Immune system-associated antigens on the surface of peripheral blood lymphocytes in patients with Alzheimer's disease

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We investigated the immune-associated antigens of peripheral lymphocytes from 13 patients with Alzheimer's disease (AD) and 13 age-matched healthy control subjects using two-color analysis with flow cytometry. Four ratios of immune-related antigens, T/B lymphocytes, CD4/CD8, CD4/CD45R and CD4/HLA-DR, were compared for the AD and control groups. The T/B and CD4/CD8 ratios did not differ between the groups, the ratio of $CD4^+CD45R^+$ subset in the AD group was lower than the ratio in the control group, and the ratios of $CD4^+CD45R^-$ and $CD4^+HLA\text{-}DR^+$ subsets in the AD group were significantly higher. Further, in the AD group, the $CD4^+CD45R^+/CD4^+$ ratio was lower and the $CD4^+CD45R^-/CD4^+$ ratio was higher than in the control group.

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Alzheimer's disease (AD) is a human-specific disease, characterized by dementia associated with brain degeneration, and its cause is still unknown. The well-known pathological characteristics of the AD brain are senile plaques and neurofibrillary tangles. Recent studies have focused mainly on these lesions and achieved encouraging results: the presence of β proteins in apposition to senile plaques was demonstrated (1) and paired helical filaments (PHF) were shown to be present in neurofibrillary changes (2, 3). However, we should also note that there are other important changes to be investigated, such as the presence of immunoglobulins around senile plaques (4) and complement factors (5, 6). These changes suggest that some immune reactions are occurring in the brain, but their meaning is not clear.

Two possible mechanisms of AD onset are suggested by the presence of these immune-related substances in the brain: (i) abnormal proteins such as β proteins in the senile plaques, PHF and A68 protein (7) are recognized as foreign substances by the immune system inside the brain, and then AD is manifested as a tissue reaction, and (ii) an immune reaction involved in the etiology of AD occurs in the brain and leads to the generation of abnormal proteins such as β proteins. The first mechanism includes 2 possibilities: (a) the immune reaction is a nonspecific reaction and not related to the etiology of AD, and (b) the immune system recognizes β proteins, PHF and A68 protein as antigens, and immunocompetent cells start to attack these antigens (including their precursors), which is similar to the autoimmune mechanism. In the second mechanism, a virus, unknown infectious factors or environmental toxins might be considered as the cause of AD.

A recent study has also revealed that the blood-brain barrier (BBB) is not as impervious as traditionally believed (8). In other words, mutual communication and information transfer can occur between the central and peripheral immune systems. Not just the brain, but the relationships between the brain and the peripheral immune system should be considered to discuss the relationship between the immune system and the etiology of AD.

From these viewpoints, we considered that an immune reaction occurring in the brain might be reflected in a peripheral immune reaction, in which case a reaction in the brain could be monitored peripherally. In this study, we investigated the expression of various immune-related antigens by the peripheral lymphocytes of AD patients and compared the findings with normal control subjects.

Material and methods

Patients and control subjects

Thirteen women, ranging in age from 42 to 75 years old (mean: 61 ± 8 years) and clinically diagnosed as having AD, were selected as the test patients. For the diagnosis of AD, dementia was first diagnosed using DSM-III-R (9), and then each patients was con-

firmed to have AD on the basis of the diagnostic standards of the NINCDS-ADRDA Work Group (10). All tests thought to be necessary for diagnosis of AD were conducted: blood tests, physiological tests such as electrocardiogram and electroencephalogram, imaging diagnosis such as computer tomographic scan, magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT) and higher brain function tests. The average disease period of the 13 patients was 5 ± 3 years, and the clinical severity measured by the FAST rating scale (10) was 5.3 ± 1.3.

As control subjects, 13 women without any disorders of intellectual function were selected. Their age range was 46–72 years old (mean: 57 ± 7 years). There was no difference in the age distribution in the AD and control groups (Mann-Whitney *U* test).

