ORIGINAL RESEARCH

MODULATION OF NEUROIMMUNE PARAMETERS DURING THE EUSTRESS OF HUMOR-ASSOCIATED MIRTHFUL LAUGHTER

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Context • *Humor therapy and the related mirthful laughter are suggested to have preventive and healing effects. Although these effects may be mediated by neuroendocrine/neuroimmune modulation, specific neuroimmune parameters have not been fully investigated.*
Objective • *To determine the efficacy of mirthful laughter to modulate neuroimmune parameters in normal subjects.*
Design • *A series of 5 separate studies based on a multivariate repeated measures design, with post hoc simple contrast analysis.*
Setting • *The schools of medicine and public health at Loma Linda University, Loma Linda, Calif.*
Subjects • *52 healthy men.*
Intervention • *Viewing of a humor video for 1 hour. Blood samples were taken 10 minutes before, 30 minutes into, and 30 minutes and 12 hours after the intervention.*
Main Outcome Measures • *Natural killer cell activity; plasma immunoglobulins; functional phenotypic markers for leukocytes including activated T cells, nonactivated T cells, B cells, natural killer cells, T cells with helper and suppressor markers, and assessment of plasma volume and compartmental shifts; plasma cytokine—interferon-γ; and total leukocytes with subpopulations of lymphocytes, granulocytes, and monocytes.*
Results • *Increases were found in natural killer cell activity (P<.01); immunoglobulins G (P<.02), A (P<.01), and M (P<.09), with several immunoglobulin effects lasting 12 hours into recovery from initiation of the humor intervention; functional phenotypic markers for leukocyte subsets such as activated T cells (P<.01), active cytotoxic T cells (P<.01), natural killer cells (P=.09), B cells (P<.01), helper T cells (P<.02), uncommitted T cells with helper and suppressor markers (P<.02), helper/suppressor ratio (P=.10) with several leukocyte subset increase effects lasting 12 hours after the humor experience; the cytokine interferon-γ (P=.02), with increases lasting 12 hours; total leukocytes (P<.05), with specific subpopulation lymphocytes during the intervention (P<.01) and 90 minutes into recovery (P<.05); and granulocytes during the intervention (P<.05) and 90 minutes following the intervention (P<.01).*
Conclusion • *Modulation of neuroimmune parameters during and following the humor-associated eustress of laughter may provide beneficial health effects for wellness and a complementary adjunct to whole-person integrative medicine therapies. (Altern Ther Health Med. 2001;7(2):62-76)*

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A merry heart doeth good like a medicine
but a broken spirit drieth the bone.
—Proverbs 17:22

Many different cultures and religious traditions over the centuries have considered humor therapy and mirthful laughter to be "good medicine."[1,2] Today, modern medicine has become intrigued with the benefits of alternative/complementary or integrative preventive and therapeutic modalities in patient care. Numerous studies support the benefits of humor and laughter in areas of cardiac rehabilitation,[3] pain perception,[4] discomfort thresholds,[5] coping with stress,[6] and immune enhancement in children and adults.[7,8] Among coronary patients who have had percutaneous transluminal coronary angioplasty, those with perceived control and expectations about their future and a positive view of themselves lessen the risk of recurring coronary problems and restenosis.[9]

In a study at Loma Linda University, 2 groups of myocardial infarction patients were followed for 1 year in their cardiac rehabilitation programs. Both groups were matched, but the experimental group was allowed to view self-selected humor for 30 minutes per day as an adjunct to standard cardiac therapy. The patients in the group that viewed the humor had fewer episodes

of arrhythmias, lower blood pressure, lower urinary and plasma catecholamines, a lower requirement for beta-blockers and nitroglycerine, and a markedly lower incidence of recurrent myocardial infarction than did the control group.[3]

Medical interest in the scientific basis for humor and laughter to positively benefit human health and wellness has been promoted by Cousins,[10,11] Black,[12] and Fry.[13] The authors of this article have previously investigated the potential of mirthful laughter to modulate specific neuroendocrine components and subsequently neuroendocrine components of the composite human stress response through a progressive series of human research investigations carried out at Loma Linda University School of Medicine.[14-16] These findings demonstrate that mirthful laughter can counter some classical biological responses associated with stress, often for a considerable period of time after the session has ended. As such, the authors have termed the effects of mirthful laughter as "eustress," a term originally used by Selye.[17] In this article, eustress is defined as a positive phenomenon that ameliorates the biological effects of distress. However, not all effects of mirthful laughter are counter to classical stress responses.

An extensive scientific literature that has developed over the last several decades elucidates the psychobiological impact of stress. Selye[17] developed a tabular construct correlating stressful events/conditions such as bereavement and divorce with increased morbidity and mortality. Cannon[18] proposed the concept of the "fight or flight response" to describe key physiological responses evoked by stressful stimuli; this description is used routinely to characterize the actions of the sympathetic nervous system. Although substantial scientific attention has been directed toward the biological impact of multiple or severe stressors (distress), limited data exist describing the resultant neuroendocrine and immunomodulatory effects of positive emotional interventions that promote eustress states such as mirthful laughter.

The field of psychoneuroimmunology examines the complex interactions between the nervous system and immune system.[19] Evidence from the past decade has demonstrated that numerous neuroendocrine signals can modulate immune responses and subsequent disease outcomes. In addition, direct nerve fiber connections have been found between the central nervous system and the parenchyma and vasculature of both primary and secondary lymphoid organs.[20,21] Postganglionic noradrenergic sympathetic nerve fibers are major contributors to this signaling, but numerous neuropeptidergic neural-immune connections also have been identified.[22] These neurotransmitters also exert extensive regulatory control over immune responses.

Cells of the immune system can synthesize "neurohormone" and neurotransmitters directly,[23] adding a new paracrine component to neural-immune signaling. Cytokines derived from lymphocytes, macrophages, and other cells of the immune system can signal neurons in the central and peripheral nervous system, altering their neuronal activity, neurotransmitter release, and the resultant behavioral activities in which they participate.[24] Many cytokines, especially IL-1β, IL-6, and tumor necrosis factor-α (TNF-α), are synthesized by glial cells and even by some neurons.[25] Therefore, neurohormones, neurotransmitters, and cytokines are shared widely by cells of the nervous system and the immune system, extensively signaling cells in both systems and providing functionally important ongoing communication that profoundly influences the behavior of both systems.

Over the past decade, the most intensive investigations of neural signaling of the immune system during stressful states have focused on the hypothalamus-pituitary-adrenal (HPA) axis and the sympathetic nervous system,[26] the 2 most prominent components of the stress response discussed by Selye[17] and Cannon.[18] The authors' investigations of mirthful laughter and immune responses also have focused on these 2 major channels of communication (cortisol, catecholamines), with additional examination of the other hormonal systems such as growth hormone, prolactin, and opioid peptides.[27]

Specific endocrine and immune measurements previously studied in the context of human stress responses were examined by the authors using mirthful laughter.[14-16] Based on knowledge of established immunomodulatory interactions of these major neuroendocrine signals, these studies of mirthful laughter were carried out to provide a logical progression of investigations focusing on modulations of immune reactivity that are evoked by mirthful laughter. The original hypothesis for these studies is that mirthful laughter as a eustress can modulate the neural-immune components of the stress response in a direction counter to that evoked by classical stressors (distress).

