

## Multiplex Analysis of Cytokines in the Serum and Cerebrospinal Fluid of Patients With Alzheimer's Disease by Color-Coded Bead Technology

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**Background and purpose:** The availability and promise of effective treatments for neurodegenerative disorders are increasing the importance of early diagnosis. Having molecular and biochemical markers of Alzheimer's disease (AD) would complement clinical approaches, and further the goals of early and accurate diagnosis. Combining multiple biomarkers in evaluations significantly increases the sensitivity and specificity of the biochemical tests.

**Methods:** In this study, we used color-coded bead-based Luminex technology to test the potential of using chemokines and cytokines as biochemical markers of AD. We measured the levels of 22 chemokines and cytokines in the serum and cerebrospinal fluid (CSF) of 32 *de novo* patients (13 controls, 11 AD, and 8 Parkinson's disease [PD]).

**Results:** MCP-1 was the only cytokine detectable in CSF, and its levels did not differ between control and disease groups. However, the serum concentration of eotaxin was significantly higher in AD patients than in the control group.

**Conclusions:** The analysis of multiple inflammatory mediators revealed marginal differences in their CSF and serum concentrations for the differential diagnosis of AD and PD. These results provide evidence that immunological responses are not major contributors to the pathogenesis of AD and PD.

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**Key Words:** Alzheimer's disease, Biomarker, Serum, Cerebrospinal fluid, Neurodegeneration

### INTRODUCTION

The promising developments in effective treatments for neurodegenerative disorders are increasing the demand and urgency for the early and accurate diagnosis of dementia.<sup>1</sup> Alzheimer's disease (AD) is the

most common form of dementia, for which the sensitivity of clinical diagnosis is relatively high; however, the specificity was lower than 60% in a multicenter clinical-autopsy study.<sup>2</sup> Even though the predictability of a clinical diagnosis of AD becomes as high as 90% in specialized institutions, very early diagnosis of AD remains a challenge in many clinical settings.

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At present, diagnosing AD and distinguishing it from other dementias depend primarily on clinical evaluation, and ultimately on clinical judgment. Knowledge of molecular and biochemical markers of AD would complement clinical approaches, and further the goals of early and accurate diagnosis. The proposed criteria for an ideal biomarker of AD includes a sensitivity >80% for detecting AD and a specificity of >80% for distinguishing it from other dementias.<sup>3</sup> Combined evaluations with multiple biomarkers significantly increase the sensitivity and specificity of the biochemical tests,<sup>4</sup> which has prompted several research groups to develop innovative methods for simultaneously quantifying multiple biomarkers in the serum and cerebrospinal fluid (CSF) using solution-based protein chip analysis.<sup>5,6</sup>

## MATERIALS AND METHODS

### 1. Patients

This study was approved by the ethics committees of Ewha University, and all patients and/or their caregivers gave their informed consent. AD and Parkinson's disease (PD) patients were diagnosed according to the criteria of the National Institute of Neurological and Communicative Disorders and the Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), and the UK Parkinson's Disease Society Brain Bank.<sup>7-9</sup> Normal healthy controls without neurological involvement were included for comparison. All patients underwent a standard battery of medical and neuropsychological tests, brain magnetic resonance imaging or computed tomography and, if indicated, positron-emission computed tomography. Patients with recent infections (occurring less than 2 months previously), signs of chronic inflammation, and using any medications related to neurologic symptoms and nonsteroidal anti-inflammatory drugs were excluded.

### 2. Multiplex analysis of cytokines

In this study, we used Luminex xMAP technology

for multiplexed quantification of 22 cytokines in the serum and CSF.<sup>6</sup> Lumbar punctures were performed with the patient in the recumbent position according to a standard procedure. The multiplexing analysis was performed using the Luminex<sup>TM</sup> 100 system (Luminex, Austin, TX, USA) by the diagnostic kit development team of Ab Frontier (Suwon, Korea). Twenty-two cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, TNF- $\alpha$ , interferon- $\gamma$ , GM-CSF, eotaxin, IP-10, MIP-1 $\alpha$ , MCP-1, and RANTES) were simultaneously measured in the serum and CSF using a Beadlyte Human Multicytokine Beadmaster kit (Upstate, Lake Placid, NY, USA) according to the manufacturer's protocol. The sensitivity of these bead sets was 0.1-10 pg/mL. Total tau and A $\beta$ 42 in serum and CSF were simultaneously analyzed using human total tau and A $\beta$ 42 antibody bead kits (Biosource, Camarillo, CA, USA) according to the manufacturer's protocol.

