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Measurement of salivary immunoglobulin A as an immunologic biomarker of job stress

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Occupational stress, particularly the relationships between work-related psychosocial stressors and health, is a growing concern. Stress may result in immune suppression, which may, in turn, lead to reduced disease resistance (1). In animal models, lowered disease resistance has resulted in infections, cancer, or autoimmunity, while clinical case studies have shown similar predilections for disease in humans who are immunocompromised due to excessive stress (2). Stress also can affect the normal homeostatic relationships between the immune, nervous, and endocrine systems (3, 4).

Psychometric instruments (questionnaires) for measuring job stress have been developed and applied to the workplace. However, biological indicators of stress (biomarkers) would be valuable objective measures to complement these questionnaires (5). Validated biomarkers could be used to measure a worker's exposure, susceptibility for developing an occupational disease, or early (preclinical, reversible) health effects resulting from occupational exposures.

Several biological indices have been studied in the past to ascertain their value in detecting physiological and health effects of various types of stress. Levels of cortisol in saliva, urine, and serum have been studied the most often (6), while other end points examined include blood pressure, heart rate, visual accommodation, adrenocorticotropin, catecholamines, blood counts, immunoglobulins, cytokines, immunocompetency, and the like (2, 7). Due to problems with methods or design, these studies have had varying degrees of success. Changes in cortisol, catecholamines, and other hormones are usually only transient responses to acute stressors and have not proved to be appropriate measures of chronic stress. Many of the transient responses have relatively large natural fluctuations due to biological rhythms (6) and are also variable in heterogeneous human populations and environments.

Immunoglobulin A (IgA) in saliva is another potential biomarker for stress-induced immunologic effects in workers (8). Salivary IgA has particular appeal as a potential biomarker because (i) it can be obtained noninvasively, easily, and frequently in comparison with blood, (ii) it is biologically relevant as a functional immune end point, (iii) it can be quantitated, and (iv) it is more stable, with a longer biological half-life, than cortisol and catecholamine. Controversy remains concerning the best way to measure salivary IgA (9—11). Therefore, total IgA levels, specific IgA titers, or both, and total salivary protein concentration or salivary flow rates have been measured in previous studies (12). Procedural variations occur in collection methods (stimulated versus nonstimulated salivation), in sampling times (biorhythms, frequency, storage), in sources (whole saliva versus parotid), in immunoassays [enzyme-linked immunosorbent assay (ELISA) versus radioimmunodiffusion, standards, antibody specificity], in IgA end points (monomeric versus polymeric, secretory component, J chain, subclass A1 or A2, specific antigen tested), and in designs (population size, replicates). Some important confounding factors that may affect salivary IgA levels include disease, nutrition, age, hormonal activity, certain medications, trauma or exertion, and biorhythms (2).

Currently, the National Institute for Occupational Safety and Health (NIOSH) is evaluating the use of salivary IgA as a noninvasive immunologic biomarker of occupational stress. This investigation is part of a larger, longitudinal, pilot study of the immunosuppressive effects of job stress. The methodology for measuring salivary IgA and preliminary results from this ongoing investigation are presented in this report.

Subjects and methods

The longitudinal pilot study used a population of 40 nurses to evaluate psychological and immunologic changes that may be related to job stress. For each subject, NIOSH job stress questionnaires were completed, saliva samples were collected on a weekly basis for eight months, and blood samples were drawn

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monthly. The subjects were adult, premenopausal women who maintained minimal health standards. Qualified individuals were randomly selected from a volunteer population and placed into one of four groups of 10 subjects per group according to their observed and reported levels of job stress. Twenty subjects had objectively defined high-stress jobs, while 20 had objectively defined low-stress jobs. Within the two main groups were two subgroups of 10 subjects each, distinguished by whether they subjectively perceived their job as having either high or low levels of stress. Saliva and blood samples were collected at similar times of the day and at least 1 h after meals for each of the nurses, during which time the psychological instruments were administered. Unstimulated, whole saliva samples were collected for 5 min into sterile 15-ml conical plastic tubes, stored at −80°C, and shipped on dry ice in weekly batches to the NIOSH laboratory for analyses. Peripheral venous blood was processed for CBC, differentials, and routine clinical chemistry to evaluate general health. Aliquots of lymphocytes and sera were evaluated with a panel of neuroendocrine and immunologic tests. Sera were tested by radioimmunoassay for cytokines (interleukin 1, interleukin 2, and gamma interferon), for neuropeptides (met-enkephalin and beta-endorphin), and for hormones (lipocortin and glucocortico-steroids). Lymphocytes were evaluated for function by natural-killer cell activity and mitogenic proliferation and by flow cytometry for phenotypes (CD3, CD4 and CD8 cells). Results of the blood tests will be correlated with the questionnaire results and with the salivary IgA results.

