BRAIN, BEHAVIOR, AND IMMUNITY 5, 339–348 (1991)

Cytokine Production and Lymphocyte Transformation during Stress

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The production of interleukin-1β (IL-1β) and interferon-γ (IFNγ) and blast transformation in peripheral blood mononuclear cells were assessed in medical students writing an academic examination. Blood samples were obtained on three occasions: (1) 1 month prior to the examination during a period of relatively low academic demand; (2) immediately after the examination; and (3) 10 days later. Results indicated that immune responses were significantly different immediately after the examination compared with the baseline and postexam measures. Lymphocyte responsiveness to both concanavalin A and pokeweed mitogen was decreased, as was the production of IFNγ, supporting earlier reports of immunosuppression after relatively commonplace stressors. In contrast to predictions, IL-1β production was significantly elevated after the examination. Cortisol levels were also measured, but did not change across the three sample points. Our finding of an increase in IL-1β production suggests that stress may have different effects on different cell populations by enhancing the responses of monocytes and depressing those of lymphocytes.

INTRODUCTION

A number of studies have shown that stressful events affect immunity in humans. For example, lymphoblast transformation in response to mitogens such as phytohaemagglutinin (PHA), concanavalin A (ConA), and pokeweed mitogen (PWM) is significantly suppressed during bereavement (Bartrop, Lazarus, Lockhurst, Kiloch, & Penny, 1977), in major depressive disorders (Schleifer, Kelly, Meyerson, Raskin, Davis, & Stein, 1984), and in medical students writing academic examinations (Dorian et al., 1982). Similarly several students have demonstrated suppressed natural killer cell activity both in medical students writing examinations (e.g., Glaser, Rice, Speicher, Stout, & Kiecolt-Glaser, 1986) and in subjects reporting high levels of life stress (Kiecolt-Glaser, Garner, Speicher, Penn, Holliday, & Glaser, 1984; Locke, 1984). Other studies on medical students writing examinations have demonstrated links between stress-related immunosuppression and virus reactivation. For example, Glaser, Kiecolt-Glaser, Speicher, and Holliday (1985) measured antibody titers to three herpes viruses (Epstein–Barr virus, Herpes simplex 1, and cytomegalovirus) before, during, and

[1] This research was supported by a Medical Research Council Studentship to J.P.D., and by a grant from the University Hospital Foundation.

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0889-1591/91 $3.00

after students had written an examination. They showed that antibody titers were highest at times following examinations stress, suggesting that cellular control of latent viruses may be impaired after stressful events.

In another study, Glaser, Rice, Sheridan, Fertel, Stout, Speicher, Pinsky, Kotur, Post, Beck, and Kiecolt-Glaser, (1987) measured a variety of immune responses in medical students across six sample points, three immediately after an academic examination and three during low stress points throughout the academic year. Higher antibody titers to Epstein–Barr virus were found during high stress periods. Furthermore, the production of interferon-γ (IFNγ) by peripheral blood mononuclear cells was substantially reduced during each of the three examination periods.

The finding of impaired IFNγ production raises questions about the production of other cytokines during stress. For example, Interleukin-1 (IL-1), a cytokine produced mainly by monocytes, has a proliferative effect on T- and B-cells and potentiates cellular responses to other cytokines. It also activates macrophages and natural killer cells either directly or indirectly by stimulating the production of other lymphokines, including IFNγ. IL-1 is the name given to the activity of two structurally different molecules IL-1α and IL-1β. Their biological effects were, until recently, believed to be indentical (Dinarello, 1988) but some difference are starting to emerge (Rasmussen, Kayser, Bech, Feeldt-Rasmussen, Perild & Bendtzen, 1990). IL-1β is the predominant form produced by human cells (Dinarello, 1988). IL-1 and IFNγ play an integrated, crucial role in the stimulation and regulation of the immune response, but have not been assessed simultaneously in stressed subjects.

Thus, the primary aim of this investigation was the concurrent assessment of IL-1 and IFNγ production, and lymphocyte blastogenesis in respone to ConA and PWM in first-year medical students writing an academic examination. Additionally, cortisol levels were assayed from both plasma and saliva to determine whether they were related to observed changes in immune function. In order to determine the magnitude of stress-related changes in these variables, a repeated measures design was employed in which subjects were tested on three occasions. The first sample provided baseline measures and was obtained during a period when subjects had not written an examination for at least 4 weeks; the second sample was 4 weeks later, immediately after subjects had written an important examination; and the third sample was obtained 10 days after the examination to evaluate recovery from any stress-induced changes.

