RAPID COMMUNICATION

Lymphocyte Subset and Cellular Immune Responses to a Brief Experimental Stressor

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To evaluate effects of acute mental stress on aspects of cellular immunity, lymphocyte populations and phytohemagglutinin (PHA)-stimulated T-cell mitogenesis were measured in 33 healthy young men, both before and immediately following subjects' performance of a frustrating, 21-minute laboratory task (Stroop test). Relative to baseline evaluations, post-task measurements showed a significant reduction in mitogenesis and alterations in various circulating lymphocyte populations; the latter included a diminished T-helper/T-suppressor cell ratio and an elevation in the number of natural killer cells. Eleven subjects assigned to a control (unstressed) condition exhibited no changes in lymphocyte populations, but did show an increase in T-cell proliferation, compared with pretask measurements.

Key words: experimental stress; psychoimmunology; cellular immunity; mitogenesis; lymphocytes; natural killer cells.

INTRODUCTION

While a number of investigations have demonstrated associations between naturally occurring stressors and alterations in cellular immune function (e.g., 1–6), interpretation of these studies is limited by their correlational design. Although few studies have examined immunologic changes to acute psychological stressors within controlled laboratory settings, there is some experimental evidence that mental stress similarly alters both quantitative and qualitative aspects of cellular immunity in human-beings, as indicated by alterations in peripheral T-suppressor/cytotoxic and natural killer cell populations (7–10), and by a decreased ability of T lymphocytes to divide when incubated with a nonspecific mitotic stimulant (7). In this report, we attempt to replicate, within a single experimental protocol, these observations regarding the effects of acute stress on circulating lymphocyte

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populations and the T-mitogenic responses of healthy young adults.

METHODS

Subjects

Forty-four undergraduate males (aged 19–25 years) were randomly assigned in a ratio of 3:1 to either a stress or control condition. In the stress condition, in vitro measurements of cellular immune function were obtained before and after subjects were administered a distinctly frustrating laboratory stressor. Heart rate (HR) and blood pressure (BP) were also assessed at baseline and during subjects' task performance. The same measurements were obtained in the control condition, although these subjects were not exposed to the experimental stressor. All subjects gave informed consent to participate in this investigation, which was approved by the Biomedical IRB of the University of Pittsburgh.

Procedures

Subjects abstained from food and caffeine for 12 hours before attending a laboratory session beginning at 08:30 A.M. Upon arrival at the laboratory, a nurse inserted an intravenous catheter into a vein in the antecubital fossa of the subject's dominant arm. The catheter was connected to an exfusion pump via a short length of heparinized, SILASTIC® tubing (Dow Corning, Midland, Michigan). Following venipuncture, an occluding cuff was placed on the subject's nondominant arm and connected to a vital signs monitor (Critikon Dinamap 8100) for automated measurement of HR (in beats per minute (bpm)) and systolic and diastolic blood pressure (in mm Hg). Following instrumentation, the subject rested for 30 minutes to achieve baseline conditions. During the last 6 minutes of this period, HR and BP (two readings) were recorded, and 30 ml of blood was drawn. At this point, the control subjects were instructed to continue to sit quietly for 21 minutes, while the stress group performed a 21-minute computerized version of the Stroop Color-Word Interference Test (11). Subjects indicated their responses by pressing one of four microswitches on a keypad under pressure of time and against a distractor (random test responses) generated by computerized voice synthesis. HR and BP were recorded every 4 minutes during task performance and a second 30 ml of blood was collected immediately after this period.

Immune Assays

A common measure of cellular immune function is the ability of the T (thymus-derived) lymphocytes to proliferate, in vitro, in response to nonspecific mitogens. Extent of mitotic division may, in turn, reflect the interrelation between two different populations of lymphocytes—T-helper lymphocytes (denoted CD4 cells) whose role is to facilitate immune response, and T-suppressor/cytotoxic lymphocytes (CD8 cells), which act, in part, to suppress immune response. In this study, we examined effects of the experimental stressor on T-mitogenic responses and on circulating lymphocyte populations.

