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Association between Cytokine Production and Disease Severity in Alzheimer's Disease

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ABSTRACT

The role of transforming growth factor (TGF)- β 1, interferon (IFN)- γ , interleukin (IL)-2, IL-3, and IL-6 in the pathogenesis of Alzheimer's Disease (AD) has long been reported in literature. In this case-control study, the concentrations of these cytokines in altered T lymphocytes, as well as serum vitamin B12, have been compared in terms of factors such as, age, the clinical course and the patients' disease risk.

40 patients who met the DSM-IV-TR criteria of AD were selected and an age- and gender-matched control group was recruited. The participants' cognitive performance was measured according to the Mini Mental State Examination (MMSE), the Global Deterioration Scale (GDS) and Clinical Dementia Ratio (CDR). The levels of cytokines were measured in supernatants of lymphocytes culture, using assays of ELISA and atomic absorption.

Higher levels of IL-6 and IFN- γ were found more in the altered T lymphocytes of the AD patients rather than in the control individuals. Furthermore, a marginal significant difference was found between the TGF- β levels of the two study groups. Regression analysis of CDR score and cytokines showed the inverse significant correlation between CDR score and IFN- γ levels. Furthermore, the relation between MMSE scores and IFN- γ was significant, meaning that by increasing MMSE score, IFN- γ level was significantly increased.

This study suggests that the levels of IL-6 and IFN- γ are significantly increased in altered T lymphocytes of AD patients, as compared to those who are not inflicted with AD, and that they are related to the patient's age. Also, IFN- γ is related to the severity stage of the AD.

Keyword: Alzheimer's Disease (AD); Cytokine; Vitamin B12

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INTRODUCTION

The inflammatory function of the immune system causes or contributes to multiple diseases, for example; atherosclerosis,¹ HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and myotonic muscular dystrophy (MMD).² Alzheimer's disease (AD) is a progressive, degenerative, and irreversible disorder, with a possibly inflammatory pathogenesis. It destroys the brain, especially affecting the higher structures. Senile plaques, neurofibrillary tangles, synaptic and neuronal loss are the characteristic neuropathologic features of the disease. There is increasing evidence that chronic inflammatory processes play a fundamental role in the progression of the neuropathological changes of AD. It has been shown that there is a strong correlation between local inflammation and development of senile plaques (SPs) and neurofibrillary tangles (NFTs). The primary cells involved in the inflammatory process in AD are thought to be microglia and astrocytes. The activation of glial cells is associated with an upregulated expression of a variety of molecules involved in inflammatory response; including cytokines, various components of the complement cascade, acute phase reactants and proteases, protease inhibitors and neurotoxic products.³ Alzheimer's disease is the most common cause of dementia, especially in the elderly, with gradual loss of cognitive functions, but the etiology of the disease still remains unknown. The neuronal damage seen in the AD brain seems to be facilitated by numerous cytokine and cellular cascades, together with an activation of the brain's innate immune system.^{4,5} There are several elusive molecular events that coordinate the inflammatory processes in AD. In 1989 Griffin et al.⁶ identified that an over-expression of interleukin-1 (IL-1) was seen in the AD brain. Based on the role of IL-1 in chronic activation of macrophages, the peripheral analogs of microglia,⁷ in regulation of antibody production and in $\alpha\beta$ -mediated neurotoxicity,^{8,9} the correlation was understandable. Recently, studies have shown that a variety of different inflammatory responses, involving different cytokines and mediators, are involved in the pathogenesis of AD.^{10,11} Important cytokines include; IL-6,¹² transforming growth factor (TGF- β 1),¹³ interferon-gamma (IFN- γ),¹⁴ IL-2 and IL-3.¹⁵ Besides the role of cytokines, dietary supplementation, especially cobalamin (vitamin B12),

has been demonstrated to affect T lymphocyte activity and cytokine production in elderly patients.¹⁶ Previous studies have reported that vitamin B12 deficiency leads to an overproduction of TNF- α in rats and humans,¹⁷ suppressing the pro-inflammatory cytokines IFN- γ , IL-1 and IL-6 in vitro reflecting Th1 activation.¹⁸ Considering the general agreement on the role of some cytokines (e.g. IL-6, IL-1, TGF- β 1, IL-4, and IFN- γ) in Th1/Th2 balance in the progression of AD, this study compares the concentration levels of these cytokines, as well as serum vitamin B12, in altered T lymphocytes in the patients of the study with those of the control individuals. Associations with age, clinical course and disease risk are evaluated.

