. Such goals are achieved via a multitude of active and passive processes, each mediated by different, but often interconnected, networks of cells and tissues. Activation of many of these processes is expensive (3, 18, 22), even in the absence of infection. Indeed, induction of various immune responses can elevate metabolic rate (4, 12, 25, 31), alter reproductive behavior (1, 2, 38), and depress somatic and reproductive growth (10, 23, 35). One issue that is yet to be resolved, however, is whether the costs of using the immune system are comparable in type and degree to the cost of maintaining the system (18).

Attempts to detect maintenance costs of immune defense have been rare, perhaps because of the diffuse nature of the immune system. In chickens (*Gallus gallus*), it has been estimated that immune cells comprise <1% of adult body mass and that the amount of lysine, a critical amino acid necessary to maintain the immune system of young birds, is <3% of the whole body requirements (19). Moreover, administration of cyclophosphamide, an immunosuppressant, to white-footed mice (*Peromyscus leucopus*) reduced circulating lymphocyte levels by >200% but had no effect on metabolic rate, which presumably would have decreased if the cost of maintaining lymphocytes was significant (7). Finally, basal metabolic rates were higher, rather than lower, in genetically engineered mice lacking T and B lymphocytes than in normal mice (36), which suggests further that the maintenance costs of lymphocytes in mice are low.

Collectively, these results imply that the costs of immunological maintenance may be modest. One component of immunological maintenance that has yet to garner attention but may be sufficiently costly as to be compromised in certain contexts is immunological memory. Immunological memory is produced in part by B cells, the major constituents of the humoral immune system. These cells combat infection, predominantly by producing small soluble proteins (i.e., antibodies) that recognize foreign proteins (i.e., antigens) and inactivate them directly or target them for destruction by other immunological mediators (16). On exposure, antibodies are rapidly generated, and these antibodies, as well as the B cells that produce them, remain in circulation for long periods of time. On subsequent exposures to encountered antigens, highly specific antibodies are generated rapidly, which allows organisms to mount more rapid and targeted control of infections than on primary encounters.

Several aspects of the antibody production process suggest that this defense, and particularly secondary responses, might be more expensive than is appreciated. *1*) Antibodies consist of protein and are typically produced and maintained in large quantities after antigen exposure. *2*) Maintenance of immunological memory is achieved via low-level ongoing proliferation of memory B cells (16, 18), which would consume resources and, presumably, require energy. *3*) Once immunological memory is generated, memory B cells actively prevent activation of subsequent primary responses to prevent generation of less specific antibodies. This phenomenon perhaps represents an effort to conserve resources for more effective defenses. *4*) Although mild food restriction enhances many aspects of immune activity, it hampers B cell- and T cell-mediated antigen recall in the few experiments that have considered these measures (32).

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In the present study, we tested the hypothesis that maintenance of B cell-mediated immunological memory was expensive by comparing secondary antibody production in animals subjected to 2 wk of food restriction (FR) (70% of ad libitum intake) with that in ad libitum-fed (AL) animals. To test our hypothesis, B cell immunological memory was generated in two groups of deer mice (*Peromyscus maniculatus*) via exposure to keyhole limpet hemocyanin (KLH), a large immunogenic molecule (4, 15, 20, 24). After the 2-wk FR period, all mice were provided ad libitum access to food in an effort to equalize the ability of mice to use their immune systems to generate secondary immune responses. We predicted that if maintenance of B cell memory was indeed expensive, then FR mice should produce less secondary IgG than AL mice, especially inasmuch as food was provided ad libitum to all animals at this point. Because FR may have also induced physiological stress, particularly a rise in corticosterone, which could influence secondary responses to KLH independent of or in conjunction with FR (5, 17, 28), additional groups of mice (both FR and AL) were briefly restrained several times during the FR period. Similar procedures have been used in rodents to elevate circulating corticosterone and approximate a largely psychologically stressful experience (9, 21, 34). Our intention in using this treatment was to obviate any potential effects of stress and, particularly, those mediated by glucocorticoids on secondary IgG production.