Prior to this study, it was confirmed that none of the subjects were taking any drugs that could influence the immune system (for example, immunosuppressants or steroids) and that none had immunological or infectious diseases or chronic infections.

Cell preparations

Peripheral blood samples (5 ml each) were collected from each subject in a syringe containing ethylenediaminetetraacetic acid (EDTA), and the lymphocytes were separated within 1 h by the Ficoll-Hypaque technique. The obtained lymphocytes were analyzed with a cell autoanalyzer following fluorescent antibody staining by a direct method.

Monoclonal antibodies

Monoclonal antibodies (mAb), corresponding to human lymphocyte surface antigens labeled by fluorescein 5-isothiocyanate (FITC) or phycoerythrin (PE), were bound to lymphocytes for analysis.

T/B Simultest® (FITC-anti-Leu 4 + PE-anti-Leu 12) was used to quantify and separate T and B cells. TH/TS Simultest® (FITC-anti-Leu 3a + PE-anti-Leu 2a) was used to quantify and separate $CD4^+$ T cells and $CD8^+$ T cells. In the same way, PE-anti-Leu 3a was used for $CD4^+$ T cells, FITC-2H4 for $CD45R^+$ T cells, and FITC-anti-HLA-DR for $HLA\text{-}DR^+$ T cells. Furthermore, for two-color analyses, antibodies labeled with PE and FITC were used in combination for quantifying each subset.

Among the mAbs used, 2H4 was purchased from Coulter (Hialeah, FL, USA), and the others were from the Leu series of Becton Dickinson (Mountain View, CA, USA). In addition, the specificity of each mAb for each antigen was already confirmed. The fluorescent-labeled mAb was used in the recommended concentration for each product, based on the number of cells analyzed (in the case of the mAbs of Becton Dickinson, 20 µl is required to analyze 1×10^6 lymphocytes).

Two-color analysis of lymphocyte subsets using flow cytometry

Quantitative two-color analysis was performed using FACScan (Becton Dickinson). Forward and right-angle scatter gates were set on the lymphocyte fraction to exclude influences from monocytes and other leukocytes and to extract data only from that area. Data were plotted as the log of PE fluorescence intensity *vs* the log of FICT fluorescence intensity, and then the plotted area was divided into 4 subsets, $(PE, FITC) = \{(+,-), (+,+), (-,-), (-,+)\}$, to obtain the percentage of each. The combinations of immune-related antigens subjected to two-color analyses were T/B cells, CD4/CD8, CD4/CD45R and CD4/HLA-DR.

Data analysis

The ratios (%) of 6 combinations of 2 antigens in the above-mentioned 4 subsets were measured for both the AD and control groups for comparison. The combinations of cell types were $(+,-)$, $(+,+)$, $(-,-)$, $(-,+)$, $\{(+,-)+(+,+)\}$ and $\{(+,+)+(-,+)\}$ in each subset.

Further, the ratio of $CD4^+/CD8^+$ (= TH/s), and the ratios of $CD4^+CD45R^-/CD4^+$ and $CD4^+CD45R^+/CD4^+$ ($CD4^+CD45R^-$ and $CD4^+CD45R^+$ are 2 types of T cells in $CD4^+$) were obtained for comparison between the AD and control groups.

All statistical analyses used the Mann-Whitney *U* test.

Results

T/B cell subset

No difference was observed in the ratios of 6 combinations of cell types between the AD and control groups (Table 1).

CD4/CD8 subset

No differences were found in the ratios of 6 combinations of cell types between the AD and control groups. In the same way, the ratios of TH/S ($CD4^+/CD8^+$) were similar in both groups ($CD4^+$ is a human lymphocyte surface antigen that defines helper/inducer T cells, and $CD8^+$ is an antigen that defines cytotoxic/suppressor T cells) (Table 2).