Saliva samples were analyzed in a "blind" fashion for (i) concentrations of total IgA, (ii) end-point titers of specific IgA against five combined strains of *Escherichia coli* cell wall antigens [lipopolysaccharides (LPS)] [lipopolysaccharides ("0 antigens") from enteropathogenic *E coli* strains 0127:B8, 0111:B4, 055:B5, 026:B6, and 0128:B12], (iii) total protein concentration, and (iv) salivary flow rate. A modified ELISA method was used to measure both total and specific IgA antibodies in the whole saliva samples. Plates with 96 wells were coated with either 50 µg of goat anti-(human IgA) antibody (01-10-01, Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, United States) in 100 µl of physiological buffered saline (PBS) to measure total IgA concentrations (8) or 10 µg *E coli* antigens in 100 µl of PBS to measure specific IgA titers (12). An eight-point standard curve for total IgA was produced for each weekly run from quadruplicate wells with the use of secretory IgA standards (human colostrum IgA, purified, I1010, Sigma Chemical Co, St Louis, Missouri, United States). Total IgA samples were run in duplicate at four twofold dilutions (500, 1000, 2000, and 4000) and reported in units of milligrams percent. Specific IgA samples were run in duplicate at seven twofold dilutions (10, 20, 40, 80, 160, 320, and 640). LPS plates were compared with PBS plates to correct for background binding of nonspecific salivary IgA to the wells. Appropriate control wells were assayed in all of the plates to assure consistent interplate readings (absorbances at 410 nm on a dual-wavelength Dynatech MR700 spectrophotometer).

Split-samples for quality control, pooled reference samples, and standardized collection, storage and processing procedures were used to reduce potential sample variation. Flow rates (ml/min) and protein content (mg/ml) (BCA kit, Pierce Chemical Co, Rockford, Illinois, United States) of saliva were determined to normalize the salivary IgA results for possible influences of either overconcentration or dilution of saliva (potentially for improving the comparisons between groups and the correlations between the blood and questionnaire results). A repeated-measures analysis of variance (ANOVA) will be used to analyze the parametric results statistically, and Duncan's new multiple range test will be used to determine significant differences ($P < 0.05$) between the groups. A Kruskal-Wallis test will be used to evaluate statistically the nonparametric data for significant group differences. An analysis of covariance will be used to determine the statistical correlations between the blood, saliva, and questionnaire data, using SAS (Statistical Analysis System, Cary, North Carolina).

Results and discussion

While the study of salivary IgA as a biomarker of occupational stress is ongoing, preliminary results suggest the following: initial experiments found the ELISA methods to be about 10 times more sensitive or less variable, more automated, and cheaper than the radioimmunodiffusion kits commonly used for total IgA analysis (11, 12). The procedures to collect, store, ship, process, and analyze the saliva samples were highly suitable. Small problems were caused by the limited shelf-life (less than four months) of the aliquoted standards and with the staggered entry of subjects into the longitudinal study. Since all of the groups were not filled with subjects until week 5 of the study, only data from weeks 5—15 have currently been evaluated. The split-samples showed good overall agreement (only a few outliers, data not shown), total IgA values averaging ±3 mg% and specific IgA titers averaging ±0.9 mg% of a twofold end-point dilution. A composite standard curve for weeks 5—15 is displayed in figure 1. A regression curve was fit weekly to the log-linear data and was used to derive the concentrations of total salivary IgA in the samples. Acceptable ELISA performance was confirmed by the inclusion of sufficient experimental control wells in each plate.