METHODS

The Loma Linda University Institutional Review Board approved the research protocol. Cost of neuroimmune assays, the large number of neuroimmune parameters assessed per study, the number of medical personnel needed to draw and prepare blood samples at the same time points for all subjects, and the true-to-life experimental setting all were factors in establishing a functional/realistic experimental algorithm and design. All subjects were volunteer medical students. A routine medical interview and history were completed before subjects were accepted into the studies. All subjects were male (mean age, 27.0 ±3.2 years). Only men were selected for these studies due to the potential confounding immune variability during different phases of the menstrual cycle in women subjects.[28-30] Fifty-two subjects were used in this series of studies. Ten subjects were selected for each of the respective 4 neuroimmune studies; however, for the natural killer (NK) cytotoxicity activity study 6 experimental subjects and 6 controls were used.

Although all variables cannot be controlled in human subjects, a detailed interview with each subject was undertaken and rigid adherence to specific conditions was maintained. All subjects were nonsmokers who were not taking prescribed or over-the-counter medications and did not consume alcohol. Exercise and sexual activity were not permitted on the day of the experiment. Subjects fasted for 6 hours prior to initial blood sampling. The protocols were conducted in similar rooms, for the same period of time, at the same time of day.

The study design and protocol were similar throughout the studies. Several days prior to the experiment, subjects received notification about selection for a study. The subjects preselected the video to be used in the studies (*Over Your Head,* with the comedian Gallagher[31]). The laughter intervention was a 60-minute humor video. Explanation of sampling procedures was provided, and a cordial relationship was established between subjects and investigators to help promote a relaxed atmosphere. As in the study by Svebak,[32] familiarity with the social and physical attributes of the experimental situation was regarded as a mode of desensitization to the settings for all subjects, allowing the subjects to be relaxed and comfortable, not anxious or stressed by the novelty of the experimental circumstances. The control group rested comfortably in chairs and had access to magazines left on a table.

Angiocathers attached to a 3-way stopcock were inserted into the subjects' antecubital vein and an infusion (drip) was run with normal saline (60 mL/h) to keep the intravenous line open for blood sampling. A resting interval of 15 minutes ensued to ensure a stable endocrine baseline. Thereafter, blood samples were drawn from the indwelling catheter, aliquoted into appropriate collection tubes, and placed immediately in crushed ice and handled according to standardized clinical laboratory protocol if needed for specific neuroimmune analysis.

Specific samplings were performed at precise intervals relative to the respective studies: (1) baseline 10 minutes before the intervention; (2) 30 minutes into the intervention; and (3) recovery 90 minutes from the beginning of the intervention. An additional recovery sampling was acquired the next day by venipuncture at 12 hours following the time of initiation of the intervention.

A repeated measures design was used for these studies. Statistical data evaluation for each study was performed by repeated measures analysis of variance using the multivariate general linear hypothesis module of SYSTAT.[33,34] Within-subject effects were analyzed across time by repeated measures multivariate analysis of variance (MANOVA). Post hoc testing by simple contrast with baseline was analyzed on the group means for the respective components. Data for NK cell activity were analyzed in a 2 x 2 time by group analysis of variance with time of measurement as a repeated measure for 2 time points: baseline and recovery at 90 minutes.

Neuroimmune Measures

Natural Killer Cell Activity. In 6 experimental and 6 control subjects, baseline samples were drawn 10 minutes prior to showing the video. Postintervention blood specimens were collected at recovery 90 minutes (30 minutes after the end of the video). Natural killer cell activity was studied at effector-to-target ratios, with doubling dilutions, from 100:1 to 3:1. Each subject's peripheral blood leukocytes were used in a standard NK cell cytotoxicity chromium release assay as previously described.[35] Natural killer cell activity was calculated using the following formula: percent NK activity = ([experimental release – spontaneous release]/[total release mean – spontaneous]) x 100.[36]

Immunoglobulins and Complement C3. Previous studies have demonstrated a significant relationship between humor and increased concentrations of salivary immunoglobulin (Ig) A.[37-39] Based on those findings, the authors measured serum IgG, IgA, and IgM as well as complement C3 levels in 10 experimental subjects (determined by nephlometric analysis). The experimental protocol was performed as previously described,[16] with the following modifications: Blood samples were drawn at baseline (10 minutes prior to showing the video), during the intervention (30 minutes into the study), following the intervention at 90 minutes (30 minutes after the video), and at 12 hours (the next day).

Functional Phenotypic Markers for Leukocytes. To further evaluate the specificity of changes within the collective immune response evoked via a mirthful laughter intervention, functional delineation of the following phenotypic markers for leukocyte subsets was performed: activated T cells ($CD3^+DR^+$), nonactivated T cells ($CD3^+DR^-$), B cells ($CD19^+$), marker to identify NK cells in purified lymphocyte populations ($CD16^+$[FcγRIII]), functionally active cytotoxic T cells ($CD57^+CD8^+$), and T cells with helper ($CD4^+$) and suppressor markers ($CD8^+$), all of which were determined by cell flow cytometry. Blood samples were drawn at baseline (10 minutes prior to showing the humor video), during the intervention (30 minutes into the study), collected after the intervention at 90 minutes (30 minutes after the video), and at 12 hours (the next day).

Levels of the Cytokine Interferon-γ in Plasma. Plasma samples from 10 experimental subjects were analyzed by enzyme-linked immunosorbent assay for measurement of interferon-γ (IFN-γ) and assayed by the Clinical Laboratories, Immunoassay Section, Loma Linda University Medical Center, Loma Linda, Calif. Blood samples were drawn at baseline (10 minutes prior to showing the video), during the intervention (30 minutes into the study), after the intervention (30 minutes after the video), and at 12 hours (the next day).

Leukocyte Populations. Alterations of circulating leukocyte populations in association with various stressful stimuli have been extensively documented.[40] To test potential changes of leukocyte subset numbers (total leukocytes, lymphocytes, granulocytes, and monocytes) with a mirthful laughter intervention, a protocol similar to that used for the measurement of immunoglobulins and complement C3 was carried out. A complete blood count was performed in 10 healthy, fasting, male volunteers. Blood samples were drawn at baseline (10 minutes prior to showing the video), during the intervention (30 minutes into the study), following the intervention (30 minutes after the video), and at 12 hours (the next day).

Assessment of Plasma Volume and Compartmental Shifts

To determine whether any neuroimmune or cellular changes observed were related to plasma volume or compartmental shifts, the authors determined blood hematocrit, serum protein, and serum glucose for the time points studied.

RESULTS

Natural Killer Cell Activity

Natural killer cell activity was assessed at effector-to-target ratios, with doubling dilutions, from 100:1 to 3:1. When the mean chromium release for the 6 E:T ratio dilutions were compared to the baseline means for the NK cell activity assay, only the experimental group showed a significant increase in NK cell activity ($P<.01$), as shown in Figure 1.

Immunoglobulins and Complement C3

Within-subject effects across time by repeated measures (MANOVA) demonstrated a significant trend toward up-modulation for IgG ($P<.02$), IgA ($P<.01$), and IgM ($P<.09$), as shown in Figure 2. In addition, a simple contrast with baseline showed significant increases in specific immunoglobulins at the following time points: (1) IgG 30 minutes into the intervention ($P<.01$), 90 minutes into recovery ($P<.05$), and 12 hours into recovery ($P<.01$); (2) IgA 30 minutes into intervention ($P<.01$), 90 minutes into recovery ($P<.01$), and 12 hours into recovery ($P<.001$); and (3) IgM 30 minutes into the intervention ($P<.05$). Although complement C3 effects across time (measured by MANOVA) did not show a significant linear trend as did the immunoglobulins ($P<.24$), the recovery at 90 minutes ($P<.01$) and 12 hours ($P<.01$) were significant when contrasted to baseline (Figure 2).

