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Memory and naive CD4+ lymphocytes in multiple sclerosis

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Summary. Helper-inducer (CD29+CD4+) and suppressor-inducer (CD45RACD4+) T-cells have been recently renamed as memory and naive T-cells, respectively. We measured cells expressing these phenotypes in peripheral blood of 46 definite multiple sclerosis (MS) patients [32 relapsing-remitting (RR-MS), 14 secondary progressive (P-MS)] and controls. CD25+ (interleukin-2-receptor-positive) cells were also evaluated in the same groups of patients. RR-MS patients showed increased levels of CD29+CD4+ and CD25+ cells compared with controls. This finding was more evident in RR-MS patients during the attack than during the stable phase of the disease. In P-MS patients we found a reduction of CD45+CD4+ cells compared with either RR-MS patients or control subjects. Our results show that RR-MS and P-MS are characterized by two different T-cell subpopulations. This finding supports the hypothesis that during the evolution from RR-MS and P-MS changes occur in the immunological status of the patients.

Key words: Multiple sclerosis – Flow cytometry – Lymphocyte subsets – Naive cell – Memory cell

Introduction

The study of cell surface markers has been widely used for understanding the role of lymphocyte subpopulations in immune regulation [1, 6, 11, 13, 15, 16, 127].

Multiple sclerosis (MS) is a T-cell-mediated autoimmune disease [12, 17, 28, 30, 31] and studies of T-cell phenotype of patients in different phases of the disease are considered useful for better clarification of the immune-system abnormalities leading to demyelination [12, 17, 28, 30, 31].

CD45R and CD29 are T-cell surface markers and have been well studied and characterized [15, 16, 25, 26]. CD45R is part of the leucocyte common antigen (LCA) and can be expressed in two different isoforms: CD45RA and CD45RO. CD45RA is the high-molecular-weight isoform (220 kDa) of LCA and CD45RO is the low-molecular-weight isoform (180 kDa). CD45RO derives from CD45RA conversion after T-cell activation [1, 11, 13, 21, 24]. CD29 belongs to the adhesion molecule family and has a molecular weight of 130 kDa [21]. All these antigens can be present at low (e.g. CD45RA/RO low or CD29 low) or high levels (e.g. CD45RA/RO high or CD29 high) [21] on T-cell membrane, and their expression characterizes different T-cell subpopulations [1, 15, 16, 24, 25]. CD45RA is considered a marker of suppressor-inducer T-cells, and CD45RO/CD29 a marker of helper-inducer T-cells [15, 16]. More recently, the co-expression of CD45RO-CD29 high has been found on CD4+ cells activated by antigen(s) and retaining memory of that; conversely, CD45RA high expression has been found only on naive (not antigen-stimulated) CD4+ cells [7, 13, 21].

Decreased levels of CD45RA high CD4+ and increased levels of CD29 high CD4+ cells have been reported in MS [8, 14, 19, 20]; however, this finding is still debated.

These controversies prompted us to re-evaluate the presence of peripheral blood cells bearing CD45RA, CD29 and CD25 in MS patients and controls.

Patients and methods

The MS patients' characteristics are summarized in Table 1.

Forty-six MS patients were selected according to the criteria for definite MS of Poser et al. [18]. Clinical course and status were defined according to the criteria of Schumacher et al. [22] and Kurtzke's disability status scale [10], respectively. None of the patients received immunosuppressive treatment before or during the study. None received steroids in the 6 months before the study. In 10 relapsing-remitting (RR-MS) patients blood samples were obtained during a relapse. Control groups included 28 healthy control subjects (HC) and 35 patients with other medical disease (OMD) (Table 1). OMD patients were affected by: senile dementia of the Alzheimer type (6), lacunar encephalopathy (3), idiopathic spastic paraparesis (3), amyotrophic lateral sclerosis (3), chronic polyneuropathy (2), hereditary neuropathy (HMSN I, II) (2), glomerulonephritis (2) and haemorrhagic cystitis, peripheral facial nerve palsy, Huntington's chorea, vitamin B12 deficiency, epilepsy, gout, herpes zoster, stroke, breast cancer, multi-infarct dementia, recurrent transient ischaemic attack, scapulohumeral dystrophy, Kugelberg-Welander syndrome, and glioblastoma (1 each).

