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Studies of Neuroimmune Markers in Alzheimer's Disease

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Abstract

Based on a suspected role of the immune system in the pathophysiology of Alzheimer's disease (AD) and the new discoveries of neuroimmune networks, the investigation of certain neuroimmune markers was performed in AD patients, healthy controls, and disease controls. In agreement with our previous immunological research on AD, the assessment of additional immune parameters revealed abnormalities of both cellular and humoral immunity in several AD patients. These include:

1. Enhanced production of cytokines, such as interleukin-1 (IL-1), interleukin-2 (IL-2), and interleukin-6 (IL-6);
2. Increase plasma level of CD8-positive lymphocyte derived soluble CD8 (sCD8) antigen; and
3. Increased incidence of autoantibodies to brain myelin basic protein (MBP) and thymic cells.

As analyzed by flow cytometry and enzyme immunoassay, the peripheral blood immunocytes from AD patients showed a significant increase in the expression of the brain-derived S-100 protein. In the cell proliferation assay, the blood immunocytes from healthy subjects responded to stimulation with beta-amyloid protein (βAP), but this response was absent in AD patients. The initial results of our research suggest that the studies of specific markers of the neuroimmune axis may be potentially important for the new development of diagnostic and therapeutic strategies for AD.

Index Entries: Alzheimer's disease; immunopathogenesis; amyloid protein; S-100 protein; brain antibodies; cytokines.

Introduction

Alzheimer's disease (AD) is a devastating brain disorder that was first described in 1907 by Alois Alzheimer, a German neurologist. In its early stages, the disease may cause subtle changes in memory and language impairments. However, when the disease becomes severe, it causes profound loss of memory (especially of recent events), intellect, and cognitive functions, often leading to incapacitation and eventual death. The neuronal degeneration is pronounced in certain brain regions such as the basal forebrain, hippocampus, and neocortex *(1)*. The neural correlates of the cognitive impairment in patients have been described *(2)*. The neuropathologic hallmarks of the disease include neofibrillary tangles, neuritic plaques, congophilic angiopathy, and Hirano bodies.

The cause of AD is not known and there is no effective treatment presently available. As formerly reviewed *(3,4)*, the disease may be caused by one or more the following factors:

1. Abnormal brain biochemistry, for example, the

problem of amyloid plaque and fibrillary triangle formation;
2. Genetic factors as in familial form of the disease;
3. Faulty immune regulation and autoimmunity as one possible mechanism;
4. An infectious agent, perhaps a virus; and
5. Exposure to toxic substances like aluminum.

Since AD appears to be a syndrome of different subsets, it is possible that different etiologies may explain the disease *(3,4)*. This is an interesting hypothesis for future exploration and necessitates the development of therapy for each subset, more or less in the same fashion as previously developed for anemia, a red blood cell disease.

Consistent with our original immune hypothesis *(6)*, recent research has demonstrated abnormalities of both the cellular and humoral immune responses *(6–11)*, suggesting a pathological association of faulty immune regulation and/or autoimmunity with AD *(4)*. Furthermore, the new discoveries of reciprocal interactions between brain and immune system (neuroimmune axis) led us to hypothesize that peripheral blood immunocytes can be used to study neuropsychiatric diseases including AD *(12)* and, indeed, there are numerous examples to support this hypothesis *(13–17)*. This article describes the studies of certain neuroimmune markers that are common to the cells of both nervous system and immune system in patients with AD.

Materials and Methods

Subject Population and Blood Separation

This study included a total of 18 patients with the clinical diagnosis of probable AD (57–95 yr) and 71 controls. The control subjects were 17 aged subjects (51–89 yr), 25 adults (20–41 yr), 6 patients with Parkinson's disease (54–62 yr), and 23 patients with Down's syndrome (2–44 yr). The diagnosis of probable AD was made by the local neurologists according to the criteria of the NINCDS-ADRDA as outlined elsewhere *(18)*, including a neuropsychiatric evaluation based on a "mini-mental state" examination and a downward 2-yr course of the disease progression. All patients entered into the study by referrals, whereas the controls were solicited from the local community. From each donor, approx 25 mL of venous blood was drawn by the standard method of venipuncture after an informed consent was obtained from various participants or a guardian if the patient was unable give consent. Preservative-free heparin (20 U/mL of blood) was used as the anticoagulant. Blood separation was carried out essentially according to our routinely used method *(17,19)*, which briefly involves the dilution of the blood sample, centrifugation through a Ficoll-Hypaque density-gradient, and the collection of mononuclear cells (MNC) or immunocytes as a milky-turbid layer at the interface between two solutions. After appropriate washings, a cell count was taken using a Neubauer's hemocyotmeter and a light microscope. The MNC were processed fresh for various studies as outlined in the following sections, whereas the plasma and serum samples were kept stored frozen at –20°C until further use.

