

Stress-Associated Modulation of Proto-Oncogene Expression in Human Peripheral Blood Leukocytes

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Changes in the cellular immune response associated with psychological stress were studied by using an academic stress model with medical students. The authors examined the expression of 2 proto-oncogenes, *c-myc* and *c-myb*, in peripheral blood leukocytes (PBLs) obtained from medical students at the time of examinations and at a baseline period approximately 1 month prior to the examinations. The level of messenger ribonucleic acid (mRNA) expression of both proto-oncogenes was significantly lower in PBLs obtained during examinations than in those from the baseline period. In addition, a significant decrease in the level of mRNA to the glucocorticoid receptor and gamma interferon was also found in the same preparations. The decrease in mRNA content of *c-myc*, *c-myb*, the glucocorticoid receptor, and gamma interferon in PBLs obtained from subjects during examinations is consistent with data from previous studies using the same model that have demonstrated a down-regulation of T-lymphocyte activation and proliferation in response to mitogens.

The interactions among the central nervous system (CNS) endocrine and immune systems are complex and operate at several levels. There is evidence that this communication is mediated through neuroendocrine peptides and hormones (Ader, Felten, & Cohen, 1991). There is also evidence suggesting direct connections between the CNS and specific subsets of lymphocytes, as demonstrated by physical contact between nerve endings and T-lymphocytes in spleens of rats (Felten & Olschowka, 1987).

The interactions among the CNS endocrine and immune systems include feedback mechanisms. One example is the feedback between the immune system and the CNS involved with interleukin-1, which is produced by macrophages and has the ability to modulate hypothalamic activity (Besedovsky & Del Rey, 1991). One result of this interaction is changes in levels of corticotropin-releasing factor secretion and adrenal corticotropin hormone stimulation (Cotman, Brinton, Galaburda, McEwen, & Schneider, 1987). There are broad changes in different aspects of the cellular immune response associated with glucocorticoids, which are modulated through the hypo-

thalamic pituitary-adrenal axis (Cotman et al., 1987).

Our laboratory is studying the mechanisms underlying these interactions and their health implications. In a series of studies, we have explored the consequences of a relatively commonplace stressful event for cellular immune function. We have used first- and second-year medical students enrolled at Ohio State University College of Medicine to investigate the effect of academic stress on immune function. Because the medical school curriculum is designed so that the students have several 2- or 3-day examination blocks across the academic year, the class cycles together through examinations and less stressful periods. At the time of examinations, we observed a down-regulation in natural killer (NK) cell activity, using two different target cells; a decrease in proliferation of peripheral blood leukocytes (PBLs) stimulated by concanavalin A (Con A) and phytohemagglutinin (PHA); a significant decrease in the ability of PBLs stimulated by Con A to synthesize gamma interferon (IFN- γ); a decrease in the expression of IL-2 receptors (IL-2R) and the synthesis of IL-2R mRNA in PBLs. Examination stress also modulated the ability of specific T-cell immunity to control the expression of two latent herpesviruses—herpes simplex virus and Epstein Barr virus (EBV) (Glaser, Kennedy, et al., 1990; Glaser, Kiecolt-Glaser, Speicher, & Holliday, 1985; Glaser, Kiecolt-Glaser, Stout, et al., 1985; Glaser, Pearson, Jones, et al., 1991; Glaser, Rice, Sheridan, et al., 1987; Kiecolt-Glaser, Garner, Speicher, Penn, & Glaser, 1984; Kiecolt-Glaser & Glaser, 1992).

We have continued these studies, exploring the impact of the physiological changes associated with psychological stress on the cellular immune response, and have found a decrease in the content of messenger ribonucleic acid (mRNA) expression of two proto-oncogenes, *c-myc* and *c-myb*, in mitogen-stimulated PBLs obtained at the time of examinations as compared with the content produced by PBLs obtained at a baseline period. We also measured the content of mRNA to the glucocorticoid receptor and to IFN- γ , and found a significant decrease in the expression of these two mRNAs in the same PBLs.

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Method

Subjects and Design

The subjects were 17 first-year medical students from Ohio State University College of Medicine who volunteered for a research project on stress and the immune response. In each of the examination periods reported, the students had a day of examinations on Wednesday, a study day on Thursday, and examinations again on Friday; all blood samples were obtained midday on Friday, immediately after the examination. Thus, the blood samples were drawn near the end of the 3-day examination sequence. Blood samples were also obtained at a low-stress baseline in October, 3 weeks before the examination period. We obtained blood samples and self-report data between 11:00 AM and 1:00 PM to control for diurnal variation; students had a break between blocks of morning and afternoon exams. The average age of the 17 subjects was 22.47 years; there were 12 men and 5 women.