Functional Phenotypic Markers for Leukocytes

Within-subject effects showed a significant increase or trend for the following: activated T cells ($CD3^+DR^+$) ($P<.01$), as shown in Figure 3; functionally active cytotoxic T cells ($CD57^+CD8^+$) ($P<.01$) and NK cells ($CD57^+CD8^-$) ($P=.09$), as shown in Figure 4; B cells ($CD19^+$) ($P<.01$) and NK cells identified in purified lymphocyte populations ($CD16^+$[FcγRIII]) ($P=.08$), as shown in Figure 5; and helper T cells ($CD4^+CD8^-$) ($P<.02$), uncommitted T cells with helper and suppressor markers ($CD4^+CD8^+$) ($P<.02$), and helper/suppressor ratio (CD4/CD8) ($P=.10$), as shown in Figure 6.

Simple contrasts with baseline showed significant increases in activated T cells ($CD3^+DR^+$) at 30 minutes into the intervention ($P<.01$) and at 90 minutes into recovery ($P<.001$), as shown in Figure 3; functionally active cytotoxic T cells ($CD57^+CD8^+$) at 30 minute into the intervention ($P<.01$) and 90 minutes into recovery ($P<.001$), as shown in Figure 4; B cells ($CD19^+$) at 90 minutes into recovery ($P<.001$) and 12 hours into recovery ($P<.001$), as shown in Figure 5; an increase in helper T cells ($CD4^+CD^-$) at 30 minutes into the intervention ($P<.01$), 90 minutes into recovery ($P<.01$), and 12 hours into recovery ($P<.05$); and an increase in T cell helper/suppressor ratio (CD4/CD8) 30 minutes into the intervention and 90 minutes into recovery ($P<.05$), as shown in Figure 6. No significant changes were detected in plasma volume shift over the time points studied (Figure 7).

Levels of the Cytokine Interferon-γ in Plasma

Within-subject effects across time by repeated measures (MANOVA) showed a significant increase in IFN-γ ($P=.02$) during the study. Simple contrasts with baseline showed significant increases in IFN-γ at 30 minutes intervention ($P<.001$), during 90 minutes recovery ($P<.001$), and at 12 hours recovery ($P<.001$) (Figure 8).

Immunocyte Populations

Within-subject effects across time by repeated measures (MANOVA) demonstrated a significant increase in total leukocytes ($P<.05$). Simple contrasts with baseline showed significant increases in total leukocytes at 30 minutes into the intervention ($P<.01$) and during 90 minutes recovery ($P<.001$); increases in lymphocytes at 30 minutes intervention ($P<.01$) and during 90 minutes recovery ($P<.05$); and increases in granulocytes at 30 minutes intervention ($P<.05$) and during 90 minutes recovery ($P<.01$), as shown in Figure 9.

DISCUSSION

Hormonal Influence on Immunoregulation

Cortisol. Immunoregulation by the nervous system occurs through 2 distinct pathways: the HPA axis and the sympathetic division of the autonomic nervous system. The latter directly innervates lymphoid organs.[26] Following the perception and processing of stressful stimuli by higher cortical and limbic forebrain structures, the corticotropin-releasing factor (CRF) neurons of the paraventricular nucleus of the hypothalamus are activated, which induces secretion of corticotropin (ACTH) from the anterior pituitary, resulting in the secretion of cortisol by the adrenal cortex into the systemic circulation.[41]

Complex feedback loops within the endocrine and immune systems serve to further regulate HPA function. Increased cortisol

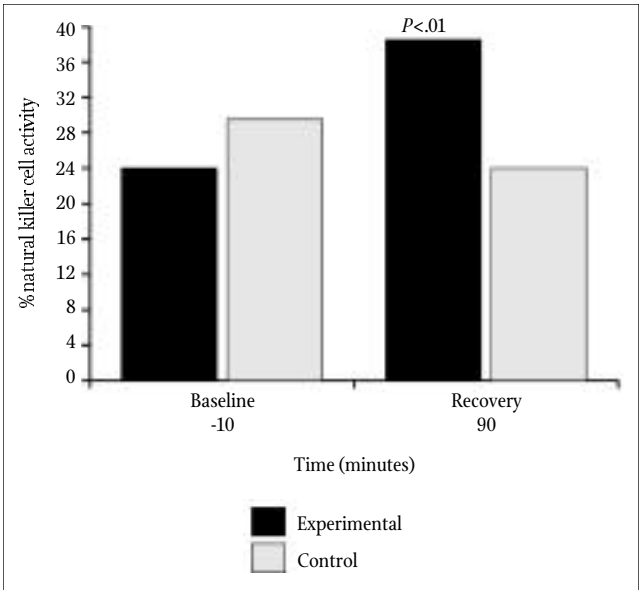
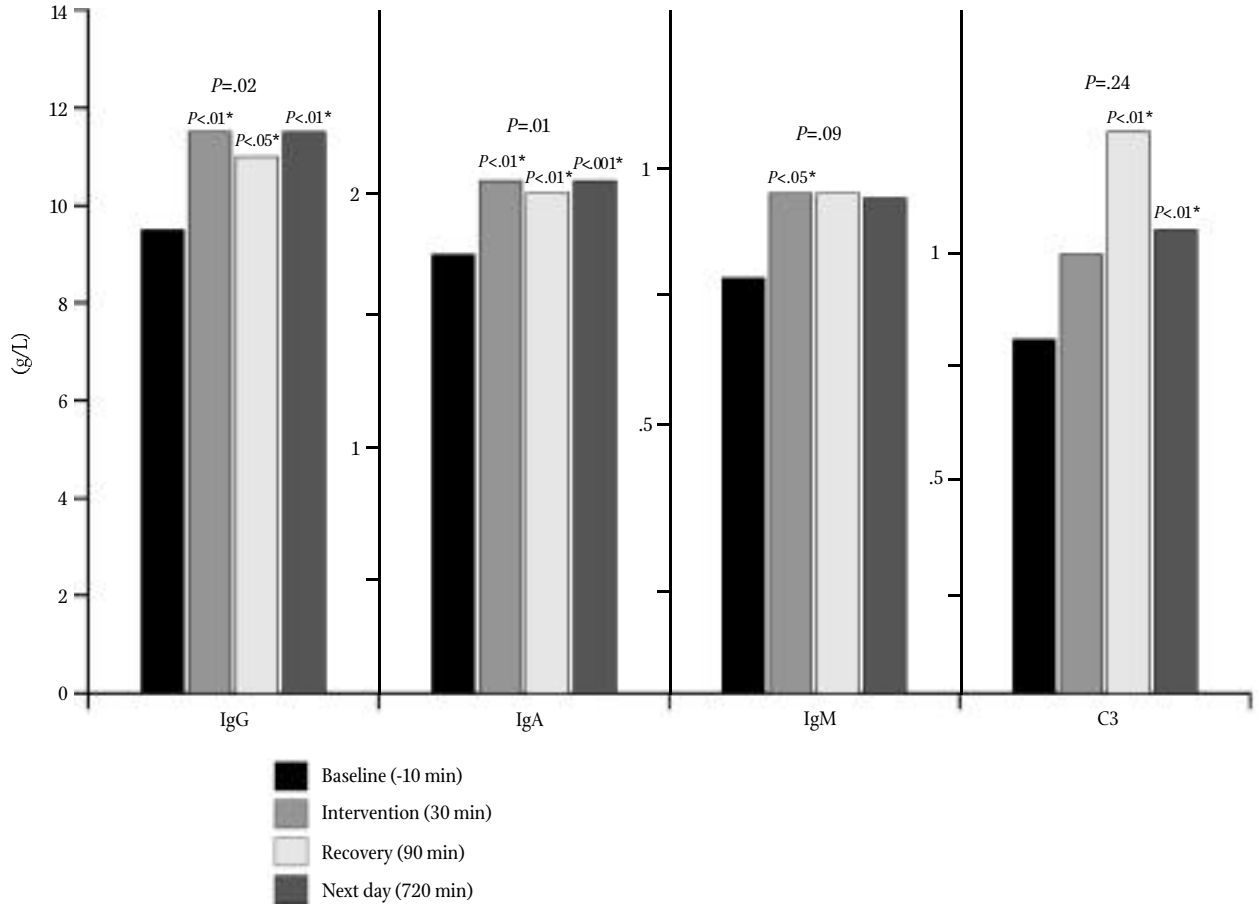