Production and Bioassay for Cytokines

The methods of production and quantitation of three cytokines, namely IL-1, IL-2, and IL-6, were essentially the same as previously described *(20,21)*. As far as possible, all procedures were performed under the sterile conditions of a laminar flowhood and a CO_2-incubator that was maintained at 37°C and supplied with 5% CO_2. For IL-1 assay *(20)*, monocytes from 5×10^6 MNC/mL were cultured in the absence (blank) or presence of 20 μg/mL of LPS (test). After 24 h, the culture supernatants were harvested by centrifugation and tested for IL-1 activity in a 72-h mouse thymocyte proliferation bioassay. The IL-1 activity (U/mL) was calculated in reference to a standard of recombinant IL-1β (Cistron Biotechnology, Pine Brook, NJ). For IL-2 assay *(20)*, MNC (2×10^6 cells/mL) were cultured for 48 h in the absence (blank) or presence of 1% (w/v) PHA (test). After the incubation, the cell-free supernatants were harvested by centrifugation and assayed for IL-2 activity using a IL-2-dependent HT-2 cell line proliferation bioassay. A standard of human recombinant IL-2 (Genzyme Corp., Boston, MA) was used to calculate the U/mL of IL-2. For IL-6 assay *(21)*, MNC (1×10^6 cells/0.2 mL) were preincubated for 48 h followed by a 24 h incubation in the absence (blank) or presence of 1 g/mL of LPS (test). Subsequently, the culture supernatants were harvested by centrifugation and assayed for IL-6 activity using a IL-6 dependent MH60.BSF2 cell line proliferation bioassay. A standard of human recombinant IL-6 (Amgen Biologicals, Thousand Oaks, CA) was used to calculate the amount of IL-6 (ng/mL). In each experiment, the blank value was subtracted from the test to obtain the net activity of each of the cytokines assayed.

Assay for CD8+ T-Cells and Soluble CD8 Antigen

The $CD8^+$ T-cells in the blood were enumerated with a monoclonal antibody (Coulter clone T8-FITC) using an EPICS C System of Coulter Corporation (Hialeah, FL). The procedure of method was essentially the same as described previously (22).

The quantification of soluble CD8 (sCD8) antigen in the plasma was carried out by an enzyme linked-immunosorbent assay (ELISA) kit available commercially (Code CK1040) from the T Cell Sciences, Inc. (Cambridge, MA). The procedure of assay method was essentially the same as described by the manufacturer of the ELISA kit. The plasma samples from various donors were tested in a double-blind fashion, and the data (plasma level of sCD8 in U/mL) are presented as the arithmatic mean ± standard error (SE).

Detection of Antibodies to Thymic Cells

This method was performed by a microplate-ELISA method as described previously (23), except that murine thymic cells instead of Raji cells were immobilized onto the polystyrene surface of a 96 well microtiter plate. Thymuses from young mice (C57B/6 strain) were dissected and manually dissociated over a polystyrene mesh to prepare thymocyte suspension in RPMI-1640 growth medium. After three washings, the cells were resuspended in PBS and adjusted to a concentration of 1×10^7 cell/mL. Two hundred microliters of the cell suspension were pipetted into each well of a flat-bottomed microtiter plate and immobilized with glutaraldehyde in the same manner as described before (23). For antibody assay, 100 µL of the twofold diluted (1:2.5, 1:5, 1:10, 1:20, 1:40) normal and patient plasmas were added in duplicate wells; 100 µL of PBS was used to set up background binding (blank). The plate was incubated for 30 min in the CO_2-incubator and was then washed four times with PBS. One hundred µL of 1:1000 diluted goat-antihuman alkaline phosphatase (Sigma, St. Louis, MO) were added to each well and the plate was incubated for another 30 min. Then the excess enzyme conjugate was decanted and the plate washed four times with PBS. Subsequently, the enzyme reaction was detected with *p*-nitrophenylphosphate substrate and the absorbance readings at 405 µm were recorded using a Microplate Reader (Bio-Rad Model 450). The data are presented as ELISA units (1 U = 0.01 OD).