MATERIALS AND METHODS

In this case-control study, patients were recruited from the Neuropsychiatry Clinic (in Ibn-e-Sina Psychiatry Hospital) of Mashhad University of Medical Sciences in Mashhad (the second most populous city of Iran and the largest city of the north-eastern part of the country). Patients who met the DSM-IV-TR criteria of AD were selected from the patients of Ibn-e-Sina Psychiatry Hospital clinic using the methods of available sampling.¹⁹ The patients cognitive performance were measured using instruments such as, Mini Mental State Examination (MMSE), Global Deterioration Scale (GDS), and Clinical Dementia Ratio (CDR). Also, the patients underwent physical examination, laboratory testing, and brain MRI to exclude causes of dementia other than AD. A control group consisting of patients from the same psychiatric clinic without signs of AD, matched for age and gender, was recruited. An MMSE was also done on the control group measuring their cognitive functions for comparison with the AD patients.

In total, 40 AD patients and 40 individuals without signs of AD were recruited. The whole blood of the participants was collected using venopuncture in vacutainer tubes containing ethylenediamine tetraacetic acid. The samples were kept at room temperature and levels of cytokines and vitamin B12 was measured within 2 hours. The AD patients did not present any major co-morbidity factors, such as cancer, symptomatic (present or previous) cardiovascular diseases, or major inflammatory diseases such as autoimmunity and infections.

Individuals with a history of allergies, autoimmune

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disorders, malignancies, malnutrition and chronic infections were excluded. In addition, participants using immune system suppressing medicines, such as corticosteroids or immune regulator therapies, including Levamisole, Cimetidine and Dapsone, were excluded from the study. The protocol used in the present study was approved by the local medical ethics committee at Mashhad University of Medical Sciences and conforms to the ethical guidelines of the World Medical Association Declaration of Helsinki. First, the details of our study were explained to the participants, and they were then asked to read and sign the written consent forms.

Cytokine Measurements

Lymphocytes were isolated from heparinized venous blood by Ficoll-Hypaque density centrifugation, washed three times in PBS and re-suspended at a concentration of 1×10^6 per ml in RPMI 1640. The cells (2×10^6 per well) were incubated overnight (12 hours) with complete medium in 24-well micro titer plates (Costar, Cambridge, MA). After 24 hours, a part of the lymphocyte was stimulated for cytokine production for 4 hours at 37°C with 10 mg/ml phorbol 12-myristate 13-acetate (PMA) in combination with 1mg/ml ionomycin (Sigma, St Louis, MO). The levels of IL-1, IL-4, IL-6 and IFN- γ were measured in supernatants culture using ultrasensitive enzyme linked immunosorbent assay

(ELISA) kits (Quantikine R&D Systems Europe Ltd, Barton Lane Abingdon, Oxon, United Kingdom). According to the manufacturers' instructions and in order to validate the reproducibility of ELISA results, all the procedures for cytokines measurements were performed twice.

Statistical Analysis

SPSS for Windows, version 16 (SPSS Inc., Chicago, IL, USA) was used in all statistical procedures. Numerical data were expressed as mean \pm SD or a proportion of the sample size. All data were checked for normality by Kolmogorov-Smirnov test (K-S test). Student's t-test or Mann-Whitney test was used to compare the consequent data. Pearson and Spearman tests were used for evaluating the MMSE and CDR clinical examinations of the cytokines measurements, as well as descriptive variables like age and gender. A *p*-value less than 0.05 was considered significant.

RESULTS

Table 1 summarizes the clinical characteristics of the participants. The results suggest that there was no significant difference in vitamin B12 status between the AD patients and the control group ($p=0.77$). The findings obtained from linear regression tests indicate that there are significant reverse correlations between CDR and MMSE in AD patients ($p=0.001$, $r=-0.77$).

Table 1. Clinical data of participants (Mean \pm SD)

| Variable | Alzheimer's Disease Patients (n=40) | Healthy Control (n=40) |
|----------------------|-------------------------------------|------------------------|
| Age | 74 \pm 7.49 | 71.5 \pm 7.72 |
| Sex; male/female | 16/24 | 16/24 |
| CDR | 2 \pm 0.87 | ---- |
| MMSE | 14.54 \pm 7.4 | 24.45 \pm 3.8 |
| Vitamin B-12 (pg/ml) | 431.2 \pm 41.9 | 362.8 \pm 42.8 |

