

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,^{1,3,4}
Janice K. Kiecolt-Glaser,^{2,3} and William B. Malarkey^{1,3,4,5}

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

Background. It has been demonstrated that growth hormone (GH) is synthesized and secreted by human peripheral mononuclear cells (PBMC), and the expression of GH mRNA can be found throughout the human immune system.

Methods. We studied a population of female caregivers of patients with Alzheimer's dementia (AD) who suffered from the stress of caring for these patients. We utilized quantitative RT-PCR to determine GH mRNA levels in T- and B-cell populations from PBMC. Subjects were nine caregivers of AD patients and nine age- and sex-matched controls.

Results. In the control group we found a threefold greater GH mRNA expression in B cells than in T cells. This finding was consistent with our previous *in situ* hybridization observation, suggesting GH mRNA in predominately B-cell areas of immune organs in humans. We also found that the expression of GH mRNA from total peripheral blood mononuclear cells and B cells in caregivers was 50% and 60% respectively less than that in the control group.

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.

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 [thymus, tonsil, lymph nodes, and spleen; (8)]. Furthermore, we have shown that lymphocyte GH mRNA occurs primarily in B-cell enriched areas in human immune organs (8). Therefore, we have been interested in evaluating GH mRNA levels in purified B- and T-cell populations from PBMCs. In this study we have used competitive RT-PCR to evaluate whether human B and/or T cells synthesize GH and in which cell type it predominates.

We chose to study a population of female caregivers of patients with Alzheimer's disease (AD). We have described numerous immune deficits in the chronically stressed AD caregiver population including an inhibition of the ability of NK cells to respond to interferon gamma (IFN- γ) and interleukin 2 [IL-2; (11)], decreased antibody and virus specific T-cell responses to influenza virus vaccination (12), and decreased expression of lymphocyte GH mRNA (13). In some caregivers of

AD patients, negative changes occur in both T- and B-cell function. 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 2×10^6 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

(PBS) without Ca^{2+} or Mg^{2+} . 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 trypsin-EDTA (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 g/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 \times first strand buffer (250 mM Tris HCl, 375 mM KCl, 15 mM MgCl_2 , 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 mRNA levels among 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 161bp in length. The sequence of sense primer was 5'-ATG ACA CCT ATC AGG AGT TTG AAG AAG-3', of the antisense primer was 5'-GAT GCG GAG CAG CTC TAG GTT AGA TTT-3'.

Competitive RT-PCR.—A competitive RT-PCR method for GH was described in our previous study (13). Briefly, a rat p53 gene fragment (241bp) was chosen for use as a competitive standard that differed in size from target gene-GH (161bp) 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 $\times 10^{-4}$ attomoles and proceeded to 10 $\times 10^{-4}$ attomoles. PCR products were generated in a volume of 12.5 μl , containing 2 μl of the serially 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 MgCl_2 , 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 terminal 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 0.78×10^{-5} to 40×10^{-4}) were empirically made and coamplified with a constant amount of sample cDNA. After acquiring this information, we were able to reduce the number of serial dilutions to six (0.312×10^{-4} to 10×10^{-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

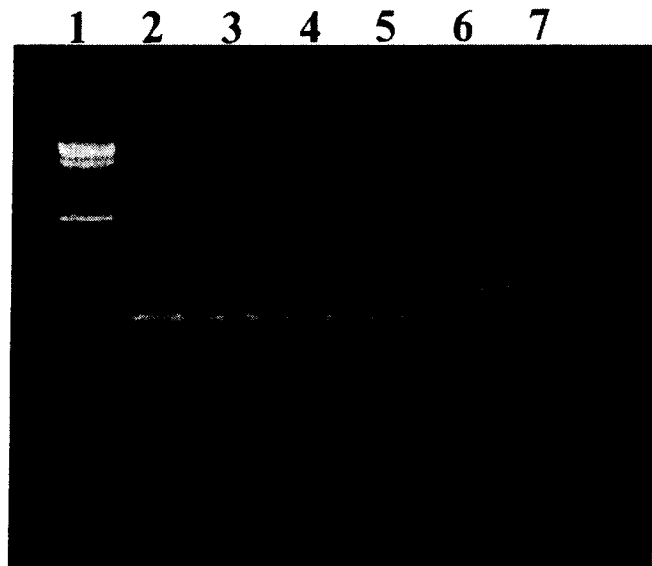


Figure 1. Ethidium bromide stained 3% agarose gel showing quantitation of GH mRNA expression in human PBMCs by competitive RT-PCR. Dilutions of competitive standard (0.312×10^{-4} – 10×10^{-4} attomoles/tube, the upper band from left to right, lanes 2–7) were added to a constant amount of mononuclear cell RNA (0.25 μ g/tube, the lower band). The point at which the upper and lower bands are of equal intensity represents equal starting amounts. Lane 1=100 bp DNA size marker.