Table 1. Two-color analysis of T and B lymphocytes in the peripheral blood of AD patients and normal controls

Group	Mean ± SD (%) T^+B^-	T^+B^+	T^-B^-	T^-B^+	T	B
AD ($n = 13$)	61.4 ± 13.1	0.4 ± 0.9	32.3 ± 14.7	3.7 ± 1.9	61.9 ± 13.0	4.1 ± 1.9
C ($n = 13$)	61.8 ± 12.6	0.1 ± 0.1	33.1 ± 13.1	5.0 ± 3.0	61.9 ± 12.6	5.2 ± 3.0

Table 2. Two-color analysis of CD4 and CD8 in the peripheral lymphocytes of AD patients and normal controls

Group	Mean ± SD (%) $CD4^+CD8^-$	$CD4^+CD8^+$	$CD4^-CD8^-$	$CD4^-CD8^+$	CD4	CD8	$T_{H/S}$ (Ratio)
AD ($n = 13$)	41.7 ± 10.2	1.3 ± 1.0	28.8 ± 11.9	28.2 ± 9.9	43.1 ± 10.2	29.5 ± 10.2	1.7 ± 0.7
C ($n = 13$)	40.8 ± 11.4	1.1 ± 0.7	27.4 ± 6.6	27.4 ± 6.6	41.4 ± 11.2	28.6 ± 6.7	1.6 ± 0.8

Table 3. Two-color analysis of CD4 and CD45R in the peripheral lymphocytes of AD patients and normal controls

Group	Mean ± SD (%) $CD4^+CD45R^-$	$CD4^+CD45R^+$	$CD4^-CD45R^-$	$CD4^-CD45R^+$	CD4	CD45
AD ($n \pm 13$)	31.3 ± 9.3	13.5 ± 11.9	24.7 ± 15.5	30.5 ± 12.1	44.8 ± 10.0	44.0 ± 19.4
C ($n = 13$)	19.9 ± 7.6	22.4 ± 12.7	34.4 ± 9.4	34.4 ± 9.4	42.3 ± 12.8	56.8 ± 13.8

Table 4. Two-color analysis of CD4 and HLA-DR(DR) in the peripheral lymphocytes of AD patients and normal controls

Group	Mean ± SD (%) $CD4^+DR^-$	$CD4^+DR^+$	$CD4^-DR^-$	$CD4^-DR^+$	CD4	DR
AD ($n = 13$)	35.4 ± 12.3	7.9 ± 4.1	36.3 ± 14.3	20.4 ± 7.1	43.3 ± 11.6	28.3 ± 10.1
C ($n = 13$)	38.8 ± 12.9	4.7 ± 2.0	39.5 ± 10.5	17.0 ± 7.3	43.4 ± 12.0	21.7 ± 8.9

CD4/CD45R subset

The ratio of $CD4^+CD45R^-$ was higher ($P < 0.01$) in the AD group than in the control group and the ratio of $CD4^+CD45R^+$ was lower in the AD group ($P < 0.05$). The ratios of the other combinations did not show significant differences between the groups (Table 3).

The ratio of $CD4^+CD45R^-/CD4^+$ was greater in the AD group, but the ratio of $CD4^+CD45R^+/CD4^+$ was clearly greater in the control group ($P < 0.01$ for both). The distributions of these two ratios for each subject are illustrated in Fig. 1. The control group presents a similar pattern of distribution for both ratios, but in the AD group the pattern was very different between the 2 ratios ($P < 0.01$).

CD4/HLA-DR subset

The ratio of the $CD4^+HLA\text{-}DR^+$ subset was greater in the AD group than in the control group ($P < 0.05$), but the other subsets in this category did not show any significant differences (Table 4).

