Short-term stress experienced at time of immunization induces a long-lasting increase in immunologic memory

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Dhabhar, Firdaus S., and Kavitha Viswanathan. Short-term stress experienced at time of immunization induces a long-lasting increase in immunologic memory. Am J Physiol Regul Integr Comp Physiol 289: R738–R744, 2005. First published May 12, 2005; doi:10.1152/ajpregu.00145.2005.—It would be extremely beneficial if one could harness natural, endogenous, health-promoting defense mechanisms to fight disease and restore health. The psychophysiological stress response is the most underappreciated of nature’s survival mechanisms. We show that acute stress experienced before primary immunization induces a long-lasting increase in immunity. Compared with controls, mice restrained for 2.5 h before primary immunization with keyhole limpet hemocyanin (KLH) show a significantly enhanced immune response when reexposed to KLH 9 mo later. This immunoenhancement is mediated by an increase in numbers of effector helper T cells in sentinel lymph nodes at the time of primary immunization. Further analyses show that the early stress-induced increase in T cell memory may stimulate the robust increase in infiltrating lymphocyte and macrophage numbers observed months later at a novel site of antigen reexposure. Enhanced leukocyte trafficking may be driven by increased levels of the type 1 cytokines, IL-2 and IFN-γ, and TNF-α, observed at the site of antigen reexposure in animals that had been stressed at the time of primary immunization. In contrast, no differences were observed in type 2 cytokines, IL-4 or IL-5. Given the importance of inducing long-lasting increases in immunologic memory during vaccination, we suggest that the neuroendocrine stress response is nature’s adjuvant that could be psychophysiological stress levels of corticosterone and epinephrine, indicating that the immunoenhancing effects of stress are mediated by physiological concentrations of these principal stress hormones (13–16, 18). In contrast, chronic stress exposure to endogenous glucocorticoids or to synthetic glucocorticoid hormones such as dexamethasone is potently immunosuppressive, as might be expected given their clinically well-known anti-inflammatory effects (15).

In this study we show that acute stress experienced during primary immunization enhances antigen-specific memory for a significant portion of the animal’s life span. We identify a key immunologic mechanism, namely, an acute stress-induced increase in effector and memory helper T cells at the time of primary immunization, as a mediator of these novel and long-lasting adjuvant-like effects of the psychophysiological stress response. It is important to note that such stress-induced immunoenhancement may promote immunoprotection in case of wounding, infection, or vaccination. However, such immunoenhancement also may contribute to stress-induced exacerbation of inflammatory and autoimmune diseases.

MATERIALS AND METHODS

Animals. Young (8–10 wk at the beginning of the study), male C57BL6 mice (Taconic, Germantown, NY) were housed in groups of five on corn cob bedding in plastic cages in the accredited (American Association of Accreditation of Laboratory Animal Care) Postle Hall vivarium at The Ohio State University. Experiments were conducted according to protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. The animal room was maintained on a 12:12-h light-dark cycle (lights on at 6:00 AM). Animals were given food and water ad libitum.

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Stress paradigm. Acute stress was administered by placing animals (without squeezing or compression) in well-ventilated wire mesh restrainers for a single session of 2.5 h beginning at 9:00 AM (lights in the animal room were turned on at 6:00 AM). This procedure mimics stress that is largely psychological in nature because of the perception of confinement on the part of the animals (3, 20). The psychological component of restraint stress is thought to arise from it mimicking a collapsed tunnel that is intrinsically stressful for these burrow-dwelling animals (9). It also has been suggested that restraint stress may have a neurogenic component due to forced positioning (2). This stressor activates the autonomic nervous system (24) and the hypothalamic-pituitary-adrenal axis (17, 35) and results in the activation of adrenal steroid receptors in tissues throughout the body (17, 35). Antigen was administered at the end of restraint (−11:30 AM).

Treatment groups. Each experiment examined three treatment groups (n = 10 per group) of mice. One group was not immunized but was exposed to keyhole limpet hemocyanin (KLH) on the pinna for the first time in parallel with the recall phase of the following two groups. This group served as the nonimmunized control. The second group of animals remained in their home cages before primary immunization as the nonstressed immunized controls and were reexposed to KLH on the pinnae 9 mo later, also under resting conditions. The third group was stressed before primary immunization and reexposed to KLH on the pinnae 9 mo later under resting conditions. This was the only group that was acutely stressed, and stress was administered only once for 2.5 h before primary immunization.