METHODS

Mice. Adult (>90-day-old) male *P. maniculatus bairdii* were obtained from the *Peromyscus* Genetic Stock Center at the University of South Carolina (Columbia, SC) and housed singly in polypropylene cages for 3 mo before the experiment. For the first 2 mo, mice were exposed to 14 h of light and 10 h of darkness each day (lights off at 1500 Eastern Standard Time), with food (Harlan TekLad 8640) and chlorinated tap water provided ad libitum. At 1 mo before the present experiment, all the mice were transferred to a room illuminated for 16 h/day. Before this transfer and for the duration of the study, ambient temperature and humidity were maintained at 22.5 ± 1°C and 50 ± 5%, respectively. At 2 wk before the first KLH injection, food intake (g dry mass food/g body mass) was measured for 4 consecutive days for each mouse. Average food consumption per day was calculated for each individual, and the data were used to establish diets (70% of ad libitum intake) to be used during the experiment. All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee before they were carried out and comply with current National Institutes of Health guidelines for animal research.

Experiment progression. Before injection, blood (~100 μl) was collected from the retroorbital sinus of each individual mouse into heparanized microcapillary tubes while mice were under deep isoflurane anesthesia; body mass was also recorded (to 0.1 g). Immediately after blood collection, each mouse was injected intraperitoneally with 150 μg of KLH (KLH in aluminum phosphate; catalog no. 374811, lot no. B30450, CalBiochem, La Jolla, CA). These injections induce antibody production but do not activate fever or other sickness responses, including reduced food intake (4). After 7, 14, and 21 days, the procedure described above was used to obtain an additional blood sample from each mouse. After blood collection, clots were allowed to form for 1 h and then removed, and samples were centrifuged at 7,000 rpm for 20 min. Serum was then removed and stored at −70°C until ELISA (see below).

Mice were then assigned randomly to one of four groups: AL-no stress, AL-restraint, FR-no stress, and FR-restraint. FR mice received 70% of the diet consumed under ad libitum conditions (as measured several weeks before the first blood sample). Use of this FR protocol significantly depresses growth and maturation of the reproductive system in this species (6, 30). All mice in the FR group consumed all the food that was provided. Restraint consisted of placing the animal individually into clean, well-ventilated polystyrene restraint tubes (50 ml) (sufficiently large for restricted movement, without compressing or squeezing the animal) for 2 consecutive hours on 6 of the 14 days of the FR period (spaced 2–3 days apart) from 0900 to 1100 each day (13). This restraint schedule was not meant to simulate the stress of FR, inasmuch as it was intended to elevate corticosterone concentrations more and for longer periods in some mice than in others and, thus, obviate stress effects on secondary antibody production. Other studies have used similar paradigms of chronic variable stress (21, 34).

After this 2-wk experimental period and for the remainder of the study, all mice were again given ad libitum access to food; 1 day after FR, a blood sample was taken (see above) and secondary antibody responses to KLH were induced via intraperitoneal injection of 30 μg of KLH. Blood samples were taken 3, 7, and 12 days later to characterize the secondary antibody response to KLH, and body mass was measured (to 0.1 g) on each day. At 12 days after secondary KLH injection, mice were decapitated while under deep isoflurane anesthesia, blood was collected, and tissues (paired testes, paired epididymal fat pads, spleen, heart, and liver) were collected, cleaned of connective tissue, and weighed to the nearest 0.01 g wet mass, inasmuch as comparable studies have detected effects of FR (6, 30) and immune challenge (7) on organ masses in this species. During all procedures and before injections or blood sampling, mice were handled and anesthetized equally.

KLH ELISA. For measurement of IgG produced against KLH, a colorimetric ELISA previously developed for *Peromyscus* was used (4). Briefly, 96-well plates were coated with KLH and then serum samples were diluted with PBS-Tween (1:80), vortexed, and added to plates in duplicate. Positive (serum samples from *Peromyscus* already determined to have high KLH IgG titers) and negative (serum from KLH-naïve mice) controls were also added to each plate in duplicate. Plates were then sealed, incubated at 37°C for 3 h, and washed with PBS-Tween, and then a secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG; code 59296, lot no. 04325, MP Biomedicals, Aurora, OH) was added to each well (1:750 dilution). Plates were again incubated (37°C for 1 h) and washed, and each well was treated with *p*-nitrophenyl phosphate. Exactly 20 min later, optical density (OD) of each well was read (405-nm filter on a Bio-Rad Benchmark microplate reader, Richmond, CA), and the mean OD of each sample was calculated. Data analysis was performed on sample OD readings expressed as a percentage of the positive controls on each plate. All samples were measured by a person blind to sample identity. Mean intraplate variation was 9%; interplate variation was 7%. Three mice mounted no significant primary antibody response to KLH (perhaps because of unsuccessful primary challenge) and were removed from analysis.

