Cerebrospinal Fluid Cytokine Dynamics Differ Between Alzheimer Disease Patients and Elderly Controls

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Abstract: The time courses of levels of multiple plasma and cerebrospinal fluid (CSF) cytokines in patients with Alzheimer disease (AD) and in age-matched control subjects were compared. Interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, granulocyte-macrophage colony-stimulating factor, interferon-γ, and tumor necrosis factor alpha levels were measured 7 times over a 24-hour period in plasma and CSF using a lumbar catheter. Baseline plasma and CSF cytokine levels were found to be similar in AD and control subjects. However, the CSF levels of all measured cytokines, except IL-6 and IL-8, diverged over time between AD and control subjects, such that CSF cytokine levels in AD subjects were higher than in controls. This difference was greatest at 24 hours after the insertion of the lumbar catheter. In contrast, no differences in cytokine trajectories were seen in plasma. These data suggest that the neuroinflammatory response to lumbar catheter placement differs between AD and control subjects.

Key Words: dementia, Alzheimer, cytokine, diurnal, cerebrospinal fluid

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Recent evidence suggests that dysregulation of neuroinflammatory signaling mechanisms plays a role in the pathogenesis of Alzheimer disease (AD; reviewed in[1,2]). Pathologically, the inflammatory response in the AD brain is manifested as plaque-associated activated microglia and astrocytes, and the degree of microglial activation parallels the severity of synaptic loss associated with plaque deposition.[3,4] Similarly, the levels of several cytokines have been found to be abnormal in the AD brain,[5] and several studies have found associations between polymorphisms in the genes for interleukin (IL)-1 and tumor necrosis factor alpha (TNF-α) and AD.[6,7] Furthermore, cytokines such as IL-1, IL-6, and TNF-α have been implicated in an inflammatory cascade that leads to the hyperproduction of amyloid-β and the hyperphosphorylation of tau.[8] These data suggest that cytokine levels of AD patients may reflect the severity of AD pathology and that cytokine measurements in either the plasma or cerebrospinal fluid (CSF) may play a role either in the assessment of the stage of the disease or in response to drug therapy.

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Cytokine levels have been measured in blood and to a much lesser extent in the CSF of AD patients. A recent meta-analysis of 40 studies demonstrated that significantly higher blood concentrations of IL-6, TNF-α, IL-1β, transforming growth factor β, IL-12, and IL-18 were observed in AD patients compared with healthy controls.[9] Despite the growing literature documenting peripheral cytokine abnormalities in AD patients, a consistent picture has not emerged for central cytokine levels, as measured in the CSF. This may be because most previous studies have focused on the measurement of individual or a few cytokines at a single time point. However, as the immune response involves the coordination of multiple cytokines, it is likely that simultaneous measurement of an array of these molecules would have a greater potential to detect a therapeutic response or to distinguish between AD and non-AD patients. Furthermore, repeated sampling paradigms in individual subjects tend to carry greater power to detect therapy-induced changes in any biomarker as this approach substantially limits intersubject variability.

Therefore, the goal of this study was to simultaneously measure multiple cytokines in the plasma and CSF of AD and control subjects and assess the changes of these levels over time. The latter issue carries significance because fluctuations in CSF cytokine levels across a 24-hour period may limit the utility of these cytokines as biomarkers. For example, CSF amyloid-β levels, when measured at a single time point, are sensitive diagnostic markers for the presence of AD.[10] However, the utility of CSF amyloid-β as an acute pharmacodynamic marker may be limited because of its fluctuations over time in CSF. This was shown by Bateman et al,[11] who demonstrated that CSF levels of amyloid-β 1 to 40 and 1 to 42 in healthy subjects, assessed using an indwelling catheter, increased over the first 18 to 24 hours after catheter insertion and then remained stable between 24 and 36 hours. We have found very similar trajectories in a separate study.[12] The mechanism for this change over time is not known, and the temporal profiles of other potential biomarkers, such as CSF cytokines, are not known. Therefore, the goal of the current study is to characterize the 24-hour profile of the levels of multiple CSF cytokines in AD and control subjects to determine their suitability for use in pharmacodynamic biomarker studies.