To assess nonspecific mitogen stimulation, a whole blood assay was conducted to establish a dose response curve at phytohemagglutinin (PHA) concentrations of 0.5, 2.5, 5.0, 10.0, and 20.0 μg/ml.[1] The difference in counts per minute between stimulated and unstimulated samples was determined separately for each concentration; these values were subjected to logarithmic (base 10) transformation prior to statistical evaluation.

Circulating populations of T-cell subtypes, B-cells and natural killer (NK) cells were assessed in whole blood using flow cytometry. Lymphocyte subsets were analyzed using monoclonal antibodies labeled with either fluorescein or phycoerythrin to quantify CD3 (total T-cells), CD4 (T-helper cells), CD8 (T-suppressor/cytotoxic cells), CD19 (B-cells), and NK

[1] Blood was diluted 1:10 with RPMI-1640 tissue culture medium, supplemented with 10 mM Hepes, 2 mM glutamine, and 50 μg gentamicin per ml. One hundred microliters of diluted blood was added to a 96-well, flat-bottomed culture plate (Costar #3596) containing 100 μl of mitogen solution added in quadruplicate (100 μl) of PHA prepared in RPMI-1640 to yield the five final PHA concentrations. The plates were incubated for 120 hours at 37°C in air and 5% CO_2. Eighteen hours before the end of incubation, the wells were pulsed with 1 mCi tritiated thymidine and harvested for counting.

cells.[2] Response was defined as the baseline-to-task change in both the number of cells of each type and the ratio of CD4 to CD8 cell subtypes. Numbers of NK cells could not be computed for one subject due to clotting, and the T-mitogenic responses of four subjects could not be determined due to bacterial contamination.

Statistical Analysis

Prior to statistical analysis, HR and BP data were reduced by calculating mean values for both baseline and experimental periods. These values, as well as the lymphocyte subset data, were subjected to 2 × 2 ($Group_{stress,control}$) × ($Period_{baseline,task}$) repeated measures analysis of variance (ANOVAs), to evaluate changes that may have occurred in response to the experimental stressor. T-cell mitogenic responses were similarly analyzed by a 2 × 2 × 5 ($Group_{stress,control}$) × ($Period_{baseline,task}$) × (PHA Concentration$_{(0.5,\ 2.5,\ 5.0,\ 10.0,\ 20.0\ \mu g/ml)}$) repeated measures ANOVA. It was predicted that a significant Group × Period interaction would emerge in each ANOVA, and that for each dependent measure, the stressed subjects would show a change in the level of the dependent variable during or following task performance, relative to corresponding pretask (baseline) measurements; it was expected that control subjects would show either no change or a change in the opposite direction. A priori comparisons among means were performed using t tests (alpha level = 0.05).

RESULTS

Task-related changes in heart rate and blood pressure were evaluated first, as an index of the physiologic effect of the experimental stressor independent of immune parameters. These analyses revealed a significant Group × Period interaction of all three measures: HR ($F(1,42) = 35.79$, $p < 0.0001$), systolic and diastolic blood pressure, ($F(1,42) = 49.74$, $p < 0.0001$; $F(1,42) = 23.04$, $p < 0.0001$). In each instance, task values were significantly higher than baseline measurements among stressed subjects, but did not differ from baseline in controls. Among stressed subjects, mean baseline-to-task changes in HR were +13 bpm, and for systolic and diastolic blood pressure, +15 and +12 mm Hg, respectively; control subjects showed a corresponding HR increase of 1 bpm, and no changes in BP.

With respect to immune parameters, the ANOVA for T-cell mitogenic responses revealed a significant Group × Period interaction ($F(1,38) = 10.04$, $p < 0.05$).[3] Comparisons among means showed that T-cell mitogenesis decreased significantly from pretask to post-task measurements in the stressed subjects, and increased significantly among controls (means and standard deviations are presented in Table 1). The absence of a Group × Period × Concentration interaction indicates that these relationships generalized across all five PHA concentrations. Similarly, analysis of lymphocyte subpopulations showed a significant Group × Period interaction for the T-helper/suppressor (CD4:CD8) ratio ($F(1,42) = 9.08$, $p < 0.005$)). Here, there was a significant decline in the CD4:CD8 ratio from baseline to post-task measure-

[2] NK cells were identified by the monoclonal antibodies CD56 and CD16, in the absence of CD3.