Detection of Antibodies to Myelin Basic Protein (Anti-MBP)

Antibody binding to MBP was detected by protein-immunoblotting technique using rabbit brain MBP (Calbiochem, La Jolla, CA) as the screening antigen. Using a Mini-Gel apparatus (Hoffler Scientific, San Francisco, CA), MBP was separated in 10% gels by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol by Lammeli's method (24). The gels were run for about 2 h at 80V, and the proteins were detected by staining with Coomassie blue. The proteins from the unstained gels were blotted onto the nitrocellulose membrane by electrotransfer for 16–18 h at 25V (25). The blots were cut into 8-mm wide strips and used for immunodetection. The blots were soaked in blocking buffer (5% nonfat dry milk in TBS buffer which contained 0.02*M* Tris-HCl, pH 7.5, 0.5*M* NaCl, and 0.05% Tween-20) for about 90 min at room temperature (22°C). The blots were washed 4 times of 5 min with each treatment of TBS buffer and incubated with patient or control sera (1:400 in phosphate-buffered saline, pH 7.4) for 90 min at room temperature, followed by 4 washings as mentioned above. The blots were washed with goat-antihuman-IgG-alkaline phosphatase (Bio-Rad, Richmond, CA) prediluted to a 1:2,000 dilution in Tris-buffer containing 0.01*M* Tris-HCl, pH 8.0, 0.05*M* NaCl, 1 m*M* $MgCl_2$ and 1% bovine serum albumin. After incubating 90 min at room temperature, the blots were washed 4 times, and developed within 10 min in the alkaline phosphatase (AP) substrate reagent solution prepared according to the kit manufacturer's recommendation (Bio-Rad, Richmond, CA). A reaction was scored positive whenever a purplish-blue protein band was detected. A mouse monoclonal antibody to MBP (1:1,000 dilution of the stock purchased from Boehringer Mannheim, Indianapolis, IN), which was detected with goat-antimouse alkaline phosphatase (1:1,500 dilution), was run simultaneously as a positive control of anti-MBP detection. All sera were coded, tested, and evaluated in a double-blind fashion.

Immunoassay for S-100 Protein in Immunocytes

The S-100 protein in immunocytes was assayed by flow cytometry and enzyme immunoassay (EIA) using rabbit-antibovine-S-100 protein (anti-S-100) (Sigma).

For flow cytometry, 1×10^6 MNC from each blood donor were incubated with 1:10 diluted anti-S-100 (25 µL) for about 45 min at room temperature followed by three washings with PBS. The cell pellet in 25 µL of goat-antirabbit-IgG-FITC (1:20 dilution) was incubated as above. After three washings, the pellet was resuspended in 300 µL of PBS and the percentage of anti-S-100-positive cells was analyzed by a flow cytometer *(22)*.

For the EIA method, MNC (1×10^6 cells/100 µL) were pipetted into duplicate tubes and incubated with 50 µL of anti-S-100 protein (1:50 dilution) for 60 min in the refrigerator. The cells were washed three times with PBS and resuspended in 50 µL of goat-antirabbit-IgG-alkaline phosphatase (1:100 dilution). After 60 min of incubation in the refrigerator, the cells were washed three times with PBS. Then 100 µL of a 1 mg/mL solution of *p*-nitrophenylphosphate (dissolved in 50 m*M* sodium carbonate buffer, pH 9.6 containing 1 m*M* $MgCl_2$) were reacted with the enzyme for about 20 min, yielding a yellow color. The optical density (OD) of the color was read at 405 µm using a Microplate Reader (Bio-Rad Model 450). The OD readings were converted to arbitrarily-defined ELISA units (1 U = 0.01 OD).