The total salivary IgA concentrations are shown in figure 2, as corrected for both protein and flow rate. The low-objective stress groups (hollow symbols) appear to have had consistently the lowest mean total salivary IgA levels, pending statistical determinations. Correction of the total salivary IgA values by protein

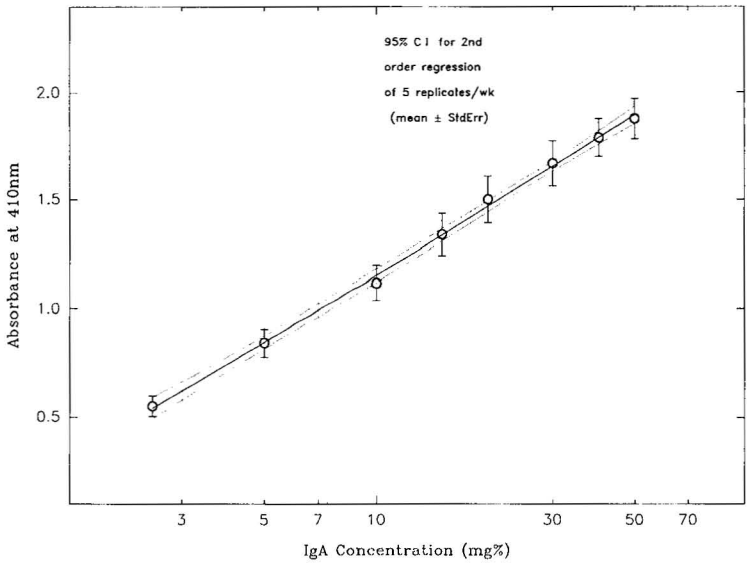

Figure 1. Composite standard curve for total immunoglobulin A (IgA) by enzyme-linked immunosorbent assay (ELISA), means over 10 weeks. (95% CI = 95% confidence interval, wk = week, StdErr = standard error)

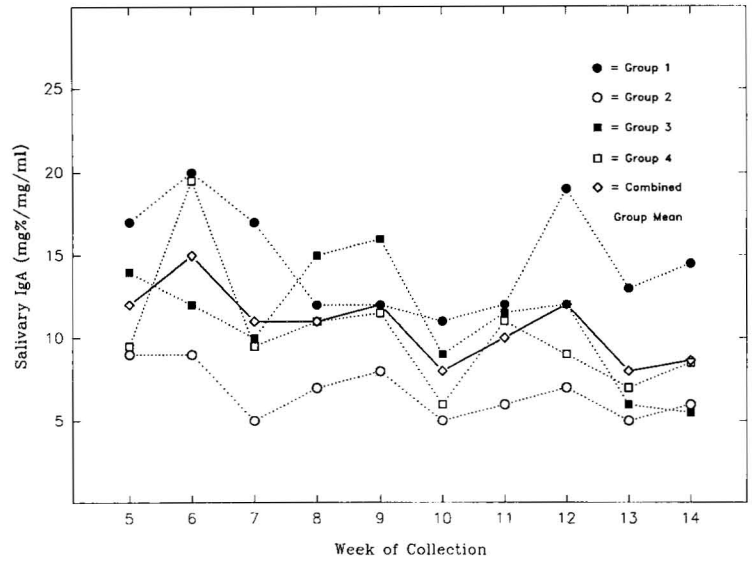

Figure 2. Group means of total salivary immunoglogulin A (IgA) concentrations per week of collection, corrected for protein (mg%) and flow rate (ml/min). (Group 1 = high objective and high subjective, Group 2 = low objective but high subjective, Group 3 = high objective but low subjective, and Group 4 = low objective and low subjective stress exposure, as assessed by objective and subjective measures)

alone tended to centralize the uncorrected group means (data not shown), while corrections by both protein and flow appeared to lower greatly the value of the low-objective but high-subjective group (hollow circles). The uncorrected salivary IgA levels were within generally reported normal ranges of 10—25 mg%. It remains to be seen if statistically significant group differences or correlations with other results occur.

Specific salivary IgA end-point titers are shown in figure 3, as corrected for both protein and flow rate. (Corrections for total protein were similar to the uncorrected values, data not shown.) It appeared that the low-objective and low-subjective stress group (hollow squares) consistently had the lowest mean specific salivary IgA titers, but the statistical significance has yet to be determined. Correction by protein and flow seemed to have slightly raised the low-objective but high-subjective group (hollow circles) to the highest values over this time. This apparent effect could possibly be due to the lowest protein and flow rates (figure 4) of this group, a phenomenon which would tend to elevate the corrected results. While normal values have not been established for specific salivary IgA titers to *E coli* antigens, our results are similar to those found in a recent doctoral research study (12).