[3] This analysis also yielded a significant main effect for PHA concentration ($F(4.152) = 4.52$, $p < 0.0001$), reflecting expected differences in cell division to the graded concentration of mitogen. Average counts per minute (log_{10}), collapsed across baseline and experimental period measurements, were 5.84 at 0.50 μg/ml of PHA, 6.10 at 2.5 μg/ml, 6.10 at 5.0 μg/ml, 5.93 at 10.0 μg/ml, and 5.52 at 20.0 μg/ml.

TABLE 1. Mean Lymphocyte Counts and PHA-Stimulated T-Lymphocyte Mitogenesis at Baseline and Following the Experimental Period among Stressed Subjects and Unstressed Controls (Standard Deviations in Parentheses)

	Lymphocytes (cells/mm^3)						T-Lymphocyte Proliferation (Δcpm)*				
	CD4/CD8	CD4	CD8	CD3	CD19	NK	0.5 (μg/ml)	2.5	5.0	10.0	20.0
Stress group											
Baseline	1.5	718	522	1209	222	215	5.84	6.12	6.11	5.94	5.54
	(0.5)	(293)	(262)	(512)	(92)	(99)	(0.25)	(0.12)	(0.13)	(0.15)	(0.20)
Experimental period	1.2	648	558	1154	204	290	5.83	6.09	6.09	5.92	5.51
	(0.4)	(245)	(247)	(479)	(80)	(124)	(0.24)	(0.13)	(0.14)	(0.18)	(0.21)
Control subjects											
Baseline	1.3	637	532	1100	203	209	5.84	6.06	6.07	5.90	5.50
	(0.4)	(154)	(147)	(183)	(50)	(94)	(0.22)	(0.08)	(0.09)	(0.09)	(0.18)
Experimental period	1.2	616	523	1071	200	232	5.90	6.10	6.12	5.95	5.54
	(0.4)	(155)	(145)	(229)	(46)	(84)	(0.22)	(0.11)	(0.10)	(0.10)	(0.18)

* Values are the differences in counts per minute ($\log_{10}$) between stimulated and unstimulated samples, at each concentration of PHA.

ments among stressed subjects, but no change in controls (see Table 1).

The ANOVAs for CD4 and CD8 populations alone revealed a significant Period main effect for CD4 lymphocytes ($F(1,42) = 9.49$, $p < 0.005$), but not for CD8 cells; in neither case was the Group × Period interaction reliable. Nonetheless, inspection of Table 1 reveals substantial baseline-to-task changes in CD4 and CD8 lymphocytes among the experimental group. Therefore, paired t tests were performed separately for each condition, comparing pretask and post-task measurements for both variables. These analyses revealed that CD8 cells increased and CD4 cells decreased significantly from baseline to post-task measurements in the stressed group ($t(32) = -2.84$, $p < 0.05$); $t(32) = 4.82$, $p < 0.0001$), but did not change among controls.

Finally, while no significant effects were observed for total T (CD3) and B (CD19) lymphocytes, the Group × Period interaction did achieve significance on analysis of NK cells ($F(1,41) = 4.99$, $p < 0.05$). Here again, the number of NK cells rose significantly between baseline and post-task measurements in stressed subjects, but not among controls.

DISCUSSION

In this study, subjects exposed to a brief experimental stressor showed suppression of PHA-stimulated T-cell proliferation, as well as alterations in various circulating lymphocyte subpopulations; the latter included a decreased ratio of CD4:CD8 lymphocytes (associated with expanded CD8 and reduced CD4 cell populations) and an increase in the number of circulating NK cells. In contrast, control subjects showed an increase in proliferative response, with no significant changes in lymphocyte populations. It is possible that the enhanced mitogenesis among the controls was due to protracted relaxation, as these subjects were instructed to continue to sit quietly during the 21-minute 'task' period. Relatedly, other studies have demonstrated augmented immune

function in response to longer-term relaxation interventions (12).