CDR, clinical dementia rating scale; MMSE, mini-mental state examination

Table 2. Measured cytokines of participants

| Cytokines (pg/ml) | Healthy Control | Alzheimer's disease Patient | P value |
|-------------------|-------------------------------|-----------------------------|---------|
| IL-1 | 29.30 \pm 2.08 ^a | 36.80 \pm 30.3 | 0.24 |
| IL-4 | 10.6 \pm 1.06 ^b | 7.50 \pm 3.90 | 0.15 |
| IL-6 | 2636 \pm 81.40 ^a | 3316 \pm 76.94 | 0.001 |
| TGF- β | 2707 \pm 29.39 ^a | 3714 \pm 18.48 | 0.07 |
| IFN- γ | 243 \pm 13.95 ^a | 386.2 \pm 22.46 | 0.001 |

Data presents as Mean \pm SD; $P < 0.05$ (^aT-Test, ^b Mann-Whitney U test)

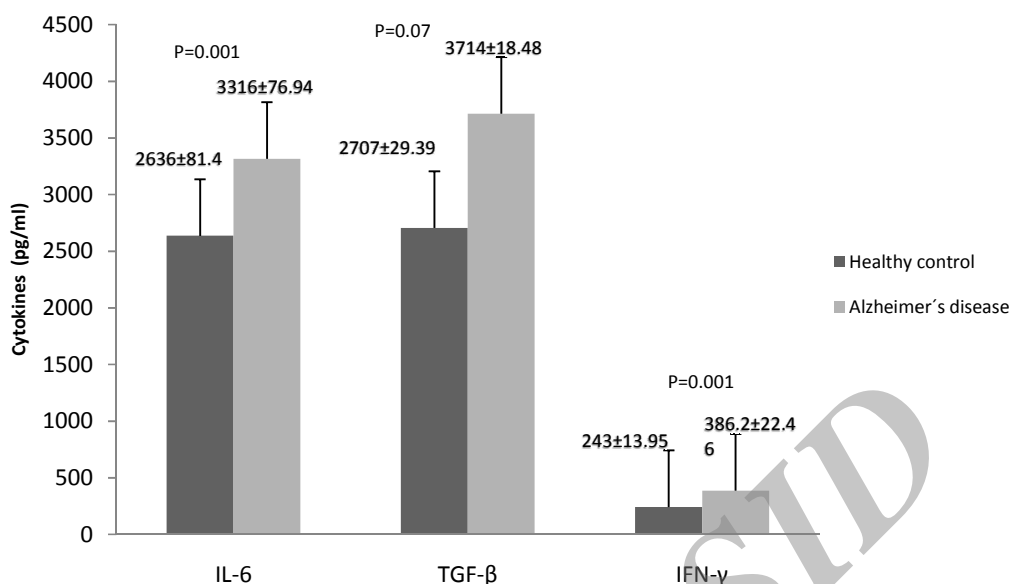


Figure 1. Comparison of levels of IL-6, TGF-β, and IFN-γ, between AD patients and healthy controls, measured in pg/ml. Error bars depicts SD.

Cytokine Levels and its Correlation with Clinical Variables

Table 2 displays the secretion of IL-1 and IL-4 in AD patients and control individuals. Levels of IL-6, TGF-β and IFN-γ are presented in Figure 1. Higher concentrations of IL-6 and IFN-γ were found in the

altered T lymphocytes of AD patients than in those of the control individuals ($p=0.001$). Furthermore, a marginally significant difference was found between TGF-β levels of the control group and AD patients ($p=0.07$). Correlation between cytokine production and clinical features of AD patients is presented in

Table 3. Correlation between PBMC cytokine production and clinical features of Alzheimer's disease patients

| Cytokines (pg/ml) | Predictors | Alzheimer's disease Patient | |
|-------------------|------------|-----------------------------|-------|
| | | P value | r |
| IL-1 | Age | 0.08 ^a | 0.27 |
| | CDR | 0.70 ^a | -0.04 |
| | MMSE | 0.49 ^a | 0.11 |
| IL-4 | Age | 0.50 ^b | -0.10 |
| | CDR | 0.32 ^b | -0.15 |
| | MMSE | 0.57 ^b | 0.09 |
| IL-6 | Age | 0.02 ^a | 0.34 |
| | CDR | 0.88 ^a | -0.2 |
| | MMSE | 0.08 ^a | 0.21 |
| TGF-β | Age | 0.14 ^a | 0.23 |
| | CDR | 0.08 ^a | 0.27 |
| | MMSE | 0.11 ^a | 0.25 |
| IFN-γ | Age | 0.004 ^a | 0.44 |
| | CDR | 0.006 ^a | -0.42 |
| | MMSE | 0.001 ^a | 0.48 |

