Social defeat differentially affects immune responses in Siberian hamsters (Phodopus sungorus)

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1. Introduction

Virtually all animals are exposed to a wide range of potential stressors during their lifetimes. These stressors can be a result of environmental perturbations (e.g., decreased food and low ambient temperatures) or social interactions (e.g., territorial conflict and male–male competition). Regardless of the type of stressor, prolonged exposure to stress can have profound, long-lasting effects on physiology and behavior [1]. These changes are mediated by specific neuroendocrine mechanisms, including the activation of the hypothalamo-pituitary-adrenal (HPA) axis [2]. Increased HPA activity in response to stress, and the resultant release of adrenal glucocorticoids (e.g., cortisol, corticosterone), can exert substantial effects on physiology, including brain remodeling [3–5,11], alterations in energy balance [6], and immune modulation [7,8]. For example, glucocorticoids have suppressive actions on cytokine production and release and can inhibit both B and T cell functioning while inducing apoptosis in B and T cell precursors. However, glucocorticoids are also implicated in the enhancement of immune measures [reviewed in [9]].

Considerable research has focused on the immunological effects of stress, particularly with respect to physical stressors (e.g., restraint or electric shock). For example, restraint stress decreases and/or redistributes the number of circulating lymphocytes in mice [10] and rats [11,12] and in rhesus macaques, natural killer cell activity is decreased by restraint, while white blood cell and neutrophil levels are increased [13]. In addition, mild foot-shock decreases anti-herpes simplex virus (HSV) antibody titers and leukocyte numbers in mouse spleens [14]. In rats, in vitro lymphocyte proliferation, cytokine production, and natural killer cell activity display marked reductions following prolonged exposure to tail-shock [15].

Although studies using physical stressors provide critical insights into the physiological mechanisms governing the stress response and subsequent effects on specific aspects of immunity, these types of manipulations are not typically experienced by animals in their natural environments. Furthermore, different types of stressors (i.e., physical or social) may activate neuroendocrine pathways via alternate mechanisms [16,17]. Thus, focusing on a single type of stressor may result in an incomplete understanding of the effects of stress on physiological coping responses. For example, non-social shock-prod stressors in rats produce increases in corticosterone and catecholamine levels that are of shorter duration and less intense than changes induced by social defeat [2].
Differential HPA activation may also lead to varying effects of social and physical stressors on immunity. Enhancement of phagocytosis following social defeat [18] differs from the suppressive effects of restraint stress on this same measure [19]. Additionally, social stress, but not restraint stress, increases mortality due to endotoxic shock in mice, perhaps via glucocorticoid resistance and increases in pro-inflammatory cytokines [20,21]. Collectively, these findings support the idea that different stressors exert differential behavioral and physiological responses in animals; thus, it is important to examine the effects of a variety of stressors.

Social stress is one of the most pervasive stressors experienced by animals in nature and thus, serves as an ecologically and behaviorally relevant form of stress [22,23]. Social conflict typically occurs between male conspecifics and often involves competition over potential mates, territory, or access to limited food. In the majority of social conflicts, resolution results in a distinct “winner” and “loser” (i.e., defeated animal). Known physiological effects of defeat include activation of the HPA axis and suppression of humoral immune measures in Syrian hamsters [23] and rats [7]. Furthermore, social stress can down-regulate T cell activity, decrease circulating T and B cell numbers, and increase granulocytes and phagocytic activity in rats [24].

Despite these and other findings, considerably less information is known regarding the effects of social stress on immune function as compared with physical stress. Social stressors (e.g., social defeat) involve multisensory stimuli that include visual and olfactory cues from a conspecific challenger [39], as well as physical interactions during the social conflict. It remains unclear, however, which specific stimulus (or combinations of stimuli) of the social interaction is responsible for triggering immunological changes. The primary goal of the present study was to examine the effects of specific components of social defeat on immune responses in Siberian hamsters. These animals provide an ideal model with which to study social stress because they are highly social, territorial animals that become aggressive during dyadic interactions [25]. The hamsters are relatively recent descendents (less than ten generations) from wild-caught individuals and, unlike laboratory-bred rodents, display considerable variability in behavioral and physiological responses. The use of Siberian hamsters in this and other studies of social stress and its effects on immune function provides insight into how a species adapted to extreme environments responds physiologically to a social stressor. A second important aim of this study is to examine the effects of social stress on multiple immune measures in order to examine physiological responses across different arms of the immune system.

