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γδ+ T cells in Wilson’s disease

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Abstract Little is currently known about the role of γδ+ T cells in disease pathogenesis. We have demonstrated elevated levels of γδ+ T cells in the peripheral blood and cerebrospinal fluid of patients with Wilson’s disease compared with other neurological diseases. The percentage of Vδ1+/γδ+ T cells was between 20% and 50% in all patient groups; γδ+ T cells in blood correlated with copper concentrations. The antigen reactivity of γδ+ T cells and how the antigens relate to the γδ+ T cells found in WD remains unknown. It remains unclear whether there is a direct reason for the elevated γδ+ T cells population found in WD. Immunohistochemistry of frozen autopsy material from brain and liver of WD patients could allow exact localization of γδ+ T cells and heat shock proteins in future studies.

Key words Wilson’s disease · γδ+ T cells · Heat shock proteins

Introduction

Wilson’s disease (WD) is an autosomal recessive disorder of copper transport described in 1912 as hepatolenticular degeneration [1]. Recently the gene for WD has been mapped to chromosome 13q14.3 [2, 3]. It has been suggested that the gene product is a copper-transporting P-type ATPase expressed in the liver. Biochemically WD is characterized by abnormally high concentrations of copper in a number of organs and tissues and deficiency of the plasma copper protein, ceruloplasmin.

γδ+ T lymphocytes were described only a few years ago [4]. The T cell receptor (TCR) of γδ type is normally expressed on a small percentage of lymphocytes [5, 6]. In the peripheral blood of normal individuals the dominant V genes and by γδ+ T cells are Vδ2 followed by Vδ1 [7]. γδ+ T cells probably represent a more primitive, early line of cellular defense, pre-programmed to recognize a limited set of specific antigens, e.g., heat shock proteins (hsp) [8, 9]. Little is currently known about the role of γδ+ T cells in the central nervous system. Selmaj et al. [10] demonstrated hsp 65 on immature oligodendrocytes at the margins of chronic lesions containing γδ+ T cells. Elevated levels of γδ+ T cells have been found in Parkinson’s disease (PD) [11].

The main aim of our study was to determine γδ+ T cells levels in patients with chronic neurodegenerative disease of known etiology to investigate their possible involvement in disease pathogenesis. We measured levels of γδ+ T cells in peripheral blood and cerebrospinal fluid (CSF) in patients with WD and controls.

Materials and methods

Patients

We studied 21 patients (9 women) with WD without evidence of other conditions likely to interfere with the study (e.g., infections, malignancies, surgical procedures within the preceding 3 weeks, or treatment with immunosuppressive drugs). The diagnosis was established on the basis of clinical examination and laboratory investigations. This study was approved by the local ethics committee.

The patients’ ages varied between 26 and 57 years (mean 39.5 years) and the duration of WD between 0.5 and 35 years (mean 11.1) years. All patients had neurological symptoms (extrapyramidal disorders); 19 had Kayser-Fleischer rings. In 16 patients the liver function was in a balanced stage, in 4 in stage of subcompensation, in 1 symptoms and signs of decompensation were observed. Nine patients were treated with penicillamine, 9 with zinc, and 3 with both drugs at the time of study. CSF was obtained from 20 patients. Mononuclear pleocytosis (>5 x 10⁶ cells/l) was not found in any patient. The CSF/serum albumin ratio [12] was normal in all patients. The IgG index [(CSF/serum IgG)/(CSF/serum albumin)] [12] was normal (<0.7) in all patients, and none had oligoclonal IgG bands in the CSF.
Two groups of control patients were included in the study. One group consisted of 15 patients (8 women) with other neurological disease. Their age varied between 16 and 47 years (mean 31.5 years); 7 of these had epilepsy, 3 neurosis, 3 discopathy, 1 patient had neuropathy, and 1 cephalgia. None had any signs or symptoms of other diseases and they had normal routine CSF variables. A second control group consisted of 17 (2 women) healthy blood donors. Their ages varied between 19 and 54 years (mean 34.5 years); their blood was collected from the local blood bank.