FIGURE 1 Natural killer cell activity response to mirthful laughter (mean ± SEM)

levels tend to suppress activation of the axis at the level of the anterior pituitary and the hypothalamus. Corticotropin release also is mediated by arginine vasopressin, oxytocin, and thymic peptides; arginine vasopressin can synergize with CRF to further activate ACTH and cortisol. Cytokines such as IL-1 and IL-6 can modulate HPA activity via activation of CRF neurons in the paraventricular nucleus.[42] Furthermore, ACTH and CRF receptors exist on some subsets of lymphocytes and other lymphoid cells, and some activated immunocytes are capable of secreting hormones including ACTH, thyroid stimulating hormone, growth hormone, prolactin, and endorphins.[43]

The decline in cortisol secretion observed during episodes of mirthful laughter in the previous study[16] is in sharp contrast to elevated secretion normally associated with stress.[17] Increased secretion of cortisol levels is considered a "hallmark" of the stress response, and traditionally is equated with immunosuppression, particularly if elevated cortisol levels persist. The accompanying attenuation of humoral and cellular immunity is characterized by attenuation of immunoglobulin synthesis, cytotoxic T lymphocyte activity, NK cell activity, and cytokine production.[44]

Cortisol is not universally immunosuppressive, nor is the result of cortisol secretion always detrimental to health. Adequate cortisol levels are essential for the maintenance of normal immune responses, and physiological levels of cortisol can influence helper T cell cytokine production in favor of humoral (T_H2) as opposed to cell-mediated (T_H1) responses. In addition, Sternberg et al[45] have demonstrated that blocking of corticosteroid activity with RU486 in rodent strains that are not susceptible genetically to induced autoimmunity (Fischer 344 rat) results in a switch in their susceptibility to an extent equivalent to the genetically susceptible strains (eg, Lewis/N-rats). As Besedovsky et al[46] noted almost 2 decades ago, corticosteroid secretion may be an essential physiological component in a normal immune response that prevents the uncontrolled proliferation of unwanted clones of immunocytes. However, mirthful laughter, when viewed as an external environmental input influencing cortisol secretion, can induce diminished secretion, directionally opposite to the elevation normally associated with stressful stimuli.[16]



* Within-subject effects across time by repeated measures multivariate analysis of variance

FIGURE 2 Plasma immunoglobulins and complement C3. Ig indicates immunoglobulin.

Epinephrine, Norepinephrine, and Dihydroxyphenylacetic Acid; Growth Hormone, Beta-Endorphin, and Prolactin. In the review of other neuroendocrine responses to mirthful laughter in the previous study,[16] the authors suggested that, conceptually, baseline differences between control and experimental populations for growth hormone levels might be explainable by an anticipatory response.[47] The control group expected a neutral stimulus while experimental subjects awaited and anticipated an entertaining, mirthful program. During the intervention, experimental subjects showed an attenuation of growth hormone secretion, in direct contrast to increased secretion characteristic of the human response to physical or psychosocial stress.[48] The subjects who later watched the humorous video showed a marked elevation of growth hormone, even before viewing began.

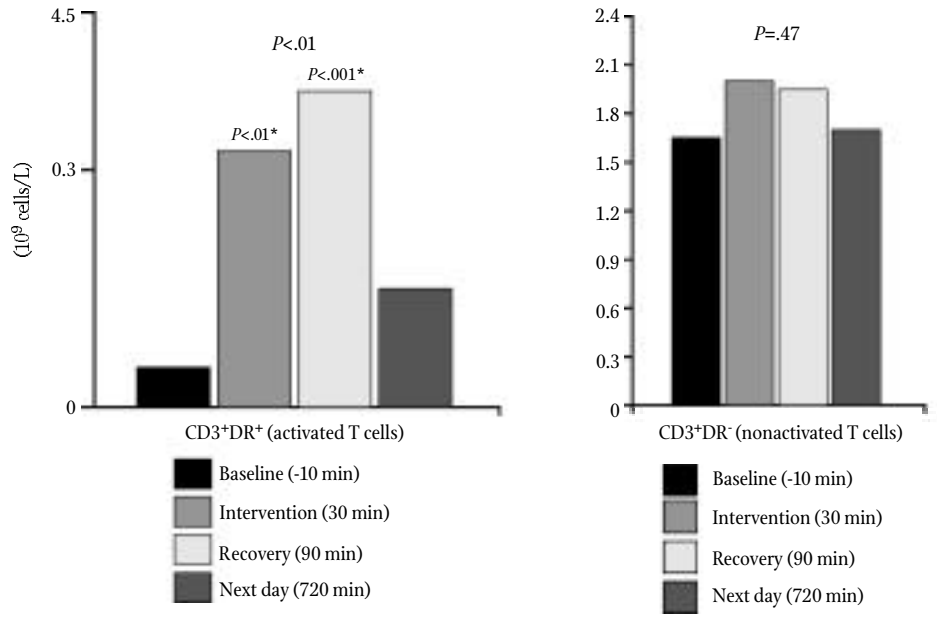
Measurement of dihydroxyphenylacetic acid showed a greater reduction from baseline in the experimental group compared to the control group. Epinephrine levels showed a marked reduction and attenuation in the experimental subjects compared with controls. The stability of norepinephrine, beta-endorphin, and prolactin levels during mirthful laughter is in sharp contrast to their expected rise in secretion noted with exercise or distress.[40]

Modulation of CRF mRNA and proenkephalin A mRNA in the paraventricular nucleus and pro-opiomelanocortin (POMC) mRNA in the anterior pituitary are stimulus dependent,[49] suggesting a disassociation among stress hormone component responses. Further support is provided by the finding that POMC, a neuropeptide precursor molecule cleaved by endopeptidases to yield ACTH, beta-endorphin, and beta-lipotrophin, undergoes tissue-specific posttranslational processing.[50] In addition, POMC-derived ACTH, beta-endorphin, and alpha-melanocyte–stimulating hormone from the anterior and intermediate lobes of the pituitary have been found to be differentially regulated.[51] It therefore appears that individual hormones in the "stress hormone" profile are subject to independent regulation and are controlled by separate signaling mechanisms. The resultant complexity of elevation, decline, or stability of those multiple hormones provides an extensive potential range of functional downstream responses in target tissues (eg, cells of the immune system) to a unique environmental input such as 1 hour of mirthful laughter. If the "stress hormones" are not all linked to a common activation mechanism, as they appear not to be, then individual environmental paradigms or inputs, such as mirthful laughter, may be useful for optimizing specific end-points in target systems of signaling, such as the immune system.

It is now accepted that lymphocytes express receptors for many hormones, neurotransmitters, and neuropeptides, including receptors for steroids, catecholamines, enkephalins, substance P, and vasoactive intestinal peptide. Expression and responsiveness of these receptors vary between different lymphocyte and monocyte subsets, so the effect of a transmitter may vary in different circumstances.[26] This signaling provides the foundation for understanding the influence of brain and behavior on functional activities of the immune system.