2. Material and methods

2.1. Animals and housing conditions

Forty adult (>60 days old) male Siberian hamsters (*Phodopus sungorus*) were obtained from the breeding colony maintained at Indiana University. An additional 20 male hamsters (>180 days old) were used as resident aggressors during social defeat. All hamsters were individually housed in polypropylene cages (27.5×17.5×13 cm) for at least 2 weeks prior to testing in a temperature-controlled (20±2 °C) room on a 16:8 h light:dark (LD) cycle with lights off at 2100 h. Food (Purina Rat Chow) and water were provided *ad libitum* throughout the experiment. All animals were treated in accordance with the Bloomington Institutional Animal Care and Use Committee (BIACUC).

2.2. Social defeat

At the start of the experiment, experimental hamsters ('intruders') were pseudo-randomly assigned to one of four groups (corrected for age and body mass). Animals in the defeat group (*n* = 10) received chronic social defeat in which each animal was placed in the cage of a singly housed dominant resident hamster ('aggressor'). Animals in the partition group (*n* = 10) were placed in the cages of a dominant, resident hamster; however, wire partitions prevented physical contact. Animals in the olfactory group (*n* = 10) were placed in cages filled with soiled bedding of older, unrelated male hamsters. In this treatment, no resident hamsters were present. Lastly, animals in the clean cage group (*n* = 10) (i.e., control group) were placed in cages filled with clean bedding. As with the olfactory group, no resident hamsters were present in the clean cage group. All treatments lasted 10 min per day across 5 consecutive days (Days 1–5).

The experiment was conducted in three separate cohorts staggered by one week. Each cohort was comprised of three or four animals from each group. Procedures were identical for all cohorts. For all groups, sessions occurred within the first 2 h of the dark phase of the light:dark cycle under red light (see below). All defeat sessions were observed in order to determine whether resident aggressors attacked the experimental animals in the defeat group. Furthermore, we determined if the experimental animals displayed any submissive behaviors. Intruders were matched with a new resident within the first 2 min if the resident did not demonstrate aggressive behaviors and the trials were restarted.

2.3. Behavioral observations and scoring

All experimental trials were performed under low illumination (25 W), red light conditions, which allowed for sufficient light during video recording and observations without disturbing the behavior of the hamsters. To identify the aggressors, small patches of fur were shaved on the dorsal surface. Behavioral interactions for the defeat group were scored using ODLog™ software (Macropod) by a trained observer blind to the treatment animals received. The number of attacks by the aggressor, bites by the aggressor, and defensive behaviors (i.e., escape and on-back submissive posture) was characterized. Attack behavior quantified lunges where high velocity body contact was made, but only sometimes did this result in the intruder getting bitten by the aggressor.

2.4. Immunizations and blood sampling

A baseline blood sample was collected two weeks prior to the start of all defeat trials. Animals were anesthetized with diethyl ether (Sigma, St. Louis, MO), and blood samples were collected via the retro-orbital sinus within 3 min of initial handling. Blood samples were allowed to clot at room temperature for 1 h, and then clots were removed and the samples were centrifuged (at 4 °C) for 30 min at 2500 rpm. Serum aliquots were extracted and stored at −80 °C until further use in the cortisol assay. Immediately following the final defeat, (i.e., within 30 min of the defeat, Day 5), an additional blood sample was collected as described above and stored until assayed for cortisol and bacteria killing ability. Following blood sampling, all hamsters received a subcutaneous injection of keyhole limpet hemocyanin (KLH; Calbiochem, San Diego, CA), 100 µg KLH in 0.1 ml saline. 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 sick (e.g., inflammation or fever) [26]. Blood samples were drawn one week and two weeks post-immunization (Days 12 and 19) as described above. These time points reflect the peak levels of serum immunoglobulin M (IgM) and immunoglobulin G (IgG), respectively. All blood samples were collected between 2200 and 2300 h. Serum aliquots were stored at −80 °C until assayed for peak anti-KLH IgM and IgG levels.
2.5. Enzyme immunoassays (EIAs)

