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Challenges in Interpreting Cytokine Biomarkers in Biobehavioral Research: A Breast Cancer Exemplar

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Abstract

Purpose—This report extends the findings of a prior study comparing the level of plasma cytokines in women with breast cancer to those of women with a benign breast biopsy with the addition of a normal comparison group. The results of this three-group comparison are presented as background for discussing several methodologic challenges for biobehavioral research in inflammatory-based conditions.

Method—This study used a descriptive, cross-sectional design to compare the levels of plasma cytokines in women with breast cancer, women with a benign breast biopsy, and a normal comparison group. The levels of 17 cytokines were measured using multiplex bead array assays (Bio-Plex®). Data analysis included a variety of descriptive and graphical techniques to illustrate between-group differences in cytokine profiles.

Results—The levels of plasma cytokines in the sample of 35 women who had recently been diagnosed with breast cancer, 24 women with a suspicious breast mass, who subsequently were found to have a benign breast biopsy, and 33 women in a normal comparison group present a background for discussing the implications of extreme between-group differences for biobehavioral nursing research. Both the levels of individual cytokines and their patterns were distinctly different in the three groups.

Conclusion—The exemplar presented from the three-group comparison has implications for planning biobehavioral nursing research in patients with conditions characterized by inflammation.

Keywords

cytokines; immune; inflammatory

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Biobehavioral research deals with interrelationships among biological, psychosocial, and behavioral factors and health (Pellmar, Brandt, & Baird, 2002). For nursing researchers, the biobehavioral paradigm that integrates biological and behavioral variables may provide significant opportunities for developing both a more holistic understanding of the phenomena of health illness and lead to the development and refinement of nursing interventions that facilitate positive biological and behavioral outcomes. It is now well accepted that the immune response and subsequent activation of inflammatory mediators may contribute to the development of psychological and behavioral alterations in both medically ill and medically healthy individuals (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). Cytokines are important components of the inflammatory response and have been associated with psychological and behavioral alterations in multiple populations. However, most biobehavioral studies have not considered how preexisting between-group differences in cytokine levels may affect relationships between cytokines and psychological and/or behavioral variables. In addition, most studies have measured levels of a small number of individual cytokines. In the study described here, we extended the findings of a prior study comparing the levels of plasma cytokines in women with breast cancer to those of women with a benign breast biopsy (Lyon, McCain, Walter, & Schubert, 2008) by the addition of a comparison group and extended analytical approaches. The results of this three-group comparison are presented as a background for discussing several methodologic concerns for biobehavioral research in inflammatory-based conditions.

Background

Biobehavioral research has achieved prominence as a paradigm that acknowledges the importance of both biological and psychosocial phenomena to health promotion and disease outcomes (Grady, 2006). Frequently, nursing interventions in a biobehavioral paradigm have focused on changing psychological, behavioral, and/or biological outcomes via modulation of a psychophysiological response, such as the stress response. Inflammatory responses affect the hypothalamic-pituitary-adrenal (HPA) axis, leading to neuroimmune disturbances including fatigue, depressive symptoms, pain, and cognitive impairment (Quan & Herkenham, 2002). Replacing more traditional functional markers, cytokine levels are increasingly being measured as biomarkers of inflammatory activation in biobehavioral research. Cytokines are small proteins that are central regulatory components of the inflammatory response. They have been of interest to biobehavioral researchers because of their association with psychological and physical responses to multiple conditions. Peripheral cytokines can access the brain and influence pathophysiological domains including effects on the HPA axis and metabolism of neurotransmitters such as serotonin (5HT) and dopamine (DA). Cytokines may also activate corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) and lead to the subsequent production and/or release of adrenocorticotrophic hormone (ACTH) and glucocorticoids (especially cortisol; Raison, Capuron, & Miller, 2006).

In conditions characterized by inflammation, such as breast cancer (Coussens & Werb, 2002), the host response to the tumor and treatments including surgery, chemotherapy, and radiation are all associated with significant tissue damage and destruction. These responses lead to an innate immune response including a predominance of proinflammatory cytokine activation (Miller, Ancoli-Israel, Bower, Capuron, & Irwin, 2008). Within the context of cancer, biobehavioral researchers have investigated the relationships of cytokines with psychological and behavioral outcomes including depressed mood, fatigue, pain, and sleep disorders. However, while there have been mostly consistent findings of increased levels of prototypical proinflammatory cytokines such as interleukin 6 (IL-6) in association with such symptoms as depressed mood and fatigue in patients with acute infections, the relationship between increased levels of circulating proinflammatory cytokines and psychological and

behavioral outcomes has been less consistent in patients with inflammatory conditions such as rheumatoid arthritis (Davis et al., 2008), Sjogren's syndrome (Hartkamp et al., 2004), and cancer (Dimeo et al., 2004). Although there may be multiple reasons for the lack of consistent relationships among symptoms and cytokines in inflammatory-based conditions, a known and significant contributor is the host response to disease. The intensity of host inflammatory activation could be attenuating relationships among cytokines and psychobehavioral variables.

