Acute and Chronic Social Defeat Suppresses Humoral Immunity of Male Syrian Hamsters (*Mesocricetus auratus*)

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Stressors, both physical and psychological, can activate the hypothalamic–pituitary–adrenal (HPA) axis, leading to a wide range of physiological responses including increased glucocorticoid release and suppression of immune function. The majority of studies published to date have focused on the effects of physical stressors (e.g., cold exposure, electric shock) on immunity. The present study examined the role of a stressor, social defeat, on humoral immune function of Syrian hamsters (*Mesocricetus auratus*). Specifically, adult male Syrian hamsters experienced social defeat (i.e., exposure to a dominant animal in that animal's home cage) that was either acute (i.e., a single exposure) or chronic (i.e., daily exposures across 5 days). A control group of animals was placed in a resident's home cage without the resident animal present and did not experience defeat. After the last encounter, blood samples were drawn and animals were subsequently injected with keyhole limpet hemocyanin (KLH). Blood samples were again taken 5 and 10 days postimmunization and serum was analyzed to determine serum cortisol and anti-KLH immunoglobulin G (IgG) concentrations. Cortisol concentrations were elevated in both acutely and chronically defeated hamsters compared with control animals. In contrast, serum IgG concentrations were significantly reduced in both groups of defeated hamsters compared with control animals. Collectively, these results demonstrate that both acute social defeat and chronic social defeat lead to activation of the HPA axis and suppression of humoral immune function. These data suggest that social defeat is an important, ecologically relevant model with which to examine stress-induced immune suppression in rodents. © 2001 Academic Press

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Individuals of most animal species are exposed to a wide range of potential stressors during their lifetimes. These stressors can be social in nature (e.g., territorial conflict, competition between male conspecifics for access to females) or due do environmental perturbations (e.g., reduced ambient temperature, reduced food availability). Regardless of their nature, exposure to stressors leads to marked physiological and behavioral changes in virtually all species examined to date. These changes are mediated, in part, through activation of the hypothalamic–pituitary–adrenal (HPA) axis and subsequent release of glucocorticoids from the adrenal cortex and catecholamines from the adrenal medulla (see Bugajski, 1999, for review).

Social stress, primarily in the form of social conflict between individuals, is one of the most pervasive forms of stress experienced by many animal species, including humans. These conflicts usually occur between male conspecifics and involve access to potential mates, territorial competition, or access to limited food availability. In the majority of social conflicts, the resolution of the conflict ends in a distinct "winner" and "loser." Defeated animals (i.e., losers) display increased activation of the HPA axis as evidenced by increased release of adrenocorticotropin, β-endorphin, cortisol, and corticosterone. Dominant animals (i.e., winners) display considerably less HPA activation compared with submissive or subordinate animals (Huhman, Bunnell, Mougey, and Meyerhoff, 1990; Huhman, Moore, Ferns, Mougey, and Meyerhoff, 1991; Huhman, Moore, Mougey, and Meyerhoff, 1992; Blanchard, Spencer, Weiss, Blanchard, McEwen, and

Sakai, 1995; Spencer, Miller, Moday, McEwen, Blanchard, Blanchard, and Sakai, 1996). Several lines of evidence suggest that social defeat also results in prolonged changes in behavioral and autonomic functions, including decreases in reproductive and aggressive behavior, decreases in food and water intake (Albonetti and Farabollini, 1994; Potegal, Huhman, and Meyerhoff, 1993; Jasnow, Banks, Owens, and Huhman, 1999), as well as decreases in immune function (Bohus, Koolhaas, Heijnen, and de Boer, 1993). For many animals living in large social groups, subordinate status results in continuous exposure to attacks from more dominant animals, creating a scenario in which the stress of social conflict becomes chronic (Blanchard *et al.,* 1995). In subordinate animals, chronic social defeat results in a prolonged stress response and chronic activation of the HPA axis that can lead to marked physiological and behavioral changes (Blanchard, Hebert, Sakai, McKittrick, Henrie, Yudko, McEwen, and Blanchard, 1998). These changes disrupt homeostatic balance and may lead to stress-induced illness that ultimately threatens survival.