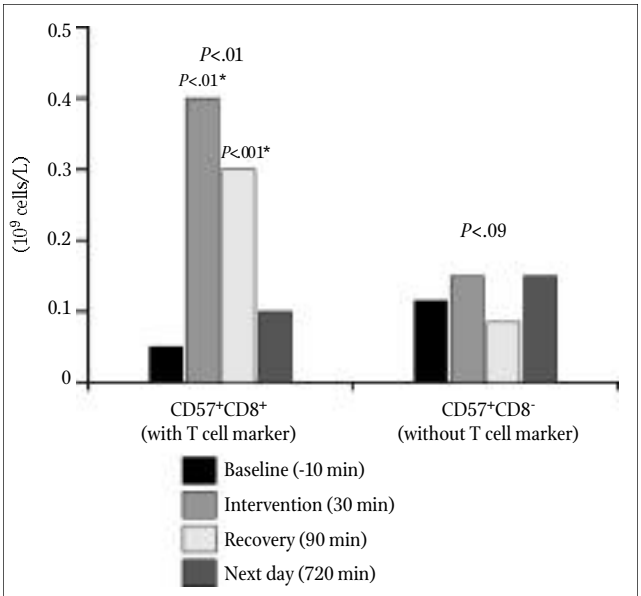
Neuroimmune Parameters

Natural Killer Cell Activity. Physical and psychosocial stressors can modify NK cell activity. The authors have previously



* Within-subject effects across time by repeated measures multivariate analysis of variance

FIGURE 3 Peripheral blood mononuclear cells expressing membrane antigens (T cells with activation markers)



* Within-subject effects across time by repeated measures MANOVA

FIGURE 4 Peripheral blood mononuclear cells expressing membrane antigens (functionally active cytotoxic T cells and natural killer cells). MANOVA indicates multivariate analysis of variance.

reported the in vitro modulation of NK cell activity associated with incubation of epinephrine, norepinephrine, and dihydroxyphenylacetic acid—the major serum neuronal catabolite of dopamine.[52] The authors also demonstrated elevated NK cell activity relative to baseline as a result of exercise stress in conditioned endurance runners. The authors observed significant NK cell activity enhancement starting from baseline to 1 hour of exercise. However, significant suppression was found below baseline at 1.5 hours after the stress run; NK cell activity did not normalize to baseline even after 21 hours of recovery.[35]

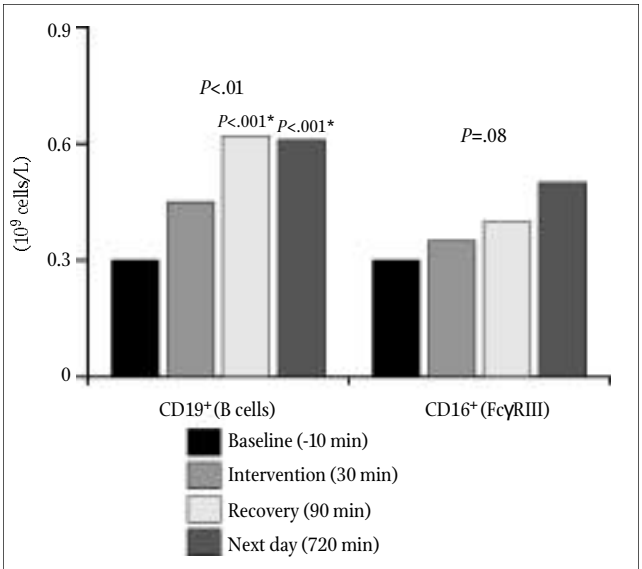
Natural killer cells represent a highly specific and efficient element of immunosurveillance against some tumor cells (especially blood-borne metastases) and virally infected cells.[53,54] Corticosteroids attenuate the enhancement of NK cell activity by interferon in vitro.[55] Independent of HPA activation, corticotropin-releasing factor reduces splenic NK cell activity through sympathetic activation and release of norepinephrine and neuropeptide Y.[56] However, most studies of stress-related neuroendocrine modulation in vivo point to catecholamines, not glucocorticoids, as the principal modulators of NK cell activity.[57]

Altered NK cell activity may be biologically significant. Diminished NK cell activity was reported in rats exposed to stressful stimuli accompanied by a 2-fold increase in lung metastasis when these same animals were challenged with tumor.[58] Reduced NK cell activity has been observed in bereaving women with depression.[59] Similar declines in NK cell activity have been reported in recently divorced or separated women compared to married controls[60] and among medical students reporting high levels of loneliness.[61]

In contrast, NK cell activity enhancement has been documented with stress reduction in women for surgical treatment for breast cancer,[62] and has been closely linked to long-term reduction in the recurrence of malignant melanoma.[63] In an experimental study in rats,[64] administration of L-deprenyl resulted in elevated NK cell activity and diminished tumor number and size.

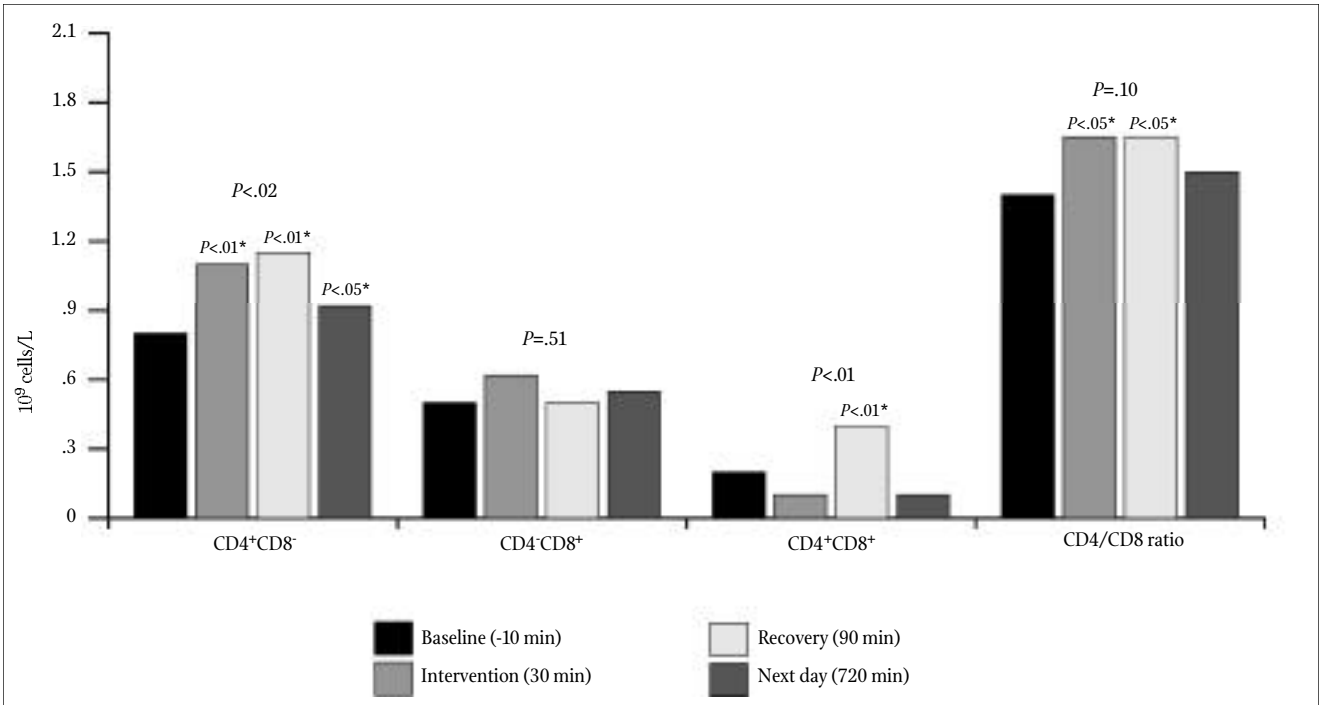
The extent to which transient elevation or repeated elevation of NK cell activity might be reflected in enhanced responses against tumors and viral infections is not yet known. It is tempting to interpret the remarkable protective effects of 4 hours of exercise per week against the incidence of breast cancer in a Finnish study[65] as the result of persistent or repeated elevation of NK cell activity compared with sedentary women. Similarly, if NK cell activity remains elevated for at least 12 hours after a 1-hour viewing of a humorous video, as noted in the present study, it is possible to envision long-term or chronic elevation of NK cell activity resulting from daily enjoyment of humor and mirthful laughter. The authors' observation of mirthful laughter on NK cell activity enhancement[66] substantiates the findings by Bennett.[67]