relates to the marked immunologic defects that characterize social isolation and chronic stress associated with caregiving of patients with AD (11,14); the second concerns our observations that lymphocyte GH may be an important autocrine/paracrine factor in the human immune system (8,13). In our caregiver studies we have found decreased immune function—not only while they are active caregivers, but interestingly, even for years after bereavement for the AD patient when they are no longer caregiving (14). Their cellular immune defects following chronic stress are characterized by a decreased proliferative response to T-cell mitogens (17) and a decreased response of NK cells to IFN- γ and IL-2 (11). Also, there is an impairment in the ability of these individuals to synthesize antibody to an influenza virus vaccine and an impairment in the virus-specific T-cell response (12). We found that fewer than 50% of AD caregivers developed a fourfold antibody response after influenza virus vaccination, which is considered the minimal effective response to prevent infection.

Meanwhile, we have been investigating lymphocyte GH production and, along with others (6,8–10), noted that it can be produced and secreted by PBMCs and that antibodies or an antisense oligodeoxynucleotide to GH mRNA in serum-free medium can decrease mitogen-stimulated lymphocyte replication (6,7). Using in situ hybridization, we have noted GH mRNA in human thymus, tonsil, spleen, and lymph nodes (8).

We observed that there was almost three times as much GH in the B cells as in the T cells. One could argue that 1%–5% contamination of the Dynabead B- and T-cell purified cell preparations could have influenced the PCR GH mRNA results. However, we feel that the consistency and the magnitude of the difference between the T-cell and B-cell group makes this argument less tenable. Also, one has to qualify the interpretation of

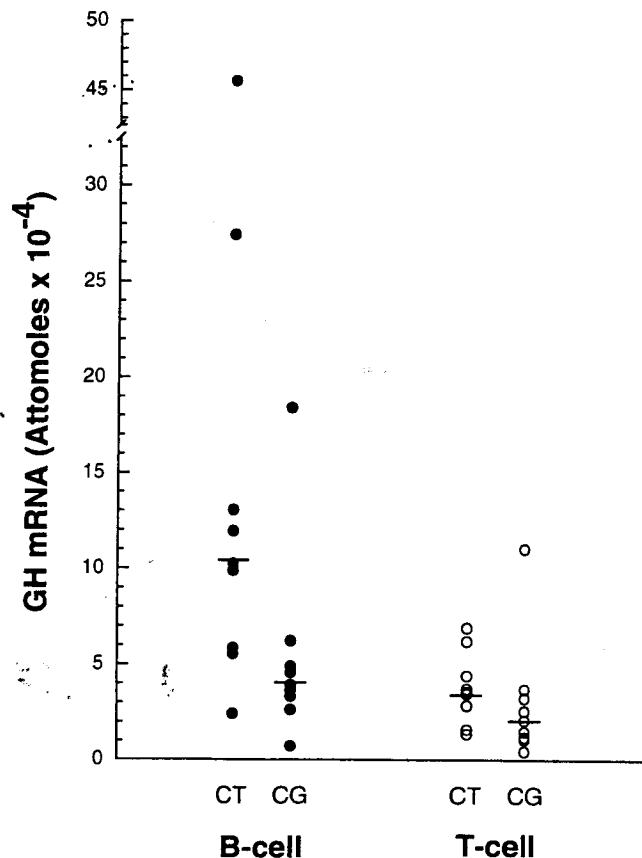


Figure 2. Quantitative analysis of GH mRNA as measured by competitive RT-PCR after correcting for the difference in size between the competitive standard fragment and cDNA. In the control (CT) group, B-cell GH mRNA was significantly ($p < .05$) higher than T-cell GH mRNA. Also, caregivers (CG) had a 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 ($p < .05$) 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-

ciation does not prove causality, and this observation could be secondary to a B- and/or T-cell defect. There are, however, several reports that make this suggestion plausible. First, Yoshida and colleagues (2) have shown that GH, when incubated with lymphocytes in vitro, increased IgG production. Also, there is an intriguing observation made by Stephenson and coworkers (18) that GH, when administered in concert with a viral vaccine, was able to dramatically improve survival in animals challenged with this encephalitic virus. Although we have not yet seen differences in serum GH levels between controls and caregivers, it is important to remember that lymphocyte GH has little if any influence on serum GH levels. The secretion of lymphocyte GH is several orders of magnitude lower than that secreted by the pituitary gland, and it usually is not measurable even by the most sensitive chemiluminescence assays (9,10). GH has been visualized by in situ hybridization in several immune organs (8), and it appears that lymphocyte GH produces its immune effects by an autocrine or paracrine mechanism.

In summary, we have demonstrated that the B cell is more active than the T cell in lymphocyte GH mRNA synthesis. Also, the decrease in levels in lymphocyte GH mRNA that occurs in chronically stressed AD caregivers occurs predominantly in B cells. These data add to the growing body of information dealing with the endocrine-immune interface and suggest that pituitary hormones that are synthesized by the central and peripheral immune system may be active participants in a wide variety of challenges to immune function.