It is well established that stressful situations can alter immune function in most mammals. For example, a variety of physical stressors including white noise (Monjan and Collector, 1977), cold water swim (Anisman, Lacosta, Kent, McIntyre, and Merali, 1998), immobilization (Gisler, 1974), and electric shock (Beden and Brain, 1982; Laudenslager, Fleshner, Hofstadter, Held, Simons, and Maier, 1988) can suppress immune function. In addition to physical stressors, social stressors also have profound effects on immune function. For instance, social defeat reduces serum antibody concentrations in rats (Fleshner, Laudenslager, Simons, and Maier, 1989). This reduction of serum antibodies is correlated directly with the amount of time animals spent in submissive postures. After chronic social defeat (i.e., introduction of a male rat into a male–female resident group), submissive males have reduced T-cell mitogen-induced lymphocyte proliferation and decreased CD4/CD8 T-cell ratios, but increases in the number of B lymphocytes and granulocytes compared with dominant animals (Stefanski, 1998). In addition, acute social stressors increase granulocyte numbers and CD4/CD8 T-cell ratios, but decrease lymphocyte numbers (Stefanski and Engler, 1998). These data suggest that both acute and chronic stressors can result in differential immunological effects depending on the type of stress and the immune parameter measured (Dhahbhar, 1998; Dhabhar and McEwen, 1997; Stefanski and Engler, 1998). In addition to altering specific immune parameters, social stressors have profound effects on susceptibility to infection and can therefore affect health and well-being. For instance, social stressors cause reactivation of herpes simplex virus type 1 in latently infected mice (Padgett, Sheridan, Dorne, Bernston, Candelora, and Glaser, 1998), as well as suppression of lymph node cellularity, virus specific interleukin-2 and interferon-γ production, and lymphocyte proliferation following inoculation with pseudorabies virus (de Groot, van Milligen, Moonen-Leusen, Thomas, and Koolhaas, 1999).

Although there has been extensive research on the effects of stress on immune function and disease susceptibility, a great deal is still unknown about the neuroendocrine mechanisms underlying social stress-induced immunological changes. The majority of studies have focused on the HPA axis, primarily the role of glucocorticoids, in mediating stress-induced immune suppression (e.g., Rook, 1999; McEwen, Biron, Brunson, Bulloch, Chambers, Dhabhar, Goldfarb, Kitson, Miller, Spencer, and Weiss, 1997) and have done so using highly controlled physical stressors (e.g., cold water swim, restraint, electric shock). Although these studies have provided important insights into the effects of stress on immunity, the relatively artificial nature of such stressors limits the generalizability of the results. Thus, the goal of the present study was to examine the effects of a more ecologically relevant stressor, social defeat, on humoral immunity in male Syrian hamsters. Specifically, if social defeat is a potent stressor, then the experience of social defeat should activate the HPA axis and suppress antibody production. Furthermore, if acute and chronic stress exerts differential effects on immunity, then antibody production should also differ between acutely and chronically stressed animals.

MATERIALS AND METHODS

Animals and Housing Conditions

Twenty-four adult (>60 days of age) male Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratories (Wilmington, MA). Initially, animals were group-housed 4 per cage. Two weeks prior to initiation of the experiments, animals were housed individually in polypropylene cages (20 $\times$ 40 $\times$ 20 cm) in colony rooms with a 24-h L:D 14:10 cycle (lights off at 1100 h EST). Temperature was kept constant at 20 $\pm$ 2°C and relative humidity was maintained at 50 $\pm$ 5. Food (Purina Rat Chow) and tap water were available *ad libitum* throughout the exper-

iment. An additional 15 animals were used as aggressive stimulus animals during social defeat.