$P < 0.05$ is considered significant (^a Pearson test, ^b Spearman test)

Table 3. A significant correlation was noted in AD patients between age and the status of IL-6 and IFN- γ ($r=0.34$, $r=0.44$, respectively), while this correlation was positive only for IFN- γ ($p=0.01$, $r=0.44$) in control individuals. There was no significant difference in cytokines concentrations between male and female participants ($p=0.45$). Besides cytokines measurements, the analysis of TH1/TH2 balance in AD patients confirms that there is not any statistical significant difference of TH1/TH2 balance between the control and AD patients ($p=0.41$). Regression analysis of CDR scores and cytokine data showed an inverse significant correlation between CDR scores and IFN- γ levels ($p=0.006$, $r=-0.42$). Furthermore, there was a significant association between increasing MMSE scores and increasing IFN- γ ($p=0.001$, $r=0.48$).

DISCUSSION

Previous studies have reported the involvement of both the innate and acquired immune systems in AD.^{20,21} The potential inflammatory biomarkers in AD have already been recognized²² while pathogenesis of AD still remains a complex issue. Data from in-vitro and in-vivo studies seem to indicate a reverse connection between vitamin B12 and inflammatory response, as low B12 level is associated with greater production of IL-6.²³ However, in this study, we found no significant difference between AD patients and healthy control individuals in this regard. Based on previous findings, the fact that IL-1 increased the neuronal acetyl cholinesterase expression and activity, possibly explains, in part, the cholinergic dysfunction characteristic of AD patients.²⁴ In addition to the microglia, over-expression of IL-1 is shown at all stages of neurofibrillary tangle formation^{25,26} as well as A β plaque formation and AD recovery processes.²⁷ Similarly, microglial activation and IL-1 over-expression have been shown in transgenic animal models of AD.²⁸ Besides this evidence, there are research suggesting that IL-1 decreases in AD patients with an increase in AD severity²², but this is still considered controversial. We found that IL-1 increased in AD patients, but that increase was not statistically significant when compared with the increase seen in the the control group. Also, we could not find any relation between IL-1 status and the clinical parameters monitored in the study.

In addition to IL-1, IL-6 is a major pro-inflammatory cytokine, the secretion of which could be induced by IL-1.³³ In AD patients, the expression of IL-6 is increased^{29,30} while the levels in the brain tissue stay normal.^{31,32} IL-6 excretion, on the other hand, has been reported at increased levels,^{12,33} normal levels,^{12,34} or even decreased³⁵ levels in CSF fluid of AD patients. In our study, IL-6 increased significantly in the experimental group vis-à-vis the control group. Also, we found that such an increase was even more significant among the older AD patients. Lee et al.³⁶ and Chao et al.³⁷ noted that the TGF- β concentration in CSF fluid of AD patients increased, as compared to the control group. Also, they reported that plasma levels of TGF- β was related to AD severity in that its status increased in relation to mild and moderate AD severity; however, it decreased for sever AD.³⁶ However, based on the ethical protocol adopted by this study, we were restricted to evaluate the CSF fluid of the participants, but we found that the difference of TGF- β increment in AD patients was not significant, as compared to that of the control group. Butovsky et al.³⁸ presented data that the cytokines IFN- γ (especially) and IL-4 as an immunosuppressive cytokine, characteristic of pro-inflammatory and anti-inflammatory T cells, respectively, can make microglia neuroprotective. Chao et al.³⁹ noted that pretreatment of cell cultures with IL-4, prevented the neuronal cell injury induced by activated microglia. IL-4 exerted its neuroprotective effects by inhibition of IFN- γ priming of microglia with a subsequent decrease in the production of TNF-alpha and nitric oxide in AD patients. Lee et al.³⁶ concluded that IL-4 concentration is decreased in AD patients, as compared to the control group. Similarly, our study found a decrease of IL-4 concentration level for AD patients, thus, confirming the findings of Lee et al. In addition to these cytokines variations, IFN- γ status in altered T lymphocytes of AD patients in this study significantly increased, too and the relation of MMSE score and IFN- γ was significant.

This study suggests that the levels of IL-6 and IFN- γ are significantly increased in AD patients and are related to the age of the patient. Also, IFN- γ is related to the stage of disease in terms of acuteness, but other cytokines under investigation, including IL-1, IL-4 and TGF- β , are not related to Alzheimer disease severity. Finally, considering the limitations of the present study, the authors recommend that further studies be carried

out, similar to present experiment, but with larger sample size intending to evaluate the cytokines simultaneously both in CSF and serum.

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