Total serum cortisol concentrations were measured as an index of defeat-induced stress. Cortisol is the predominant glucocorticoid produced in Siberian hamsters at ▼100× that of corticosterone [27]. Serum cortisol was measured at 3 time points: baseline (2 weeks prior to defeat), 15 min following defeat (Day 5), and Day 12 after a week of recovery. Cortisol concentrations were determined with a commercially available enzyme immunoassay (EIA) kit (Correlate-EIA™, Assay Designs, Ann Arbor, MI). This assay was previously validated for use with Siberian hamsters [28] and is highly specific for cortisol. The cross-reactivity of corticosterone is 27.68% and other steroid hormones is ▼0.1%. The sensitivity of the assay is 5.672 pg/ml. Intra-assay variability was ▼10.0%.

2.6. Enzyme-linked immunosorbent assays (ELISAs)

To assess humoral immunity, serum anti-KLH antibody concentrations were assayed using an enzyme-linked immunosorbent assay (ELISA). For measurement of anti-KLH IgM and IgG concentrations (see [29]) microtiter plates were coated with antigen by incubating overnight at 4 °C with 0.5 mg/ml KLH in sodium bicarbonate buffer (pH 9.6). Plates were washed with phosphate buffered saline (PBS) (pH 7.4) containing 0.05% Tween 20 (PBS-T) at pH 7.4, then blocked with 5% non-fat dry milk in PBS overnight at 4 °C to reduce non-specific binding, and washed again with PBS-T. Thawed serum samples were diluted 1:20 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 sera from hamsters previously determined to have high levels of anti-KLH antibody, similarly diluted with PBS-T) were added in duplicate. Plates were sealed, incubated at 37 °C for 3 h, and then washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated-anti hamster IgG diluted 1:500 with PBS-T (Rockland, Gilbertsville, PA)); alkaline phosphatase-conjugated-anti mouse IgM diluted 1:500 with PBS-T (Cappel, Aurora, OH)) was added to the wells, and the plates were sealed and incubated for 1 h (for IgG) or 1.5 h (for IgM) at 37 °C. Plates were then washed again with PBS-T and 150 µl of the enzyme substrate p-nitro-phenyl phosphate (Sigma, St. Louis, MO: 0.1 mg/ml in diethanolamine substrate buffer) was added to each well. Plates were protected from light during the enzyme–substrate reaction. The optical density (OD) of each well was determined using a plate reader (Bio-Rad, Benchmark Richmond, CA) equipped with a 405 nm wavelength filter, and the mean OD for each sample was expressed as a ratio of its plate positive control OD for statistical analysis.

