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Psychological research documents that the psychosocial burdens following breast cancer are notable in number, severity, and scope. A biobehavioral model of cancer stress and disease course has been proposed (see Andersen, Kiecolt-Glaser, & Glaser, 1994) and provides a conceptual basis for the proposed research. We are testing the model with a clinical trial: 235 women with stage II or III breast cancer who have been diagnosed and recently surgically treated are randomized between two conditions: (1) assessment and intervention, or (2) assessment only (control). In addition to documenting the quality of life benefits of a psychological intervention, this study provides an experimental test of the psychological and behavioral variables which may influence health outcomes directly. Further, we test specific mechanisms--alteration in immune and endocrine functions--to achieve beneficial health effects for women with breast cancer.

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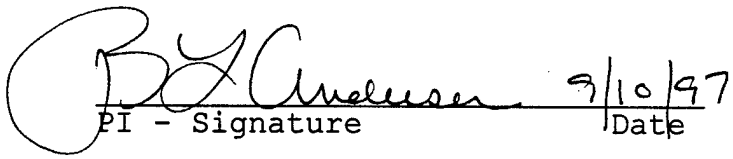

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Table of Contents

INTRODUCTION

Background of Previous Work

We have proposed a biobehavioral model of cancer stress and disease course (see Andersen, Kiecolt-Glaser, & Glaser, 1994, for a full discussion). The model identifies the psychological and behavioral factors and the biologic mechanisms by which health outcomes and cancer progression might be influenced. This model provides the conceptual basis for the proposed research (see **Fig. 1**). The present study is a randomized clinical trial testing the model.

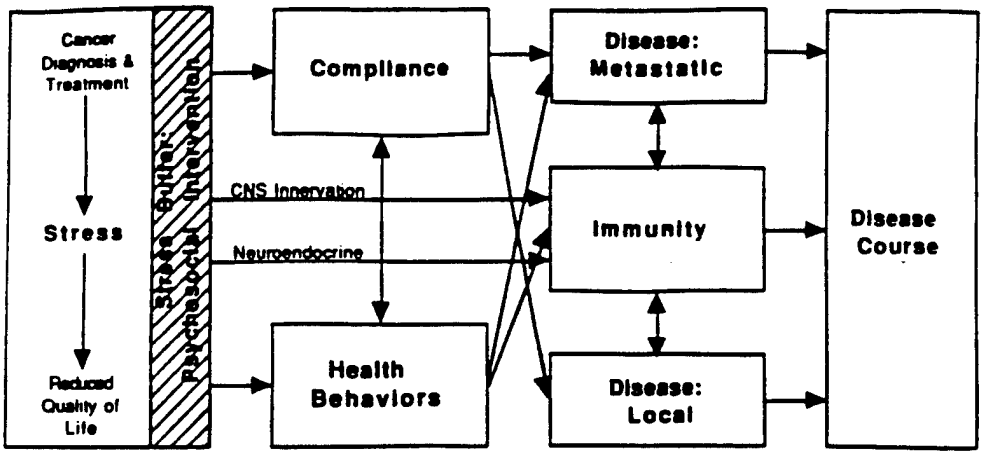

Figure 1: A biobehavioral model of the psychological (Stress and QoL), behavioral (compliance and health behaviors), and biologic pathways from cancer stressors to disease course. (CNS = Central Nervous System).

In late 1994 the US Army initiative in breast cancer (1 year sabbatical award for training in psychoneuroimmunology) and research funds from the American Cancer Society (ACS; a two year award and a one year renewal) and were awarded to the PI for preliminary studies and pilot data collection. In the fall of 1994 the sabatical award funded a one course reduction and the PI was able to enroll in the graduate course in Molecular and Cellular Immunology (Dent 513) at the Ohio State University. These were the activities funded by the sabatical. The research funds from the ACS enabled the randomized trial to begin, although every component of the study was significantly under funded. Nevertheless, the monies were maximally stretched to pilot the intervention, hire and train personnel, and approximately 40 subjects were randomized and followed. Having completed the immunology coursework, the sabatical funds enabled the PI to manage this very large project and also serve as the co therapist for the intervention groups for the trial.

Purpose of the Present Work (DAMD17-96-1-6294)

We are conducting a randomized clinical trial (see **Table 1**). Women with stage II or III breast cancer are randomized within strata to one of two arms: psychological/behavioral intervention or a no intervention (assessment only) condition. The intervention consists of two phases; an intensive phase of weekly meetings for four months and a subsequent maintenance phase of monthly meetings for an additional eight months. Our hypothesis is that women being treated with the psychological intervention regimen will show lowered stress, increased QoL, improved health behaviors and compliance, and an increase in immune functioning. And, in turn, women treated with the intervention will show a doubling (ratio of median durations = 2.0) in time to recurrence with a .05 level of significance and power of 0.80, one-sided test.

Army funding in 1996 enabled this large, important effort to continue beyond the pilot phase. Full funding has enabled us to move agressively ahead on subject accrual, complete the backlog of previously unfunded tasks, and, importantly, expand the biologic aspect of the project to include a breast cancer specific immune assay mucin-1 (MUC-1) and an endocrine panel of measures (e.g. serum coritsol, catecholamines, prolactin, growth hormone). Three types of preliminary data are provided, per the statement of work: Task 1 (Recruitment), Task 2 (Intervention Groups), Task 3 and 4 (Data Collection and Analysis).

Table 1: Schematic diagram of the research design for subjects across the 4 years of study participation.