METHODS

Subjects

This exploratory biomarker study was a single-center study, conducted at California Clinical Trials, Glendale, California. The study protocol and informed consent forms were approved by an independent ethics committee [California Institutional Review Board (IRB) Inc., IRB #

07-021], and written informed consent was provided by all participants. All subjects underwent a screening period from day −28 to day −2, during which eligibility was assessed and written informed consent was provided. Subjects (AD patients and controls) were recruited from the local population and were compensated for their participation. Subjects were admitted to the unit on day −1, and study samples were collected from day 1.

Screening involved a full medical history, a physical examination, an electrocardiogram (ECG), a lumbar spine x-ray (unless the subject had such an x-ray taken within 1 y of screening), and either a computed tomography or magnetic resonance imaging of the brain (unless the subject had such imaging within 28 d of day −1). Samples for laboratory safety assessments (including serum biochemistry, hematology, urinalysis, and serology) and drug and alcohol abuse tests were taken after an 8-hour fasting period. Female subjects undertook urine and serum pregnancy tests.

Subjects between 55 and 90 years of age were recruited for this study. AD subjects met the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association criteria for probable AD during screening.[13] In addition, they had a Mini-Mental Status Examination (MMSE) score of 15 to 26 (inclusive) and a Modified Hachinski Ischemic Scale score of ≤ 4 during screening. Two subjects were taking a cholinesterase inhibitor during the study. No subjects were taking memantine. Control subjects had an MMSE score ≥ 27 during screening. With the exception of the presence of stable medical conditions, the subjects were in general good health, based upon the results of a medical history, physical examination, vital signs, laboratory profile, and a 12-lead ECG. All subjects, or their proxies, voluntarily signed and dated an informed consent, approved by an Independent Ethics Committee/IRB before the initiation of any study-specific procedures, including withdrawal of medications to qualify for the study.

Subjects were excluded from study enrollment if they had: a positive urine drug screen during screening for nonprescribed drugs of abuse, including but not limited to alcohol, cocaine metabolites, phencyclidine, opiates, barbiturates, benzodiazepines, marijuana metabolites, amphetamines, methadone, methaqualone or propoxyphene; a history of drug or alcohol disorder (abuse/dependence) (excluding nicotine) within 1 year before screening; current diagnosis of major depression or other major psychiatric disorder; an abnormally low vitamin B12 (cobalamin), an abnormally high thyroid stimulating hormone at screening, or a positive rapid plasma reagin test at screening that was confirmed by a positive fluorescent treponemal antibody absorption test (vitamin B12, thyroid tests, and syphilis tests applied to subjects with AD only); a positive test result for human immunodeficiency virus performed during screening; a history of any significant neurological disease (other than AD for those subjects enrolled in the AD group) including Parkinson disease, multi-infarct or vascular dementia, Huntington Disease, normal pressure hydrocephalus, brain tumor, progressive supranuclear palsy, seizure disorder, subdural hematoma, multiple sclerosis, or a history of significant head trauma followed by persistent neurological defaults or known structural brain abnormalities; a history of stroke or transient ischemic attack within the 6 months before study day −1; or the presence of any uncontrolled medical illness.

Study Procedures

The 11-item version of the Alzheimer's Disease Assessment Scale-cognitive subscale(ADAS-Cog)[14] was administered to all AD subjects at screening. After successful screening, subjects were admitted to the study unit on day −1 for a baseline safety assessment (vital signs, ECG and laboratory safety assessments, and drug/alcohol abuse and pregnancy tests). On day 1, after an overnight fast, all subjects were catheterized in the lumbar region (approximately L3 to L4) for CSF sampling. This was performed using the dynabridging technique (California Clinical Trials Inc., CA), in which the catheter is connected to a peristaltic pump for automatically timed withdrawal of CSF samples (approximately 8 mL per withdrawal). Venous blood samples (10 mL per withdrawal) were taken at the same time as CSF samples. Blood and CSF samples were taken 7 times over a 24-hour period. Samples were obtained at 0, 1, 4, 8, 12, 18, and 24 hours after lumbar catheter insertion. Time 0 = 5:30 AM. After collection of the 24-hour samples on the morning of day 2, the lumbar drain was removed. All study procedures were identical for AD and control subjects. Subjects were monitored for an additional 48 hours and discharged on the morning of day 4.