Corticosterone assay. Total serum corticosterone was quantified using double-antibody ^{125}I kits (MP Biomedicals, Costa Mesa, CA; cross-reactivity with other steroids <1%, lower detection limit 5 ng/ml). Corticosterone was measured in serum samples collected 1 day after the FR period, which was 1 day after all mice were given food ad libitum. The assay was conducted following the guidelines set by the manufacturer, except all samples were diluted 1:1,000, because *Peromyscus* have much higher corticosterone concentrations than domestic mice (13). Intra-assay variability was <10%.

Data analysis. Before analysis, data were tested to ensure that requirements of parametric statistics were met; when necessary, data were square root (ratio of KLH IgG titers on *day 21* to all values during secondary IgG responses) to improve variance distributions. To compare antibody production, repeated-measures general linear models (GLM) were used, with FR and restraint treatment and their interaction as factors. Body and organ masses were compared using univariate GLM with the above-mentioned factors. GLM could not be used to compare corticosterone among groups, because only 45% of

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the samples from the time point immediately after FR were available after ELISAs. Because some of these samples were unavailable and because tube stress did not affect antibody production, an independent *t*-test was used on data from available samples to determine whether FR alone affected corticosterone concentrations. Means ± SE are provided where relevant.

RESULTS

Antibody production. Mice mounted significant primary IgG responses to KLH ($F_{3,57} = 82.4$, $P < 0.001$). Unexpectedly, primary antibody production was greater in FR than in AL mice, even though no differential treatment had been administered to groups (Fig. 1*A*; $F_{3,57} = 6.3$, $P = 0.001$). This difference was not apparent to us until the completion of ELISAs (many weeks later). To account for this difference, titers for each day of the secondary IgG response were divided by the IgG titer of the primary response on *day 21*, which was the highest titer for all individuals. This ratio quantifies the percent change in antibody titer in each sample relative to the last time point of the primary response. Mice mounted significant secondary antibody responses to KLH ($F_{3,57} = 2.8$, $P < 0.05$). Only FR ($F_{3,57} = 3.2$, $P < 0.05$), but not restraint ($F_{3,57} = 0.29$, $P = 0.83$) or the interaction of factors ($F_{3,57} = 1.1$, $P = 0.36$), affected secondary IgG production in response to KLH. As predicted, FR mice exhibited less secondary IgG production than AL mice (Fig. 1*B*).

Body and organ masses and corticosterone. Body mass was not different among treatment groups before the primary KLH injection ($F_{3,24} = 0.21$, $P = 0.89$) or immediately after the 2-wk FR-stress treatment ($F_{3,23} = 0.51$, $P = 0.68$). Most organs and final body mass and testosterone concentrations were not affected by FR or restraint (all $P > 0.05$). Paired epididymal fat pad mass, however, varied among groups ($F_{3,24} = 3.0$, $P < 0.05$). Restraint marginally enhanced final epididymal fat pad mass ($F_{1,24} = 3.9$, $P = 0.06$), and there was a significant interaction of FR and stress ($F_{1,24} = 7.2$, $P = 0.01$). Student-Newman-Keuls post hoc tests indicated that epididymal fat pad mass of FR mice that did not undergo stress was larger than that of any other group: 0.15 ± 0.02 g for AL-no restraint, 0.16 ± 0.02 g for AL-restraint, 0.22 ± 0.02 g for FR-no restraint, and 0.14 ± 0.01 g for FR-restraint. At 1 day after the 2-wk FR period, there was no difference in circulating corticosterone between AL and FR mice: 174.0 ± 73 and 90.0 ± 19 ng/ml, respectively ($t_{11} = 1.03$, $P = 0.33$).