	YEAR 1				YEARS 2-4	
	Dx./Ca. Trt	Follow up (months)			Cont. Follow up (months)	
Grp	0	4	8	12	6	12
1	x----------Inten------x----Maintenance---x--Maintenance--x				x	x
2	x----------None-----x--------None-------x-------None-----x				x	x

Note: *Dx.* = Cancer diagnosis and *Ca.Trt.* = Beginning of initial cancer treatment; Inten(sive) = Weekly (x18) intervention sessions with reliability/validity checks on intervention integrity; Maintenance = Monthly (x8) intervention sessions with reliability/validity checks; *x* = Psychological, health behavior, compliance, and immune and endocrine assessments and disease endpoints.

BODY

Task 1: Recruitment and Accrual.

Eligible women are newly diagnosed and/or recently treated (i.e. < 3 months post surgery) women with Stage II or III invasive breast cancer who are ≥ 20 years of age. Considering accrual thus far, "up front" refusal rates are running 28%, and 12 month dropout rate is extremely low, 6%. The literature suggests that refusal rates in psychosocial intervention studies have ranged from 10 to 25% (Andersen, 1992). In considering the drop out rate, studies of low and moderate risk patients (i.e. Stage I - III: Andersen, 1992) were considered. The literature suggests that dropout rates range from 9% to 27% for the studies which have provided data for initial to 12 month assessments. Thus, the actual rates in this study are extremely good. Also, we have conducted analyses examining the potential for biases between the groups on sociodemographic, disease or health variables, and current analyses find no significant group differences on any variable at the intial assessment between the study arms.

Below in **Table 2** is an actual and projected accrual tally for the accrual rate and resulting numbers of psychological/medical/immune assessments for grant years 1-4. This summary is based on an annual projected rate of 120 potential subjects approached for recruitment per year, 90 Ss actually recruited and assessed per year, with 68 Ss retained after 3 years of study participation. Per subject, there are 4 assessments during year 1 of participation (90 Ss x 4 = 360) and 2 assessments/year for years 2-4 (assuming 90 Ss x 2 = 180 for years 2 and 3; 68 Ss x 2 = 136 for year 4) of participation. Rates designated with an asterisk (*) are projections.

Table 2: Accrual: Actual rate and projections for remainder of grant period.

Grant year	Accrual	Assessments by Accrual Year				Assessment Summary
		Pilot	1	2	3	N/year
Pilot	45	180	—	—	—	180
1st year	90	90	180	—	—	270
2nd year	90*	90*	90*	180*	—	360*
3rd year	90*	90*	90*	90*	180*	450*
4th year	—	90*	90*	90*	90*	360*

Task 2: Intervention Group

Six cohorts of intervention groups have been conducted. There is presently a 12% drop out rate in the intervention arm. Because this rate is so low, it is difficult to single out particular reasons for drop out, however clinical data would suggest that women with prior psychiatric histories (e.g. major depression, agorophobia) are at greatest risk, and we are vigorously attending to these conditions to meet the needs of these women and retain them in the study.

Task 3 and 4: Data Collection and Analysis

These tasks have gone extremely well with the added funding. The first major paper from the project, entitled "Stress and immune responses following surgical treatment for regional breast cancer," is in press at the Journal of the National Cancer Institute and is provided in its entirety in the appendix. This paper provides a complete documentation of the methods, procedures, results, and discussion of the data from the project thus far, and so those areas are not repeated below, however we do provide a brief summary of the findings.

We examined the relationship between stress and several aspects of the cellular immune response in the context of the diagnosis of breast cancer and the post surgery period. Women (N = 116) recently surgically treated for Stage II (70%) or III (30%) invasive breast cancer participated. Prior to beginning adjuvant therapy, all completed a validated questionnaire assessing stress about the cancer experience and provided a 60cc blood sample. A panel of natural killer (NK) cell and T-cell assays were conducted: 1) NK cell lysis; 2) the response of NK cells to recombinant gamma interferon (rIFN-γ) and recombinant interleukin-2 (rIL-2); 3) blastogenic response of peripheral blood leukocytes (PBLs) to phytohemagglutinin A (PHA) and concanavalin A (ConA) and the proliferative response of PBLs to a monoclonal antibody (MAb) to the t-cell receptor (T3).

Multiple regression models were used to test the contribution of psychological stress in predicting immune function. We hypothesized a negative relationship between stress and immunity, and expected this relationship to be replicated between assays and within a single assay [i.e. replicated across effector to target (E:T) cell ratios for NK cell lysis, for example]. All regression equations controlled for variables which might also be expected to exert short or long term effects on these responses, such as age, stage of disease, and length of time of surgical recovery, and ruled out other potentially confounding variables (e.g. nutritional status) that might also be influential. These controls reduced the plausibly of alternative, rival hypothesis for the findings.

Significant effects were found and replicated between and within assays, including the following: 1) stress significantly ($p < .05$) predicted NK cell lysis; 2) stress significantly ($p < .01$) predicted the response of NK cells to rIFN-γ; 3) stress significantly predicted the response of PBLs to ConA ($p < .05$) and PHA ($p < .05$), and the proliferative response to the T3 MAb ($p < .05$). The cells from 62% of the sample did not respond to rIL-2, but stress was not a factor in predicting the response for the remainder of the sample (38%). The data show that the physiologic effects of stress inhibited a panel of cellular immune responses, cancer relevant NK cell cytotoxicity and T cell responses. Our longitudinal studies determine the stability of this effect, its health consequences, and the biobehavioral mechanisms for any adverse health outcomes.