In addition to consideration of the host response, another potential methodologic issue affecting study results is that the measurement of cytokines has been limited by available technology. Enzyme-linked immunosorbent assay (ELISA) techniques require relatively large amounts of sample volume for each measure, thus limiting the number of cytokine analyses that can be done per sample. Consequently, in most studies using a biobehavioral framework, investigators have measured single cytokines or a few cytokines representing a predefined immune response and examined these values to determine whether they correlate with a psychological or a behavioral variable of interest. This limitation may have led to false negatives in interpreting relationships among cytokines and psychobehavioral variables. In addition, though ELISA assays have good reproducibility and offer researchers a basis for comparing values across studies, the differences among ELISA kits and the use of different techniques (e.g., cell stimulation) and different samples (serum, plasma, and cell culture) lead to some difficulty with across-study comparisons.

With the advent of proteomic methods such as multiplex technology, researchers have an opportunity to simultaneously measure multiple cytokines in small sample volumes (Leng et al., 2008). Because the activity of an individual protein is dependent not only on its abundance but also on the effects of its interaction with and/or modification by antagonistic and synergistic proteins (Kingsmore, 2006), measuring multiple cytokines simultaneously permits both an absolute measure of levels of individual cytokines and a relative measure of each cytokine in proportion to others in the system. Innovation in measuring cytokines is consistent with the move toward “systems science” in its assertion that the nature of a system cannot be understood by studying the component parts in isolation (Mabry, Olster, Morgan, & Abrams, 2008).

To provide an empirical basis for a discussion of emerging issues in biobehavioral research methods related to cytokine measurement, we use as an exemplar an extension of a prior study investigating differences in cytokine patterns in women with breast cancer compared to women who underwent a breast biopsy and were subsequently determined to not have breast cancer (Lyon et al., 2008) to which was added a “normal” female comparison group. These three groups were selected to compare one with breast cancer to characterize the inflammatory response associated with cancer, one prior to a biopsy found to be benign to characterize a group with heightened psychological distress and no breast cancer, and a normal comparison group. We show baseline group differences in cytokine patterns, graphically display the levels of cytokines grouped into three standard classifications, and discuss the implications for biobehavioral research methods in inflammatory-based conditions.

Method

Participant Procedure

The procedure for collecting and processing the samples from women with breast cancer ($n = 35$) and women with a benign breast biopsy ($n = 23$) has been described elsewhere (Lyon et al., 2008). An additional sample of “normal” women was recruited for a comparison group, after permission was received from the institutional review board for the addition to

the original protocol. Inclusion criteria were female, between the ages of 30 and 65, not pregnant and no history of cancer. In addition to having a 5 cc sample of blood collected between the hours of 8 and 9 a.m., each participant completed a demographic and lifestyle questionnaire. Participants received a \$10 gratuity for participating.

Cytokine Levels—Levels of cytokines in plasma were determined using a Bio-Plex suspension array system (Bio-Rad, Inc.) with a standard 17-plex cytokine detection kit according to the manufacturer's protocol. The Bio-Plex Human Cytokine 17-Plex panel includes human interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN- γ), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 β (MIP-1 β), and tumor necrosis factor alpha (TNF- α). Samples with cytokine concentrations below the detection limit were assigned an averaged value between 0 and the lowest detectable level in each assay plate before log transformation so that all samples were retained in the data set. The Bio-Plex assay combines fluorescent flow cytometry and ELISA technology. In a liquid suspension array, 5.6 μ m beads were prepared by the assay manufacturer so that each bead is internally dyed with different ratios of two spectrally distinct fluorophores to assign it a unique fluorescent signature. In addition, each bead with a particular fluorescent signature is conjugated with a specific anti-cytokine antibody (e.g., anti-IL-2 and anti-IL-4) that binds only a specific cytokine. The beads were mixed and incubated with the subject's sample in a microplate well, permitting each bead to bind to the specific cytokine it recognized in proportion to the amount of that cytokine in the sample. Following incubation, biotinylated anti-cytokine antibodies (reporter antibodies) were added and also bound to cytokines in the sample. The samples were washed to remove unbound antibodies and streptavidin was added to activate the reporter antibodies. The sample was then drawn into the flow cytometer of the Bio-Plex array reader, where two lasers excited the beads individually. One laser excited the dyes in each bead, identifying that bead's unique fluorescent signature, and the other laser excited the reporter antibody associated with the bead-bound cytokine. For each of the 17 cytokines in every sample, 100 beads specific for that cytokine were assayed and the mean cytokine binding for the sample was determined. Thus, the Bio-Plex runs 100 “duplicates” of each analyte. The manufacturer reports that the assay accurately measures cytokine values in the range of 1–2,500 pg/ml (well within the required limits of detection for this project), is precise (intra-assay CV <10%, interassay CV <15%), and shows less than 1% cross-reactivity among cytokines or with other molecules. This process provided simultaneous quantification of each of the 17 cytokines being assayed in the sample.