Biochemical Analysis

The measurement of CSF and plasma cytokines was performed using the MesoScale Discovery (MSD) (Gaithersburg, MD) 96-well MULTI-SPOT Human Cytokine Assay as outlined by the manufacturer's protocol. Briefly, the MULTI-SPOT plate coated with an array of cytokine capture antibodies [granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, and TNF-α)] was blocked with 25 μL diluent 2 (provided in the kit) for 30 minutes with vigorous shaking at room temperature. Next, 25 μL of sample (CSF or plasma) and calibrator were added and the plate was incubated 2 hours with vigorous shaking at room temperature. The plate was washed with 3× phosphate buffer saline containing 0.05% Tween-20. A volume of 25 μL of the detection antibody solution was added to the plate and incubated for 2 hours with vigorous shaking at room temperature. The plate was washed with 3× phosphate buffer saline containing 0.05% Tween-20 before adding 150 μL 2× MSD Read Buffer, and the plate was immediately read on an MSD Sector Imager 6000. Analysis was performed using MSD workbench version 3.0.17.3 (Gaithersburg, MD). A 4-plex assay was also run for the same samples for IL-1β, IL-6, IL-8, and TNF-α. We found that the values for the cytokines for both assays were correlated and took their average to enhance accuracy. A fit curve was generated for each analyte using the standards, and the concentration of each sample was calculated. CSF and plasma samples were analyzed in duplicate and randomized on each plate.

The measurement of CSF total protein was taken using a Pierce BCA Protein Assay Kit as outlined by the manufacturer's microplate procedure. Briefly, 12 μL of the standard and CSF sample (diluted 1:5 in phosphate buffer saline) was added to a clear 96-well plate, followed by the addition of 100 μL of working reagent. The plate was mixed on a plate shaker for 30 seconds before covering and incubating at 37°C for 30 minutes. The plate was allowed to cool briefly, and absorbance at 562 nm was measured on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA);

analysis was performed using Softmax 5.2 software. CSF samples were randomized and measured in duplicate.

CSF hemoglobin concentrations were measured in randomized CSF samples using an enzyme-linked immunosorbent assay kit (catalogue number E88 to 135) purchased from Bethyl Labs (Mongomery, TX). All procedures were according to the manufacturer's protocol. The detection range was found to be 0.5 to 200 ng/mL for undiluted standards with a detection range of 5 to 2000 ng/mL corresponding to the 1:10 dilution used for CSF samples. Samples with signals below the detection range were assigned concentrations equivalent to the lower limit (5 ng/mL), whereas samples with signals above the highest standard were assigned a concentration of 2000 ng/mL.

Data Analysis

Baseline continuous variables were compared with AD patients and controls using the Mann-Whitney test. To assess differences in cytokine measurements over time, we used a mixed-effects model. In this model, group (AD versus Control), time, and group × time interaction were included as fixed effects, baseline body weight and body surface area were included as covariates, and subjects nested within groups were included as random effects, with the compound symmetry covariance structure. Log transformation was necessary for all cytokines to ensure adequate symmetry of the distribution. To verify the symmetry of the distribution and to identify outliers, studentized residuals were derived from this mixed-effects model for each cytokine; samples whose studentized residuals were less than $Q1 - 1.5 \times (Q3 - Q1)$ or greater than $Q3 + 1.5 \times (Q3–Q1)$, where Q1 and Q3 represent the first and third quartiles of the distribution, were flagged as statistical outliers. These outliers were then removed before fitting the above mixed-effects model. In addition, IL-12p70 plasma values from 2 AD subjects were removed because they were 3 times greater than the standard deviation above the mean. A total of 52 data points out of 2772 (2.0%) were removed as outliers. Additional analysis was performed without removal of outliers, and the results were similar. Inference was made specific to each time point, and family-wise error rate was not controlled in the comparisons. We have verified the validity of the assumptions of the mixed model in this analysis (distribution, variance structure). These analyses were performed using JMP version 8.0.2. Comparisons with P-value < 0.05 were deemed statistically significant.