CONCLUSIONS

To summarize, we view stress, QoL, health behaviors, and compliance as the major factors in a conceptual model of adjustment to the cancer stressor. Also part of the model are the physiological systems--the endocrine and immune systems--which may be important ones for moderating the effects of stress on disease processes. More specifically, we would look to NK cell function and antibody production to MUC-1 as providing an important "windows" on the immune response and a panel of endocrine measures--but cortisol in particular--as an important stress hormone marker. The literature confirms that QoL benefits accrue from psychological interventions. In contrast, health behaviors and compliance have rarely been an intervention target, although data suggest that such a broadened approach would be effective and provide added power in the examination of stress/endocrine/immunity question.

The context of randomly assigning individuals to conditions that will result in differential psychological and behavioral outcomes (Andersen, 1992) provides one of the necessary conditions for an experimental test of the biobehavioral model. A "simple" experimental design--treatment vs. no treatment--was the strategic next step, as such a design provides cause--effect data for the presence of an intervention producing enhanced psychological and behavioral outcomes, immune responses, and health effects. In addition to the biobehavioral model, the specific design of the intervention, with intensive and maintenance phases, is novel as a maintenance component may be critical to achieve the longterm psychological /behavioral gains necessary to effect endocrine and immune responses and/or disease progression. In sum, the biobehavioral model provides a testable, conceptualization for PNI research in breast cancer and provides an opportunity to test for specific biologic or health consequences of psychological/behavioral interventions for breast cancer patients.

Tasks 1-4 in the Statement of Work have been accomplished. Accrual is proceeding at the projected rate and the refusal rate is in the range projected and the drop out rate is exceedingly low for a study as intensive as the present one. Six cohorts of intervention groups have been conducted with only a 12% drop out rate. Adequate funding has provided staffing appropriate for the high demand of this project, including the need for psychological/behavioral and medical assessors, laboratory technicians, and data management. The additional army grant funds have enabled us to expand the biological assessment to include a critical breast cancer specific immune assay (MUC-1) and an endocrine panel. Finally, our first empirical paper from the project has been accepted in a top tier journal, Journal of the National Cancer Institute. It shows that the physiologic effects of stress inhibited a panel of cellular immune responses, cancer relevant NK cell cytotoxicity and T cell responses. The longitudinal data from this study will be able to determine the stability of this effect, its health consequences, and the biobehavioral mechanisms for any adverse health outcomes.

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Stress and immune responses following surgical treatment for regional breast cancer

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Abstract

Background: Adults undergoing chronic stressors experience high rates of adjustment difficulties and important biologic effects--including effects on the immune system. For individuals with cancer, the immune system may be relevant to host resistance against progression and metastatic spread. *Purpose:* We examined the relationship between stress and several aspects of the cellular immune response in the context of the diagnosis of breast cancer and the post surgery period. *Methods:* Women (N = 116) recently surgically treated for Stage II (70%) or III (30%) invasive breast cancer participated. Prior to beginning adjuvant therapy, all completed a validated questionnaire assessing stress about the cancer experience and provided a 60cc blood sample. A panel of natural killer (NK) cell and T-cell assays were conducted: 1) NK cell lysis; 2) the response of NK cells to recombinant gamma interferon (rIFN-γ) and recombinant interleukin-2 (rIL-2); 3) blastogenic response of peripheral blood leukocytes (PBLs) to phytohemagglutinin A (PHA) and concanavalin A (ConA) and the proliferative response of PBLs to a monoclonal antibody (MAb) to the t-cell receptor (T3). *Results:* Multiple regression models were used to test the contribution of psychological stress in predicting immune function. We hypothesized a negative relationship between stress and immunity, and expected this relationship to be replicated between assays and within a single assay [i.e. replicated across effector to target (E:T) cell ratios for NK cell lysis, for example]. All regression equations controlled for variables which might also be expected to exert short or long term effects on these responses, such as age, stage of disease, and length of time of surgical recovery, and ruled out other potentially confounding variables (e.g. nutritional status) that might also be influential. These controls reduced the plausibly of alternative, rival hypotheses for the findings. Significant effects were found and replicated between and within assays, including the following: 1) stress significantly ($p < .05$) predicted NK cell lysis; 2) stress significantly ($p < .01$) predicted the response of NK cells to rIFN-γ; 3) stress significantly predicted the response of PBLs to ConA ($p < .05$) and PHA ($p < .05$), and the proliferative response to the T3 MAb ($p < .05$). The cells from 62% of the sample did not respond to rIL-2, but

stress was not a factor in predicting the response for the remainder of the sample (38%). *Conclusions:* The data show that the physiologic effects of stress inhibited a panel of cellular immune responses, cancer relevant NK cell cytotoxicity and T cell responses. *Implications:* Longitudinal studies will need to determine the stability of this effect, its health consequences, and the biobehavioral mechanisms for any adverse health outcomes.

Stress and immune responses following surgical treatment for regional breast cancer

A cancer diagnosis and cancer treatments are objective, negative events. Although negative events do not always produce stress and lowered quality of life, data from many studies document severe, acute, stress at cancer diagnosis (1) and during recovery (2). The negative psychological responses of individuals with cancer are important in their own right, as they are targets for cancer control efforts (3,4). In addition, data suggest that stress responses are accompanied by non random (i.e. correlated) negative changes in a broad range of immune responses. The present study examines the biobehavioral relationship between stress and immunity in the context of the diagnosis of breast cancer and the post surgery period (5).

Meta analyses (6, 7) conclude that psychological stress and the experience of life stressors are reliably associated with immune alterations in non cancer subject samples. Further, the direction of this linear relationship is negative. That is, "higher" levels of stress (e.g. self reports of stress or negative affects such as sadness or clinical diagnoses of depression) are related to "lower" indicators of cellular immunity, including both quantitative and functional responses, such as lowered natural killer (NK) cell lysis. This effect has been regularly found for individuals in the midst of chronic stressors, with some of the largest responses and changes found for lengthy stressors and ones which have interpersonal components.