Immunoglobulins and Complement C3. The potential benefits of enhancement of the humoral component of the immune system are multiple. Immunoglobulin G, with a half-life of 23 days, represents approximately 15% of the total protein in serum. This versatile immunoglobulin causes agglutination of insoluble antigens; opsonizes antigenic determinants (epitopes) on microorganisms;



* Within-subject effects across time by repeated measures MANOVA

FIGURE 5 Peripheral blood mononuclear cells expressing membrane antigens (B cells and natural killer FcγRIII markers). MANOVA indicates multivariate analysis of variance.



* Within-subject effects across time by repeated measures multivariate analysis of variance

FIGURE 6 Peripheral blood mononuclear cells expressing membrane antigens (T cells with CD4 and CD8 markers)

participates in antibody-dependent, cell-mediated cytotoxicity; activates the complement cascade; neutralizes toxins and viruses; and immobilizes bacteria. Immunoglobulin M, with a half-life of 5 days, is found primarily in intravascular spaces; it is also an efficient agglutinator that can form macromolecular bridges between epitopes too distant to be bridged by IgG antibodies.

Immunoglobulin M is also an excellent complement-fixing or -activating antibody, with components that include the isohemagglutinins against the red cell antigens of the ABO blood groups. Immunoglobulin A, with a half-life of 5.5 days, is present primarily in tears, saliva, sweat, colostrum, and mucus. It is a potent agglutinator, and plays key bactericidal and antiviral roles against local infections of the respiratory and gastrointestinal tracts.[68] Complement C3 is a beta-globulin, with a molecular weight of 180000 d, secreted as pro-C3 by macrophages, the activation of which produces anaphylatoxins, chemotaxins, opsonization, and the phenomenon of immune adherence.[69]

The present finding of increased Ig levels and C3 levels with 1 hour of mirthful laughter is consistent with the down-regulation of cortisol associated with this intervention,[16] and may reflect some of the other neuroendocrine changes (eg, decreased epinephrine and elevated growth hormone) elicited by mirthful laughter.

Functional Phenotypic Markers for Leukocytes. Leukocyte subsets demonstrate changes following stressful stimuli. Following a 6-minute naturalistic speaking stressor, human subjects demonstrated significant decreases in B cells, helper T cells, and T cell helper/suppressor ratios.[70] Leukocyte subset changes also occur in a wide range of stressors including examination stress in medical students,[44] caregiving,[70] divorce and separation, and bereavement or depression.[60]

Meticulous attention to plasma volume effects must be considered in enumeration of lymphocyte subsets. In one evaluation of a stressor in young adults, when hemoconcentration was factored into the analysis the magnitude of measured stress-related NK cell increases was diminished, whereas significant decreases in $CD4^+$ and $CD19^+$ cell concentrations persisted, suggesting that NK cell increases with acute stress may partially be attributable to hemoconcentration.[71,72]

The changes in cellular subsets with specific phenotypic markers found in the present study did not depend on hemoconcentration or plasma volume shifts, suggesting that they reflect actual changes in cellular compartmentation. These changes contrast with the attenuation of such subsets characteristic of the composite stress (distress) response noted in many reports cited above.

There are several notable observations in the phenotypic marker leukocyte study. Natural killer cells account for up to 15% of blood lymphocytes and express neither T cell nor B cell antigen receptors. Most surface antigens detectable on NK cells by monoclonal antibodies are shared with T cells or monocytes/

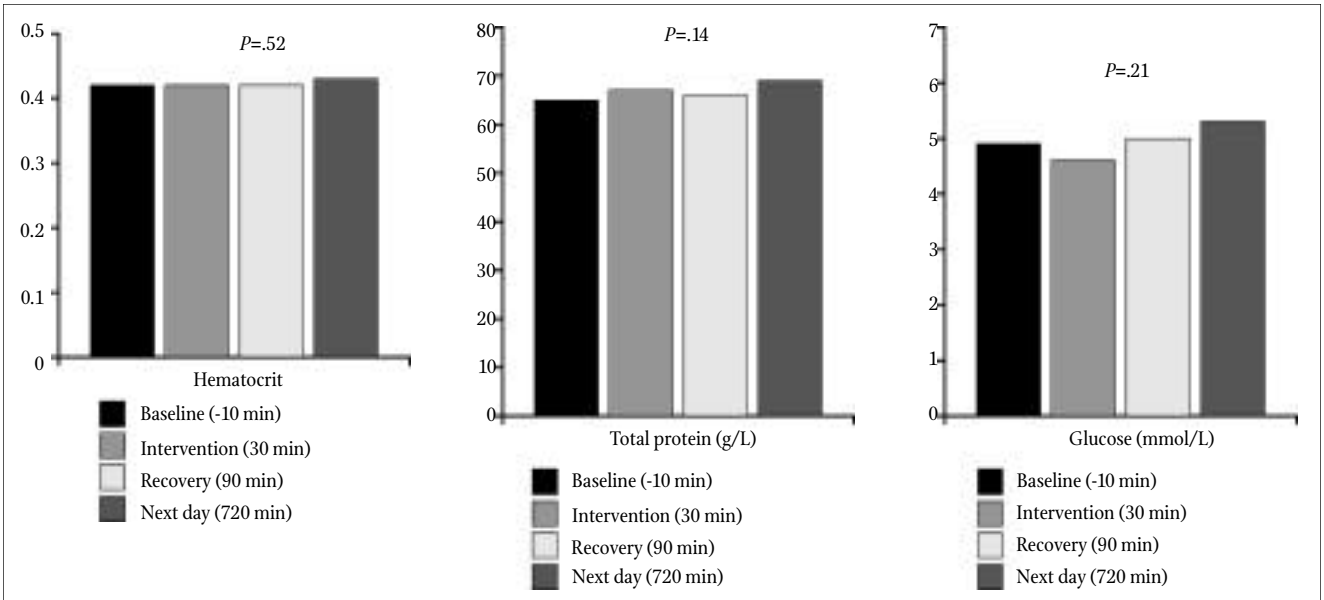


FIGURE 7 Hematology and chemistry parameters used to assess plasma volume shifts. MANOVA indicates multivariate analysis of variance.

macrophages. Both phenotypes—CD16 (FcγRIII), which is used to identify NK cells in purified lymphocyte populations, and $CD57^+CD8^-$ NK cells, which are devoid of T cell markers—showed a tendency to increase. This parallels the present results showing a significant increase in NK cell activity.

Levels of the Cytokine Interferon-γ in Plasma. Cytokines have a central role in positive and negative regulation of innate and acquired immune responses, and in integrating them with other physiological compartments such as the endocrine and hemopoietic systems. Cytokines can best be defined as small proteins (approximately 8-80 kd molecular weight) that usually act in an autocrine (ie, on the cell that produced them) or paracrine manner (ie, on cells close by). The production of these potent molecules is usually transient and tightly regulated. More than 100 different human cytokines have been identified. Cytokines act by binding to specific receptors at the cell membrane, setting off a cascade that leads to induction, enhancement, or inhibition of a number of cytokine-regulated genes in the nucleus of the affected cell.