Assay of βAP-Induced Proliferation of Immunocytes

This method was performed in the same fashion as it is routinely used in our laboratory *(19)*, except for minor changes to account for the response to βAP. Briefly, this procedure involved setting up of triplicate cultures of MNC in a flat-bottomed 96 well microtiter plate. An aliquot of 100 µL/well of MNC suspension (1×10^6 cells/mL) in complete growth medium (RPMI1640 streptomycin/penicillin mixture–10% fetal bovine serum) was mixed with 100 µL well of growth medium alone (blank) or prediluted concentrations of βAP. The plate was incubated for 5 d in the CO_2-incubator at 37°C. Afterward, the cell proliferation was measured in terms of the uptake of [methyl-^{3}H]-thymidine (0.5 µCi/well; SA = 20 Ci/mmol) precursor into DNA as described previously *(19)*. The disintegrations per minute (DPM) of radioactivity were used to assess the proliferative response of βAP relative to the blank. The βAP used was a 99% pure synthetic peptide of beta-amyloid protein$_{(1-28)}$ (Bachem, Torrance, CA).

The statistical significance ($p < 0.05$) of the data was evaluated by the Student's *t*-test.

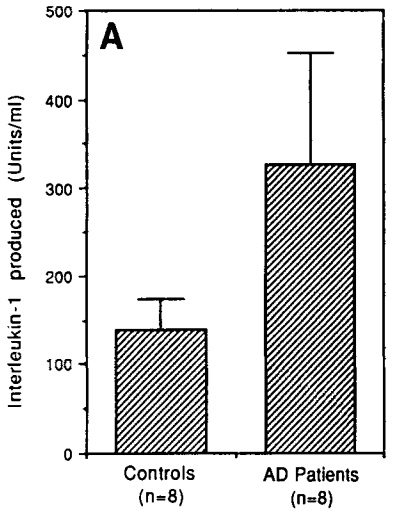
Results

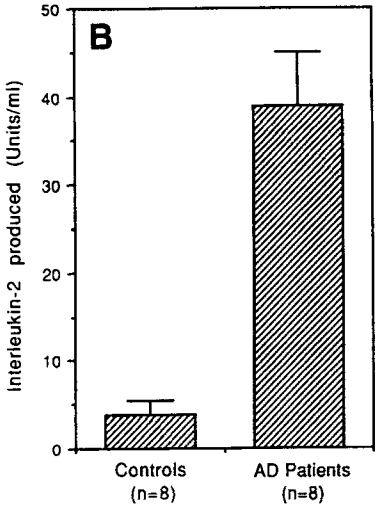
The results of cytokine production in AD patients and age-matched controls are shown in Fig. 1. As compared to controls, the patients produced higher levels of IL-1 (Fig. 1A), IL-2 (Fig. 1B), and IL-6 (Fig. 1C). As a group, the increase was approx 2.3-fold for IL-1, tenfold for IL-2, and threefold for IL-6. Despite a larger viability in the IL-1 and IL-2 data, the mean of the patient group was considerably greater than the mean of the control group. As summarized in Table 1, the blood levels of $CD8^+$ cytotoxic/suppressor T-cells were unchanged but there was a significant ($p = 0.003$) increase in the plasma level of sCD8 antigen in AD patients ($n = 11$) as compared to controls ($n = 9$).

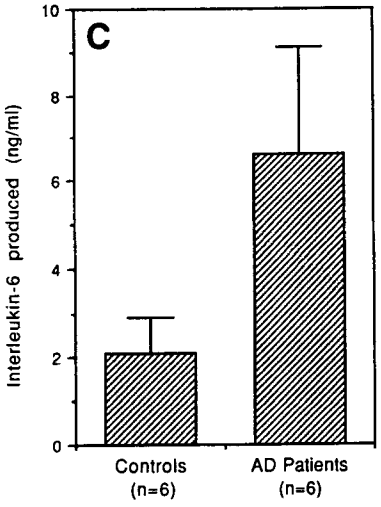
Using an ELISA system, elevated levels of antibodies to thymic cells were found in all AD patients. As shown in Fig. 2, the mean values of antithymic antibodies (ELISA units) at different serum dilutions for the patient group were significantly higher than the corresponding values for the control group ($p \leq 0.0001$). Furthermore, there was an increased incidence of serum antibodies to brain myelin basic protein (anti-MBP) among AD patients. As detected by protein-immunoblotting technique, a typical illustration of anti-MBP positive reaction is shown in Fig. 3. Using this approach, the screening for anti-MBP revealed a positive reaction with 16 of 18 (90%) sera of AD patients as compared to only 5 of 71 (7%) sera of control subjects (25 adults and 17 aged healthy subjects, 6 Parkinson's disease patients, and 23 Down's syndrome patients). This result in patients was statistically different from the controls ($p < 0.0001$).