RESULTS

CSF and plasma were obtained at 7 time points over 24 hours from 15 subjects with AD and 7 age-matched control subjects. Time 0 = 5:30 AM. As shown in Table 1, the subjects were well matched in terms of age, sex, and ethnicity. Subjects in the AD group had a significantly lower MMSE score than the control group (22.1, SD = 3.2 vs. 29.1, SD = 0.9; $P = 0.0002$). The majority of AD subjects had mild AD, with 13/15 having MMSE scores > 20.

There were no differences between baseline (time 0) plasma cytokine levels of AD and control subjects (Table 2), and there were no significant correlations between the ADAS-Cog and baseline values of plasma cytokines (not shown). There was substantial variability in the cytokine levels of AD subjects, with many cytokines (IL-1, IL-2, IL-10, and IL-12) showing a coefficient of variation > 100%. Therefore, despite the trend for baseline levels of a few cytokines to be elevated in the plasma of AD subjects (eg, IL-10 and IL-12), these differences were not significant. Levels of these cytokines were sampled 7 times over a 24-hour period. Figure 1 illustrates the 24-hour changes in plasma levels of IL-1β, IL-2, TNF-α, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, and GM-CSF. There were trends for higher levels of IL-2, IL-10, and GM-CSF plasma levels over time in the AD versus control subjects, and this difference reached significance for the 4- and 8-hour time points for IL-2 and for the 4-hour time point for GM-CSF. The trend for IL-10 elevation was driven by 2 subjects, both of whom had sustained plasma IL-10 levels between 40 and 50 pg/mL, but did not reach significance overall.

CSF cytokine levels were also measured. Similar to plasma, no statistically significant differences in baseline levels of any cytokine (Table 3) were observed, and we found no significant correlations between baseline ADAS-Cog and baseline CSF cytokine levels (not shown). Unlike plasma, however, the concentration over time for many CSF cytokine levels differed between AD and control subjects. Significant increases in IL-1β, IL-2, IL-10, IL-12p70, GM-CSF, IFN-γ, and TNF-α were observed, compared with controls (Fig. 2); the difference between these cytokine levels in AD and controls tended to increase over time, even in the cytokines that did not reach statistical significance. No correlation was found between the increase in any cytokine level in plasma or CSF (measured as the difference between baseline and maximum increase) and MMSE or ADAS-Cog score (data not shown).

To ensure that differences in CSF cytokine levels were not because of a nonspecific effect related to fluid dynamics that would affect all proteins similarly, we examined the levels of total protein in CSF over time. As shown in Figure 3, the levels of CSF total protein were similar in AD and control subjects and did not substantially change over the course of 24 hours of CSF sampling. CSF hemoglobin values were measured to determine the degree

TABLE 1. Baseline Demographic Features of Subjects in This Study

Metric	AD Subjects (n = 15)	Control Subjects (n = 7)	*P*
Age	70.2 (7.4)	65.0 (5.2)	0.12
MMSE score	22.1 (3.2)	29.1 (0.9)	0.0002
ADAS-Cog score	21.9 (8.7)	—	—
No. males/females	12/3	5/2	0.66
No. white/black/Asian/others	10/3/1/1	7/0/0/0	0.39

Values for age, MMSE, and ADAS-Cog are expressed as mean and standard deviations and are shown in parentheses. *P*-values for age and MMSE scores were generated using the Mann-Whitney test. *P*-values for sex and ethnicity differences were generated using the χ^2 test.

ADAS-Cog indicates Alzheimer's Disease assessment scale-cognitive subscale; AD, Alzheimer Disease; MMSE, minimental status examination.