Illustrative data comes from Kiecolt-Glaser, Glaser and colleagues, who have followed individuals with the long, stressful experience of care giving for a spouse diagnosed with Alzheimer's Disease (8-11). Not surprising, care givers report high levels of distress and negative affect as they cope with their relative's difficult behavior and mental deterioration (8). Moreover, these researchers have found, for example, that NK cells obtained from care givers are less responsive to the cytokine recombinant gamma interferon (rIFN-γ) and recombinant interleukin-2 (rIL-2) than are cells obtained from matched community controls (9), there is a poorer proliferative response to mitogens (8), there are significant deficits in the antibody and virus-specific T-cell

responses to an influenza virus vaccine (10), and there are stress related defects in wound repair (11).

There are fewer data on the relationship between stress and immunity in the context of cancer. Levy, Herberman and colleagues (12) reported on these relationships at 3 months post treatment (lumpectomy or mastectomy with or without adjuvant therapy) for 66 women with Stage I or II disease. In addition to estrogen receptor (ER) status predicting NK cell lysis, social support--a variable hypothesized to *reduce* stress--added significantly to a regression model predicting *higher* NK cell activity. These data suggest that how a person responds to stress may also influence the impact of stress on the immune response.

There is considerable evidence that tumor-bearing patients express abnormal cellular immune responses; this has been found for many different types of malignant disease (13-15), including breast cancer (16, 17). Stressors are not generic, and they would not be expected to have identical physiologic outcomes. So too, the immune response involves a cascade of responses and events that can occur over time. For reasons such as these, we used a homogeneous breast cancer subject sample and timing of assessment to test the relationship between stress and several components of the cellular immune response, including NK cell and T-cell functions.

Women who had been diagnosed and surgically treated were studied prior to the initiation of adjuvant therapy. Since we were interested in the contribution of stress in predicting an immune response above and beyond known correlates, we conducted our statistical analyses controlling for naturally occurring factors that effect the immune response, specifically age, disease burden (nodal status), and recovery (days since surgery; 18). As the immune system contains a considerable amount of redundancy, we focused on three components that would provide individually important, but complimentary, information.

First, because NK cells are believed to act early in the immune response and they have been demonstrated to play an important role in immune surveillance against tumors and virally infected cells (19-21), we measured NK cell lysis. Second, we measured the ability of the NK cells to

respond to rIFN-γ and rIL-2. It has been shown that lymphokine-activated killer (LAK) cells are highly cytotoxic against a wide variety of tumor cells in comparison to those lysed by resting NK cells (22), an effect found in patients with breast cancer (23). Finally, to obtain information on the T-cell response, we measured the proliferation of peripheral blood leukocytes (PBLs) to two mitogens [phytohemagglutinin (PHA) and concanavalin A, (ConA)], and we induced proliferation by stimulating the T-cells with a monoclonal antibody (MAb) to the T-cell receptor.

Subjects and Methods

Patient eligibility and data collection

Participants were 116 women who were diagnosed and recently surgically treated (i.e. < 4 months post surgery) for invasive breast cancer, but they had not yet begun adjuvant treatment. Women were from 14 to 101 days (mean = 37, median = 33) post surgery for Stage II (70%) or III (30%) invasive breast cancer. They ranged in age from 31 to 84 years (mean = 52 years). Recruited consecutively from mid 1994 to early 1997, the majority (82%) were being treated at a NCI designated University affiliated Comprehensive Cancer Center, and the remainder (18%) were receiving treatment at local community hospitals. All women came to the General Clinical Research Center at the university for the collection of psychological, behavioral, and medical data and a 60cc blood draw. Assessments were conducted between 8:00 am and 12:00 am to reduce diurnal variability.

Stress measure

The Impact of Events Scale (IES; 24) is a standardized self report questionnaire used to examine intrusive thoughts ("I had dreams about being a cancer patient," "Other things kept making me think about cancer") and avoidant thoughts and actions ("I tried not to talk about it," "I was aware that I still had a lot of feelings about cancer, but i didn't deal with them") concerning cancer. Fifteen items are used and women rate each event or feeling in terms of the frequency (i.e. "not at all," "rarely," "sometimes," and "often") of occurrence during the previous seven days. Scores can range from 0 to 75. For this sample, descriptive statistics were: range from 0 to 65, mean = 26, median =25, and standard deviation = 15.2. The scale has satisfactory reliability with

internal consistency of .78-.82 and two-week test-retest reliability of .79-.89, respectively. The validity of the measure is suggested by data indicating that individuals who experience involuntary, distress-related cognitions following traumatic life events are also those who psychologically suffer the greatest negative effects (e.g. 2).

Immune assays

Blood cell separation. From 60 cc of venous blood, PBLs were isolated using Ficoll (Pharmacia) gradients. The isolated leukocytes were then washed in calcium and magnesium free phosphate buffered saline (PBS) and counted on a Coulter Counter. Aliquots of 8 x 10^6 isolated PBLs were suspended again in 0.8ml of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 0.75% sodium bicarbonate, 2 mM L-glutamine, and 10 ug/ml of Ciproflaxacin.

Quantification of total T lymphocytes, T cell subsets, and NK cells. Isolated PBLs were absorbed with MAbs conjugated to either fluorescein isothiocyanate (FITC) or rhodamine (RDI) according to the cell surface marker being studied: total T cells (CD3, FITC), T4 subset (CD4, RDI), T8 subset (CD8, FITC), and NK cells (CD56, RDI). All MAbs were purchased from Coulter Corporation. Briefly, 0.5 x 10^6 cells were incubated with the MAb for 15 minutes at room temperature. After the incubation, the cells were fixed and the red cells lysed with Optilyse (Coulter), following the manufacturer's instructions. Samples were analyzed using a Coulter EPICS Profile II flow cytometer as previously described (8).