2.7. Hemolytic complement assay

To measure hemolytic complement activity, a component of the innate immune system, a modification of the methods provided in Sinclair and Lochmiller [30] was used. Briefly, 5 µl of serum was diluted (1:320) in veronal buffer and was serially diluted twofold in a 96-well round-bottom microtiter plate. Twenty-five µl of 0.6% suspension of washed sheep red blood cells (SRBC; Sigma, St. Louis, MO) in veronal buffer and 25 µl of 1:40 dilution a rabbit-anti-SRBC antibody (hemolysin; Sigma, St. Louis, MO) in veronal buffer were added to each well. Plates were vortexed for 5 min and incubated for 1.5 h at 37 °C and centrifuged for 5 min at 500 rpm. The absorbance of each well was determined using a plate reader (Bio-Rad, Benchmark Richmond, CA) equipped with a 405 nm wavelength filter. Hemolytic complement activity was expressed as CH50 units/ml serum, where 1 CH50 unit equals the amount of serum required to lyse 50% of the SRBC in culture. Matson et al. [31]. This assay quantifies the relative number of E. coli colony forming units (CFU) that grow after incubation with serum. Differences in CFU presumably represent differences in serum proteins. Briefly, E. coli (ATCC #8739, Microbiologics, St. Cloud, MN) (1 pellet ▼107 CFU) was added to 40 ml 1 M sterile PBS warmed to 35–37 °C and vortexed to create a bacterial stock solution, which was activated by incubation for 30 min at 37 °C. Serum samples were diluted 1:40 in glutamine enriched CO2-independent media (Invitrogen Corp., Carlsbad, CA). This dilution was validated for serum with a dose response curve prior to the experiment. The stock bacteria solution (500,000 CFU/ml) was diluted with sterile 1 M PBS to create a 50,000 CFU/ml working solution. To obtain estimates of bacterial numbers (i.e., positive control), the working solution was diluted 1:10 with glutamine enriched CO2-independent media. For each sample, the working solution was added at a 1:10 ratio to the diluted serum sample. The bacteria/serum cocktails were incubated for 30 min at 37 °C. All samples were vortexed and 50 µl was added to Petri plates in duplicate and spread with a flame-sterilized spreader. All plates were stored upside down overnight at 37 °C. Following incubation, bacteria colonies were counted on each plate, and duplicates were averaged. The mean value for each sample was expressed as a percent of bacteria killed relative to the control plates in which no killing occurred.

2.9. Statistical analyses

All statistical tests were performed using Minitab 14 (State College, PA). There were no significant differences between cohorts; therefore, cohort data were combined for all statistical tests. Differences in cortisol values and body mass over time were assessed using a repeated measures general linear model (GLM) (time × treatment). Differences in immune measures were tested via one-way GLM (treatment). Treatments were defined as clean cage, olfactory, partition, or defeat. Post hoc comparisons between pair-wise means were conducted using Tukey’s honestly significant difference (HSD) when overall GLMs were significant. The significance level (α) for all tests was set at P = 0.05 and tests were two-tailed. Some animals died from the anesthesia (death n = 2, control n = 1) during blood sampling, resulting in reduced numbers for statistical tests.

3. Results

3.1. Behavior

The defeated animals responded to the attacks with a variety of behavioral reactions, including on-back submission, fleeing, or in some cases fighting back. All hamsters in the defeated group were subjected to attacks by the aggressor hamster. However, in the few cases where intruders attacked the aggressor, at the end of 10 min it was not clear which animal was dominant. Other behaviors observed were grooming and investigating the novel cage. The intruder animals in the partitioned cages primarily spent time on three behaviors: investigating the resident through the mesh partition and investigating and scent marking the bedding in the new cages. Both the olfactory and clean cage animals spent time investigating and scent marking the bedding of the new cages as well, however, only the defeat group was videotaped, thus a quantitative breakdown of time for the control groups was not possible. We examined whether the behaviors exhibited by the animals in the defeat group correlated with any of the immune measures. Bacteria killing ability positively correlated with the total number of attacks the intruder received from the aggressor (r2 = 0.458, P < 0.05) and the total number of seconds that intruders displayed submissive postures (r2 = 0.545, P < 0.05). Bacteria killing ability, however, did not correlate with the number of bites by the aggressor (r2 = 0.260, P = 0.161).
3.2. Endocrine and immune effects

There was no overall effect of time (P=0.75) on serum cortisol (Table 1). However, when the post-stress time point was analyzed alone, cortisol was significantly increased in defeated animals compared with partitioned animals (F_{3,34} = 3.378, P<0.05). Anti-KLH IgG antibody levels were significantly decreased in defeated hamsters compared with both clean and olfactory control groups (F_{3,33} = 6.437, P<0.01; Fig. 1A). Anti-KLH IgM antibody levels did not differ between groups (F_{3, 34} = 1.704, P = 0.185; Fig. 1B). Hemolytic complement activity was not affected by the social treatment of the animals (F_{3, 34} = 0.05; P>0.05). Bacteria killing ability was significantly greater in defeated animals compared with all other groups (F_{3, 34} = 6.120, P<0.05; Fig. 2).