Social Defeat

Experimental animals were randomly divided, matched for age and body mass, and assigned to one of three groups. Animals in Group 1 received chronic social defeat in which the animals were placed in the cage of a dominant, aggressive animal for five consecutive defeat sessions, one session per day across 5 days. Each defeat session lasted for 15 min. Animals in Group 2 received a single, 15-min acute social defeat on the last day of training after 4 consecutive days of being placed in the dirty cage of a stimulus animal without the animal present. Finally, control animals in Group 3 were removed from their home cages and placed in the dirty cage of an unfamiliar stimulus animal on 5 consecutive days, for 15 min each day, but were not defeated. Control animals were placed in the cages of the same dominant, aggressive animals that were used to defeat animals in Groups 1 and 2. All defeat sessions occurred during the first 2 h of the dark phase of the daily light:dark cycle. Experimental animals (Groups 1 and 2) were paired with novel aggressors and control animals were placed in novel cages for each defeat session. All sessions were observed in order to determine whether resident aggressors attacked experimental animals and that experimental animals displayed submissive and defensive behaviors. All resident aggressors attacked experimental animals and these attacks were characterized by frequent displays of upright and side offense, chasing, and lunging attacks. All experimental animals displayed high levels of submissive and defensive behaviors, including upright and side defense, tail lifts, fleeing, tooth chattering, and full submissive posture. No experimental animals were wounded during the defeat procedure.

Immediately following the last day of social defeat (i.e., day 5), animals were brought into the surgery room one at a time and anesthetized lightly with methoxyflurane vapors (Metophane, Pitman-Moore Inc., Mundelein, IL), and blood samples ($\sim$500 μl) were drawn into capillary tubes via the retro-orbital sinus. Handling time was kept consistent and to a minimum; the time from initial removal of the animal from the cage to the end of bleeding was $<$3 min. Blood samples were allowed to clot for $\sim$1 h and then the clots were removed and the samples centrifuged at 3500 rpm for 1 h. Serum aliquots were extracted and stored at $-80°$C until assayed for cortisol. The following day, all hamsters received a single subcutaneous

injection of 100 μg of the novel antigen keyhole limpet hemocyanin (KLH), suspended in 0.1 ml sterile saline (Day 0), and the animals were then returned to the colony room. KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Megathura crenulata*). KLH was used because it generates a robust antigenic response in rodents, but does not make the animals ill (e.g., inflammation or fever) (Dixon, Jacot-Guillarmod, and McConahey, 1966). Additional blood samples were drawn on Days 5 and 10 postimmunization as described above. These time points were chosen because they reflect the postimmunization times when serum IgG levels are basal (Day 5) or at their peak levels (Day 10). Serum aliquots were stored at $-80°$C until assayed for anti-KLH IgG.

Cortisol Radioimmunoassay

Serum cortisol concentrations were determined in a single radioimmunoassay (RIA) using the Diagnostics Products Corp. (Inter-Medico, Markham, Ontario, Canada) [125]I double antibody kit. Previous studies have validated this kit for measuring cortisol, the predominant glucocorticoid in hamsters (Reburn and Wynne-Edwards, 2000). Procedures recommended in the kit were followed except that half of the volume of all the reagents was used, and the volume of standards and samples was reduced from 25 to 10 μl. The cortisol assay was highly specific, cross-reacting at less than 1% with other hormones (Inter-Medico, Markham, Ontario, Canada). Serum cortisol values were determined in a single RIA. The intra-assay coefficients of variation were all $<$10%.

Enzyme-Linked Immunosorbent Assay for IgG

Anti-KLH IgG was determined for day 10 serum postimmunization serum samples using an enzyme-linked immunosorbent assay. Microtiter plates were coated with antigen by overnight incubation at 4°C with 0.5 mg/ml KLH in sodium bicarbonate buffer, washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), blocked with 0.5% nonfat dry milk in PBS-T overnight at 4°C, and washed again with PBS-T. Thawed serum samples from Syrian hamsters were diluted 1:20, 1:40, 1:80, and 1:160 with PBS-T, and 150 μl of each serum dilution was added in duplicate to the wells of the antigen-coated plates. Positive control samples (pooled serum from hamsters previously determined to have high levels of anti-KLH antibodies, similarly diluted with PBS-T) and negative control samples (pooled serum from hamsters never immunized with KLH) were also added in

duplicate to each plate. The plates were sealed, incubated at 37°C for 3 h, and then washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG diluted 1:2000; Cappel, Durham, NC) was added to the wells and plates were sealed and incubated at 37°C for 1 h. Plates were again washed with PBS-T and 150 μl of the enzyme substrate *p*-nitrophenyl phosphate (1 mg/ml in diethanolamine substrate buffer; Sigma Chemical, St. Louis, MO) was added to each well. Plates were protected from light during the enzyme substrate reaction, which was terminated after 15 min by adding 50 μl of 1.5 M NaOH to each well. The optical density (OD) of each well was determined using a plate reader equipped with a 405-nm wavelength filter (Bio-Rad Model 3550, and the mean OD for each set of duplicate wells was calculated. To minimize intra-assay variability, the mean OD (i.e., percentage plate positive) for each sample was expressed as a percentage of its plate positive control OD for statistical analyses.