### 3. Statistical analysis

Biomarkers in the patient groups were analyzed statistically using ANOVA with Tukey's honest significant-difference post-hoc test applied to significant main effects (SPSS version 12.0K for Windows, SPSS, Chicago, IL, USA).

## RESULTS

A total of 32 patients were enrolled, comprising 13 controls, 11 AD, and 8 PD patients (Table). Before analyzing multiple cytokines, we first examined the levels of total tau and A $\beta$ 42 in the CSF samples to confirm whether our study protocol could reproduce the same results for the levels of tau and A $\beta$ 42 in AD and PD. It has been well documented that the CSF levels of A $\beta$  decline while those of total tau increase in AD patients.<sup>4,10,11</sup> Consistent with previous reports, we also observed a significant decrease in A $\beta$ 42 but no significant increase in total tau (data not shown).

Since the A $\beta$ 42 and tau profiles were compatible

with previous reports, we next applied the multiplexed analysis of 22 cytokines and chemokines to the samples. In the CSF, only MCP-1 was detectable, with the levels not differing significantly between the control and disease groups. Fifteen of the 22 analyzed cytokines were detectable in the serum samples. Interestingly, the serum concentration of eotaxin (which is a chemotactic factor for eosinophil) was significantly higher in AD patients than in controls. IL-12p40 was also elevated in both AD and PD, while IL-12p70 did not differ significantly between the disease groups. Interferon- $\gamma$ , MIP-1 $\alpha$ , and IP-10 were elevated in both AD and PD patients, but the differences from controls were not statistically significant.

## DISCUSSION

Among 22 cytokines and chemokines, only eotaxin

was significantly elevated in the serum of AD patients compared to the controls. This is not consistent with a previous report that the CSF levels of IL-8, IP-10, and MCP-1 were increased in AD patients.<sup>12</sup> This discrepancy might be partly due to differences in the sensitivities of the different assays used and in patient characteristics. Since we enrolled *de novo* patients who had no history of medication related to neurologic symptoms, our cohort might have comprised patients with relatively mild conditions and in the early stage compared to previous studies. To our knowledge, increased levels of eotaxin have not been related to any neurologic disorders without autoimmune components. Increased levels of eotaxin have been reported in ischemic coronary diseases, obesity, and allergic disorders such as asthma, parasitic infections, and allergic respiratory diseases.<sup>13,14</sup> The significance of increased eotaxin in AD is currently unclear. IL-12p40 was also elevated in both AD and PD, while IL-12p70