TABLE 2. Mean Baseline Plasma Cytokine Concentrations in pg/mL

Markers	AD (n = 15)	Control (n = 7)	*P* (AD vs. Control)	Lower Limit of Detection
IL-1β	1.02 (2.07)	0.71 (0.21)	0.1586	0.36
IL-2	0.51 (0.60)	0.77 (0.63)	0.3593	0.35
IL-6	2.44 (2.03)	2.15 (0.40)	0.3595	0.27
IL-8	7.82 (3.66)	5.71 (1.75)	0.1210	0.09
IL-10	8.65 (16.21)	2.24 (1.14)	0.5758	0.21
IL-12	9.32 (28.47)	1.97 (1.57)	0.6816	1.4
GM-CSF	1.42 (1.40)	1.18 (0.99)	0.8879	0.2
IFN-γ	1.64 (1.29)	2.05 (1.64)	0.8879	0.53
TNF-α	7.58 (1.51)	7.79 (2.02)	0.7780	0.50

Numbers in parentheses represent standard deviations. *P*-values determined using the Mann-Whitney test.
AD indicates Alzheimer Disease; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon γ; IL, interleukin; TNF-α, tumor necrosis factor α.

to which blood contamination contributed to CSF cytokine changes. The values of CSF hemoglobin were similar (lowest *P*-value = 0.66) in AD versus control patients. In addition, CSF hemoglobin was found not to be a significant covariate with any cytokine except IL-8 in the mixed-effects model.

DISCUSSION

To our knowledge, this is the first continuous CSF sampling study that examined matched CSF and plasma cytokine levels in AD versus control subjects. We demonstrated that the dynamics of CSF cytokine levels after lumbar catheterization differ between AD and age-matched