In a previous investigation, we observed a similar decrease in lymphocyte proliferation and a selective increase in CD8 lymphocytes among stressed subjects, but only in those individuals who showed the most pronounced sympathoadrenal activation on exposure to the laboratory stressor (i.e., "high" sympathetic reactors, as evidenced by marked rises in HR, BP, and venous plasma catecholamine concentrations). Interestingly, the average HR and BP response elicited by the Stroop task in the present experiment approximated that of the "high" sympathetic reactors in our earlier study. The relatively small HR and BP responses of subjects identified in the previous study as "low" sympathetic reactors, in turn, were matched here by only six of 33 experimental subjects; moreover, as in our earlier investigation, these few, less responsive individuals showed no changes between baseline and task periods in comparable measures of immune response.[4] Overall, then, we believe that the cellular-immune effects of acute mental stress presented here are consistent with findings reported previously by ourselves (7) and others (8–10). That our results are also similar to those obtained following epinephrine and isoproterenol infusions in humans (i.e., reduced lymphocyte proliferation and CD4:CD8 ratios, and increased NK cell populations) further suggests that the effects of acute psychological stress on cellular immune function is sympathetically mediated (13–15).

Other recent studies reveal that naturalistic stressors (e.g., stressful life events, marital distress, and caregiving for a terminally ill family member) are also associated with diminished lymphocyte proliferation and CD4:CD8 cell ratios (1–3). However, while NK cell numbers consistently rise following brief laboratory challenges (8–10), naturalistic stressors have generally been associated with a smaller NK cell population (4). Differences in the chronicity of stress may partially account for the varying effects of naturalistic and laboratory stressors (8). It is interesting to note that the effect of restraint stress on NK activity in rats depends on the duration of the stressor, with NK activity falling after repeated, but not acute, exposure to immobilization (16). The mechanisms underlying differential immune changes under acute and chronic stressor conditions are not well understood. It is possible that the distinct immunologic responses during chronic stress reflect the influence of a more complex neuroendocrine milieu.

Antigen-induced lymphocyte proliferation is one of the most basic immune system functions. Consequently, there is considerable interest in the mechanisms underlying altered proliferative responses associated with stress. It has been suggested that decreases in peripheral T-cell mitogenesis may be due, in part, to concomitant shifts in lymphocyte populations, specifically, an increase in CD8 cells and/or a diminished CD4:CD8 ratio. In this regard, experimental subjects here showed a relatively large reduction in the CD4:CD8 ratio, accompanied by a significant, albeit minimal, decline in mitogenesis following the task. Responses of control subjects, on the other hand, were

[4] For these six individuals, mean baseline and post-task values of PHA-stimulated mitogenic response [$\log_{10}$ (across five concentrations of PHA)], were 5 91 and 5 90 cpm, respectively; corresponding mean CD8 counts were 514 and 527 cells/mm^3.

characterized by a relatively large increase in T-lymphocyte proliferation *without* corresponding changes in cell numbers. Hence, the relationship between mitogenesis and acute alterations in lymphocyte populations remains unclear. It is possible, too, that true associations between these quantitative and functional measures are partly obscured by the fact that CD8 lymphocytes themselves subsume two distinct cell populations (true-suppressor and cytotoxic cells), and that only the former is immune-suppressive. Future assessments of true-suppressor cell populations would perhaps yield more consistent associations with proliferative changes. Finally, alterations in T-cell mitogenesis may be influenced by numerous other factors as well. For instance, examination stress has been shown to reduce IL-2 receptor expression on peripheral blood leukocytes (5, 6), and both IL-2 production and the expression of IL-2 receptors by activated T lymphocytes (neither of which were evaluated here) are necessary for antigen-stimulated proliferative responses.

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