NK cell cytotoxicity. To determine NK cell activity, a microtiter ^{51}Cr-release cytotoxicity assay was used as previously described (9, 28). The target cells used were K-562 cells, an NK cell sensitive myeloid cell line. Target cells, labeled overnight for 16 hr with ^{51}Cr, were placed in triplicate wells of 96-well V-bottom plates and PBLs were added, resulting in effector to target (E:T) cell ratios of 100:1, 50:1, 25:1, 12.5:1, and 6.25:1.

NK cell response to cytokines. Procedures for treatment of PBLs with rIFN-γ and rIL-2 involved preparing isolated PBLs at a concentration of 3 x 10^6 cells/ml in complete RPMI 1640 medium, then seeding into 3 replicate tissue culture tubes (Falcon) at 6 x 10^6 cells/tube. Cells were

incubated in complete RPMI 1640 medium alone, complete medium supplemented with 250 international units (IU)/ml rINF-γ or 60 IU/ml of rIL-2 (Genzyme Corporation). Cell suspensions were gently mixed and then incubated at 37C in an atmosphere of 5% CO_2 for 65 hours. For the assay, triplicate aliquots of cell suspensions were placed in wells of V-bottom plates, with E:T cell ratios of 50:1, 25:1, 12.5:1, 6.25:1, or 3.13:1. In addition, 6 wells with target cells and medium only, and target cells with detergent (5% SDS in PBS) were prepared to determine spontaneously released chromium and maximal lysis, respectively. The plates were centrifuged at 300xg for 5 minutes to bring the effector and target cells into close contact and then incubated at 37 C in an atmosphere of 5% CO_2 for 5 hours. Following this incubation, the plates were centrifuged at 300xg for 5 minutes, 100ul of supernatant was collected from each well, and counts per minute (CPM) were determined using a Beckman 9000 gamma counter as previously described (9, 25).

Blastogenic response to PHA, ConA, and MAb to the T3 Receptor. The concentrations for PHA and ConA used were 2.5, 5.0, and 10.0 ug/ml. To measure the blastogenic response to the MAb to the T-cell receptor, three dilutions of the purified MAb were used; 1:32, 1:64, and 1:128. For all three assays isolated PBLs seeded in triplicate at 0.5 x 10^5/wells were incubated for 68 hours at 37 C in 96-well flat-bottom plates, and then labeled for 4 hours with MTS (Promega) to measure proliferative response. Briefly, the MTS procedure is a non-radioactive calorimetric procedure which labels metabolically active cells via reduction of a colored substrate. The amount of proliferation was determined by optical density (OD) of the suspension in the well. OD determinations were performed using a Titertek Multiscan MCC microplate reader at 492nm as the determination wavelength, and 690nm as the reference wavelength as has been noted (26, 27).

Statistical Analyses

Preliminary. Prior to conducting the principal analyses, we checked the data for the contribution of "nuisance" variables (covariates) that could potentially be related to psychological stress, immune outcomes, or both (see 28 for a discussion). The variables examined were measures of aspirin, alcohol, caffeine and nicotine intake, sleep, plasma albumin level (as an

indicator of nutritional status), incidence of recent infectious illness, and the Karnofsky performance status rating. We examined the relationships between these variables and each of the three sets of outcome variables: NK cell lysis, ability of NK cells to respond to rIFN-γ and rIL-2, and the blastogenic response of PBLs to ConA, PHA, and the T3 MAb. Analysis of variance was used for the categorical, and simple correlations were used for numerically scaled, independent variables.

Screening of these potential covariates involved examination of relationships between 11 covariates and 20 dependent variables, or a total of 220 bivariate associations. Of these 220, 15 were found to be statistically significant using a .05 significance level. This number of significant effects is only slightly more than would be expected by chance alone (i.e., 220 x .05 = 11). Inspection of the significant relationships showed that many of them were attributable to the influence of a few outliers. Yet, to be conservative, all regression analyses described below were run twice, once including and once excluding those covariates with significant bivariate associations with the relevant dependent variables. In no case were results of the regression analyses significantly altered by the inclusion of the covariates. Given this fact and the consistently weak relationships of the covariates to the dependent variables, we do not report further results involving the covariates.

Principal. The principal analyses assess the relationship between the IES measure of psychological stress and three sets of outcome measures: (a) NK cell lysis at five E:T ratios; (b) response of NK cells to rIFN-γ and rIL-2 stimulation at five E:T ratios each; and (c) the PBL blastogenic response to PHA and ConA and proliferative response to the T3 MAb at three concentrations/dilutions each.

We were interested in the role of stress in predicting these outcomes beyond the contribution of disease and recovery variables which may impact on the immune response. Thus, three variables were chosen for control: (1) age, as it is associated with down-regulation of the immune system; (2) disease stage, as an indicator of extent/burden of disease; and, (3) days since

surgery, as an indicator of the duration of recovery from surgical stress and related factors (e.g. anesthesia).

We tested the contribution of psychological stress in predicting the immune outcomes with hierarchical multiple regression (29). This procedure enters variables in a specified sequence and, at the final step, provides a test of the variance of the dependent variable (immune outcome) due to the predictor (stress) above and beyond the contribution of the control variables (age, stage, and days since surgery). In these regression analyses, age, days since surgery, and IES were considered as numerical variables. Stage was a categorical variable with 2 levels: II vs. III.