Discussion

This study was performed to determine whether any immune-related abnormality is expressed in the peripheral blood of AD patients and if an abnormality is detectable, whether it shows any correlation with the changes occurring in the brain. We speculated that, since Rogers et al. (10) proved the existence of abnormal immune reactions in the postmortem brain of AD patients, such abnormal immune reactions in

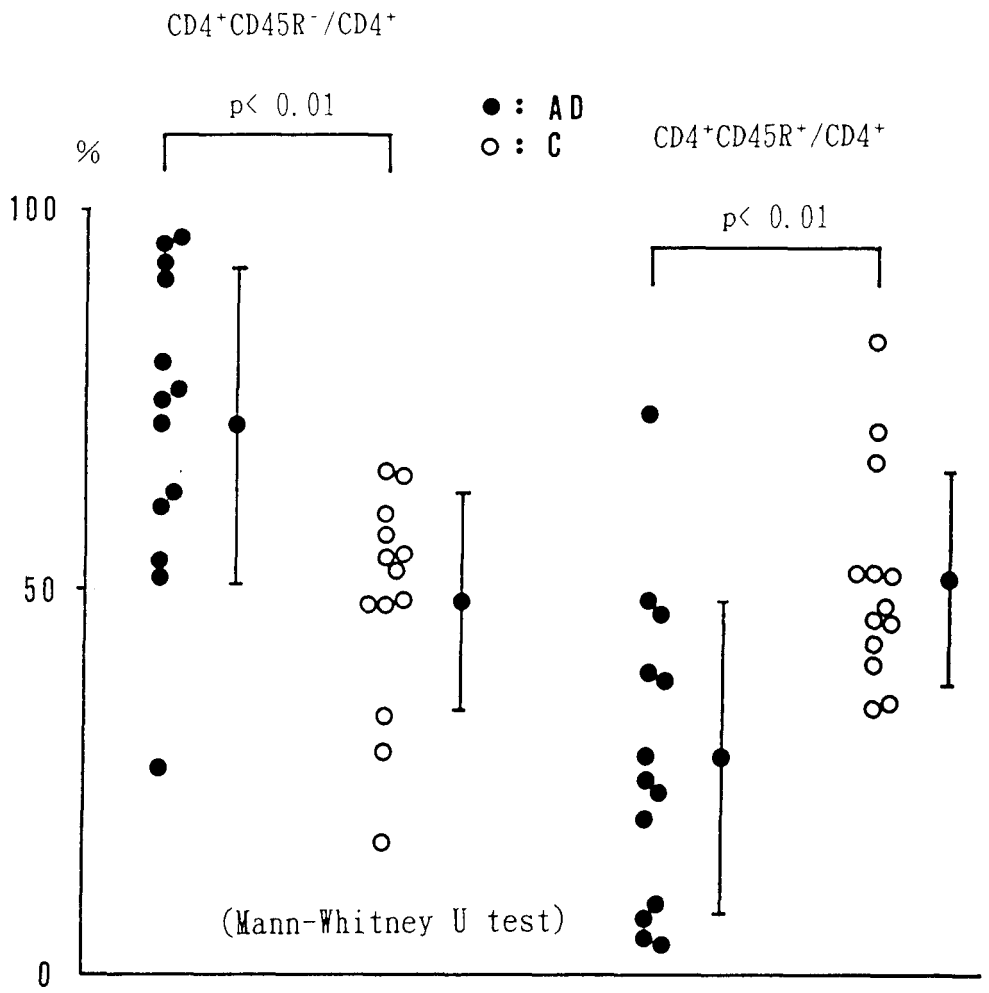

Fig. 1. Ratios of CD4 positive, CD45R positive or negative T lymphocytes in the peripheral blood of AD patients and normal control subjects.
AD: $CD4^+CD45R^-/CD4^+$ *vs* $CD4^+CD45R^+/CD4^+$ ($P < 0.01$); C: $CD4^+CD45R^-/CD4^+$ *vs* $CD4^+CD45R^+/CD4^+$ (NS) (Mann-Whitney *U* test).

the brain might be reflected in the peripheral blood of AD patient. We based our reasoning on the belief that the traditional concept of complete sequestering of the brain from the immune system by the BBB is no longer tenable for several reasons. First, the BBB is weak or virtually absent at several key points in the nervous system (8). Second, it was demonstrated that, even when the BBB functions normally, activated T-cells can penetrate the BBB to perform surveillance of the brain parenchyma (13). Further, endothelial cells of the blood vessels in the brain, pericytes and astroglias can express HLA-DR or Ia (in the rat) (14, 15).