The data we present in this paper suggest that while the functions of T- and B-cell populations were suppressed during stress, IL-1 production by monocytes was significantly enhanced.

METHODS

Subjects

Twenty-nine first-year medical students (16 males and 13 females) attending the University of Western Ontario participated in this study. The mean age was 24.4 and ranged from 21 to 29 years.

Procedure

Subjects participated on three occasions between January and March and at the same time of day, 1600 h, on each occasion. At time 1, subjects had not written an examination for at least 4 weeks, had no examinations scheduled for at least 4 weeks, and reported that other academic demands were at a minimum. At time 2, 4 weeks later, the subjects were studied immediately after having written an important academic examination. At time 3, approximately 10 days later, subjects were again relieved of academic demands and had just returned from a 1-week vacation.

On each occasion, subjects provided blood and saliva samples and completed questionnaires. Thirty milliliters of blood was drawn from an antecubital forearm vein with a 19-gauge needle into 10-ml sterilized, heparinized vacutubes.

Since salivary cortisol values are independent of salivary flow rate, untimed 5-ml samples of saliva were collected into 10-ml sterilized, plastic tubes.

Cell Separation

Peripheral blood mononuclear cells (PBMC) were separated from heparinized whole blood using Boyum's (1968) method of Ficoll/Hypaque centrifugation.

Lymphocyte Transformation

PBMC were stimulated by four different dosages of ConA (1, 5, 10, 100 μg/ml) and PWM (0.1, 0.5, 1, and 5%), and each test was run in triplicate. Control cultures were set up using RPMI and were also run in triplicate. One hundred microliters of mitogen was added to 5×10^4 cells in 100 μl RPMI with 20% human group AB serum in 96-well flat-bottom plates and incubated for 66 h in 5% CO_2 at 37°C. Twenty microliters of tritiated thymidine (0.5 μCi) was then added to each well, and cells were incubated for an additional 6 h.

The cells were harvested with a Skatron Titertek harvester onto filter paper, resuspended in scintillation fluid, and counted on a beta scintillation counter.

Lymphocyte proliferation responses were assessed at each of the previously mentioned concentrations of ConA and PWM. A dose–response curve with mitogen concentration along the abscissa and counts per minute (cpm) along the ordinate was computed for each subject at each sample point. In order to compare lymphocyte responsiveness to each mitogen across time, the area under the curve was calculated using the trapezoidal rule (Gibaldi & Perrier, 1982), producing a single value for each mitogen at each sample point, for each subject.

Monocyte Stimulation

Monocytes produce IL-1 in the presence of lipoprotein polysaccharide (LPS; Drakes, Harth, Galsworthy, & McCain, 1987). In order to compare the amount of IL-1 produced during direct stimulation versus baseline production, LPS-stimulated monocytes were compared with non-LPS-stimulated control monocytes. The methods used for culturing monocytes and assaying IL-1β have been described previously (Harth, McCain, & Cousin, 1990a).

Briefly, PBMC were diluted to a concentration of 5×10^6/ml in RPMI, divided

into aliquots, and allowed to adhere to plastic petri dishes for 2 h in 5% CO_2 at 37°C. Since monocytes adhere to plastic while lymphocytes do not, the nonadherent cells were washed off with RPMI. Monocytes were then stimulated with 20 μg LPS/ml in a culture medium of 1 ml of 5% AB serum, 1% L-glutamine, and 94% RPMI. Supernatants were removed with a Pasteur pipette, passed through a 0.2-μm filter, and frozen at −70°C until assayed.

ELISA for Interleukin-1β

The materials for the enzyme-linked immunoabsorbent assay (ELISA) for IL-1 were purchased from Cistron chemicals. The ELISA is a procedure conducted in microtitration wells coated with monoclonal antibody specific for IL-1β. A reference curve was constructed from the standards and sample values of IL-1β were reported in pg/ml. Each assay was run in duplicate. The values of IL-1 fell within the expected ranges, and the correlation between duplicates was 0.97.