Two self-report measures provided data on stress and anxiety: the Profile of Mood States (POMS; McNair, Lorr, & Dropelman, 1981) and the Perceived Stress Scale (PSS; Cohen & Williamson, 1988). The POMS is one of the best self-report measures for identifying and assessing transient, fluctuating mood states. The measure is widely used, has excellent normative data, and psychometrically is very strong in terms of both reliability and validity. We were particularly interested in the tension-anxiety scale, the most responsive scale in our medical student population to the short-term increases in distress associated with examinations. The POMS was administered each time blood was drawn.

The PSS is a 14-item scale that assesses global perceptions of stress and measures the degree to which an individual appraises situations in his or her life as unpredictable, uncontrollable, and overloading. Normative data are available from a national probability sample (Cohen & Williamson, 1988). The PSS was also administered each time blood was drawn.

Students were asked a number of health-behavior questions at baseline and again during examinations (e.g., they were asked to indicate how many hours they had slept within the last 3 days, any change in weight within the last week, and the number of alcoholic drinks consumed during the last 48 hr). They were also asked to list any medications taken in the last week, either prescription or nonprescription.

Preparation of Samples for mRNA Analysis and mRNA Measurements

We measured the content of mRNA to *c-myc* and *c-myb*, the glucocorticoid receptor, and IFN- γ in PBLs obtained from whole blood by using Ficoll-Hypaque density gradients. The PBLs were stimulated in culture with Con A and placed in culture within 6 hr following the drawing of the blood samples. Equal numbers of PBLs (7.5×10^6) were cultured at 37 °C for 48 hr at a concentration of 1×10^6 cells/ml in RPMI 1640 media with 5% FBS and 5 ug/ml Con A. RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987). The concentration of RNA was determined by spectrophotometric measurements at 260 nm.

To measure the levels of specific mRNA, RNA slot blots were prepared with 2.5 ug of RNA per slot. The RNA samples were diluted to a volume of 50 ul in water treated with dimethyl pyrocarbonate (DEPC). Thirty ul of 20 \times SSC and 20 ul of formaldehyde were added to each sample, and the sample was heated at 65 °C for 15 min. Duplicate samples of each RNA preparation were applied to a nitrocellulose membrane by using a Minifold II slot-blot apparatus

Table 1
Mean ($\pm SE_M$) Stress and Anxiety Ratings

| Scale | Baseline | | Examination | |
|------------------------|----------|-----------|-------------|-----------|
| | <i>M</i> | <i>SD</i> | <i>M</i> | <i>SD</i> |
| POMS Tension/Anxiety | 46.40 | 1.67 | 56.33 | 2.56 |
| Perceived Stress Scale | 20.94 | 1.23 | 25.82 | 1.86 |

Note. POMS = Profile of Mood States. All *ps* < .01.

(Schleicher and Schuell, Keene, NH). For each student, both baseline and stress samples were applied to the same membranes. For each cDNA probe, all membranes were prehybridized and hybridized together. Blots were prehybridized overnight at 42 °C in 5 \times SSPE, 5 \times Denhardt's solution, 0.50% SDS, 50% formamide, and 100 ug/ml heat denatured herring sperm DNA. Hybridization was then carried out for 16 hr in fresh prehybridization buffer with 10% dextran sulfate and 1×10^6 cpm/ml of 32 P-radiolabeled oligonucleotide probes. Oligonucleotide probes (40 mer), specific for human *c-myc*, *c-myb*, glucocorticoid-receptor, and IFN- γ genes, were obtained (Oncogene Science, Manhasset, NY). Each oligonucleotide was of the antisense orientation and was derived from sequences corresponding to the N-terminus of each protein. Oligonucleotide probes were radiolabeled by 5' end labeling with T4 polynucleotide kinase and purified with NENSORB 20 nucleic acid purification cartridges (NEN, Boston, MA). Blots were washed twice in 2 \times SSPE, 0.50% SDS at 42 °C. Blots were then exposed to X-ray film for 4-8 days. X-ray films were scanned with a Biorad densitometer and the integrated area (OD \times mm) was obtained for each sample.

Results

Self-Report Data

The data were analyzed by using repeated-measure analyses of variance with one within-subjects variable: the change from baseline to examinations. Consistent with our interpretation of the examination periods as higher stress periods, subjects reported significantly greater anxiety, $F(1, 14) = 18.89, p < .01$, and stress, $F(1, 16) = 10.81, p < .01$, during examinations compared with at baseline (see Table 1).