For all of the analyses described below, missing data were managed using the pairwise deletion technique, wherein each bivariate association is estimated using all subjects for whom measures on both variables are available. This approach allows for complete usage of available data in comparison to alternative procedures (e.g. listwise deletion). For all dependent variables except response of NK cells to rIFN-γ, the quantity of missing data was small, with never more than 10 observations missing for any bivariate association. Effective sample sizes for regression analyses ranged from 113 for NK cell lysis ratios to 103 for T3 values. For rIFN-γ measures, sample sizes varied from 85 to 49 across the range of concentrations employed.

For the analyses, three regression models are provided. Model A includes only the control (independent) variables (i.e. age, nodal status, and days since surgery) in predicting the immune outcome (e.g. NK cell lysis). Predictors in Model A were introduced simultaneously because we had no basis nor strong interest in investigating their effects in any particular sequence. Model B, includes the three control variables as well as the psychological stress variable (IES) in the prediction of the immune outcome. Of particular interest in this analysis was the increment in the squared multiple correlation (R^2) from Model A to Model B (i.e. R^2_{B-A}), indicating variance in a dependent variable (e.g. NK cell lysis) attributable to stress (IES) beyond that explained by the control predictors. In addition, the standardized regression beta (ß) for the psychological stress variable (IES) in Model B (i.e. $ß_{IES}$) indicates the magnitude and direction of the influence of this

predictor on the dependent variable. The significance of the β weight was also tested; a one-tail test was used as we a priori hypothesized a *negative* influence of psychological stress on the immune parameters. Finally, Model C indicates the contribution of psychological stress as the lone predictor; this third model provides the simple association between psychological stress and immune function.

Results

Analyses predicting NK cell lysis

Table 1 provides the results from the three models, A-C, predicting NK cell lysis. For Model A, using age, stage, and days since surgery as independent variables, R^2_A was small and non-significant for every E:T ratio (all F-ratios were <1.0). Since the percentage of NK cells available would be a contributing factor in total NK cell activity as measured by lysis, we next added the percentage of NK cells as determined by flow cytometry into the analyses as an additional, independent control variable as shown by the step labeled Model AA. Across all E:T ratios the R^2_{AA} suggested that indeed, significant variance was added, as predicted, yielding the R^2_{AA} values, ranging from .085 to .25.

More importantly, the effect of the addition of the stress variable (IES) as a predictor is shown in Model B. The R^2_B was noticeably larger than R^2_{AA}, and it provided a significant increment in prediction across all E:T ratios for lysis. These data indicate that the measure of psychological stress accounted for significant variance in NK cell lysis above and beyond that explained by age, nodal status, days since surgery, and percentage of NK cells. Moreover, the sign of the regression β coefficient for IES is negative, as predicted, indicating that an increases in measured stress is associated with a decline in NK cell lysis. The t-tests for these coefficients are significant at every E:T ratio. Also, no other predictor in Model B had a significant regression coefficient.

For elaboration, we also provide the regression results when only IES is used as a predictor, eliminating the control predictors from the model, presented as Model C in Table 1.

These results show that the simple association between IES and each E:T ratio for NK cell lysis is statistically significant.

Analyses predicting the response of NK cells to cytokines

Results for the NK cell response to rIFN-γ are provided in Table 2 and show a similar pattern. For Model A, using age, stage, and days since surgery as independent variables, R^2_A was small to moderate, ranging from .025 to .138. Notably, the addition of stress (IES) to the Model B regression shows that in all but one E:T ratio (50:1) the R^2 values are statistically significant. Considering the increments due to IES, R^2_{B-A}, these are significant and range from .054 to .119, reflecting the proportion of variance in the cell response accounted for by stress (IES) beyond that explained by the control variables. Again, the negative β weights for IES in Model B indicate a negative influence of psychological stress on the response of the NK cells to rIFN-γ. Again, no other predictor in Model B had a significant regression coefficient. Finally, results for Model C in Table 2 show the simple association of IES with the rIFN-γ response. These correlations are significant at four of the five E:T ratios, with proportions of variance accounted for being in the range of .077 to .149.

A parallel set of regressions were attempted for the response of NK cells to rIL-2. A large portion of the sample (62%) had no response to rIL-2. When the regressions were conducted on the remainder of the sample (38%), the addition of stress (IES) in Model B produced a significant R^2 value at the 25:1 E:T ratio only. It appeared that the majority of the subjects NK cells were not responsive to treatment with rIL-2.

Analyses predicting blastogenic response of PBLs to ConA, PHA, and the T3 MAb

Table 3 shows regression results for the ConA and PHA blastogenic responses across three concentrations each. As the findings are similar for both assays, they will be discussed together. For Model A, using age, stage, and days since surgery as independent variables, R^2_A for ConA

ranged from .035 to .054, and of similar magnitude for PHA, ranging from .022 to .033. Since the number of total T-cells available will affect the blastogenesis values, we next added the number of T3 cells into the analyses as an additional, independent control variable as shown by the step Model AA. Across all concentrations for each mitogen, the R^2_{AA} suggested that indeed, additional variance was added, yielding the R^2_{AA} values ranging from .105 to .125 for ConA and R^2_{AA} values ranging from .023 to .033 for PHA.

Notably, the addition of stress (IES) to the regression added significant variance as indicated in Model B. All of the R^2 values are statistically significant. Considering the increments in R^2 due to stress (IES), these are significant and range from .032 to .061 for ConA, and from .074 to .083 for PHA, reflecting the proportion of variance in the cell response accounted for by IES beyond that explained by the control variables. Again, the negative β weights for IES in Model B indicate a negative influence of psychological stress on the blastogenic responses across concentrations. Again, no other predictor in Model B had a significant regression coefficient. Finally, results for Model C in Table 3 show the simple association of IES with the blastogenic responses. These correlations are significant for each concentration of ConA and PHA.