Data Analysis

Sample demographics were computed by group for race, menopausal status, age, and body mass index (BMI). Associations between race and group and menopausal status and group were tested using Fisher's exact test. Significant differences in age by group were analyzed using analysis of variance (ANOVA). The Wilcoxon rank sum test was used to test for differences in BMI by group because BMI was not normally distributed. Descriptive statistics, including median, mean, and standard deviation, were computed for each of the cytokines. Because the cytokine values were not normally distributed, significant differences between groups were tested using the nonparametric Kruskal-Wallis test on the median values. Family-wise error rate was set to $\alpha = .05$. Thus, individual p values were corrected using the Bonferroni procedure, and median values were compared using the $\alpha .05/17 = .0029$ level of significance. Furthermore, the levels of cytokines were examined through the use of profile plots (often called spaghetti plots) to visually depict the pattern of cytokines. These plots are generated by graphing each individual's values for the cytokine measures, using the cytokines as identifiers on the x-axis and the value for the cytokine measure on the

y-axis. Each individual's measurements are connected with a line that creates the “spaghetti” look. These plots were generated for each group by cytokine categorization.

Results

Demographic characteristics are presented in Table 1. Average age ranged from 46.6 to 50.0 years (p value for group differences = .61). A majority of the women in each group were White and most of the rest were Black. There were no significant associations between race and group (p = .58). Menopausal status and group were significantly associated (p .01); women in the comparison group were more likely to be premenopausal than women in the benign biopsy or cancer groups.

Cytokine values are presented by group in Table 2. All cytokine values were significantly different between the groups, with the exception of IFN- γ . The differences remained after using a Bonferroni corrected α (.05/17 = .0029). In general, most of the cytokines exhibited lower median levels in the comparison group and higher median levels in the women with cancer than those in women in the benign biopsy group. Median levels of IL-5 and G-CSF levels were similar in the benign biopsy group and cancer group, while median levels of IL-8 and MCP-1 were slightly higher for the comparison group than the benign biopsy group. IFN- γ median levels were similar across all three groups.

Profile plots of the cytokine levels in the three groups are shown separately. Figure 1 depicts the 17-cytokine panel, Figure 2 depicts the proinflammatory cytokines, and Figure 3 depicts the anti-inflammatory cytokines. The graphical pattern analyses depicted in the profile plots revealed a different overall pattern of cytokines and different pro- and anti-inflammatory patterns across the groups. Different patterns by groups may be noted when plotting all 17 cytokines together (Figure 1), such as the striking differences seen in elevated IL-1 β levels in the benign biopsy and breast cancer groups as compared to the normal comparison group.

In addition, in Figure 1, visible differences in the patterns can be discerned, with the plot in the cancer group having a taller shape with more jagged peaks than the other two groups. The plot in the comparison group had the most variability. Participants in the comparison group had higher levels of IL-8, IFN- γ , and IL-17 and lower values of IL-1 β and TNF- α than those in the other two groups. From the anti-inflammatory plots in Figure 2, we see that most women had higher values for IL-4, IL-10, and IL-12 in the benign biopsy and cancer groups, although two participants from the normal comparison group had exceptionally high levels of these cytokines. Overall, Figure 3 demonstrates that the proinflammatory profile for the women in the benign biopsy group was relatively suppressed as compared to women in the normal and breast cancer groups.

Discussion

The examination of between-group differences in cytokine levels and cytokine patterns using a breast cancer exemplar demonstrates several important research considerations for biobehavioral research in inflammatory-based conditions. For biobehavioral researchers, consideration of the underlying condition is important prior to selecting inflammatory biomarkers. For patients with cancer, conceptualizing the nature and intensity of cytokine responses to the tumor and malignant process is necessary prior to selecting inflammatory biomarkers to correlate with psychological and behavioral variables. Further description of the effects of the host response on biological markers of inflammation is important for better understanding of possible a priori differences within and between groups. As demonstrated by results from this study, the extreme group differences could have attenuated the linear relationships among the cytokines and psychosocial variables of interest, such as depressive

symptoms. Linear techniques may have yielded ceiling or floor effects on single-cytokine measures.