Cell preparation

Procedures for cell preparation and staining have been described previously [13]. Venous blood was collected in heparinized glass tubes and diluted 1:2 with phosphate-buffered saline, pH 7.2, containing 1% albumin and 0.1% sodium azide (PBS-BSA). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Nye-gaard, Oslo, Norway) at 400 x g for 30 min at room temperature. After washing twice in PBS-BSA for 10 min at 200 x g and 4°C, equal volumes of suspension containing 5 x 10^6 PBMC were transferred to siliconized glass tubes. CSF was obtained by lumbar puncture on the same day. After cell counting and centrifugation for 10 min at 200 x g, the CSF cells were washed once in PBS-BSA for 6 min at 200 x g and 4°C. The suspension was aliquoted into tubes for staining. The minimum number of CSF cells analyzed was 5,000. CSF samples with more than 10^7 erythrocytes/l were excluded from the study. The viability of blood and CSF cells was always greater than 95%, as measured by trypan blue exclusion.

Staining procedures

Both PBMC and CSF cells were pelleted by centrifugation at 200 x g for 6 min at 4°C. 50 μl of monoclonal antibodies were added to the pellet. The following two staining procedures were performed: (i) anti-Vβ7 (IgG1 isotype) fluorescein (FITC) conjugate (T-Cell Sciences, Cambridge, USA), anti-CD3 (anti-human Leu-4) phycoerythrin (PE) conjugate, anti-CD3 (anti-human Leu-4) conjugated with biotin; (ii) anti-CD25 (anti-IL-2R) FITC conjugate, anti-γδ (anti-TCR-γδ1) PE conjugate, anti-CD3 (anti-human Leu-4) conjugated with biotin. All reagents were purchased from Becton-Dickinson (Mountain View, Calif., USA).

Stock solutions of the antisera were diluted 1:10, 1:20, and 1:40, respectively, in PBS-BSA before use. As negative controls, FITC conjugated and PE conjugated monoclonal antibodies of IgG1 isotype (Dako, Glostrup, Denmark) were used. All monoclonal antibodies were applied as saturating concentrations. Cell suspensions were incubated on ice for 30 min. PBMC were washed twice and the CSF cells once.

Streptavidin-Tri-Color (Caltag, San Francisco, Calif., USA) served as a secondary reagent for visualization of biotinylated anti-CD3 (diluted 1:200 v/v) from stock solution. After an additional 30-min incubation on ice, the PBMC were washed twice and CSF cells once in 1 ml PBS-BSA. For fixation, cells were resuspended in PBS-BSA containing 1% paraformaldehyde.

Flow cytometry

Flow cytometric analysis was performed by FACSort (Becton-Dickinson). Forward and side light scatter were used for cell gating. The gated cells comprised all mononuclear cells. For fluorescence analysis of three-color staining, Lysys II software was used. At least 5,000 CSF cells and 10,000 PBMC were analyzed. Windows for calculation of the percentage of cell subsets labelled with monoclonal antibodies were set according to the histograms of negative isotype controls. Calculations were done by comparing the percentage of γδ T cells with the total CD3+ T cells and the percentage of CD25+ cells with the γδ T and total CD3+ T cells, respectively. CD25 expression on γδ T cells was not always detectable.

Results

Flow cytometry

The mean percentage of γδ T cells in peripheral blood was 4.6 ± 4.4% (median 4.4) in patients with WD compared with 2.4 ± 1.5% (median 2.6) in those with other neurological disorders (P < 0.05). Values exceeding 8% were found in 2 WD patients, but not in those with other neurological disorders (Fig. 1).

In CSF, the mean percentage of γδ T cells was 7.1 ± 6.0% (median 4.8) in patients with WD compared with 1.7 ± 1.4% (median 2.1) in other neurological disorders (P < 0.05). Values exceeding 8% were observed in 7 patients with WD, but never in controls (Fig. 1).

There was no correlation between the percentages of γδ T cells in blood and CSF in the individual patient groups. Also, there was no correlation between the percentages of γδ T cells in blood and CSF in the patients with WD. Three patients with high percentages in CSF (18.0, 16.3, 16.1) had different values in blood (1.9, 19.5, 2.3, respectively).

The percentage of Vδ1+/γδ T cells was in the range 20%–50% in all patients (data not shown). Nine (of 17) WD patients and 5 (of 15) patients with other neurological disorders had CD25+ γδ T cells in CSF. CD25+ γδ T cells were also found in blood, but less frequently and at lower levels than in the CSF of patients with WD (data not shown).

![Fig 1 γδ T cells in peripheral blood and cerebrospinal fluid (CSF) measured by three-color immunofluorescence in patients with Wilson's disease (WD) and other neurological diseases (OND). Scattergrams represent percentages of γδ T cells out of all T cells](image-url)