Table 4 shows regression results for the proliferative response of t-cells to three different dilutions of the T3 MAb. For Model A, the control R^2 values were not significant for any dilution. Addition of number of T3 cells available as a control increased the variance accounted for as shown by the step Model AA. The R^2_{AA} values ranged from .088 to .143. However, increments in R^2 due to the addition of stress (IES), as shown by $R^2_{B\text{-}AA}$, were significant, ranging from .056 to .067. This indicates that about 6% of the variance was accounted for by stress (IES) beyond that explained by the control variables. Once again, no other predictor in Model B had a significant regression coefficient. Results for Model C again show the simple, significant association of stress (IES) with the response to the T3 MAb at all dilutions, with R^2 values of .09 to .10.

Discussion

Any immune response involves a complex cascade of events that occur over time. Studies

suggest that peripheral products of stress can play numerous roles in regulating this response, and so the effects of stress will, necessarily, be variable. Current research suggests, for example, that the occurrence of acute stressors, both real (e.g. parachute jumps; 30) and artificial (e.g. experimental tasks including speech or math stress; 31) are correlated with the mobilization (increase) of NK cells. These changes are thought to be a result of changes in cell trafficking. In contrast, studies of chronic stressors (e.g. bereavement, care giving, divorce; 7, 9) suggest, in the main, an effect on the ability of NK cells to lyse a target cell, the ability of NK cells to respond to rIFN-γ and rIL-2 in vitro, and other aspects of the cellular immune response.

The results obtained in this study suggest that stress, as assessed via a self report measure of intrusive and avoidant thoughts and behaviors about cancer, was related to a negative effect on NK cell lysis, the ability of NK cells to respond to two cytokines, the blastogenic response of PBLs to two mitogens, and to the MAb response to the T-cell receptor. These effects were inhibitory and of similar magnitude (i.e. reliable), both between the assays and within an assay (i.e. across E:T ratios, mitogen concentrations). The analyses controlled for variables which might also be expected to exert short or long term effects on these responses, such as age, stage of disease, and length of time of surgical recovery, and ruled out other potentially confounding variables (e.g. nutritional status) that might also be influential. These controls reduce the plausibly of alternative, rival hypotheses for these consistent findings.

It is recognized that NK cells mediate natural immunity, but, some would suggest their role in health has generally been underestimated (32). For example, there is evidence to suggest that the NK cells participate either directly or indirectly in multiple developmental, regulatory, and communication networks of the immune system. Further, NK cells are efficient effector cells which are not only equipped for cell killing but also capable of rapid responses to exogenous or endogenous signals by producing cytokines and other factors involved in interactions between immune and non immune cells (20).

The ability to spontaneously lyse a broad range of infected targets or tumor cells is the best-known functional attribute of NK cells (20, 22). Consistent with previous reports, these data

suggest that stress may impair this important process. These findings highlight the specific effect of cancer stress on immune function, whereas prior data by Levy (33) had suggested that women's reports of fatigue were related to lower levels of NK cell lysis. Chronically low levels of NK cell activity occur in patients with cancer, particularly when there are large tumor burdens or disseminated metastases (32). In general, patients with low NK cell activity appear to be at higher risk for infections, to have more prolonged diseases, or to suffer more severe symptoms than those patients whose NK cell activity remains normal (32, 34).

A variety of agents (e.g. biological response modifiers) are known to increase the activation, proliferation, or cytotoxicity of NK cells (20). Among the best-known activators of NK cells are IL-2 and IFN-γ. Our data show that the physiological changes associated with psychological stress inhibited NK cell lysis. Stress also effected the ability of NK cells to respond to rIFN-γ, consistent with two previous reports involving other life stressors (i.e. caregiving for a spouse with Alzheimer's Disease; 9, 25). Interestingly, NK cells from 62% of the women did not respond at all to rIL-2. In post hoc analyses of women who did have an rIL-2 response contrasted with those who did not, no stress or disease variable differentiated the groups. Further studies will need to be preformed in order to explore this result, however, it is possible that the lack of responsiveness of NK cells to rIL-2 may be due to an over production of prostaglandin E2 by monocytes. It has been suggested that PGE2 decreases IL-2 production in effector cell populations resulting in the down regulation of the expression of the IL-2 receptor on NK cells obtained from breast cancer patients (23). Follow up studies will need to pursue and clarify this difference in cytokine responses.

It has been shown that the ability of PBLs to respond to PHA is reduced, in general, in cancer patients (35); this lowered response is related to tumor burden and declines with disease progression (36). The negative effect of stress on blastogenesis was replicated in this study across two mitogens, PHA and ConA, as well as the response of T-cells to a MAb to the T-cell receptor. These findings are consistent with correlational and experimental studies indicating that stress

impairs the blastogenic response of PBLs to mitogens and virus specific T-cell responses (e.g. 8, 10, 37-39). Mitogen induced proliferation has been used to indicate the immune system's ability to respond to antigens (pathogens). Data from chronically stressed but healthy individuals showing decrements in the cellular immune response (including NK cell lysis and the response of the PBLs to mitogens across proliferative responses) also subsequently report a higher incidence of infectious illness (8). If this effect is reliable, these data would suggest that cancer patients evidencing high levels of stress, lowered levels of responsive T-lymphocytes, and decreased NK cell function may be at greater risk for infectious illness as they go on to adjuvant therapy.