Lastly, body mass was measured at the time each blood sample was taken. There were no differences between treatment groups in mean body mass over time (F_{3, 37} = 1.760, P>0.2).

4. Discussion

Social defeat is a commonly occurring, environmentally relevant form of social stress that can exert marked effects on physiology and behavior. Social defeat involves multiple sensory modalities, from visual and olfactory cues to physical interactions with conspecifics (i.e., defeat). We wanted to determine whether the physical interaction is necessary to elicit the well-documented effects on immunity, or alternatively, if specific sensory aspects are sufficient to elicit the stress response. The secondary aim of the experiment was to determine the effects of social defeat on innate and acquired immune responses.

Our results demonstrate that immunity is affected by social defeat. Whereas animal receiving physical defeat displayed altered immune function, the visual and olfactory components of a social stressor did not by themselves alter immune responses. In contrast, the olfactory group and partition group did not differ from the clean cage control group for any immune measures. These results are consistent with a recent study in mice where social stress that involved fighting over 7 days increased levels of interleukin-6 (IL-6) on Day 7, but mild social stress where fighting was prevented did not [32]. However, both treatments increased IL-6 on Day 1 of treatment. Overall, these findings provide initial support for the idea that the physical component of social defeat is necessary to modulate the immune measures tested here. This study, however, did not determine the precise stimuli underlying these physiological changes. Future studies in which specific cues are manipulated (e.g., the use of anosmic animals to further test olfactory components) should provide further insight into the mechanism by which social defeat alters immune parameters in this and other species.

As expected, social defeat affected immunity; unexpectedly, however, defeat had opposite effects on the acquired and innate immune measures. Whereas serum anti-KLH IgG levels were suppressed by defeat, bacteria killing ability was enhanced in defeated animals. Within the defeated group, behaviors related to defeat (i.e., the number of attacks an intruder received by the resident and the time spent in a submissive posture) were positively correlated with bacteria killing ability. Bacterial killing ability was the only immune measure that significantly correlated with any behavioral interactions. This suggests that the degree of immune enhancement may be related to either the intensity of the attack or to the coping behaviors demonstrated by the intruder. Specifically, animals that were attacked

<table>
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<tr>
<th>Group</th>
<th>Baseline</th>
<th>Post-stress (Day 5)</th>
<th>Recovery (Day 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>83.15 (5.29)</td>
<td>75.74 (6.59)</td>
<td>80.77 (7.62)</td>
</tr>
<tr>
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<td>75.34 (5.94)</td>
<td>67.52 (4.50)</td>
<td>72.29 (6.20)</td>
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<tr>
<td>Partition</td>
<td>74.94 (7.86)</td>
<td>60.27 (4.17)</td>
<td>77.25 (6.19)</td>
</tr>
<tr>
<td>Defeat</td>
<td>78.91 (6.70)</td>
<td>92.96 (12.29)</td>
<td>73.01 (7.97)</td>
</tr>
</tbody>
</table>

**Table 1**

Mean (±SEM) baseline, post-stress and recovery serum cortisol concentrations (ng/ml) in male Siberian hamsters experiencing social defeat (defeat, n=10), exposure to a conspecific (partition, n=9), exposure to the odors of a conspecific (olfactory, n=10) or control animals (n=9). All samples were taken between 2200 and 2300 h. Significant differences between the defeat group and the partition group at the post-stress time point (P<0.05) are indicated by the symbol (*).
more and in a submissive posture longer had the greatest bacterial killing ability. It is possible that the defeated animals could have received bites that stimulated inflammatory factors that affect bacteria killing. Although this possibility cannot be ruled out because wounding was not scored in this experiment, previous studies from our laboratory have found similar results (i.e., enhanced bacteria killing and suppressed humoral immunity) following a metabolic stressor. The results are also consistent with a study where social disruption enhanced bacteria killing by splenocytes, at least in part, via activation of Toll-like receptor-dependent pathways. These pathways are responsible for producing reactive nitrogen and oxygen intermediates which kill bacteria [33]. Another possibility is that the physiological response to the stressor could mediate both the behavior and immune response. These findings suggest that enhancement of bacterial killing ability is due to specific aspects of the stress response per se, rather than any mild wounding that may have occurred during the social interaction. Further studies will be necessary to determine the mechanisms underlying the variation in behavior and immunity in response to a repeated social stressor.