CD45R is a lymphocyte surface antigen with a molecular weight of 190 kDa (16); it is expressed on 42% of T-cells in the peripheral blood (17). With regard to CD4 and CD45R, the ratio of $CD4^+CD45R^-$ was significantly higher in the AD group but the ratio of $CD4^+CD45R^+$ was lower in the AD group. However, since these figures represent only the percentage of this subset and not actual numbers, we measured the $CD4^+CD45R^-/CD4^+$ and $CD4^+CD45R^+/CD4^+$ subset ratios for comparison of the groups. The results are illustrated in Fig. 1. The difference between the groups becomes more apparent: $CD4^+CD45R^-$T cells and $CD4^+CD45R^+$T cells exist in almost the same proportion in the peripheral blood of the control group, whereas in the AD group the ratio of $CD4^+CD45R^+$T cells was decreased while the ratio of $CD4^+CD45R^-$T cells was increased.

Rose et al. (18) reported almost the same changes for patients with multiple sclerosis (MS): they demonstrated that the number of $CD4^+CD45R^+$T cells decreased while $CD4^+CD45R^-$T cells increased preceding acute exacerbations of MS, and they postulated that these 2 ratios can be indicators of active phases of MS. To date, $CD4^+CD45R^-$T cells are known to take a major role in the helper activity of B cells for IgG production (19), while

$CD4^+CD45R^+$ T cells *in vitro* non-specifically induce $CD8^+$ suppressor T cells (20). These points suggest the involvement of $CD4^+CD45R^+$ T cells in cellular immunity, and $CD4^+CD45R^-$ T cells in humoral immunity.

Therefore, the decrease in $CD4^+CD45R^+$ T cells and the increase in $CD4^+CD45R^-$ T cells in AD patients may indicate the change in the relative balance in immunity *in vivo*, i.e., an activated humoral immunity and decreased cellular immunity.

In this study, the ratio of $CD4^+$ HLA-DR^+ T cells was high in the AD group. HLA is a group of major histocompatibility complex antigens in man, and HLA-DR in the group is a controlling antigen of the HLA-D region as well as a cell surface receptor that starts immune reactions by binding antigens to T cells (21). Therefore, the $CD4^+$ HLA-DR^+ cell group represents the expression of activated T cells and indicates as well as CD45R dose that an immune reaction is occurring peripherally.

The increase in $CD4^+$ HLA-DR^+ T cells observed in this study may correspond to the immune reaction in the brain reported by Rogers et al. (12). They reported that HLA-DR positive cells in the postmortem brains were increased as a change associated with aging, but this number was substantially higher in AD patients, the reactivity of cells to HLA-DR was significantly accelerated in the gray matter (the primary lesion) in AD, while it was rarely found in normal control subjects and patients with Parkinson's disease and most glial cells in apposition to senile plaques were HLA-DR positive.

The above-mentioned points suggest that some immune-related phenomenon is occurring in the AD brain, and also suggest that it is possibly related to the etiology of AD. Therefore, the high percentage of $CD4^+$ HLA-DR^+ T cells in the peripheral lymphocytes of AD patients was possibly caused by changes in the central nervous system that are translated by communication between the brain and the peripheral immune systems.

Our findings relating to HLA-DR expression in the peripheral circulation were able to be compared with those of Rogers et al. in the brain, but comparison was impossible for CD45R. There have been no reports concerning CD45R in the brain. We expect that there are parallel changes in the brain and the peripheral circulation even for CD45R, and this will be a subject for further study.

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