Primary immunization. Nonstressed immunized and stressed immunized animals were given a subcutaneous injection of 100 μg of KLH (Calbiochem, La Jolla, CA) in 0.1 ml of 0.9% sterile saline on their dorsum.

Antigen reexposure and elicitation of CMI. Nine months after primary immunization, all animals were exposed to KLH (25 μg of KLH in 25 μl of saline) at a novel site (pinnae) under resting conditions, to elicit a skin CMI response that is also known as a delayed type hypersensitivity response. KLH was administered intradermally on the dorsal surface of the pinnae. At the same time, the previously nonimmunized group was exposed to KLH for the first time. The immune reaction at the site of antigen reexposure was characterized by an increase in the thickness of the pinnae, which was measured using a constant-loading dial micrometer (Mitutoyo, Tokyo, Japan) and by histological and cytokine analyses 24 h after KLH administration.

Histological analyses. Animals were euthanized with carbon dioxide at 24 h after antigen reexposure. Pinnae were immediately collected, frozen, and stored at −80°C. Sections (10 μm) were obtained from each pinna, processed, and analyzed using hematoxylin (Sigma Aldrich, St. Louis, MO) and eosin (Fisher Diagnostics, Middletown, VA) staining. Stained sections from nonimmunized, nonstressed immunized, and stressed immunized groups were also analyzed for enumeration of leukocyte subpopulations. Two sections were analyzed for every animal from each treatment group. Three representative regions (circles, 33-μm radius) were identified for each section analyzed, and the total number of macrophages and lymphocytes was counted in a blinded fashion.

Cytokine analyses. The mouse Th1/Th2 cytokine cytometric bead array (CBA; BD-Pharmingen, San Diego, CA) was used to detect TNF-α, IFN-γ, IL-2, IL-4, and IL-5 levels in KLH-challenged mouse pinnae from nonstressed immunized and stressed immunized groups. Skin punches from each individual animal’s pinna were homogenized in a PBS buffer containing phenylmethylsulfonyl fluoride (Sigma Aldrich). Tissues from different animals were run separately. The homogenates were centrifuged (12,000 rpm, 15 min, 4°C), and the resulting supernatant was used for cytokine analysis. Each sample was mixed with cytokine capture beads and phycoerythrin detection reagent, and the mixture was incubated for 2 h at room temperature according to the manufacturer’s instructions. Data were acquired on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using CBA software (BD-Pharmingen). Standard curves for each cytokine were generated, and the concentration of each cytokine in each assay sample from each animal was determined.

Preparation lymph node cell suspension. In a separate experiment, animals belonging to the three treatment groups were euthanized at 72 h after primary immunization, and scapular lymph nodes draining the site of immunization were collected. Briefly, cell suspensions were prepared by squeezing lymph nodes between the frosted ends of glass slides. Cells were then filtered through a 70-μm nylon sieve. The cell suspension thus obtained was washed twice and resuspended in Hank’s buffered salt solution (Sigma Aldrich). Cell numbers were counted on a hematology analyzer (Hemavet, Oxford, CT), viability was tested using Trypan blue (Sigma Aldrich) exclusion, and remaining cells were stained for flow cytometric analysis.

Flow cytometry. Scapular lymph node lymphocyte subtypes were measured using immunofluorescent antibody staining and analyzed using multicolor flow cytometry (FACSCalibur). Cell suspensions were incubated with Fc Block (BD-Pharmingen) for 15 min on ice to block nonspecific binding. Cells were then incubated with specific monoclonal antibodies for 30 min at room temperature, washed with PBS, and analyzed on the FACSCalibur. Each panel consisted of either single or multiple antibodies. Fluorochrome-conjugated rat anti-mouse CD3 (clone 145–2C11), CD4 (RM4–5), CD44 (IM7), CD45RA (14.8), and CD62L (MEL-14) (BD-Pharmingen) were used. Approximately 10,000 events were counted per sample. Control samples matched for each fluorochrome and antibody isotype were used to set negative staining criteria. Data were analyzed using CellQuest software (Becton Dickinson). Lymphocytes were identified and gated using forward- vs. side-scatter characteristics. CD4+CD3+ cells were identified as helper T (Th) cells.