Culturing Cells to Product IFNγ

PBMC were cultured to produce IFNγ. The methods for culture and for assaying IFNγ by a radioimmunoassay (RIA) have been described previously (Harth, Cousin, & McCain, 1990b). Briefly, PBMC were stimulated with ConA to produce IFNγ. A 0.5-ml suspension of 500,000 cells in 10% AB serum, 2% L-glutamine, 1% Hepes, and 94% RPMI was placed in a 12 × 75-mm tube, to which 5 μg of ConA in 0.5 ml RPMI was added. The cells were stimulated for 48 h in 5% CO_2 at 37°C. After 48 h the cells were centrifuged at 800*g* and the supernatants were stored at −70°C until assayed.

Assay of IFNγ

IFNγ was measured by RIA with materials purchased from Centocor (Malvern, PA). Each assay was run in duplicate, and several discrepant duplicates were reassayed. Values of IFNγ fell within expected ranges and the correlation between duplicates was 0.92.

Measurement of Salivary Unbound and Plasma Total Cortisol

Both saliva and plasma samples were frozen at −70°C until assayed. Plasma was obtained from blood samples after Ficoll/Hypaque cell separation. Both salivary free and plasma total cortisol levels were measured by RIA (Walker, Riad-Fahmy, and Read, 1978). Each assay was run in duplicate, and the intra-assay correlation was computed to be 0.92.

RESULTS

The data analyses assessed the effects of within-subject changes across the three sample periods (baseline, examination stress, and recovery) on lymphocyte transformation, IFNγ production, IL-1β production, and plasma and salivary cortisol levels. The dependent variables (DVs) were grouped together on the basis of similarity, and multivariate analyses of variance (MANOVAs) were employed to test the magnitude of variance accounted for by linear combinations of DVs. Three such groups of DVs were formed: cortisol levels (salivary and plasma

control), lymphocyte transformation (ConA and PWM), and cytokine production (IL-1β and IFNγ). The MANOVAs were computed using SPSS^x, analyzing the unique sums of squares where cell sizes were unequal.

Lymphocyte Transformation

A multivariate repeated measures analysis of variance was computed with ConA and PWM as DVs, each measured on the three occasions. Using Wilk's criterion (Kirk, 1982), the combined values of ConA and PWM changed significantly across time, $F(4,98) = 6.32$, $p < .001$, $\eta^2 = 0.37$.

Since Mauchley's sphericity test was significant ($p < .001$), the Geisser–Greenhouse conservation F test was used to evaluate the univariate effects (Kirk, 1982). This analysis revealed that both ConA ($F(1,25) = 9.81$, $p < .01$) and PWM ($F(1,25) = 9.88$, $p < .01$) changed significantly across time.

Scheffe's S test (Kir, 1982) revealed that there was a significant decrease in transformation from time 1 to time 2 for both ConA and PWM, a decrease that did not return to baseline levels at time 3.

These results are presented in Figs. 1 and 2. These figures depict the area under each dose–response curve, for each sample point, calculated using the trapezoidal rule (Gibaldi & Perrier, 1982). Univariate analyses of variance assessing changes across time for each dose in the response curve demonstrated the same pattern of change as depicted in Figs. 1 and 2. These analyses also revealed that all but one of these changes were statistically significant; the smallest dosage of ConA (0.1 μg) did not change significantly across time.

Cytokine Production

A multivariate repeated measures analysis showed that cytokine production changed across time ($F(4,94) = 11.88$, $p < .001$, $\eta^2 = 0.66$), and the Geisser–Greenhouse conservation F test revealed that this change was significant for both IL-1β ($F(1,24) = 18.42$, $p < .01$) and IFNγ ($F(1,24) = 5.48$, $p < .05$).

An evaluation of the means using Scheffe's S test revealed that the productions

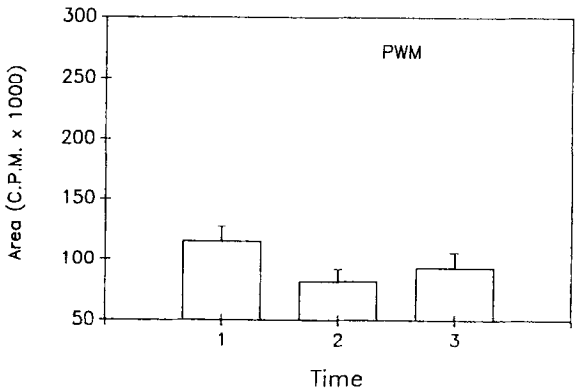

FIG. 1. Lymphocyte transformation in response to PWM at three different sampling times. Values along the ordinate represent the mean areas under the dose–response curves (blocks) +1 standard error of the mean (bar) and are expressed as cpm ×1000.