DISCUSSION

FR dampened secondary IgG responses to a novel protein in male deer mice, even though mice were fed ad libitum during the secondary response. Periodic restraint meant to induce a mild, chronic stress did not further affect secondary antibody responses, nor did restraint alone depress secondary antibody production. Also, 2 wk of FR did not elevate corticosterone concentrations or induce body mass loss, indicating that mice were able to compensate somewhat for reduced resource availability over the FR period. Finally, organ masses were not negatively affected by FR at the end of the secondary antibody production measurement period, indicating that *1*) FR influenced the immune, but not other organ, systems, *2*) compensation for shrinkage occurred for most organs, but not within the compartments of the immune system responsible for generating secondary antibody responses, or *3*) activity and/or metabolic rate was depressed to levels that hindered antibody production but promoted maintenance of organ size. Together, these data provide evidence that the benefits of immunological memory are not free. Functionally, compromised secondary antibody responses to FR suggest that when food resources are low, the benefits of immunological memory would go unrealized. They also indicate that psychological stress, which can elevate the immunosuppressive hormone corticosterone, does not have the same effects on secondary antibody production as FR.

Several mechanisms could have led to the outcomes detected in this study. *1*) The activity or abundance of helper T cells, which are critical for antigen presentation to naïve B cells, might be involved. The effectiveness or the presence of these cells may have been compromised by FR. *2*) FR individuals may have remained in a glucoprived or lipoprived state after FR, which would have prevented them from mounting responses against secondary antigen exposure in much the same way that FR might inhibit primary antibody responses. *3*) High primary antibody responses in the FR group, which were unexpected and unknown until the time of assay, may have

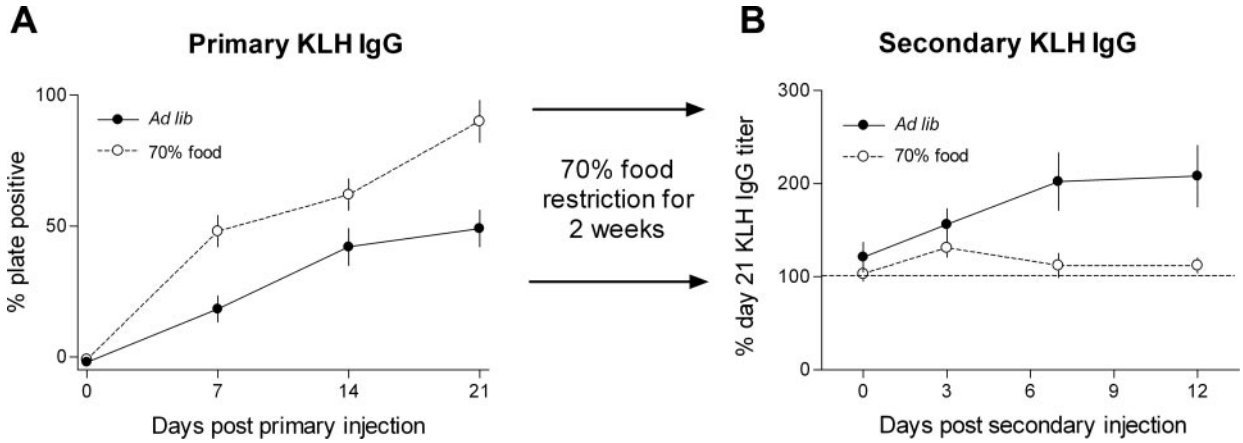

Fig. 1. Food restriction decreases secondary antibody production in response to a novel protein [keyhole limpet hemocyanin (KLH)] in male deer mice, *Peromyscus maniculatus*. *A*: primary antibody response to KLH, expressed as percentage of plate-positive controls, in samples collected before food restriction. *B*: secondary antibody responses to KLH, expressed as proportion of antibodies produced at each time point relative to titer on the last day of the primary response. Values are means ± SE.

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prevented strong secondary responses later. This would seem unlikely, given that the rate and intensity of secondary responses are often related to the size of the primary response. Larger primary responses and, hence, greater proliferation of memory B cells typically lead to larger secondary responses (16). *4*) Fewer KLH-responsive B cells persisted in circulation (or IgG production per B cell was lower) in FR than in AL mice. Either early cessation of B cell proliferation (and, hence, memory generation) during the primary response or increased B cell death in FR mice may have led to fewer cells in circulation to produce antibodies in response to a second antigen challenge. Inasmuch as body mass was not reduced 1 day after FR, this mechanism seems most likely (although all 4 possibilities remain viable).

