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ORIGINAL

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$\gamma\delta^+$ T cells in Wilson's disease

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Abstract Little is currently known about the role of $\gamma\delta^+$ T cells in disease pathogenesis. We have demonstrated elevated levels of $\gamma\delta^+$ T cells in the peripheral blood and cerebrospinal fluid of patients with Wilson's disease compared with other neurological diseases. The percentage of $V\delta 1+/\gamma\delta^+$ T cells was between 20% and 50% in all patient groups; $\gamma\delta^+$ T cells in blood correlated with copper concentrations. The antigen reactivity of $\gamma\delta^+$ T cells and how the antigens relate to the $\gamma\delta^+$ T cells found in WD remains unknown. It remains unclear whether there is a direct reason for the elevated $\gamma\delta^+$ T cells population found in WD. Immunohistochemistry of frozen autopsy material from brain and liver of WD patients could allow exact localization of $\gamma\delta^+$ T cells and heat shock proteins in future studies.

Key words Wilson's disease · $\gamma\delta^+$ 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.

$\gamma\delta^+$ T lymphocytes were described only a few years ago [4]. The T cell receptor (TCR) of $\gamma\delta$ 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 $\gamma\delta^+$ T cells are $V\delta 2$ followed by $V\delta 1$ [7]. $\gamma\delta^+$ 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 (hsps) [8, 9]. Little is currently known about the role of $\gamma\delta^+$ T cells in the central nervous system. Selmaj et al. [10] demonstrated hsp 65 on immature oligodendrocytes at the margins of chronic lesions containing $\gamma\delta^+$ T cells. Elevated levels of $\gamma\delta^+$ T cells have been found in Parkinson's disease (PD) [11].

The main aim of our study was to determine $\gamma\delta^+$ T cells levels in patients with chronic neurodegenerative disease of known etiology to investigate their possible involvement in disease pathogenesis. We measured levels of $\gamma\delta^+$ T cells in peripherial 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 ($>5 \times 10^6$ 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.

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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 cephalea. 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% bovine serum albumin and 0.1% sodium azide (PBS-BSA). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Hypoaque (Lymphoprep, Nyegaard, Oslo, Norway) at $400\times g$ for 30 min at room temperature. After washing twice in PBS-BSA for 10 min at $200\times g$ and 4 °C, equal volumes of suspension containing 5×10^6 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 $200\times g$, the CSF cells were washed once in PBS-BSA for 6 min at $200\times g$ 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 $200\times g$ 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-Vδ1 (Identi-T, δTCS-1) fluorescein (FITC) conjugate (T-Cell Sciences, Cambridge, USA), anti-$\gamma\delta$ (anti-TCR-γ/δ-1) phycoerythrin (PE) conjugate, anti-CD3 (anti-human Leu-4) conjugated with biotin; (2) anti-CD25 (anti-IL-2R) FITC conjugate, anti-$\gamma\delta$ (anti-TCR-γ/δ-1) 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 $\gamma\delta^+$ T cells with the total CD3+ T cells and the percentage of CD25+ cells with the $\gamma\delta^+$ T and total CD3+ T cells, respectively. CD25 expression on $\gamma\delta^+$ T cells was not always detectable.

Statistical analysis

Nonparametric statistics were used, since data were not normally distributed. Differences between pairs of groups were assessed by Mann-Whitney's U-test. Associations between various parameters were sought by Spearman rank correlation analysis. P values <0.05 were considered significant.

Results

Flow cytometry

The mean percentage of $\gamma\delta^+$ 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 $\gamma\delta^+$ 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 ($P<0.01$). Values exceeding 8% were observed in 7 patients with WD, but never in controls (Fig. 1).

There was no correlation between the percentages of $\gamma\delta^+$ T cells in blood and CSF in the individual patient groups. Also, there was no correlation between the percentages of $\gamma\delta^+$ 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$^+$/$\gamma\delta^+$ 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+$\gamma\delta^+$ T cells in CSF. CD25+ $\gamma\delta^+$ 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).

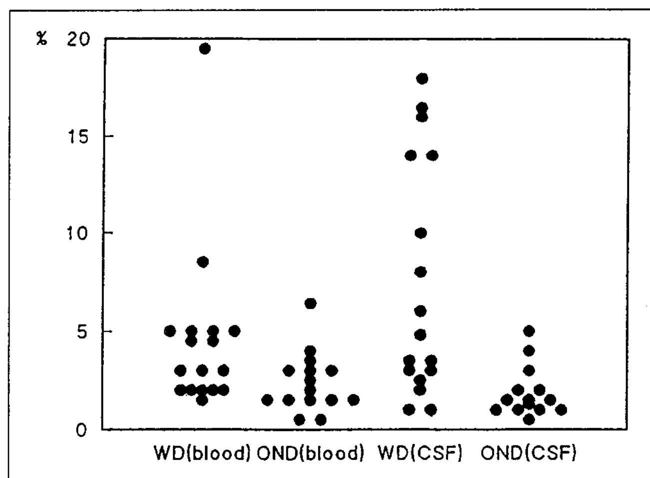

Fig. 1 $\gamma\delta^+$ T cells in peripheral blood and cerebrospinal fluid (*CSF*) measured by three-color immunofluorescence in patients with Wilson's disease (*WD*) and other neurological diseases (*OND*). Scattergrams represent percentages of $\gamma\delta^+$ T cells out of all T cells

The percentages of $\gamma\delta^+$ T cells in blood correlated with copper concentrations ($r=0.7$, $P<0.01$, data not shown). There was no correlation between the percentage of $\gamma\delta^+$ T cells and the duration of disease or ceruloplasmin concentrations in blood in WD patients. Also, there was no correlation between the percentages of $\gamma\delta^+$ T cells and the duration of disease or copper concentrations in the CSF of WD patients or between copper concentrations in blood and CSF.