Students reported significantly less sleep in the 3 days before an examination blood draw than they had experienced before the low-stress baseline period. The students reported a mean ($\pm SE_M$) of 21.41 ± 0.57 hr of sleep at the baseline data collection compared with 16.12 ± 1.28 hr before examinations, $F(1, 16) = 14.62, p < .01$. Sleep was not significantly correlated with the mRNA levels, however (all *rs* less than .32). None of the students were ill at the time blood was drawn.

Minimal alcohol use was reported, with a maximum of six drinks reported by any subject within the previous 48 hr. Plasma albumin levels were within normal limits for all subjects at each data point, and weight did not fluctuate significantly, suggesting that nutritional deficits were not a factor in the observed cellular changes. Gender differences were not found for content of *c-myb*, glucocorticoid receptor, or IFN- γ mRNAs in the PBL samples. Levels of *c-myc* mRNA were significantly lower, however, in PBLs obtained from men than in those from women, $F(1, 16) = 5.62, p < .05$.

Effect of Academic Stress on the Expression of *c-myc* and *c-myb*

RNA was extracted from PBLs stimulated with Con A for 48 hr as previously described. DNA probes to *c-myc* and *c-myb* mRNA were then used to determine the content of mRNA to both gene products as a measure of activation of resting lymphocytes. The content of mRNA expression was calculated as relative intensity by using densitometry scanning as described earlier. Table 2 provides data on the means and SE_M s for each of the four probes, whereas Table 3 shows both the number and percentage of students showing a decrease in mRNA levels. The mRNA content was assayed in mitogen-treated PBLs; *c-myb*, $F(1, 16) = 21.63, p < .001$, decreased significantly in PBLs obtained during examinations compared with those obtained at baseline (see Table 2). The analysis also showed a trend toward a significant decrease for *c-myc*, $F(1, 16) = 3.31, p < .09$, but because several extreme outliers were present in the data, we performed a Wilcoxon matched-pairs signed-ranks test, the results of which were significant, $z = -1.96, p < .05$. The majority of subjects showed declines in mRNA content during examinations (see Table 3).

Expression of the Glucocorticoid Receptor and IFN- γ mRNA in PBLs

We then examined the same PBLs for expression of the glucocorticoid receptor and IFN- γ mRNAs in the same RNA preparations that had shown a decrease in mRNAs to both proto-oncogenes. As shown in Table 2, we found a significant decrease in the content of mRNAs to both products at the time of examinations as compared with baseline levels: glucocorticoid receptor, $F(1, 16) = 17.67, p < .001$; IFN- γ , $F(1, 16) = 36.36, p < .001$.

Discussion

T-lymphocytes are normally quiescent and only become activated on contact with their cognate antigens. Activation requires interaction of the T-cell receptor with processed antigen in association with self-histocompatibility molecules on antigen-presenting cells (Altman, Coggeshall, & Mustelin,

Table 2
Mean ($\pm SE_M$) mRNA Levels as Measured by OD of Slot-Blot Hybridizations by Using mRNA Prepared From Mitogen-Stimulated Cells (48 hrs)

| Probe | Relative intensity, $M \pm SE_M$ | | | |
|---------------------------|----------------------------------|-----------|-------------|-----------|
| | Baseline | | Examination | |
| | <i>M</i> | <i>SD</i> | <i>M</i> | <i>SD</i> |
| <i>c-myc</i> * | 0.29 | .04 | 0.20 | .05 |
| <i>c-myb</i> ** | 1.52 | .21 | 0.46 | .06 |
| Glucocorticoid receptor** | 0.17 | .03 | 0.04 | .01 |
| IFN- γ ** | 0.86 | .09 | 0.24 | .04 |

Note. mRNA = messenger ribonucleic acid; OD = optical density; IFN- γ = gamma interferon.

* $p < .05$ (Wilcoxon). ** $p < .001$.

Table 3

Subjects Showing a Decline in mRNA Levels From Baseline to Examination as Measured by OD of Slot-Blot Hybridization

| Probe | Number decreasing | % |
|-------------------------|-------------------|------|
| <i>c-myc</i> | 13/17 | 76.5 |
| <i>c-myb</i> | 16/17 | 94.1 |
| Glucocorticoid receptor | 14/17 | 82.4 |
| IFN- γ | 16/17 | 94.1 |

Note. mRNA = messenger ribonucleic acid; OD = optical density; IFN- γ = gamma interferon.

1990; Samelson, 1989). This interaction stimulates a sequence of gene activations that results in T-lymphocyte proliferation and lymphokine secretion (Crabtree, 1989). T-lymphocytes can also be activated in vitro by binding lectins, such as Con A and PHA, and by binding anti-CD3 monoclonal antibody to the T-cell receptor/CD3 complex.