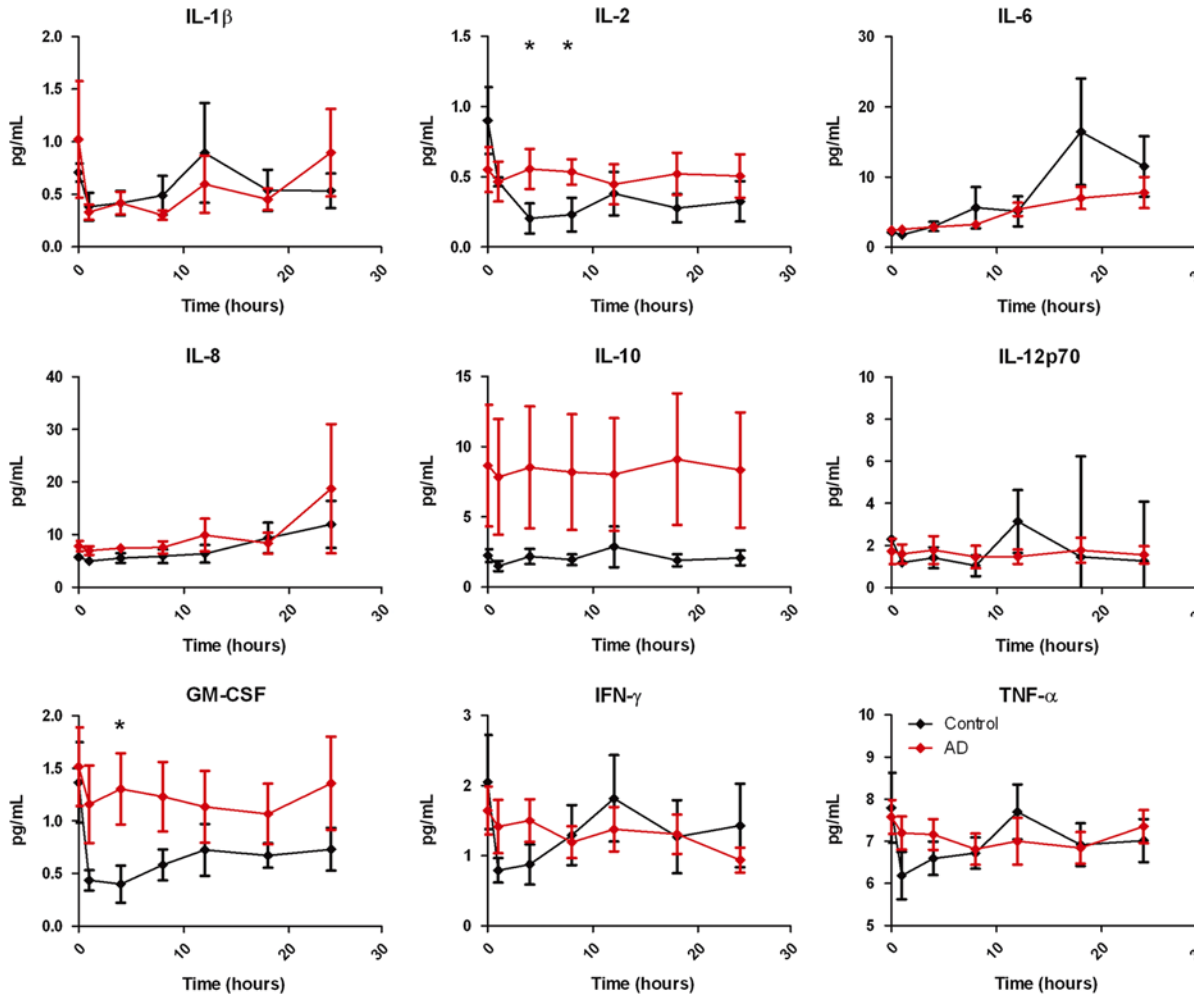

FIGURE 1. Mean concentration versus time plots for Alzheimer disease (AD) subjects (red) and control subjects (black) for plasma levels of 9 different cytokines. Error bars = standard error. *$P < 0.05$.

TABLE 3. Mean Baseline CSF Cytokine Concentrations in pg/mL

Markers	AD (n = 15)	Control (n = 7)	*P*	Lower Limit of Detection
IL-1β	0.77 (1.18)	0.97 (1.36)	0.5446	0.36
IL-2	2.21 (2.83)	2.57 (3.21)	0.8047	0.35
IL-6	2.46 (2.14)	3.04 (1.88)	0.2433	0.27
IL-8	36.72 (9.62)	39.84 (11.52)	0.5728	0.09
IL-10	2.45 (2.31)	2.60 (3.19)	0.7779	0.21
IL-12	2.07 (3.52)	3.22 (4.28)	0.8320	1.4
GM-CSF	1.09 (1.21)	1.26 (1.58)	0.6467	0.2
IFN-γ	3.81 (5.64)	5.40 (6.61)	0.9438	0.53
TNF-α	2.43 (3.38)	3.70 (4.00)	0.3232	0.50

Numbers in parentheses represent standard deviation. *P*-values determined using Mann-Whitney *U* test.
AD indicates Alzheimer Disease; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon γ; IL, interleukin; TNF-α, tumor necrosis factor α.

control subjects. Specifically, we found that there are sustained increases in the levels of multiple cytokines in CSF in AD subjects compared with control subjects and that these increases are not seen in plasma. These data suggest that the neuroinflammatory response to lumbar catheterization and subsequent CSF sampling differs in AD versus control subjects.

Our finding of similar baseline levels of CSF and plasma cytokine levels across AD and control subjects is similar to several previous reports for mild-to-moderate AD. For example, many studies have found similar CSF cytokine levels in AD and control subjects [(IL-10, IL-12),[15] (IL-6),[16] (IL-8, IL-10),[17] and (IL-1β)[18]]. Other studies have observed increased levels of various cytokines in the CSF of

