Chronic Stress Associated With Spousal Caregiving of Patients With Alzheimer’s Dementia Is Associated With Downregulation of B-Lymphocyte GH mRNA

Hong Wu,1 Juan Wang,1 John T. Cacioppo,2,3 Ron Glaser,1,4 Janice K. Kiecolt-Glaser,2,3 and William B. Malarkey1,4,5

Departments of 1Medicine, 2Psychology, 3Institute for Behavioral Medicine Research, 4Medical Microbiology and Immunology, and 5Comprehensive Cancer Center, The Ohio State University.

GROWTH hormone (GH) is involved in immunomodulation of both cellular and humoral immunity (1). Of importance to the work performed in this investigation, GH has an effect on B-cell proliferation and also increases IgG and IgE production (2). Snell-Bagy dwarf mice, which are deficient in GH, have a reduced ability to synthesize antibodies to particular antigens (3), a decreased number of splenic T and B cells (4), and a cellular depletion of the bone marrow (5). Also, lymphocyte proliferation is blocked by specific antibodies to GH (6), and an antisense oligodeoxynucleotide to GH mRNA (7).

It has been demonstrated that GH is synthesized (6,8) and secreted (9,10) by human peripheral mononuclear cells (PBMCs), and the expression of GH mRNA can be found throughout the human immune system. Furthermore, we have shown that lymphocyte GH mRNA occurs primarily in B-cell enriched areas in human immune organs (8). Therefore, we were interested if there was a downregulation of GH gene expression in T and B cells as measured by GH mRNA. Such a finding would support our hypothesis that decreased lymphocyte GH mRNA is involved in the poor immune response to the influenza virus vaccine that we have seen in these individuals (12).

Materials and Methods

Subjects.—Our study population consisted of nine elderly women who suffered from the chronic stress of caring for a relative with AD, and nine age- and sex-matched control subjects. The stress involved with spousal caregiving has been well characterized (14). The weights of both groups were comparable (controls 71±4 kg vs 73±5 kg for the caregivers). Their ages were similar (controls 68±2 yr vs 64±1.8 for the caregivers). The inclusion criteria for participation in the study included good general health, body mass index <31, and normotensive; no history of a chronic illness; postmenopausal and not on estrogen replacement; and not on any pharmacologic or tobacco products. Participants were asked not to ingest any medication or alcohol 24 h preceding the test day.

T- and B-lymphocytes separation.—Starting with 30 ml of whole blood, approximately 2 × 10⁷ PBMCs were obtained. Lymphocytes were separated by density gradient centrifugation over ficoll-sodium diatrizoate (specific gravity 1.077; Sigma, St. Louis, MO) and washed twice with phosphate buffered saline. Products. Participants were asked not to ingest any medication or alcohol 24 h preceding the test day.

Conclusions. Because the B-cell population is the source of antibody production, our findings suggest that the decrease in B-cell GH mRNA may contribute to the poor immune response to influenza virus vaccination that has been reported previously in chronically stressed caregivers.
CHRONIC STRESS AND SYNTHESIS OF GH mRNA

M213

(PBS) without Ca²⁺ or Mg²⁺. CD2 (T-cell surface marker) and CD19 (B-cell surface marker) positive cells were prepared using appropriate antibody coated magnetic beads (Dynal, Lake Success, NY) according to the procedure provided by Dynal. To check for purity of the separation CD19-positive cells prior to extraction, cells were detached from the beads by using Detach-a-Bead (Dynal). The purity of CD2-positive cells was evaluated following trypan blue exclusion (GIBCO/BRL, Gaithersburg, MD) for 15 min, a PBS wash, and overnight culture at 37°C in RPMI 1640 supplemented with 500 U/ml penicillin G, 500 μg/ml streptomycin, 1.5 mM L-glutamine, 25 mM HEPES (15). Immunostaining, using monoclonal antibodies (Coulter, Hialeah, FL), and flow cytometry were then performed as previously described (12). All B- and T-cell preparations were at least 95% pure.

RNA extraction and reverse transcription.—Total RNA obtained from human T and B cells was isolated using the single-step isolation method (16). The reverse transcription from RNA to cDNA was performed as previously described using equal amounts of RNA from B or T cells (8). Briefly, a total volume of 25 μl contains 3 μg total RNA, 1 μl random primer (90 units/ml), 1 μl first strand buffer (250 mM Tris HCl, 375 mM KCl, 15 mM MgCl₂, pH 8.3), 200 μM of deoxynucleotide triphosphate, 0.5 μl DTT (0.1M), and 1 unit of RNase inhibitor. The reaction mixture was incubated at 65°C for 10 min and cooled on ice for 5 min. Afterwards, 200 units of RNase H reverse transcriptase (GIBCO/BRL) was added, and this mixture was incubated at room temperature for 10 min, at 42°C for 30 min, and at 90°C for 10 min. For comparisons of RNA levels from different RNA samples, reverse transcription was performed simultaneously using reagents from a single master mix.

Oligonucleotides.—GH primers flanking exons 2, 3, and 4 in human GH gene were synthesized as mentioned previously (8). The sense primer (5’ end) is located within the last 20 nucleotides of exon 2 and the first 7 nucleotides of exon 3 of the human GH gene. The antisense primer (3’ end) is complementary to the last 6 and the first 21 nucleotides of exons 3 and 4 so that contamination by genomic DNA would be avoided (8). The amplified fragments were 161 bp in length. The sequence of sense primer was 5’-ATGACA CCT ATC AGG AGT TTG and of the antisense primer was 5’-GAT GCG GAG AAG AAG-3’, of the antisense primer was 5’-GAT GCG GAG AAG AAG-3’, of the antisense primer was 5’-GAT GCG GAG AAG AAG-3’, of the antisense primer was 5’-GAT GCG GAG AAG AAG-3’.