In previous studies from our laboratory, we demonstrated that proliferative response of PBLs to Con A and PHA, as measured by ^3H TdR incorporation, was significantly lower in medical students at the time of examinations compared with baseline. Similarly, in an unpublished study, we found that monoclonal antibody to the CD3 component of the T-cell receptor complex is also less able to stimulate PBL proliferation at the time of examinations. Although these studies used experimentally induced polyclonal activation of T-lymphocytes, the sequence of gene activation is the same for antigen-activated T-lymphocytes (Crabtree, 1989).

The purpose of this study was to determine the impact of examination stress on gene expression of the proto-oncogenes *c-myc* and *c-myb*, as measured by the content of their mRNAs. As already discussed, we had previously demonstrated a down-regulation of a variety of immune measures, including mitogen-stimulated blastogenesis. In this study, we found a significant reduction in the content of *c-myc* and *c-myb* mRNA in PBLs obtained at the time of examination and a decrease in the specific memory (blastogenic) T-cell response to EBV polypeptides (Glaser, Pearson, Bonneau, et al., in press). A down-regulation of the level of gene expression to the glucocorticoid receptor and to IFN- γ , as measured by mRNA levels to both of these markers, was also found. The decrease in mRNA to IFN- γ is consistent with two earlier studies from our laboratory showing a decrease in the synthesis of INF- γ by PBLs treated with Con A, obtained at the time of examinations, compared with baseline control values (Glaser, Rice, Sheridan, et al., 1987; Glaser, Rice, Speicher, et al., 1986). We also found a significant decrease in mRNA to the glucocorticoid receptor.

Glucocorticoids were not measured in these medical students; however, we have measured plasma (mean hourly day and night values) and urinary glucocorticoids in other similarly designed medical-student studies in our Clinical Research Center. In those studies, we found no evidence of an increase in glucocorticoids in blood samples during examination days compared with baseline periods, even though we found an increase in stress and anxiety and evidence of negative immune

changes (Glaser, Pearl, et al., in press). In addition, no significant changes were found from baseline concentration when 24 urine-free cortisol levels were measured on a weekly basis from the 4 weeks between baseline and examinations. There are several other recent reports of a dissociation between glucocorticoid secretion and stress-associated immune changes (Cunnick, Lysle, Kucinski, & Rabin, 1990; Irwin, et al., 1991; Irwin, Vale, & Rivier, 1990; Jain et al., 1991; Sundar, Cierpial, Kilts, Ritchie, & Weiss, 1990).

Although the function of the *c-myc* and *c-myb* proto-oncogenes is not fully understood, these nuclear-localized proto-oncogenes have been implicated in mediating the intracellular events that regulate cellular proliferation. Expression of *c-myc* mRNA in T-lymphocytes can be detected within 30 min following mitogen stimulation (Kelly, Cochran, Stiles, & Leder, 1983; Reed, Alpers, & Nowell, 1986). T-lymphocytes cultured with antisense oligonucleotides complementary to *c-myc* RNA have delayed entry into S phase following mitogen stimulation, implying that *c-myc* is involved in the entry of T-lymphocytes into the S phase of the cell cycle (Heikkila et al., 1987). Expression of *c-myb* mRNA is first detected in T-lymphocytes 6-8 hr following mitogen stimulation and requires interaction of IL-2 with its receptor (Reed et al., 1986; Stern & Smith, 1986). Induction of *c-myb* precedes DNA synthesis, and culturing of T-lymphocytes with *c-myb* antisense oligonucleotides also prevents entry of T-lymphocytes into S phase and proliferation (Gewirtz et al., 1989; Venturelli, Travali, & Calabretta, 1990). Thus, the down-regulation of *c-myc* and *c-myb* mRNA levels by examination stress observed in this study is consistent with our previous studies that demonstrate a stress-induced down-regulation of T-lymphocytes to proliferate in response to mitogens, to an anti-CD3 monoclonal antibody, and to EBV polypeptides.

The stress-associated down-regulation of the level of glucocorticoid-receptor mRNA levels may also represent an effect of stress on T-cell activation. The number of glucocorticoid receptors (measured by dexamethasone binding) increased following T-cell activation, from a low of 2,700 receptors/cell in unstimulated human peripheral T-lymphocytes to 13,244 receptors/cell after stimulation with PHA for 20 hr (Wang, Joncourt, Kristensen, & DeWeck, 1984). This study, however, did not measure mRNA levels, so it is uncertain whether this receptor increase is the result of increased glucocorticoid receptor gene expression.

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