It is interesting to note that in contrast to bacterial killing, we found no effect of defeat on hemolytic complement activity. While the hemolytic complement and bacteria killing assays are both complement-dependent measures of immunity, the bacteria killing assay requires additional components, including natural antibodies, to kill the E. coli. It provides a functional measure of immunity not limited to one branch of the immune system, thus the results can be independent of the measure of innate immunity.

Prolonged elevations of glucocorticoid levels have been repeatedly shown to cause immunosuppression [35–37]. However, post-interaction serum cortisol levels were not significantly elevated in the defeated group compared to the control group. That the increase in cortisol was modest in the defeated animals indicates that we did not capture the peak elevation and likely the hamsters habituated to the repeated exposure to a novel aggressor. Acute exposure to stressors may result in the redistribution and enhancement of certain measures of immunity. For example, leukocytes migrate from the peripheral blood supply to the lymph nodes and skin compartments, likely as an adaptive response to acute stressors [12]. In the current study, hamsters were exposed to an acute defeat repeated over five consecutive days. Bacterial killing ability may be enhanced in response to the acute stimulation provided by each defeat session. In contrast, humoral immunity may be diminished due to the chronic administration of the treatments. Other factors, such as the sympathoadrenal system or endogenous opioids, were not assessed in this study and likely the hamsters habituated to the repeated exposure to a novel aggressor. Acute exposure to stressors may result in the redistribution and enhancement of certain measures of immunity. For example, leukocytes migrate from the peripheral blood supply to the lymph nodes and skin compartments, likely as an adaptive response to acute stressors [12]. In the current study, hamsters were exposed to an acute defeat repeated over five consecutive days. Bacterial killing ability may be enhanced in response to the acute stimulation provided by each defeat session. In contrast, humoral immunity may be diminished due to the chronic administration of the treatments. Other factors, such as the sympathoadrenal system or endogenous opioids, were not assessed in this study and may provide alternative mechanisms of immunomodulation.

Regardless of the precise neuroendocrine mechanisms, these results support the idea that social stress can exert marked effects on both innate and acquired immune responses. In addition, these findings raise an interesting question: why would an innate immune measure be stimulated following exposure to a stressor, whereas humoral responses are suppressed? The physiological mechanisms underlying humoral immunity may simply be more sensitive to stress than those of the innate immune system, leading to a reduction in antibody production. However, if this were simply the case, then we would not predict the enhancement of bacteria killing ability. Cells and proteins involved in the innate immune response are constitutively expressed, destroy a broad array of pathogens, and provide the first line of defense against invaders [34]. It is thought that the energetic “cost” of the development, maintenance, and use of these defenses is relatively low with respect to acquired immunity, based on low cell proliferation or turnover rates and low tissue and protein masses [38,40]. In contrast, developing specific antibodies necessary for acquired humoral defense to a novel pathogen requires cell differentiation and proliferation, is presumably costly [38]. When an animal is in a “stressed” state, it may be adaptive to shift energy allocation towards constitutive innate immunity, rather than the more “costly” acquired immunity. As a result, the first line of immune defense would be enhanced, while limiting resource depletion. Although these ideas are intriguing, additional studies are necessary.

In summary, the results of the present study suggest that: 1) social stress exerts differential effects on innate versus acquired immunity, enhancing the former and suppressing the latter and 2) physical defeat is a critical component of social stress-induced changes in immunity, though other cues may contribute to this effect. Lastly, these results support the notion that social defeat in Siberian hamsters is an important model for stress-induced immune modulation. The present findings highlight the importance of measuring multiple facets of immune function; while exposure to social stress suppresses certain measures of immunity, the system may compensate for this suppression through the enhancement of other measures.

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References