The analytical data of S-100-containing lymphocytes by two different methods are shown in Fig. 4. By flow cytometry, the percentage of $S\text{-}100^+$ lymphocytes was found to be significantly greater in AD patients ($p = 0.011$) as compared to controls (Fig. 4A). The EIA method also detected a significantly

Fig. 1. *(opposite page)* Cytokine production by MNC from AD patients and controls. As described in the Methods 1×10^6 MNC were used for the production of IL-1 **(A)**, IL-2 **(B)**, and IL-6 **(C)**. The cells in cultures were stimulated with either LPS (for IL-1 and IL-6) or PHA (for IL-2) for different intervals and centrifuged to collect the supernatants. They were bioassayed for IL-1 (murine thymocyte proliferation), IL-2 (HT-2 cell line proliferation), and IL-6 (MH60 BSF2 cell line proliferation).







higher level of S-100 protein in AD patients (p = 0.008) than the level in controls (Fig. 4B).

As shown in Fig. 5, three concentrations of βAP elicited a small but significant ($p \leq 0.05$) proliferative response to immunocytes (or MNC) from the peripheral blood of healthy controls. In contrast, the immunocytes isolated from the blood of AD patients did not respond to βAP and the difference between the patients and controls was statistically significant ($p \leq 0.01$) especially at the lowest concentration of βAP tested.

Discussion

Alzheimer's disease (AD) has been considered as a degenerative disease primarily involving the neurons of the brain. Although extensive research is currently underway in various laboratories around the world, the cause of the disease remains poorly understood. A role for the immune system has also been suggested *(6,16)* in the pathophysiology of the disease. Laboratory investigations have revealed abnormalities of both cellular and humoral immunity in approx 45–60% of patients with AD *(4)*, which may represent a subset, since AD is a clinically heterogeneous syndrome *(5)*. As described in this report, there was an increased production of IL-1, IL-2, and IL-6 cytokines in AD patients, suggesting abnormal cellular immunity in these patients. The pathological relevance of increased production of cytokines by peripheral immune cells remains unclear, but IL-1 and IL-6 have been implicated in AD-associated amyloidogenesis *(26)* and an increased level of IL-1 was detected in AD brain *(27)*. Consistent with our previous report *(4)*, the blood level of $CD8^+$ cytotoxic/suppressor T-cells was normal (Table 1). The sCD8 antigen is a soluble form of the CD8 antigen of the cytotoxic/suppressor T-cells and its plasma levels are known to increase under conditions of immune activation that accompanies virus infections and autoimmunity *(28,29)*. Thus the increase of sCD8 antigen (Table 1) in the plasma of AD patients may indicate immune activation, presumably caused by an autoimmune response and/or virus infection. This finding may be relevant to the formerly described abnormal functioning of suppressor T-cells in patients with senile dementia of the AD *(8,9)*.

As the proponent of an autoimmune hypothesis *(16)*, there has been extensive search for autoantibodies that react with brain tissue *(16,30–33)*. Our former study with indirect immunofluorescent

Table1
Blood Levels of CD8+ T-cells and sCD8 in AD Patients

Aged controls			Alzheimer's patients		
Code	CD8+ cells, %	sCD8, U/mL	Code	CD8+ cells, %	sCD8, U/mL
1	16	335	1	27	495
2	22	285	2	27	590
3	19	325	3	22	495
4	19	400	4	47	500
5	17	325	5	43	900
6	25	309	6	25	500
7	34	435	7	21	560
8	27	350	8	22	425
9	24	365	9	19	495
			10	20	300
			11	16	480
$n = 9$	23 ± 2	348 ± 16	$n = 11$	27 ± 3, ns	522 ± 44, $p = 0.003$