Interestingly, there is accumulating evidence that suggest that psychological/behavioral stress reduction interventions may *enhance* certain aspects of the cellular immune response, including NK cell lysis. An important early effort was that by Kiecolt-Glaser, Glaser and colleagues (40) who studied 61 healthy retirement home adults. Following a one month of training in progressive muscle relaxation, intervention subjects evidenced a 30% increase in NK lysis in comparison with no treatment and social contact only control groups. Fawzy and colleagues (41) studied 61 melanoma patients, and reported that at 6 month post treatment, intervention subjects had significantly greater levels of IFN-α augmented NK cell activity than no treatment controls. These data suggest that if behavioral interventions can reduce stress and enhance the cellular immune response, then health outcomes might be, in turn, positively impacted.

In conclusion, the data presented here show a down regulation of different aspects of the cellular immune response associated with the psychological stress accompanying cancer diagnosis and initial surgical treatment. We note that these study participants are part of a larger effort testing the biobehavioral aspects of stress, immunity, and disease course (5). It will be important to document the longitudinal nature of these findings, and future studies will provide such data. Moreover, half of the women are randomized to receive a psychological/behavioral intervention specifically designed reduce stress, enhance quality of life, and test for the biologic mechanisms, such as immune responses, which may mediate any positive effects on health and disease outcomes.

Notes

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Table 1. Results of Regression Analyses for Predicting NK Lysis

	Dependent Variable					
	NK100	NK50	NK25	NK12.5	NK6.25	NK3.125
Model A						
R^2_A	.005	.007	.012	.015	.020	.023
Model AA						
R^2_{AA}	.085	.148	.185	.233	.250	.241
Model B						
R^2_B	.135	.212	.238	.268	.275	.253
$R^2_{B\text{-}AA}$	.050	.064	.053	.035	.025	.012
β_{IES}	−.234	−.265	−.240	−.194	−.165	−.115
t (df=110)	−2.462	−2.921	−2.672	−2.223	−1.892	−1.280
p (1-tail)	.008	.002	.004	.014	.031	.102
Model C						
R^2_C	.067	.091	.084	.066	.056	.032
t (df=110)	−2.826	−3.338	−3.199	−2.811	−2.558	−1.867
p (1-tail)	.003	.001	.001	.003	.006	.033

Model A includes age, days since surgery, and stage of disease.
Model AA includes Model A variables plus the percentage of NK cells.
Model B includes Model AA variables plus IES (score on impact of events scale).
Model C includes IES only.

Table 2. Regression Analyses for Predicting Outcomes of NK Cells Stimulated with Gamma Interferon across 5 E:T ratios

	Dependent Variable				
	50:1	25:1	12.5:1	6.25:1	3.17:1
Model A					
R^2_A	.025	.097	.080	.138	.124
Model B					
R^2_B	.041	.151	.199	.257	.208
R^2_{B-A}	.016	.054	.119	.119	.084
β_{IES}	-.128	-.244	-.358	-.358	-.301
t	-1.104	-2.190	-3.203	-3.084	-2.083
df	82	81	74	65	46
p (1-tail)	.137	.016	.001	.002	.022
Model C					
R^2_C	.015	.077	.149	.149	.088
t	-1.128	-2.586	-3.581	-3.343	-2.080
df	82	81	74	65	46
p (1-tail)	.132	.006	.001	.001	.022

Model A: Includes age, days since surgery, and stage of disease as predictors
Model AA: Includes Model A variables plus NK cell percent as predictors
Model B: Includes Model AA variables plus stress (IES) as predictors
Model C: Includes stress (IES) only as a predictor

Table 3. Results of Regression Analyses for Predicting the Blastogenic Response to ConA and PHA across 3 concentrations each

	Concentrations					
	ConA			PHA		
	10 ug/ml	5 ug/ml	2.5 ug/ml	10 ug/ml	5 ug/ml	2.5 ug/ml
Model A						
R^2_A	.035	.043	.054	.022	.024	.033
Model AA						
R^2_{AA}	.105	.125	.115	.023	.024	.033
Model B						
R^2_B	.166	.174	.147	.083	.074	.080
$R^2_{B\text{-}AA}$	.061	.049	.032	.060	.050	.047
β_{IES}	-.255	-.229	-.187	-.256	-.234	-.229
t (*df*=103)	-2.668	-2.401	-1.927	-2.521	-2.299	-2.254
p (1-tail)	.005	.009	.029	.007	.012	.013
Model C						
R^2_C	.053	.065	.053	.070	.054	.052
t (*df*=108)	-2.443	-2.724	-2.443	-2.857	-2.489	-2.441
p (1-tail)	.008	.004	.008	.003	.007	.008

Model A includes age, days since surgery, and stage of disease.
Model AA includes Model A variables plus T cell count.
Model B includes Model A variables plus IES (score on impact of events scale).
Model C includes IES only.

Table 4. Results of Regression Analyses for Predicting T3 Cell Response Across 3 Dilutions

	Dilutions		
	128:1	64:1	32:1
Model A			
R^2_A	.026	.052	.064
Model AA			
R^2_{AA}	.088	.104	.143
Model B			
R^2_B	.155	.160	.200
R^2_{B-A}	.067	.056	.057
β_{IES}	-.273	-.249	-.252
t(df=101)	-2.747	-2.514	-2.604
p (1-tail)	.004	.007	.006
Model C			
R^2_C	.102	.092	.094
t(df=101)	-3.452	-3.255	-3.307
p (1-tail)	.001	.001	.001

Model A includes age, days since surgery, and stage of disease.
Model AA includes Model A variables plus T cell count.
Model B includes Model AA variables plus IES (score on impact of events scale).
Model C includes IES only.