Competitive RT-PCR.—A competitive RT-PCR method for GH was described in our previous study (13). Briefly, a rat p53 gene fragment (241 bp) was chosen for use as a competitive standard that differed in size from target gene-GH (161 bp) PCR product. This competitive standard consists of a nonhomologous DNA fragment with primer templates that "mimic" and are thus recognized by GH gene-specific primers. The competitive standard 1:2 dilution series began at 0.312 x 10⁻⁴ attomoles and proceeded to 10 x 10⁻⁴ attomoles. PCR products were generated in a volume of 12.5 μl, containing 2 μl of the serial diluted competitive standard, 1.5 μl of CDNA (equivalent to about 0.25 μg of total RNA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl 2.5 mM MgCl₂, 25 pmol of each primer, 0.2 mM of each dATP, dGTP, dCTP, and dTTP (GIBCO/BRL), and 0.6 μl of Taq-Start mix. The Taq-Start mix containing 80 μl Taq DNA polymerase (GIBCO/BRL), 80 μl Taq antibody and 320 μl dilution buffer (Clontech, Palo Alto, CA) was utilized to minimize PCR amplification of nonspecific fragments. Amplification was carried out in an automated Perkin-Elmer/Cetus 9600 thermal cycler for initial denaturation for 1 min at 94°C, then for 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 62°C and extension for 30 sec at 72°C, followed by a final incubation for 7 min at 72°C.

The products from each reaction were examined on ethidium bromide-stained 3% agarose gel. A typical photograph of a competitive standard dilution series with one human PBMC RNA sample is shown in Figure 1. The intensities of target and mimic fragments were determined using a digital IS-1000 imaging system (Alpha Innotech, San Leandro, CA).

Two independent competitive RT-PCR studies were performed for each sample, and each independent experiment gave similar results. These data were analyzed using the nonparametric Wilcoxon-Kruskal-Wallis test, and a p < .05 was considered significant.

RESULTS

Separation of PBMCs into T- and B-cell populations.—PBMCs from subjects were separated into CD2 (T cell) and CD19 (B cell) by using the appropriate magnetic bead-coated antibodies. The purity of the separated cells was tested by immunostaining and flow cytometry. Both T- and B-cell populations were greater than 95% pure (data not shown).

Competitive PCR of GH mRNA.—To determine appropriate dilutions of competitive standard for competitive PCR, we did preliminary titration experiments in which 10 twofold serial dilutions from 0.78 x 10⁻⁵ to 10 x 10⁻⁴ were empirically made and amplified with a constant amount of sample cDNA. After acquiring this information, we were able to reduce the number of serial dilutions to six (0.312 x 10⁻⁴ to 10 x 10⁻⁴), which allowed both competitive standard and target GH fragments to be amplified and visualized on an agarose/EtBr gel.

To compare GH cDNA levels in T-cell and B-cell populations from the two experimental groups, we performed PCR amplification of four samples at the same time. One fifth of the amplified product from these four samples was then examined on a single agarose/EtBr gel. A typical photograph of a competitive standard dilution series with one of the human PBMC RNA samples is shown in Figure 1.

The expression of GH mRNA in T and B cells.—Chronic stress-related changes in GH mRNA levels in human T-cell and B-cell populations were measured using a competitive RT-PCR technique. In the control group we found threefold greater GH mRNA expression in B cells than in T cells (p < .05), even though the total T-cell population is 3-4 times greater than the B-cell population in man (Figure 2). We also noted that B- and T-cell GH mRNA levels were highly correlated (R = 0.5, p < .05). Further analysis showed that the expression of GH mRNA from B cells was 60% less (p < .05) in the AD caregiver group compared with the control women, whereas the expression of GH mRNA from T cells was similar between the two groups (p > .05).

DISCUSSION

In this study we combined two areas of investigation in which our laboratory has been active for several years. The first
Figure 2. Quantitative analysis of GH mRNA as measured by competitive RT-PCR after correcting for the difference in size between the competitive standard fragment and eDNA. In the control (CT) group, B-cell GH mRNA was significantly \((p < 0.05)\) higher than T-cell GH mRNA. Also, caregivers (CG) had a 60% decrease in B-cell GH mRNA when compared to controls, whereas T-cell GH mRNA concentrations were similar between the two groups. When the 3 highest B-cell GH mRNA data points (2 in the controls and 1 in the caregivers) are eliminated from the analysis, significant \((p < 0.05)\) differences persist.

The decreased GH mRNA reflecting a decrease in lymphocyte GH production, as the lower GH mRNA could also reflect increased turnover of mRNA and possibly even an increase in GH production. If we accepted the latter interpretation, however, it could be the first example of human stress increasing an immune peptide; thus, we believe the most parsimonious interpretation is that it is reflective of decreased GH production.

Recently, we have found that immune defects in caregivers include suppression of lymphocyte GH levels. Utilizing quantitative PCR techniques, we observed that caregivers have a 50% decrease in lymphocyte GH mRNA (13). In this study, we replicated these findings as we observed approximately a 50% decrease in the caregivers’ GH mRNA compared to the age-and sex-matched control women. Of marked interest was our finding that although GH mRNA levels in T cells were similar between caregivers and control subjects, there was a marked 60% decrease in GH mRNA levels in B cells in the caregiver population. This observation suggests the possibility that a diminution in lymphocyte GH could be contributing to the defective antibody response to repetitive influenza vaccine that we had previously reported in the caregiver population (12). Obviously, asso-
CHRONIC STRESS AND SYNTHESIS OF GH mRNA

M215

Columbus, OH 43210.
University Medical Center, N1105 Doan Hall, 410 West 10th Avenue,