Statistical Analyses

Data were analyzed using a one-way between-groups analysis of variance (ANOVA). All pairwise comparisons of mean differences were performed using Tukey HSD comparisons (Sigma Stat, Jandel Scientific, San Rafael, CA) when the overall ANOVA was significant. A Pearson product-moment test (Sigma Stat) was used to determine the correlation between serum cortisol and IgG in animals experiencing social stress. Differences between group means were considered statistically significant if $P < 0.05$. The 1:40 serum dilution was used in subsequent analysis of the IgG data because this dilution was determined to be optimal. Three animals (one from the acutely defeated group, one from the chronically defeated group, and one from the control group) were removed from the statistical analysis because the reliability of these serum samples run in duplicate was less than 90%.

RESULTS

Serum cortisol concentrations in both chronically and acutely defeated animals were significantly increased compared with control animals ($F_{2,23} = 8.78$, $P < 0.05$) (Fig. 1a). There was no difference in serum cortisol concentrations between chronically and acutely defeated animals (Fig. 1a). Anti-KLH IgG antibody production was significantly reduced in both chronically and acutely defeated hamsters compared with control animals ($F_{2,20} = 4.81$, $P < 0.05$) (Fig. 1b).
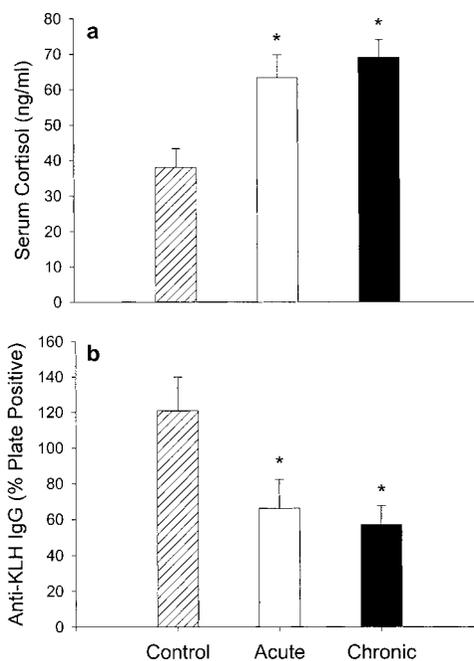

FIG. 1. (a) Mean [± standard error of the mean (SEM)] serum cortisol concentrations (ng/ml) in control hamsters ($n = 8$) and hamsters experiencing acute ($n = 8$) or chronic ($n = 8$) social defeat. (b) Mean (±SEM) serum immunoglobulin G (IgG) concentrations (% plate positive) in control hamsters ($n = 8$) and hamsters experiencing acute ($n = 7$) or chronic ($n = 7$) social defeat. Significant differences between pairwise means are indicated by an asterisk ($P < 0.05$).

There was no difference in anti-KLH IgG antibody production between chronically and acutely defeated animals. Raw absorbances for control, acutely stressed, and chronically stressed animals were 0.831 ± 0.131, 0.455 ± 0.111, and 0.392 ± 0.074 nm, respectively. There was a nonsignificant, positive relationship between serum cortisol and anti-KLH IgG antibodies within animals experiencing social defeat ($R^2 = 0.178$; $P > 0.05$).

DISCUSSION

The present results demonstrate that both chronic social defeat and acute social defeat suppress humoral immune function in Syrian hamsters. In the present experiment, hamsters that were defeated demonstrated increased serum cortisol concentrations as well as suppressed anti-KLH IgG production compared with control animals that were not defeated. The extent of social defeat (i.e., acute versus chronic), however, did not affect either serum cortisol or antibody production; the levels of both dependent measures did not differ between acutely and chronically stressed

animals. Furthermore, the degree of immune suppression was not significantly correlated with the serum cortisol concentrations *within* defeated animals. Collectively, the present data are consistent with previous results showing suppressed immune function in socially defeated animals (Fleshner *et al.,* 1989). Moreover, these results suggest that both chronic defeat and acute defeat act similarly to increase cortisol secretion and suppress humoral immune function.