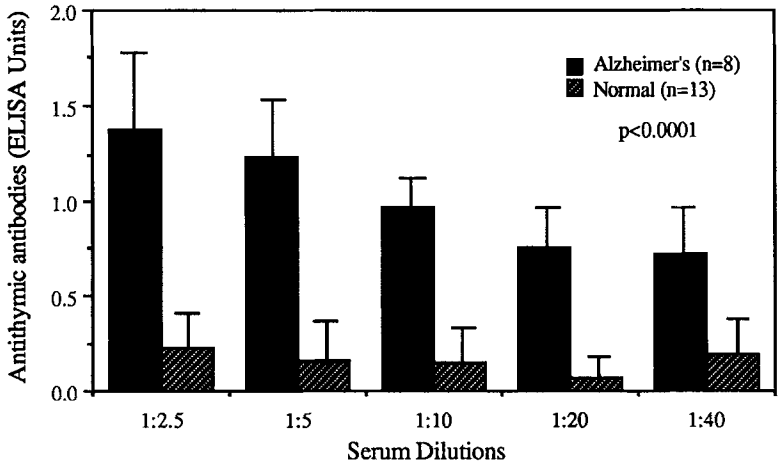


Fig. 2. ELISA detection of antithymic antibodies. As shown, different dilutions of patient or control sera were assayed for autoantibodies (IgG isotype) to thymocytes from murine thymus. The data were presented in terms of arbitrarily defined ELISA units.

technique *(16)* showed that the brain antibody staining of AD serum was preabsorbed with thymus homogenate, indicating crossreactivity of autoantibodies with thymic cells. This finding has now been confirmed by our ELISA method (Fig. 2) of antithymic antibody detection in the sera of AD patients. In addition, nearly 90% of AD patients as compared to only 7% of controls had anti-MBP. This approx 13-fold increase in the incidence of anti-MBP suggests that this immune parameter may potentially serve as a diagnostic tool for AD. With regard to myelin, a marked increase of MBP in neuronal fractions *(34)* and accumulation of oligodendroglial microtubular masses (OMM) *(35)* would clearly suggest a defect of brain myelin in AD patients. Whether these alterations are the cause or effect of the disease is not known, but we suggest that anti-MBP may be involved in the immune attack of brain myelin that somehow accompanies the neuropathology in AD.

Recent studies of interconnections between the nervous system and immune system have suggested a neuroimmune axis *(3,36)* that has been hypothesized to provide a new target for therapeutic and diagnostic developments in neuropsychiatric diseases including AD *(4)*. Since both the nervous

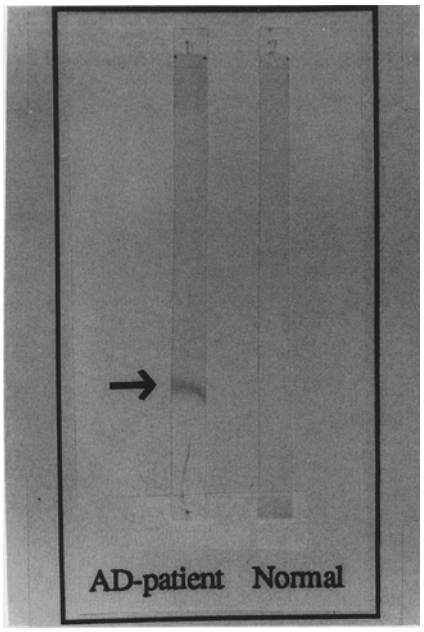


Fig. 3. Photograph showing anti-MBP as detected by protein-immunoblotting. Approximately 2 μg/lane of rabbit brain MBP were separated by SDS-polyacrylamide electrophoresis, transferred to nitrocellulose membranes, followed by the immunodetection of anti-MBP in various sera prediluted to a screening dilution of 1:400. A reaction was recorded positive whenever a purplish-blue protein band was seen in immunoblotting assay.