Data analysis and statistics. One-way ANOVA was used to test for differences in pinna thickness (Fig. 1), lymphocyte and macrophage numbers in antigen-exposed pinnae (see Fig. 3), and T cell phenotype analysis in draining lymph nodes (see Fig. 5). Tukey’s honestly significant difference (HSD) or Tamhane’s post hoc test was used to determine significant differences.

Fig. 1. Acute stress experienced at the time of immunization induces a long-lasting enhancement of antigen-specific immunity. Nonstressed (NS) or acutely stressed (STR) mice were immunized with keyhole limpet hemocyanin (KLH; 100 μg, subcutaneous, dorsum) while nonimmunized controls remained in their home cages (n = 10 mice per treatment group). Nine months later, all mice were exposed to KLH at a novel site (25 μg, intradermal, pinnae) under resting conditions. Pinna thickness was measured 24 h after antigen reexposure and compared with baseline thickness measured before antigen reexposure. Animals that had been acutely stressed before primary immunization showed significantly greater pinna swelling than the other 2 groups. Results are expressed as means ± SE. †Significantly different from nonimmunized group; *significantly different from NS before immunization group [P < 0.05, Tukey’s honestly significant difference (HSD)].

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identify statistically significant differences among the nonimmunized, nonstressed immunized, and stressed immunized groups. For data shown in Fig. 4, where only two groups are compared, the unpaired Student’s t-test was used to test for significant differences in cytokine levels between the nonstressed immunized and stressed immunized groups. Data are expressed as means ± SE. Differences are considered significant when \( P < 0.05 \); means that differ significantly are indicated. The computer statistics package SPSS (SPSS, Chicago, IL) was used for statistical analyses.

**RESULTS**

Acute stress experienced during primary immunization enhances recall response upon antigen reexposure 9 mo later. Nine months after primary immunization with KLH, animals were tested under resting conditions for a recall response following antigen reexposure at a novel site (pinna) (Fig. 1). ANOVA revealed a significant difference between groups in the magnitude of the immune response to KLH \( [F(2, 37) = 13.15, P < 0.01] \). Tukey’s HSD further showed that animals acutely stressed at the time of primary immunization had a significantly larger immune response compared with animals that had not been stressed at the time of primary immunization \( (P < 0.01) \). Importantly, this stress-induced immunoenhancement was observed 9 mo after acute stress administration and primary immunization.

Acute stress experienced during primary immunization enhances macrophage and lymphocyte infiltration at site of antigen reexposure 9 mo later. Histological changes were analyzed by examining hematoxylin and eosin-stained pinna sections. Compared with nonimmunized controls, both groups of previously immunized animals showed more leukocyte infiltration, as would be expected (Figs. 2 and 3). Moreover, ANOVA revealed a significant difference among groups in the numbers of infiltrating macrophages \( [F(2, 19) = 7.63, P < 0.01] \) and lymphocytes \( [F(2, 19) = 17.86, P < 0.01] \) (Fig. 3). Specifically, acutely stressed animals showed significantly higher macrophage numbers compared with both nonimmunized \( (P < 0.01, \text{ Tamhane's}) \) and nonstressed immunized \( (P < 0.05, \text{ Tamhane's}) \) animals, as well as significantly higher lymphocyte numbers compared with the other two groups \( (P < 0.01 \text{ for both, Tukey’s HSD}) \).

Acute stress experienced during primary immunization enhances IL-2, IFN-\( \gamma \), and TNF-\( \alpha \) levels at a novel site of antigen reexposure. Compared with nonstressed animals, animals acutely stressed at the time of primary immunization showed higher levels \( (P < 0.05, \text{ unpaired \( t \)-test}) of the type 1 cytokines IL-2 and IFN-\( \gamma \) and the proinflammatory cytokine TNF-\( \alpha \) after reexposure to antigen at a novel site 9 mo later (Fig. 4). Interestingly, no differences were observed between nonstressed and acutely stressed animals in levels of the type 2 cytokines IL-4 and IL-5 (Fig. 4). These results, taken together with the finding that memory lymphocytes mediate protection in peripheral tissues by rapidly producing type 1 cytokines (19), suggest that acute stress experienced during primary immunization increased the numbers and/or activity of memory T cells. This enhanced antigen-specific immunity over a time period that encompasses a major portion of the animals’ life span.