Future plans

Aliquots of the saliva (stored at −80°C) are being kept for possible follow-up studies of potential biomarkers that may be identified in blood (cytokines, hormones, or neuropeptides) and which may be accessible in a less invasive fluid such as saliva. If significant relationships

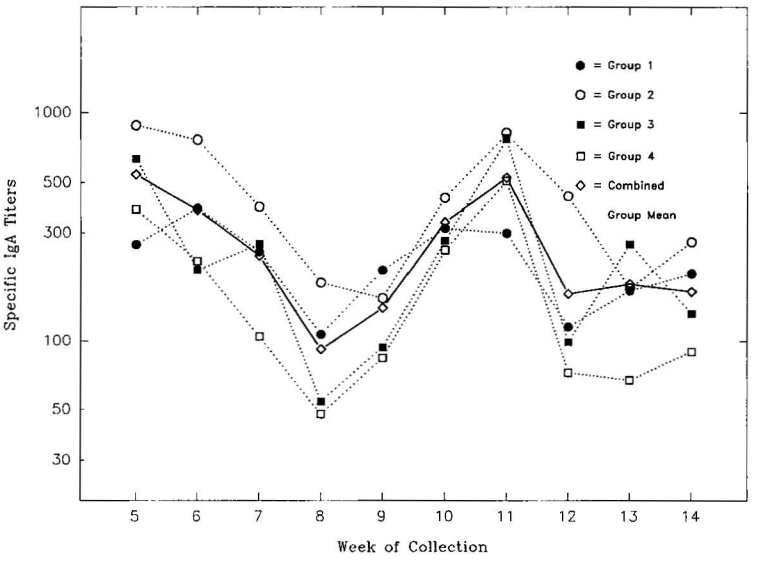

Figure 3. Group means of specific salivary immunoglobulin A (IgA) end-point titers per week of collection, corrected for protein (mg%) and flow rate (ml/min). (Group 1 = high objective and high subjective, Group 2 = low objective but high subjective, Group 3 = high objective but low subjective, and Group 4 = low objective and low subjective stress, as assessed by objective and subjective measures)

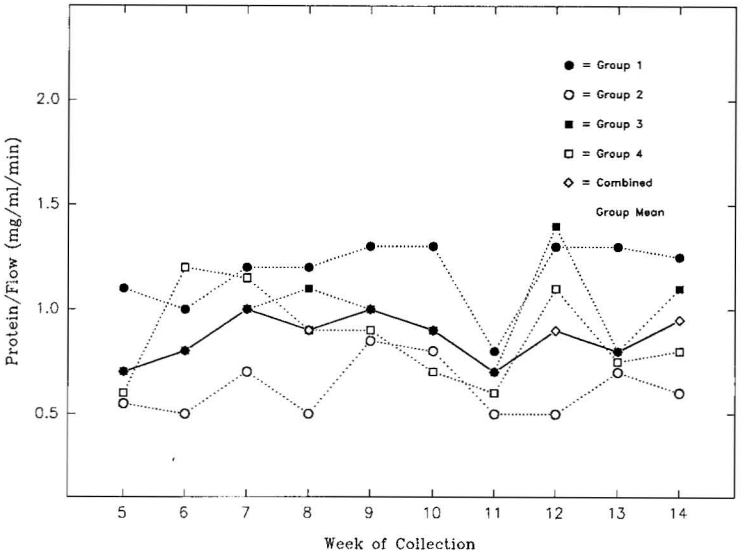

Figure 4. Salivary protein and flow rate group mean correction factors for immunoglobulin A (IgA) per week of collection. (Group 1 = high objective and high subjective, Group 2 = low objective but high subjective, Group 3 = high objective but low subjective, and Group 4 = low objective and low subjective stress, as assessed by objective and subjective measures)

between the questionnaire data and the blood and salivary IgA results are found in this pilot study, a larger and more definitive field study will be carried out.

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