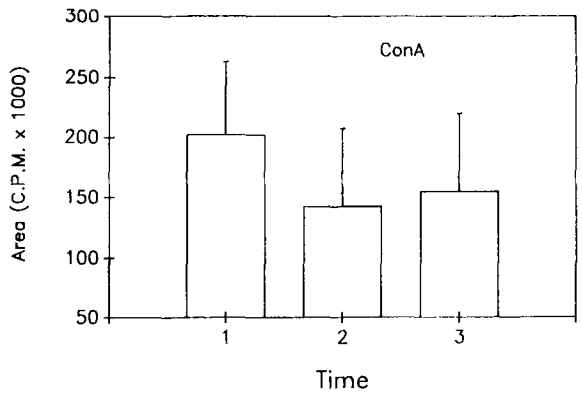

FIG. 2. Lymphocyte transformation in response to ConA at three different sampling times. For explanation of blocks and bars see the legend to Fig. 1.

of IL-1 and IFNγ were quite different across time (Fig. 3). Consistent with prior research, the production of IFNγ was significantly lower after the examination than at times 1 and 3 ($p < .05$). However, in contrast to predictions, the production of IL-1 was significantly higher during time 2, than at times 1 and 3 ($p < .01$).

Cortisol Production

A repeated measures multivariate analysis of covariance (MANCOVA) was performed on salivary and plasma cortisol levels, with oral contraceptives (coded as dichotomous variable) as a covariate. Although oral contraceptive use accounted for a significant proportion of variance in plasma control levels, the combined effects of salivary and plasma cortisol values did not change across time, $F(4,102) = .91$, ns.

DISCUSSION

The main purpose of this study was to replicate earlier reports of stress-related changes in immune functioning and extend these findings by assessing cytokine

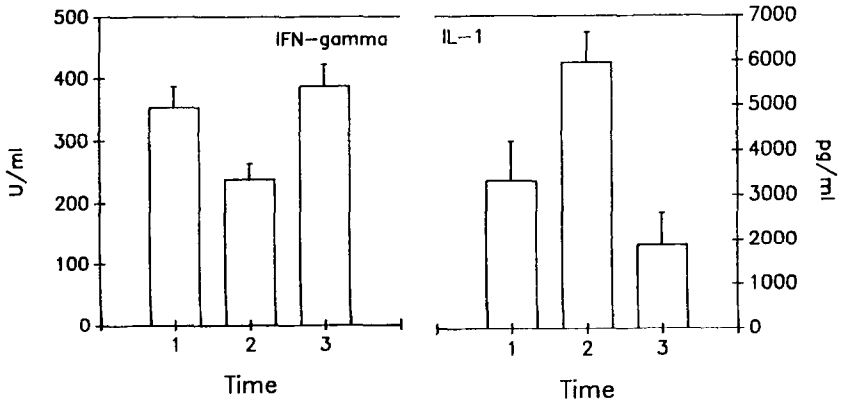

FIG. 3. Production of interferon-γ (IFNγ) and interleukin-1β (IL-1) at three sampling times. Mean values (blocks) + 1 SEM (bars) of IFNγ and IL-1 are expressed as units/ml or pg/ml, respectively.

production in addition to lymphocyte transformation. The results support the hypothesis that changes in the immune system occur with stress, but raise questions about the nature of these changes and whether they are necessarily immunosuppressive.

Consistent with earlier reports was the finding that lymphocyte transformation to both ConA and PWM was significantly impaired during stress. This result has been demonstrated previously with various populations including medical students (e.g., Kiecolt-Glaser, Glaser, Strain, Stout, Tarr, Holliday & Speicher, 1986), the bereaved (e.g., Bartrop et al., 1977), and astronauts (Kimzey, 1975).

A unique aspect of the present study was the reassessment of immune function 10 days after the examination. Previous studies have reported recovery from stress-induced immune suppression after periods of 4 and 8 weeks (Bartrop et al., 1977; Glaser et al., 1987).

In the present study, lymphocyte responsiveness to both ConA and PWM, assessed 10 days after the examination, showed that, although transformation at time 3 was greater than at time 2, the former was still significantly suppressed compared to baseline levels. Of special interest to this finding is that the third sample was obtained immediately after the medical students had returned from spring vacation. One conclusion is that cells are slow to recover after any stress-induced changes in immune function. It is also possible that a return from spring vacation may have itself induced stress. However, this second explanation is inconsistent with our findings that mood changes and subjective perceptions of stress had returned to baseline levels at time 3 (data not shown).