Acute stress experienced at the time of primary immunization increases numbers of effecter and central memory-like T cells in lymph nodes draining the site of immunization. Long-term immunoenhancement induced by a single acute stressor experienced before primary immunization suggested that stress...
CD62L\textsuperscript{low}, and CD45RA\textsuperscript{−}CD62L\textsuperscript{+}, phenotypes, suggesting that the observed changes in the draining lymph nodes of immunized mice were caused by antigen exposure. These results suggest that acute stress experienced during primary immunization increased the numbers of effector and Tcm-like Th cells in sentinel lymph nodes during the early phase of the primary immune response.

**DISCUSSION**

The studies described introduce a novel concept, namely, that a psychophysiological stress response is nature’s fundamental survival mechanism that could be therapeutically harnessed to augment immune function during vaccination, wound healing, or infection. We report the novel finding that short-term stressors experienced at the time of primary immunization can induce long-lasting increases in immunological memory that may be mediated by increased numbers of memory T cells.

These studies are important because they are specifically designed to model naturally experienced temporal relationships between stressor and antigen exposure. For example, when a gazelle escapes from a charging lion, the gazelle is acutely stressed during the attack, after which it is exposed to antigens and pathogens that enter wounds that are likely to arise during the stressful chase and frantic scuffle that enable the gazelle to escape. Similarly, when a child sees a nurse approaching with a vaccine-loaded syringe, it remembers a prior painful injection and mounts a full-blown stress response before and during the process of being immunized. Adults also may mount similar psychophysiological stress responses before and/or during medical or surgical procedures. Stressors experienced during
many such conditions are acute and temporally proximal to accidental or surgical wounding and antigen exposure. Our experiments, which are designed to mimic such temporal relationships between stressor and immune challenge, suggest that the physiological changes accompanying an acute stress response may have long-lasting immunoenhancing effects that are established early during the development of a primary immune response.

These results show that acute psychophysiological stress exerts adjuvant-like effects on the immune system. Animals acutely stressed before immunization showed increased numbers of activated-effector (CD62Llow and CD44hi) and Tcm-like (CD45RA−CD62L+) T cells during the early phase of the primary immune response (Fig. 5). Although several weeks may be required to generate a full repertoire of memory lymphocytes (38), phenotypic changes that occur soon after antigen exposure are critical for determining the ultimate number and efficacy of the memory pool (23, 34). Effector cells that subsequently transform into Tcm cells are conferred with an ability to persist long term and offer better protection during recall (40, 45). Thus stress-induced increases in numbers of effector and Tcm-like Th cell populations soon after antigen exposure may mediate the long-lasting enhancement of immunity.

The only stress manipulation that was performed in these experiments occurred 9 mo before the recall CMI response was measured. Interestingly, acutely stressed animals mounted a significantly larger recall response that was marked by a robust increase in macrophages and lymphocytes infiltrating the novel site of antigen reexposure (Figs. 1–3). The enhanced CMI response was accompanied by increased levels of type 1 cytokines IL-2 and IFN-γ and the proinflammatory cytokine TNF-α at the site of antigen reexposure (Fig. 4). Interestingly, we did not see a difference between nonstressed and acutely stressed animals in levels of the type 2 cytokines IL-4 and IL-5, indicating that acutely stressed animals showed a shift toward type 1 cytokine production. IFN-γ, IL-2, and TNF-α are important cytokine mediators (18, 19), and macrophages and T cells are important effector cells for CMI (5, 10). Memory lymphocytes mediate protection in peripheral tissues by rapidly producing type 1 cytokines (19). Our results suggest that acute stress experienced during primary immunization induces a long-term increase in memory T cells that results in an enhanced secondary cell-mediated immune response upon antigen reexposure. Memory T cells are likely to be the source of the enhanced IL-2 and IFN-γ levels observed at the site of antigen reexposure (Fig. 4), although intracellular cytokine staining coupled with surface marker flow cytometry is required to provide definitive evidence for this. IL-2 is a principal T cell growth factor that, together with IFN-γ, promotes CMI (1, 27), although its crucial role in T cell tolerance is increasingly being appreciated (30). IFN-γ is a potent activator of macrophages and effector CD8 T cells (1, 27). Th1 T cells (26) and activated macrophages (1) are the major sources of TNF-α that was also observed in higher levels in acutely stressed animals. TNF-α is crucial for inducing endothelial cell activation, vasodilation, leukocyte recruitment, and Langerhans cell activation and migration during the primary and secondary phases (48) of a cell-mediated immune response (1, 22) and for promoting effector cell function (36). Therefore, our results suggest that the acute stress-induced increase in memory T cells at the time of primary immunization translated into an enhanced effector response driven by IL-2, IFN-γ, and TNF-α and mounted by macrophages and T cells at the time of antigen reexposure at a novel site.