In addition to considering the context of the host inflammatory response, particularly in inflammatory-based conditions, development of biobehavioral science depends on conceptual and methodological clarity regarding the measurement of biomarkers, such as cytokines, that function in a complex and dynamic system. The cytokine network is characterized by interdependence, pleiotropy (cytokines have multiple effects), and redundancy (multiple cytokines have the same effect). Thus, the effects of cytokines may dampen or amplify the effects of other cytokines, making the context of the cytokine within the system as important as any individual measurement (Ozaki & Leonard, 2002). The current pro- and anti-inflammatory cytokine taxonomies are limited by the dichotomous distinction between cytokine types. This simplistic classification system does not account for the context of cytokine functioning within a particular system and a particular host response pattern. However, dichotomous classification systems are still used to categorize cytokines and are used in biobehavioral research to classify correlates of behavioral and psychosocial phenomena. These categorical classifications may limit further understanding of the interplay among cytokines and psychosocial phenomenon.

Although multiplexed methods do not yet have established reference ranges for specific populations, the measurement of multiple cytokines simultaneously enables assessment of relative proportions of cytokines within a system. Furthermore, while assays have become increasingly sensitive, individual values may still be below the level of detection. With the relative novelty of proteomic methods and reference ranges still in development, there is little basis for comparison. For example, in a recent study evaluating four multiplex kits (Bio-Plex, LINCOplex, Fluorokine, and Beadlyte) for the analysis of immunomodulators in plasma of patients with rheumatoid arthritis (RA), profiles of significantly elevated immunomodulators were detected by at least three of the four kits (BioPlex, LINCOplex, and Beadlyte; Khan et al., 2008). Further studies using multiplex platforms are needed to better define concordance across different kits and instruments. Notwithstanding the limitations, multiplex assays have demonstrated good correlations, but often variable concurrence, in comparison to individual ELISA measurements (Elshal & McCoy, 2006). Recent investigators have suggested that some of the measurement issues with multiplex assays can be addressed with bead count intraplexing and multi-well sample replicates to improve precision and confidence (Hanley, 2008).

To understand cytokines as components of an integrated biological network instead of as single measures of inflammatory activation, a systems science approach to conceptualizing and analyzing cytokines is needed. Research designs that examine linear relationships between single cytokines, or even representatives from a class such as proinflammatory cytokines, do not represent the complexity of the biological system and may lead to interpretation errors. Further documentation of relevant patterns certainly should include consideration of more recently identified markers such as Type 17 (Th17) cells, a new subset of CD4+ effector T cells that predominantly produce IL-17 and induce autoimmunity (Annunziato, Cosmi, Liotta, Maggi, & Romagnani, 2008), regulatory T cells (Treg; Iwakura & Ishigame, 2006) and chemotactic ligands, all immunomodulators of cytokine responses that do not fit the traditional pro- and anti-inflammatory frameworks.

Limitations

The exemplar study was limited by the small sample size, which did not permit analyses of the relationships of cytokine levels with potentially relevant variables such as medication usage, BMI, age, or variations in menopausal status. In addition, in the premenopausal

participants, the timing of data collection in relationship to the luteal or follicular phase of the menstrual cycle was not controlled, another potentially important consideration for future research (O'Brien et al., 2007).

Conclusion

Innovations in measurement that allow a greater appreciation of multiple components of complex biological systems have important implications for biobehavioral nursing science. Multiplex innovations permit a better characterization of the innate host response, which may lead to a better understanding of the relationships among cytokines and behavioral and psychosocial phenomena. To continue this line of research, further characterizations of inflammatory patterns in cancer and over the course of cancer treatments are needed to promote a clearer understanding of the interrelationships between biological and psychological phenomena.

Multiplex cytokine measures offer a relative comparison of cytokines in a network, an important conceptual foundation for biobehavioral research in integrated networks. Innovations in measures offer the potential for a more complete appreciation of the integrated systems of biological phenomena such as cytokines. Further development of cytokines as biomarkers in biobehavioral science depends on a greater appreciation of the effects of both the innate host response on cytokine levels and on the adaptation of nonlinear statistical approaches to better examine the complexity of cytokine networks.

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Figure 1.