In the present study, it is important to point out that antibody concentrations of chronically and acutely defeated animals were low compared to similarly treated control animals that did not undergo physical defeat. Specifically, control animals were removed from their home cages and placed in the soiled cage of an unfamiliar stimulus animal (i.e., animals used to induce social defeat in the experimental animals). The purpose of this group was to control for novel cues (e.g., odor, handling) that may induce fear or anxiety and thus explain the changes in cortisol and humoral immunity independent of actual social defeat. No animals in the present study experienced the physical presence of an aggressive animal in the absence of direct physical contact. It is important to note, however, that cortisol concentrations in control animals in the present study were similar to those reported in previous studies in which hamsters were exposed to an aggressive animal in the absence of any physical interactions (Huhman *et al.,* 1992). The increase in cortisol and decrease in immune function in defeated hamsters compared with control animals suggests that actual physical defeat, rather than nonphysical cues of aggressive animals, was responsible for the lowered antibody levels observed in defeated animals in the present study.

The primary goal of the present study was to examine the effects of social defeat on humoral immunity; we did not examine the possible neuroendocrine mechanisms underlying suppressed immune function in socially defeated animals. One likely explanation for suppressed immune function in the present study, however, is increased activation of the HPA axis leading to increased cortisol secretion. Consistent with this hypothesis, both chronically and acutely defeated animals in the present study had similar increases in serum cortisol concentrations. The ability of glucocorticoids to modulate immune function is well known. For example, *in vivo* administration of exogenous glucocorticoids suppresses immune function in a variety of species (reviewed in Rook, 1999). The ability of glucocorticoids to suppress immune function appears to be direct; glucocorticoid receptors have been localized on several lymphoid tissues, including splenocytes and thymocytes, as well as circulating lymphocytes (Miller, Spencer, Pearce, Pisell, Azrieli, Moday, Rhee, and McEwen, 1998). An increasing amount of evidence, however, suggests that glucocorticoids affect many neuroendocrine systems; changes in these neuroendocrine systems, and not the effects of glucocorticoids directly on lymphoid tissue, may mediate changes in immune function. Therefore, the notion that the direct action of glucocorticoids alone influences immune function following social stress may be too simplistic.

One possible alternative mechanism for the lowered antibody response observed in the present study is through stress-induced changes of the sympathetic nervous system (SNS). In support of this hypothesis, connections between the immune system and the SNS have been demonstrated using retrograde tract tracing methods (Drazen, Nelson, Bartness, and Demas, 2000; Elmquist and Saper, 1996). For example, injections of the trans-synaptic retrograde tract tracer pseudorabies virus (PRV) into the spleens of Siberian hamsters revealed infected neurons in areas traditionally implicated in SNS regulation (e.g., the A5 and C1 cells groups and the locus coeruleus of the brainstem) (Drazen *et al.,* 2000). Importantly, a *functional* role of the SNS in mediating immune responses has been identified. For example, surgical denervation of SNS nerves innervating the spleen leads to decreased immune function (Liu, Mustafa, Li, Nuortio, Mustafa, and Bakhiet, 2000; Demas, Drazen, Jasnow, Bartness, and Nelson, in press). Thus, decreased humoral immunity seen in socially defeated animals in the present study may be due to changes in sympathetic activity.

Regardless of the precise mechanisms, the present results indicate that social defeat increases serum cortisol and suppresses antibody formation in Syrian hamsters. In addition, both chronic defeat and acute defeat appear to elicit similar neuroendocrine and immune responses. Interestingly, there was a lack of a significant correlation between serum cortisol concentrations (presumably reflecting the extent of stress experienced) and the degree of immune suppression. This finding indicates that animals experiencing "more stressful" defeats (indicated by higher cortisol concentrations) do not necessarily display greater reductions in antibody formation than less stressed animals. Collectively, the results of the present study suggest that social defeat can have a significant impact on humoral immunity, and possibly subsequent disease susceptibility, in rodents. These results also suggest that social defeat provides a valuable, ecologically relevant model for the study of stress-induced changes in immune function in rodents.

ACKNOWLEDGMENTS

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