Because the magnitude of an immune response is known to decline with age (28), it is possible that the enhancement of the recall response observed 9 mo after acute stress preceding primary immunization may reflect an acute stress-induced maintenance of immunological memory that resists an age-related decline. Such a memory-maintaining effect of acute stress would counter an age-related decrease in the primary immune response that may be present in animals that were not stressed at the time of primary immunization.

One important weakness of these studies is that we did not directly enumerate KLH-specific T cells. It will be necessary to examine the effects of acute stress on formation of antigen-specific T cells in future studies. However, the observation that the nonimmunized animals failed to show the increase in effector and Tcm-like lymph node Th cell numbers that was seen in immunized animals (Fig. 5) suggests that the changes seen in immunized animals were a direct result of antigen stimulation. Moreover, when animals were exposed to antigen 9 mo after primary immunization, the previously immunized groups showed more leukocyte infiltration at the site of reexposure than did the nonimmunized group (Fig. 2). This strongly indicates that the recall response was driven by antigen-specific T cells. Importantly, animals that were acutely stressed before primary immunization showed a significantly larger recall response than nonstressed immunized controls (Figs. 1–3). Because the two groups of previously immunized animals were reexposed to KLH under identical resting conditions, the only difference between them was that the stressed immunized group had been acutely stressed at the time of immunization 9 mo earlier. The fact that the acutely stressed group showed an enhanced recall response upon antigen reexposure 9 mo after stress and immunization strongly suggests that stress experienced at the time of primary immunization resulted in the formation of higher numbers of antigen-specific memory T cells that were able to orchestrate a more robust immune response at the time of antigen reexposure.

We recognize that it is important to further elucidate the mechanisms by which acute stress enhances a primary immune response. We suggest that macrophages and dendritic cells that lie at the nexus between innate and adaptive immune responses, as well as skin surveillance T cells, may contribute to a stress-induced enhancement of a primary immune response (44a). It has been shown that acute stress enhances innate and adaptive immune responses (7, 11, 16, 18, 25, 29, 39, 46, 47). The findings described in this study set the stage for future studies that must specifically examine the effects of stress on macrophages, dendritic cells, and effector and memory subtypes of Th cells and cytolytic T cells across a time frame that examines multiple time points after immunization. Studies also are needed to examine the mechanisms by which memory function enhanced by stress at the time of primary immunization translates into greater T cell and macrophage effector function during a recall CMI response.

These studies suggest that it may be possible to design therapeutic interventions that involve behavioral manipulations, and/or administration of cocktails of physiological mediators, to maximally recruit the body’s natural army (the
immune system) in the fight against disease. Taken together with our earlier findings identifying corticosterone and epinephrine as mediators of the immunoenhancing effects of acute stress (15), our results suggest that stress hormones administered in physiological concentrations may be used to enhance immune function during vaccination, wound healing, or infection. These results are novel because they show for the first time that a psychological manipulation can be used to increase numbers of effector and memory Th cells at the time of primary immunization and to endogenously induce long-lasting immunoenhancement. They are intriguing because they show that this adjuvant-like manipulation involves stress, which is widely believed to be immunosuppressive. They are important because they suggest that behavioral manipulations or endogenous stress hormones administered in physiological concentrations that are easily metabolized may be used as natural adjuvants with minimal side effects. Moreover, the cutaneous immunization model used in this study is especially relevant given recent studies showing that immunization through skin may be a highly efficient route of vaccination requiring lower concentrations of antigen and conferring more robust and longer lasting immunoprotection (21, 34).

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