The production of IFNγ and IL-1β was affected by examination of stress in different and unexpected ways. A suppression of IFNγ levels after examinations has been previously reported (Glaser et al., 1987) and is consistent with evidence of a depressed lymphocyte proliferative response, since IFNγ is produced mainly by T-lymphocytes (Murray, 1988). However, in contrast to predictions, IL-1 levels were significantly higher after the exam.

IFNγ has a wide variety of immune-activating functions, including antimicrobial activation of macrophages, increased NK cell cytotoxicity, and stimulation of B-cell differentiation. Several acquired defects in IFNγ production are known to correlate with an increased incidence of opportunistic infections (Murray, 1988). It is unknown if stress-related suppression of IFNγ or stress-related decreased NK cell activity, previously reported (Glaser et al., 1986), lead to increased susceptibility to disease.

The lymphocyte-activating effects of interleukin-1 and its diverse roles as an inflammatory mediator are crucial responses to infection and injury. The increased IL-1 production could result in enhanced responses to infection and may represent an adaptive response to stress, perhaps compensating for other suppressed systems.

Our findings show a paradoxical dissociation between IFNγ and IL-1 production. IL-1 enhances T-cell activation, and IFNγ enhances endotoxin-induced IL-1 messenger RNA expression (Ucla, Roux-Lombard, Dayer, & Mach, 1990). One would expect the production of these two cytokines to parallel each other. Divergence between IFNγ and IL-1 production in our study suggests that stress

affects IL-1-producing cells such as monocytes in a very different manner from the way it affects IFNγ-producing lymphocytes.

There are numerous other examples of enhanced immunity during stress: for instance, enhanced T- and B-cell transformation in mice exposed to auditory stress (Monjan & Collector, 1977), increased interferon production during and after sleep deprivation (Palmblad, Cantell, & Strander, 1976), and enhanced salivary IgA levels after an academic examination (McClelland, Ross, and Patel, 1985). Recently, MacQueen et al. (1988) have demonstrated that mast cell function in anaphylaxis can be elicited by Pavlovian conditioning in response to an audiovisual cue.

It is not clear whether such processes are adaptive ("healthy") or maladaptive ("unhealthy"). While IL-1 plays a crucial immune-activating role during microbial infection, it also has a pathologic role when found in synovial fluid and may account for a variety of symptoms of rheumatoid arthritis (Firestein & Zvaifler, 1990).

Cortisol Production

Neither salivary unbound cortisol nor plasma total cortisol levels changed across the three sample points. A similar lack of association between salivary and plasma total cortisol production under experimental conditions, similar to ours has been previously reported (e.g., Allen, Batty, Dodd, Herbert, Hugh, Moore, Seymour, Shiers, Stacey, & Young, 1985). Properties of cortisol make it difficult to quantify, even within relatively short time frames. The clearance rate of free cortisol is relatively high, while that of cortisol bound to serum protein is relatively slow (Gorbman, Dickoff, Vigna, Clark, & Ralph, 1983). Salivary free cortisol is a better measure of immediate hormonal activity than total plasma levels (e.g., Walker et al., 1978; Williams & Dluhy, 1987). However, in the present study, the assessment of free and total cortisol levels after the examination, which lasted 2 h, may have been too late to reflect a stress response.

Limitations of this Study

The results of the present study warrant some caution with respect to their generalizability given the relatively unrepresentative population from which subjects were drawn (medical students), and the type of stress employed. Medical students are very familiar and highly skilled at dealing with a specific type of stress such as an examination; this is uncharacteristic of other populations experiencing stressors such as bereavement or changes in marital status.

This study was conducted in the late winter and early spring. While we cannot entirely rule out a seasonal factor, we think that it is unlikely; previous work from this laboratory on production of IFNγ and IL-1 by leukocytes from normal controls, sampled over the course of the year, did not suggest any trend toward a seasonal variation (Harth et al., 1990a,b).

In conclusion, the results of the present study provide additional support for the hypothesis that stress affects immune functioning. The relationship between stress and immunity however is complex; stress may stimulate some immune

responses, while depressing others. Other investigations are required to determine whether these alterations in immunity can affect the states of health.

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Received February 12, 1991