ACKNOWLEDGMENTS

This work was supported in part by Training Grant MH-18831 and Grants MH-42096 and MH-50538 from the National Institute of Mental Health, Program Project Grant AG-11585 from the National Institute on Aging, The General Clinical Research Center Grant M01 RR-00034 from the National Institutes of Health, and Core Grant CA-16058 from The Ohio State University Comprehensive Cancer Center.

We express appreciation to Carolyn Cheney for providing technical assistance, David Phillips for outstanding editorial services, Dr. H. N. Nagaraja for statistical assistance, and the nurses at the General Clinical Research Center.

Address correspondence to Dr. William B. Malarkey, The Ohio State University Medical Center, N1105 Doan Hall, 410 West 10th Avenue, Columbus, OH 43210.

REFERENCES

- Kooijman R, Hooghe-Peters EL, Hooghe R. Prolactin, growth hormone and insulin-like growth factor-1 in the immune system. *Adv Immuno*. 1996;63:377-454.
- Yoshida A, Ishioka C, Kimata H, Mikawa H. Recombinant human growth hormone stimulates B-cell immunoglobulin synthesis and proliferation in serum-free medium. *Acta Endocrinol*. 1992;126:524-529.
- Kelly KW. Growth hormone in immunobiology. In: Ader R, Felten D, Cohen N, eds. *Psychoneuroimmunology*. 2nd ed. New York: Academic Press; 1991:377-396.
- Gala RR, Shevach EM. Influence of prolactin and growth hormone on the activation of dwarf mouse lymphocytes in vivo. *Proc Soc Exp Biol Med*. 1993;204:224-230.
- Hooghe-Peters EL, Hooghe R. *Growth Hormone, Prolactin and IGF-I as Lymphohemopoietic Cytokines*. Austin, TX: R.G. Landes; 1995.
- Weigent DA, Baxter JB, Wear WE, Smith LR, Bost KL, Blalock JE. Production of immunoreactive growth hormone by mononuclear leukocytes. *FASEB J*. 1988;2:2812-2818.
- Weigent DA, Blalock JE, LeBoeuf RD. An antisense oligodeoxynucleotide to growth hormone messenger ribonucleic acid inhibits lymphocyte proliferation. *Endocrinology*. 1991;128:2053-2057.
- Wu H, Devi R, Malarkey WB. Localization of growth hormone messenger ribonucleic acid in the human immune system—a clinical research center study. *J Clin Endocrinol Metab*. 1996;81:1278-1282.
- Varma S, Sabharwal P, Sheridan JF, Malarkey WB. Growth hormone secretion by human peripheral blood mononuclear cells detected by an enzyme-linked immunoplaque assay. *J Clin Endocrinol Metab*. 1992;76:49-53.
- Hattori N, Ikekubo K, Ishibara T, Moridera K, Hino M, Kurahachi H. Spontaneous growth hormone (GH) secretion by unstimulated human lymphocytes and the effects of GH-releasing hormone and somatostatin. *J Clin Endocrinol Metab*. 1994;79:1678-1680.
- Esterling B, Kiecolt-Glaser JK, Glaser R. Psychosocial modulation of cytokine-induced natural killer cell activity in older adults. *Psychosom Med*. 1996;58:264-272.
- Kiecolt-Glaser JK, Glaser R, Gravenstein S, Malarkey WB, Sheridan J. Chronic stress alters the immune response to influenza virus vaccine in older adults. *Proc Natl Acad Sci*. 1996;93:3043-3047.
- Malarkey WB, Wu H, Cacioppo JT, et al. Chronic stress down-regulates growth hormone gene expression in peripheral blood mononuclear cells of older adults. *Endocrine*. 1996;5:33-39.
- Kiecolt-Glaser JK, Glaser R. Caregivers, mental health, and immune function. In: Light E, Niederhe G, Lebowitz B, eds. *Advances in Alzheimer Disease Caregiving and Family Stress*. New York: Springer; 1994:64-74.
- Galileo DS, Gee AP, Linser PJ. Neurons are replenished in cultures of embryonic chick optic tectum after immunomagnetic depletion. *Dev Biol*. 1991;146:278-291.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162:156-159.
- Castle S, Wilkins S, Heck E, Tanzy K, Fahey J. Depression in caregivers of demented patients is associated with altered immunity: impaired proliferative capacity, increased CD8+, and a decline in lymphocytes with surface signal transduction molecules (CD38) and a cytotoxicity marker (CD56+ CD8+). *Clin Exp Immunol*. 1995;101:487-493.
- Stephenson JR, Lee JM, Bailey N, Shepherd AG, Melling J. Adjuvant effect of human growth hormone with an inactivated flavivirus vaccine. *J Infect Dis*. 1991;164:188-191.

Received August 22, 1997

Accepted July 15, 1998