It is now understood that $CD4^+$ helper T cells have 2 different profiles of cytokine production (T_H1 and T_H2) and these patterns select between the 2 basic types of acquired immune responses mediated by $CD4^+$ helper T cells: cell mediated and humoral, respectively. Typical human T_H1 cytokines include IFN-γ, TNF-β, and IL-12; T_H1 cells are involved in cell-mediated responses. Several T_H1 cytokines activate cytotoxic, inflammatory, and delayed hypersensitivity reactions. By contrast, T_H2 cells are typified by the production of IL-4 and IL-5, with IL-6, IL-9, IL-10, and IL-13 also commonly produced. Furthermore, T_H2 cells encourage production of antibodies, especially IgE, and T_H2 cytokines are associated with regulation of strong antibody and allergic responses. Cytokines from T_H1 cells usually can inhibit the actions of T_H2 cells, and vice versa. Immune responses often are characterized as T_H1- or T_H2-dominant responses.

The T_H1/T_H2 decision is crucial to effective immunity; it is likely that many interlocking factors contribute to that decision. Many signals may influence the differentiation of $CD4^+$ helper T cells. Relevant to the neuroendocrine findings is the activity of co-stimulatory molecules and hormones present in the local environment. For example, glucocorticoids such as cortisol tend to drive the developing response toward T_H2. Derivatives of dehydroepiandrosterone tend to oppose this effect and favor a T_H1 response.[73] Levels of these adrenal hormones are regulated both systemically and by local metabolism within target organs. Therefore, the balance between cortisol and dehydroepiandrosterone concentrations within lymphoid organs and sites of pathology may be a critical determinant of the response type.

The authors studied IFN-γ because it has numerous important immunoregulatory actions and plays a major role relative to T_H1 cell-mediated reactions. Its primarily sources are antigen-specific T cells and NK cells, and its principal targets are lymphocytes, monocytes, NK cells, and tissue cells. Its effects are exerted through specific saturable binding to a single class of high-affinity receptors found in myelomonocytic cells, lymphoid cells, mast cells, endothelial cells, fibroblasts, neuronal cells, and melanocytes. It is, however, the most potent inducer of macrophage activation and class II molecules on tissue cells. In

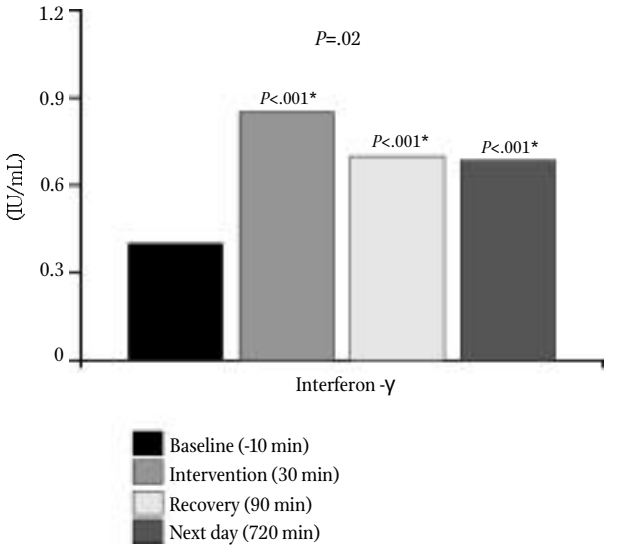
this and other functions, it synergizes with TNF-α and TNF-β. Interferon-γ also can regulate proliferation, differentiation, and activation of T and B cells, and can activate NK cell activity.[74]

Cytokine levels have been analyzed in various stressful paradigms. Increased levels of cytokines have been measured in athletes and the active elderly.[75] Markedly diminished production of IFN-γ (in response to mitogen or endotoxin) has been noted following strenuous exercise[76] and in stressful conditions in humans, such as examination stress.[77]

In an experimental study of rats exposed to inescapable tail shock, reduced levels of concanavalin A-stimulated IFN-γ were found in mesenteric lymphocytes and splenocytes immediately after inescapable shock termination, accompanied by long-term reductions in antikeyhole limpet hemocyanin IgM and IgG.[78] Spousal caregivers of patients with Alzheimer's disease showed diminished NK cell activity in response to IFN-γ or IL-2 compared to aged-matched controls; higher NK cell activity to each cytokine in the caregivers was found in individuals with positive and tangible social support.[79]

Type 1 cytokines (T_H1) such as IFN-γ are immune modulators that mainly induce cell-mediated immunity. Interferon-γ appears to play a significant role and can be effective in activating cytotoxic T lymphocyte and NK-mediated cytolytic functions associated with effective antitumor defense mechanisms. Interferon-γ enhances the presentation of antigenic peptides to helper T lymphocytes. In addition, IFN-γ directly inhibits the growth of cervical carcinoma cell lines.[80] The present findings of increases of the T_H1 cytokine IFN-γ in conjunction with mirthful laughter suggest a potential interplay in cytokine modulation of the immune responses via the T_H1/T_H2 paradigm.

Immunocyte Populations. Although many subsets of cells participate in cellular and humoral immune responses, it is not possible to correlate alterations in numbers of circulating immunocytes alone with heightened immunocompetence. The underlying mechanisms of stress-induced suppression of lymphocyte functions are multifactorial, with some factors showing corticosteroid, catecholamine, or opioid peptide dependence.[36] Stress-induced lymphopenia in general, however, has been reported to be adrenal-dependent based on results of adrenalectomy in rats.[81] Radioactive labeling studies in rabbits, using cortisol-induced lymphopenia, demonstrate lymphocyte redistribution from peripheral blood, spleen, and bone marrow to other lymphatic tissue.[82] Intravenous injections of hydrocortisone and epinephrine in humans have demonstrated increases of all leukocyte subsets at 10 minutes, moderate lymphopenia and monocytopenia at 1 hour, and neutrophilia and eosinopenia at 6 hours.[83] The present data documenting relative increases in total leukocytes and specific leukocyte subsets are consistent, at least in part, with mirthful laughter acting through attenuation of circulating cortisol.



* Within-subject effects across time by repeated measures MANOVA

FIGURE 8 Plasma cytokine interferon-γ response to mirthful laughter. MANOVA indicates multivariate analysis of variance.

CONCLUSIONS

The molecular and cellular events underlying differential stress responses are a puzzle that is still in the construction phase. The biological concept of stress—a term Selye borrowed from physicists more than 60 years ago—is well established yet not completely understood. Today, integrative medical researchers such as neuroimmunologists and psychoneuroimmunologists are not only studying negative stress (distress), but positive stress (eustress) modulators as well.

Medical scientists believe they have a reasonable knowledge of some triggering events that can disrupt homeostatic balance. A term used to describe the process of the human body adapting to regain homeostasis is *allostasis.* According to McEwen,[84,85] the biological cost in regaining homeostasis is called the "allostatic load." It is the price the human body pays to adapt in response to a stressor. An individual's response to stress is multifactorial. It is now accepted that genetics does not account for all variability. However, 2 factors that appear to play major roles in response to stress are perception of a situation or circumstance and general health (including behavior and lifestyle choices such as diet, smoking, drinking, and exercise).[85]

In addition, research has shown that early life experiences may influence and set an individual's response to subsequent stress.[86] In humans, high allostatic loads can suppress the immune system, decrease bone mineral density, weaken muscles, promote atherosclerosis, increase insulin resistance, and accelerate memory loss.[85] In the reciprocal situation of low allostatic loads, the failure to produce adequate neuroendocrine hormones can result in elevated autoimmune and inflammatory responses.[85] Optimal exercise, a balanced optimal diet, and appropriate social interaction appear to contribute toward keeping allostatic load in check.