The lack of effects of restraint on secondary IgG production and the absence of an elevation in corticosterone or decrease in body mass after FR were unexpected. Previous studies have shown that FR can increase corticosterone in rodents (27). Similarly, chronic, variable restraint elevates glucocorticoid concentrations (13), which, if elevated over long periods, suppress immune activity, including antibody responses (8). Although the lack of effects of FR on corticosterone in *P. maniculatus* has precedence (6), the lack of restraint effects on immunological memory does not. Perhaps the transient effects of circulating corticosterone on the immune system (8, 26) explain the results of our study. Indeed, our experimental design does not eliminate corticosterone as an important mediator of depressed secondary antibody production in response to FR. It is likely that FR and restraint induce a different pattern of corticosterone secretion, which may have distinct effects on secondary antibody responses. Had we been able to obtain larger serum samples during the experiment (without imparting additional stress to mice), we would have been able to address this possibility. Because we could not, further study is warranted.

Irrespective of the mechanisms, suppression of immunological memory after FR represents a design constraint of secondary antibody responses or an intentional and, perhaps, adaptive strategy in this species. Cessation of B cell proliferation during the primary response (at the onset of FR) may have led to a lower secondary baseline in FR than in AL mice. Thus, unless B cells from FR mice were able to produce antibodies at a greater rate than AL mice once they were returned to an ad libitum diet, they may have been unable to catch up to AL mice during the secondary IgG response. There was no indication that FR mice would have caught up to AL mice, even if serum collection had continued over several weeks; IgG in the final secondary samples was lower at all time points in FR than in AL mice. Perhaps B cells generate antibodies at their maximum capacity at all times, leaving no room for compensation. One way to resolve this issue would be to expose mice to KLH several days (weeks) after returning them to ad libitum feeding. If compromised secondary antibody production is transient, then secondary antibody responses should be comparable in FR and AL mice given more time after FR. The persistence of a reduced antibody response after this period would suggest that immunological memory itself had been permanently compromised.

If the effects of FR on immunological memory are indeed permanent, a dampening of immunological memory after FR may represent a strategy whereby mice promote survival of short-term resource shortages. In other words, when *P. maniculatus* are energetically or nutritionally stressed over the course of days, they may sacrifice immunological memory in favor of other more immediately critical processes (e.g., thermogenesis). Studies of immunosenescence, or how the immune system changes with age, support this possibility (32). Mild FR generally enhances, rather than depresses, immune activity. Indeed, immune enhancement is thought to be one mechanism whereby FR prolongs lifespan in rodents and other species. The majority of that work, however, focused on innate defenses, such as those involved in the initiation and perpetuation of acute-phase (i.e., fever) responses (32). In the few studies that considered the effects of FR on immunological memory, B and T cell-mediated memory was depressed in response to FR (11, 37). Given this pattern, FR may not be compromising the immune system inasmuch as it is inducing a shift in priority. Indeed, a similar argument has been proposed for the short-term enhancing but long-term suppressing effects of glucocorticoids on the immune system (8).

It is apparent from the previous discussion that the effects of FR on secondary antibody responses detected here provide extensive opportunities for future work. Although the mechanisms by which FR compromises immunological memory have not been reconciled, it is apparent that benefits of immunological memory are expensive and, hence, may be important in ecological and evolutionary contexts. One might predict, therefore, that animals investing heavily in reproduction should avoid such immune defenses, which may be prohibitive of intensive breeding. Similarly, one might predict that immunological memory might be weak in animals residing in resource-limited habitats (33) or in winter vs. summer, when resources are scarce (14, 29). Finally, consideration of the currencies used to pay the costs of immunological memory would be a valuable line of investigation (22). Whether calories, specific amino acids, micronutrients, or all three prohibit strong secondary IgG production after FR remains unknown. Titration of each of these substances could yield insight into this issue.

As highlighted in this study, new approaches to the study of costs of the immune system are critical. The maintenance costs of the immune system are generally thought to be small. Leukocytes generally possess little cytoplasm when quiescent (16); thus, in the absence of infection, they probably require modest resources to persist and occasionally engage in housekeeping activities (e.g., controlling intestinal microflora and/or clearing apoptotic and necrotic cells). Why then does FR compromise secondary antibody responses? As with prior attempts to assess immunological maintenance costs, the outcomes of this study may partly be due to methodology. For example, FR induces many physiological changes besides those considered here. Perhaps measurement of other hormones, neurotransmitters, or cytokines could have elucidated the mediators of this study. Regardless of these limitations, on the organismal level, it is apparent that food availability impinges on the ability of animals to use the immunological memory they generate. Consequently, the benefits of immunological memory in these and perhaps other species may come at a cost.

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