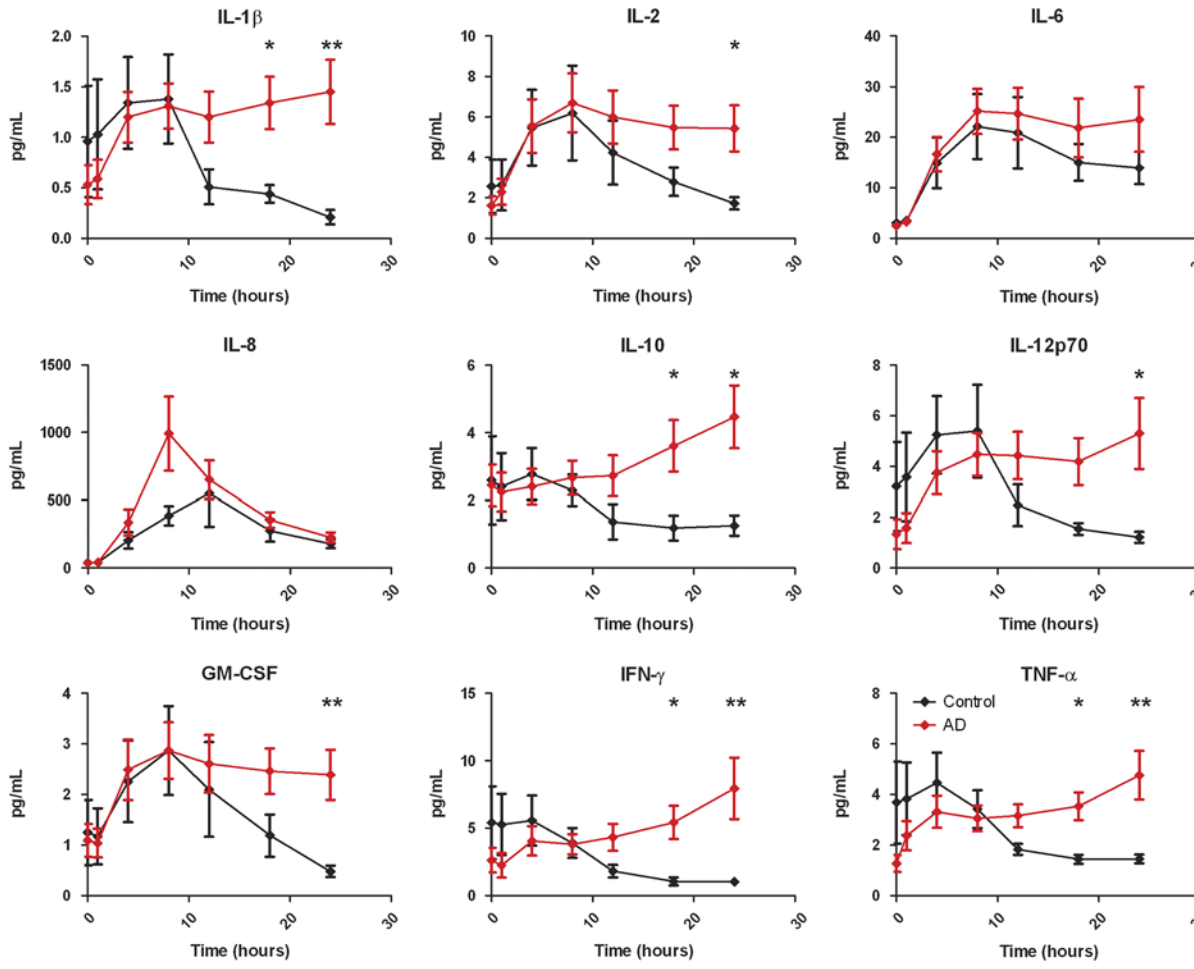

FIGURE 2. Mean concentration versus time plots for Alzheimer disease (AD) subjects (red) and control subjects (black) for cerebrospinal fluid levels of 9 different cytokines. Error bars = standard error. *$P < 0.05$, **$P < 0.005$.

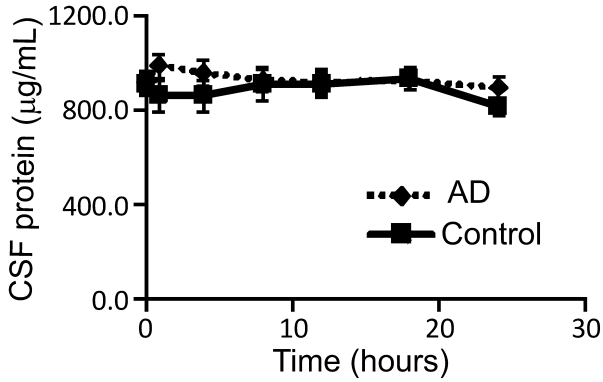

FIGURE 3. Mean values over time in cerebrospinal fluid protein concentrations in Alzheimer disease (AD) subjects (dotted line) versus healthy control subjects (black). Error bars = standard error.