**Table.** Demographic data and analysis of serum and CSF levels of biomarkers

	Control	AD	PD	$F_{2,31}$ , $P^*$
Number (M/F)	13 (5/8)	11 (2/9)	8 (0/8)	1.94, 0.16
Age (years)	68.5 $\pm$ 7.2	73.5 $\pm$ 4.0	73.0 $\pm$ 8.6	1.42, 0.26
Serum levels of cytokines (ng/mL)				
IL-1 $\alpha$	6.1 $\pm$ 21.6	n.d. <sup>†</sup>	1.8 $\pm$ 5.0	0.60, 0.56
IL-4	14.4 $\pm$ 2.8	13.8 $\pm$ 0.6	13.6 $\pm$ 0.3	0.66, 0.53
IL-5	9.4 $\pm$ 0.8	9.1 $\pm$ 0.3	9.1 $\pm$ 0.2	0.65, 0.53
IL-7	40.7 $\pm$ 11.1	37.2 $\pm$ 3.1	37.0 $\pm$ 1.0	0.91, 0.41
IL-8	11.8 $\pm$ 5.2	11.0 $\pm$ 1.3	10.6 $\pm$ 0.3	0.38, 0.69
IL-12p40	39.3 $\pm$ 19.3	60.8 $\pm$ 22.6	60.4 $\pm$ 32.7	2.98, 0.07
IL-12p70	22.7 $\pm$ 9.8	21.8 $\pm$ 6.7	23.1 $\pm$ 12.8	0.05, 0.96
IL-13	19.6 $\pm$ 11.0	32.1 $\pm$ 21.5	23.6 $\pm$ 8.2	2.09, 0.14
Interferon- $\gamma$	22.2 $\pm$ 13.7	34.0 $\pm$ 28.4	31.3 $\pm$ 10.4	1.19, 0.32
GM-CSF	24.1 $\pm$ 13.9	25.5 $\pm$ 9.8	24.5 $\pm$ 12.5	0.04, 0.96
Eotaxin	6.9 $\pm$ 10.0 <sup>‡</sup>	23.0 $\pm$ 24.8 <sup>‡</sup>	21.9 $\pm$ 19.3	3.42, 0.047
IP-10	2.2 $\pm$ 0.2	4.3 $\pm$ 0.4	3.7 $\pm$ 0.4	2.40, 0.11
MCP-1	11.4 $\pm$ 7.7	22.3 $\pm$ 31.9	15.9 $\pm$ 10.5	0.87, 0.43
MIP-1 $\alpha$	5.1 $\pm$ 3.1	14.2 $\pm$ 17.6	13.3 $\pm$ 21.0	1.37, 0.27
RANTES	9.9 $\pm$ 0.2	1.1 $\pm$ 0.9	1.1 $\pm$ 0.2	1.90, 0.17
TNF- $\alpha$	26.8 $\pm$ 18.7	34.7 $\pm$ 22.8	30.6 $\pm$ 22.2	0.43, 0.66
CSF concentrations of cytokines (ng/mL)				
MCP-1	1.8 $\pm$ 0.6	2.0 $\pm$ 1.1	3.4 $\pm$ 4.3	1.63, 0.22
A $\beta$ <sub>42</sub>	0.4 $\pm$ 0.2	0.3 $\pm$ 0.1 <sup>‡</sup>	0.6 $\pm$ 0.4 <sup>‡</sup>	3.27, 0.04
tau	0.9 $\pm$ 0.5	1.2 $\pm$ 0.9	0.4 $\pm$ 0.2	1.71, 0.19

F, females; M, males.

\*ANOVA (between groups) <sup>†</sup>n.d., not detected <sup>‡</sup>Tukey's post-hoc test applied to significant effect of group ANOVA,  $p < 0.05$ .

Data are mean $\pm$ SD values.

did not differ significantly between the disease groups. IL-12 has been implicated in proinflammatory responses to bacterial infection and in recovery after interferon treatment.<sup>15,16</sup> However, confirming these preliminary results requires longitudinal analysis of a larger group of patients.

Interestingly, the levels of A $\beta$ 42 and tau differed between the PD group and the control and AD groups, with a decrease in CSF tau and increase in A $\beta$ 42 in PD. This finding is opposite those of previous studies showing that tau protein levels were significantly higher and A $\beta$ 42 lower in PD patients than in AD patients and controls.<sup>17,18</sup> This discrepancy might be due to the small numbers of patients and the selection of nondemented patients in our study. The combined analysis of total tau and A $\beta$ 42 not only facilitated the clear distinction between AD and controls but also better differentiation between AD and PD.

Since CSF is directly contiguous with the central nervous system, we initially expected that any changes in immunological responses related to neurodegeneration would be predominantly reflected in the CSF. However, we instead observed significant differences in the pattern of a proinflammatory biomarker in the serum (and not in the CSF) between control and AD patients. Even though the lumbar puncture is a relatively easy procedure with a low incidence of complications, it is highly preferable to use blood samples for biochemical analysis, especially for screening purposes.<sup>1</sup>

Since the present study demonstrated differences in a proinflammatory mediator in AD and PD patients, the further exploration and development of surrogate biomarkers for neurodegenerative disorders might yield useful tools for the differential diagnosis of dementia, for monitoring disease progression and the therapeutic efficacy of potential treatments, and also for improving the understanding of the underlying pathological mechanisms.

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