Profile plots of 17 cytokines by group. G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; IL = interleukin; IFN- γ = interferon-gamma; MCP-1 = monocyte chemotactic protein-1; MIP-1 β = macrophage inflammatory protein-1 beta; TNF- α = tumor necrosis factor-alpha. *Highest IL-12 value was 29,000 but graphed at 18,000.

Figure 2.
Profile plots of anti-inflammatory cytokines by group. IL = interleukin. *Highest IL-12 value was 29,000 but graphed at 3700.

Figure 3.
Profile plots of proinflammatory cytokines by group. IL = interleukin; IFN- γ interferon-gamma; TNF- α = tumor necrosis factor-alpha.

Table 1

Participant Demographics

Variable	Benign Biopsy (<i>n</i> = 24)	Cancer (<i>n</i> = 33)	Comparison (<i>n</i> = 33)	Total (<i>n</i> = 90)
Race				
White	19 (79.2%)	21 (63.6%)	21 (65.6%)	61 (68.5%)
Black	5 (20.8%)	9 (27.3%)	11 (34.4%)	25 (28.1%)
Other	0	3 (9.1%)	0	3 (3.4%)
Total	24 (26.9%)	33 (37.1%)	32 (36.0%)	89 (100%)
Age, <i>M</i> (<i>SD</i>), years	50.0 (11.0)	47.8 (7.6)	46.6 (8.2)	47.9 (8.8)
BMI, <i>M</i> (<i>SD</i>), kg/m ^{2a}	—	27.6 (5.7)	31.7 (10.0)	29.9 (8.6)
Menopausal status				
Premenopausal	8 (33.3%)	14 (46.7%)	22 (66.7%)	44 (50.6%)
Peri/postmenopausal	16 (66.7%)	16 (53.3%)	11 (33.3%)	43 (49.4%)
Total	24 (27.6%)	30 (34.5%)	33 (37.9%)	87 (100%)

Note. BMI = body mass index. Values provided are *n* (%), unless otherwise noted

^aBMI data were not collected for benign biopsy group.

Table 2

Cytokine Levels in the Benign Biopsy, Cancer, and Comparison Groups

Cytokine (pg/ml)	Benign Biopsy (n = 24)			Cancer (n = 33)			Comparison (n = 33)			p Value
	Mdn	M	SD	Mdn	M	SD	Mdn	M	SD	
IL-2	36.75	73.86	107.82	151.50	327.61	493.85	3.00	327.52	749.57	.0002
IL-4	236.75	241.48	121.54	508.00	491.38	111.96	12.00	76.64	202.47	<.0001
IL-6	50.75	90.75	109.49	138.00	296.73	498.91	32.00	114.55	302.77	<.0001
IL-8	108.7	110.97	30.56	230.00	632.21	977.69	127.39	1985.74	4175.24	<.0001
IL-10	76.5	74.88	23.92	122.00	228.89	372.47	5.23	125.90	613.53	<.0001
GM-CSF	96	109.38	85.81	228.00	332.58	370.00	1.50	95.82	165.44	<.0001
IFN- γ	34	38.97	19.46	38.00	107.86	247.08	35.00	279.33	503.17	.2734
TNF- α	133	124.53	64.42	177.50	205.47	192.32	0.10	16.13	37.09	<.0001
IL-1 β	49	70.58	71.60	65.50	184.50	363.95	1.39	7.67	23.47	<.0001
IL-5	23	24.76	7.33	26.00	36.33	37.34	0.78	12.82	48.68	<.0001
IL-7	63	63.33	15.05	91.00	122.98	122.97	18.42	440.59	2176.38	<.0001
IL-12	65	63.64	16.48	87.50	185.02	414.61	30.00	1111.89	5130.50	<.0001
IL-13	34.5	36.10	13.70	48.00	55.91	36.67	5.00	182.27	826.49	<.0001
IL-17	130	118.92	44.73	167.00	167.95	26.98	10.00	195.73	405.19	<.0001
G-CSF	85.5	98.59	43.89	85.00	101.88	58.78	32.00	94.30	132.34	.0008
MCP-1	178.75	322.76	630.51	679.00	748.55	437.11	379.38	408.60	266.62	<.0001
MIP-1 β	1416	1392.56	514.71	5150.00	6057.35	3543.58	293.17	322.85	164.79	<.0001

Note. G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN- γ = interferon-gamma; IL = interleukin; MCP-1 = monocyte chemoattractant protein-1; MIP-1 β = macrophage inflammatory protein-1 beta; TNF- α = tumor necrosis factor-alpha. Differences in median values between groups were tested using the nonparametric Kruskal-Wallis test with a Bonferroni corrected alpha level of .0029.