AD subjects compared with controls [(TNF-α),[19] (TNF-α and IL-6),[20] (IL-8),[21] (IL-8),[22] (GM-CSF),[17] and (IL-6)[18]]. The latter group of studies tended to recruit subjects with a greater severity compared with our study. Most subjects in the current study were mildly affected (average MMSE score = 22.1). In plasma, a clear pattern has not emerged from the literature about baseline differences in cytokine levels in the mild-to-moderate AD population. Although several groups have reported elevations of baseline levels of IL-1β, IL-6, or TNF-α in AD subjects, these subjects tended to have more advanced disease compared with those studied here.[23–25] A recent meta-analysis of 14 studies concluded that baseline levels of IL-1β, IL-6, and TNF-α were not different between AD and control subjects, which is consistent with the current findings.[9] The same group did observe a consistent increase in transforming growth factor β in AD patients, but this cytokine was not measured in the current study. The data for other cytokines are quite heterogenous, with some studies showing elevations, decreases, or no differences (recently reviewed by Reale and colleagues[1]).

A novel feature of the current study was the simultaneous measurements of CSF and plasma cytokines over a 24-hour period. Although no substantial differences were seen over time in the plasma cytokine profile between AD and control subjects, we observed differences in CSF. Over time, the levels of all measured cytokines tended to increase in AD patients relative to control subjects; this effect could not be due to a general concentrating effect that might be seen only in AD subjects, as total CSF protein concentrations did not differ over time between AD and control subjects. One interpretation of these findings is that AD subjects produce a more robust inflammatory response to the insertion of the lumbar catheter. Although lumbar catheterization is generally considered to be a benign procedure, protracted lumbar catheterization carries risk of infection, bleeding, damage to underlying neural structures, and low-pressure phenomena, such as headache or cranial neuropathy.[26] It is possible that the protracted exposure to a foreign body, the introduction of minute amounts of blood products, and/or changes in pressure dynamics of the CSF space can induce inflammatory or other responses in the central nervous system. Given the exaggerated inflammatory responses seen in mononuclear cells from AD subjects, subjects at risk for AD,[27] and microglia from AD animal models,[28] the elevation in cytokines may represent an exaggerated (but subclinical) inflammatory response to an otherwise benign intervention. Future studies looking for a broader panel of peripheral inflammatory markers that include the cellular fraction of blood, such as mononuclear cell composition, may provide further data to evaluate this hypothesis. It is also possible that differences between AD and control subjects may reflect disrupted diurnal neuroinflammatory rhythmicity in AD subjects. This is supported by findings that central nervous system inflammatory mediators have diurnal fluctuations in their expression levels[29] and that AD patients have disruptions in diurnal fluctuations of multiple metabolic parameters.[30,31] Another potential explanation for the differences between AD and control subjects is differential clearance of cytokines induced by lumbar shunting of CSF. As the CSF volume is larger in AD versus control subjects as a consequence of brain atrophy,[32] any given volume of CSF removed from AD subjects represents a smaller proportion of total CSF compared with control subjects. Therefore, CSF collection may cause a greater rundown in CSF cytokine levels in control than in AD subjects. Finally, it is possible that the known disruption of the blood-brain barrier in subjects with AD[33] may have contributed to the increase in CSF cytokine levels in AD patients. However, similar hemoglobin concentrations were found in CSF samples from AD and control subjects. Furthermore, inclusion of CSF hemoglobin as a covariate in the mixed-effects model demonstrated that only IL-8 was a significant covariate. However, CSF IL-8 levels did not differ between AD and control subjects. Therefore, it is unlikely that blood contamination of CSF contributed to the observed differences in CSF cytokine levels.

Irrespective of the underlying mechanism for the differential cytokine response, the findings in the current study have implications for the design of pharmacodynamic biomarker studies that use CSF cytokine levels as an outcome measure. Although healthy subjects are often used in biomarker studies for recruitment purposes, the difference in the dynamics of cytokine levels observed in AD versus control subjects in the current study would suggest that subject population is an important consideration for study design. In addition, given the fluctuations of cytokines over the course of a day after catheter insertion (eg, CSF IL-6 and IL-8 levels), it will be critical to time-match sample collection across different treatment arms to detect small changes superimposed on the large changes in cytokine levels. Given the relatively small sample size of this study, more work will be needed to confirm these results and to clarify the underlying mechanisms of the changes of cytokines or other markers in CSF over time. Future work may help to mitigate these changes, which will enhance their utility as pharmacodynamic biomarkers.

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