Discussion

In this study we have demonstrated that elevated levels of $\gamma\delta^+$ T cells are found in the peripheral blood and CSF of patients with WD compared with those with other neurological disorders. No correlations were found between the numbers of $\gamma\delta^+$ T cells in the peripheral blood and CSF. The percentages of $\gamma\delta^+$ T cells in blood correlated with copper concentrations. Treatment with penicillamine and zinc or sex of the patients had no obvious influence on the results, either in CSF or in blood.

It has been proposed that $\gamma\delta^+$ T cells are involved in the immunopathology of such conditions as multiple sclerosis (MS) [10], rheumatoid arthritis [14], infection [7], and celiac disease [15]. Wen et al. [16] found an elevation of activated $\gamma\delta^+$ T cells in patients with autoimmune chronic liver disease (autoimmune chronic active hepatitis and primary sclerosing cholangitis). Both diseases are characterized by mononuclear cell infiltration of the liver and the authors speculated that high levels of these cells in the peripheral blood simply reflect increased numbers in the liver infiltrate. Ohteki et al. [17] suggested that the liver may be another example of a localized site for $\gamma\delta^+$ T cells. Roark et al. [18] suggested that hsp-60-reactive $\gamma\delta^+$ T cells in adult murine liver and spleen are independent of each other and may be resident in their respective sites.

WD is characterized by failure to incorporate copper into ceruloplasmin in the liver and failure to excrete copper from the liver into bile. This results in toxic accumulation of copper in the liver, which affects liver functions. However, the higher numbers of $\gamma\delta^+$ T cells in CSF (7.1%) than in blood (4.6%) may indicate local accumulation or expansion of such cells in the CSF in WD, a similar tendency was also observed in PD [9]. In WD copper accumulates in the brain and causes neuronal damage in brain tissue. However, little is currently known of the mechanism by which neuronal damage occurs in brain tissue, notably in basal ganglia. Hartard et al. [19] found that in WD the concentration of copper in the CSF was also changed. In the present study there was no correlation between copper concentrations and the percentages of $\gamma\delta^+$ T cells in the CSF of WD patients. The above phenomenon suggests that there are other factors which may influence the increase in $\gamma\delta^+$ T cells in WD.

Little is currently known of the role of $\gamma\delta^+$ T cells in neuronal damage. Freedman et al. [20] demonstrated that $\gamma\delta^+$ T cells are capable of directly inducing injury to human hsp-expression oligodendrocytes. The antigen reactivity of $\gamma\delta^+$ T cells and the possible role of this T cell subpopulation in the immune response are the subjects of intensive study [21, 22]. Whether any of the antigens relate to the $\gamma\delta^+$ T cells found in WD remains unknown. To examine activation of $\gamma\delta^+$ T cells, we estimated the percentages of CD25-expression cells. Slightly more patients with WD (50%) had CD25+$\gamma\delta^+$ T cells in CSF compared with those with other neurological diseases (30%); in blood the percentages were lower than in CSF, indicating no special chronic activation of $\gamma\delta^+$ T cells in WD. Similar findings have been reported in MS patients [13], PD patients [11], and chronic autoimmune liver disease [16]. The percentage of $V\delta1^+/\gamma\delta^+$ T cells was between 20% and 50% in all patient groups. This is in accordance with a report of Selmaj et al. [10], who found very few $V\delta1^+$ T cells in blood and demyelinating plaques of MS patients. Mix et al. [13] demonstrated that 20%–40% of $\gamma\delta^+$ T cells in CSF and blood belonged to the 'epithelial' $V\delta1^+$ subtype in MS patients.

Członkowska and Milewski [23] reported immunological abnormalities in WD. They suggested that an increase in humoral immune response (i.e., a higher level of IgG and IgM) and a depressed cell-mediated immunity are caused by cirrhosis, but they did not exclude an inhibitory effect of copper ions upon the immune response. They concluded that free copper could have a suppressive effect on T cells and could lead to disturbances in the interaction between the T and B cell, and in consequence to overactivation of B cells. Copper has been shown to be involved in many functions of the immune system [24, 25]. Our observations could be a secondary effect of copper toxicity.

It remains unknown whether there is a direct reason for the elevated $\gamma\delta^+$ T cells population in WD. Immunohistochemistry of frozen autopsy material from brain and liver of WD patients could allow exact localization of $\gamma\delta^+$ T cell and hsps in future studies.

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EDITORIAL NOTE

We deeply regret to inform our readers that Dr. Tibor Diamantstein, member of the Advisory Board of this journal, deceased on 5 December 1995.