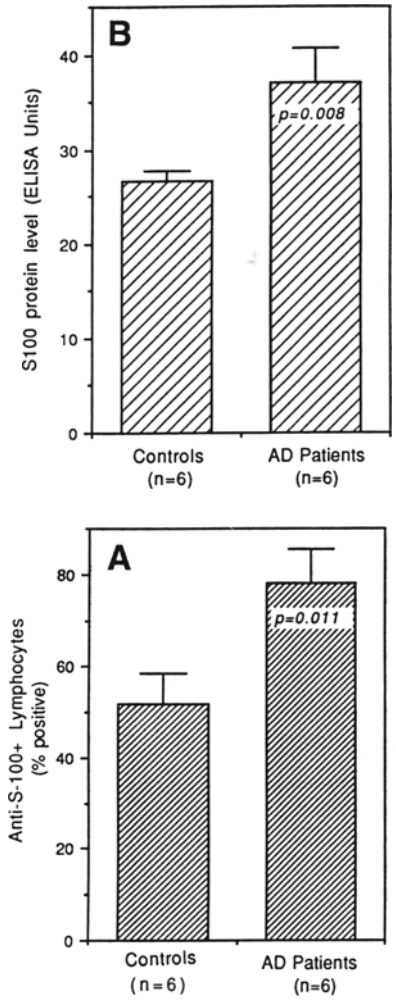


Fig. 4. Detection of S-100 protein in immunocytes from AD patients and controls. Approximately 1×10^6 MNC/tube were incubated with anti-S-100 followed by detection with either goat-antirabbit-IgG-FITC (flow cytometry) or goat-antirabbit-IgG-alkaline phosphatase (EIA method). The flow cytometry data were presented as the percentage of cells enumerated positive for S-100 protein, whereas the data of EIA method were given in terms of the arbitrarily defined EIA units.

and immune system share certain common proteins, it was postulated that the peripheral blood immunocytes can be used to study neuropsychiatric diseases *(12)*. In support of this approach, several abnormalities have been resolved in the blood of AD patients:

1. Reduction of muscarinic cholinergic receptors in lymphocytes *(13)*;
2. Diminished uptake of Ca^{2+} by mitogen-activated lymphocytes *(15)*;
3. Decreased membrane fluidity of blood platelets *(37)*;
4. Depressed lymphocyte activity of acetylcholinesterase *(14)*;
5. Marked reduction of immunocyte-associated CRF receptors *(16)*;
6. Decrease of serotonin receptors in immunocytes *(17)*;
7. Increase of lymphocyte-localized S-100 protein (this report); and
8. Lack of βAP-induced immunocyte proliferation (this report).

The S-100 protein, a brain-derived protein with Ca^{2+}-binding and neurotrophic activities, is now localized in the blood immunocytes. The βAP is a small mol wt (4.2 kDa) peptide found mainly in the brain and has been pathologically linked by many to AD. Interestingly, we have found that this peptide causes a small but significant proliferation of normal blood immunocytes in vitro, which is deficient in the blood immunocytes of AD patients; the reason and significance of this deficiency is not presently know. As reviewed elsewhere *(3)*, some

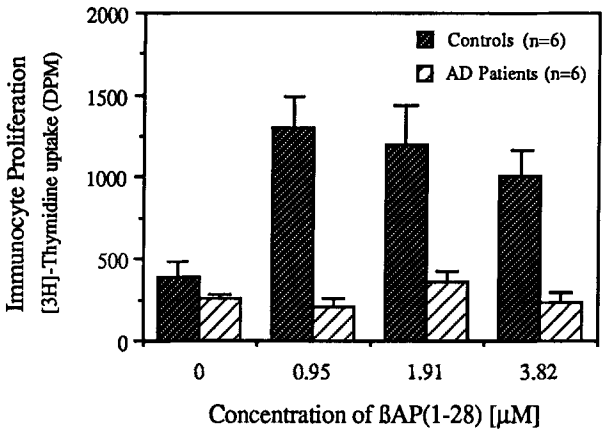


Fig. 5. βAP-induced proliferation of immunocytes from AD patients and controls. As described in the Methods, approx 1×10^5 MNC/well were incubated with different concentrations of βAP. After 5 d, the cell cultures were labeled with [^{3}H]-thymidine, and the data were presented as the radioactivity (DPM) of [^{3}H]-thymidine taken up by the cells as an index of cell proliferation.

of these lymphocyte-derived abnormalities (e.g., acetylcholine muscarinic receptors, acetylcholinesterase activity, and CRF and serotonin receptors) are also found in the brain of AD patients. Furthermore, an increased level of mRNA for S-100 protein was found in AD brain *(27)*.

Although AD is commonly believed to be a degenerative disease of the central nervous system neurons, there are numerous nonneuronal manifestations of the disease *(3,4)*. Some of them, as mentioned above, are associated with the immune system in the periphery *(4)*. The present nonneuronal abnormalities raises the possibility that AD is a systemic disease affecting many body organs but with clinical effects confined primarily to the brain. As reviewed elsewhere *(4)*, the accumulating immunological data supports the hypothesis of a faulty immune regulation and/or autoimmunity as a viable mechanism of pathogenesis of the disease *(11,16)*. Our initial results of neuroimmune axis research (e.g., anti-MBP in 90% of patients and nonresponsiveness to βAP-stimulation in all patients) may have some potential for the new development of diagnostic and therapeutic strategies for AD.

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