The data from the authors' studies of mirthful laughter show some similarities with exercise (immune reactivity), but

without identical changes in mediators such as beta-endorphin, norepinephrine, and neuropeptide Y.[16] The authors show some changes directionally opposite to the response of stressors (distress), again without directionally opposite changes in all the "stress mediators." Some responses are unique to laughter, such as the surge of growth hormone in anticipation of the intervention.[16] Resultant immunological changes induced by the eustress of mirthful laughter are likely to be mediator specific and temporally specific, and may be useful in a clinical context to aid in the appropriate modulation of specific measures (eg, NK cell activity, immunoglobulin secretion, and IFN-γ secretion) that are thought to be desirable to optimize selected immunological responses in the prevention and/or amelioration of some specific disease states. Music therapy has been shown to increase serum melatonin levels.[87] Melatonin is a hormone capable of modulating neuroimmune parameters.[88] The authors have shown further neuroendocrine and neuroimmune (NK cell activity) modulation in subjects participating in music therapy.[89]

In another study with high-stressed patients, the authors used 3 eustress metaphors in a video format to present music, nature imagery, and positive affirmations to elicit marked reduction in neuroendocrine stress hormones associated with immunosuppression.[90] Perhaps alternative or complementary behaviors (eustress metaphors) such as optimal exercise, optimal dietary lifestyle, appropriate social interaction, enjoyment of music, spirituality, and now mirthful laughter with its related neuroendocrine and neuroimmune responses may represent a modulation of biological parameters toward tempering or modifying the allostatic load. Modulating perception and/or enhancing lifestyle wellness for optimal health benefit can accomplish this.

Voltaire, the 18th-century French philosopher, is credited with the statement, "[T]he art of medicine consists of amusing the patient while nature cures the disease." Norman Cousins,[91] a man of great insight into the personal understanding of the influence between mind and body, aptly stated that

> the best physicians are not just superb diagnosticians but men who understand the phenomenal energy (and therefore curative propensity) that flow out of an individual's capacity to retain an optimistic belief and attitude toward problems and human affairs in general. It is a perversion of rationalism to argue that words like "hope" or "faith" or "love" or "grace" (and "laughter") are without physiological significance. The benevolent emotions are necessary not just because they are pleasant, but because they are regenerative.

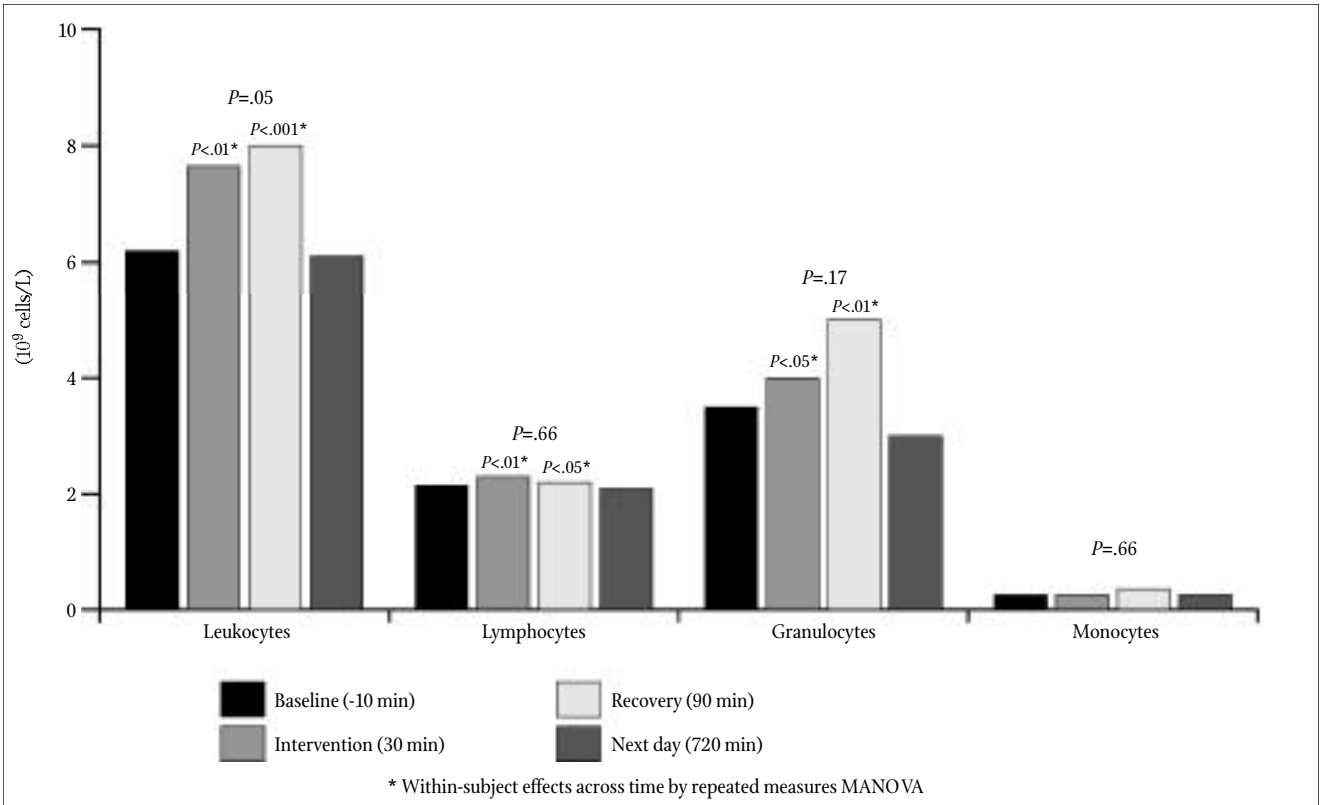


FIGURE 9 Hematology parameter response to mirthful laughter. MANOVA indicates multivariate analysis of variance.

An Apache myth tells that the creator endowed human beings, the 2-leggeds, with the ability to do everything—talk, run, see, and hear. But he was not satisfied until the 2-leggeds could do just one more thing—laugh. And so men and women laughed and laughed and laughed! Then the creator said, "Now you are fit to live!"[92] With the commencement of the 21st century, the tools of molecular biology will provide further discoveries in neuroimmunology and, as a result, a greater appreciation and use of alternative/complementary and integrative (whole-person) medicine.

Dossey[93] has appropriately stated that science raises more questions about humor than it answers. Now, perhaps, we may begin to understand parts of those questions. As we begin to see more pieces of the puzzle and understand the function of the parts from the whole, we must never forget to place that knowledge back into the integrative whole. It is our challenge in medicine to remember that the patient as a whole is more than the sum of his or her parts. In treating the integrative whole, we can move toward not only more optimal wellness, but synergize therapies in the healing process. Perhaps medical science is beginning to catch up to intuition.

Although limited in sample size due to the complexity of experimental logistics and cost constraints, these studies require further research to fully elucidate mirthful laughter's effects on the specific components and composite immune response. The changes in the indices measured are consistent and compelling enough to suggest that the humor-associated interventions of mirthful laughter may be capable of immunomodulation via specific neuroendocrine and neuroimmune parameters. Indeed, mirthful laughter and the associated eustress emotions may be the intuition and music of the soul that confirm the biblical wisdom that "[a] merry heart doeth good as medicine but a broken spirit drieth the bones" (Proverbs 17:22).

Acknowledgments

The authors dedicate this paper to the memory of the late Norman Cousins, who provided his insight and support through the years. The authors also wish to remember Joseph Berk and Arlene Spencer, who, as they encountered cancer, added quality and quantity to their days with a merry heart and mirthful laughter, leaving us with a greater appreciation for the understanding of the biology of a merry heart.

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