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Table 1. Characteristics of the patients. EDSS, Expanded Disability Status Scale; HC, healthy controls; OMD, other medical disease patients; RR-MS, relapsing-remitting MS patients; P-MS, secondary progressive MS patients. Mean (SD)

	No. of patients	Sex (F/M)	Age (years)	EDSS	Age at onset (years)	Disease duration (years)
HC	28	12/16	45 (15)			
OMD	35	19/16	57 (13)			
RR-MS	32	19/13	33 (8)	2.0 (1.1)	27 (7.3)	6.0 (4.6)
P-MS	14	9/5	44 (10)	4.0 (1.5)	34.2 (10.6)	11.5 (6.6)

Sample preparation

Peripheral blood was collected in ethylenediamine tetra-acetic acid test tubes for all the experiments.

CD4-, CD25-, CD29- and CD45RA-bearing lymphocytes were measured in each blood sample after a 30-min incubation in an ice-bath with monoclonal antibodies (mAb) directed against CD4 (T4), CD25 (IL-2R), CD29 (4B4) and CD45RA (2H4). Each mAb was conjugated either with fluorescein-5-isothiocyanate (FITC) (T4 and IL-2R, Coulter Immunology, Hialech, Fla., USA) or with phycoeritrin (PE) (4B4 and 2H4, Coulter Immunology). Each experiment was performed according to the company's recommendations. Briefly, erythrocytes were lysed with a solution containing formic acid (0.1%). Leucocytes were stabilized and fixed by successive passages in sodium carbonate (50 m*M*), sodium chloride (250 m*M*) sodium sulphate (220 m*M*), and paraformaldehyde (330 m*M*) [5]. The samples were then centrifuged at 1200 rpm for 5 min. Supernatants were then discarded and each pellet was washed in phosphate-buffered saline solution (pH 7.2) and counted.

Flow cytometry

Single and two-colour fluorescence analyses were performed to quantify the CD25-bearing lymphocytes and the CD29 high CD4+ or CD45RA high CD4+ lymphocytes using an EPICS PROFILE II (Coulter Electronics) equipped with single 15 mW argon laser and 488 nm excitation wave length. Non-specific background fluorescence was evaluated with isotypic controls.

Initial instrument alignment was performed with FITC-labelled (green) and PE-labelled (orange red) microspheres (DNA Check, Coulter Immunology).

Amplification of the red and green photomultiplier tubes was adjusted so that signals generated from the green positive FITC region minimally exceeded background in the red positive PE detection system (and vice versa). The remaining overlapping sensitivity to both dyes was compensated for by electronic subtraction.

To exclude monocytes, other leucocytes and debris, forward and right-angle scatter gates were set on lymphocytes. Signals of forward angle light scatter, orthogonal side angle light scatter, green fluorescence (530 nm) and red-orange fluorescence (585 nm), obtained when the cells traversed the laser beam area, generated two parameter histograms. Linear amplification was used for light scatter signals and logarithmic amplification for fluorescence signals. Data were plotted in histograms: (1) forward angle light scatter versus orthogonal side angle light scatter, (2) red fluorescence versus green fluroescence, (3) cell number versus log of green fluorescence and (4) cell number versus log of red fluorescence. Five thousand cells per sample were analysed and the results were recorded on floppy disk.

Statistical evaluation

Statistical evaluation was performed using Student's *t*-test and analysis of variance.

Results

The percentages of lymphocyte subpopulations are summarized in Table 2.

RR-MS patients (stable plus attack) showed a significant increase of CD29 high CD4+ cells (42.8, SD 6.24%; $P < 0.001$ vs HC and $P < 0.01$ vs OMD) and CD25+ cells (1.40, SD 0.85%; $P < 0.003$ vs HC and $P < 0.01$ vs OMD). MS patients during the attack showed levels of CD29 high CD4+ and CD25+ cells higher than the stable MS patients.

Figures 1 and 2 show the distribution of CD29 high CD4+ and CD45RA high CD4+ cell percentage values in MS patients and controls, respectively. Six out of

Table 2. T-lymphocyte subset percentage values in patients with MS or other medical diseases and in healthy controls [mean (SD)]. *1 $P < 0.03$ vs HC; *2 $P < 0.01$ vs HC; *3 $P < 0.007$ vs HC; *4 $P < 0.001$ vs HC; *5 $P < 0.05$ vs OMD; *6 $P < 0.03$ vs OMD; *7 $P < 0.007$ vs OMD

	CD29 high CD4+	CD45RA high CD4+	CD25+
HC	34.8 (10)	26.0 (6.3)	0.7 (0.5)
OMD	37.3 (10)	22.3 (8.8)	0.8 (0.6)
Stable RR-MS	42.0 (6.3)*1	26.5 (7.7)	1.4 (0.9)*3,6
Attack RR-MS	44.6 (5.5)*2,5	28.6 (9.6)	1.5 (0.6)*4,7
P-MS	40.9 (5.8)	21.1 (7.1)*1,5	0.7 (0.3)

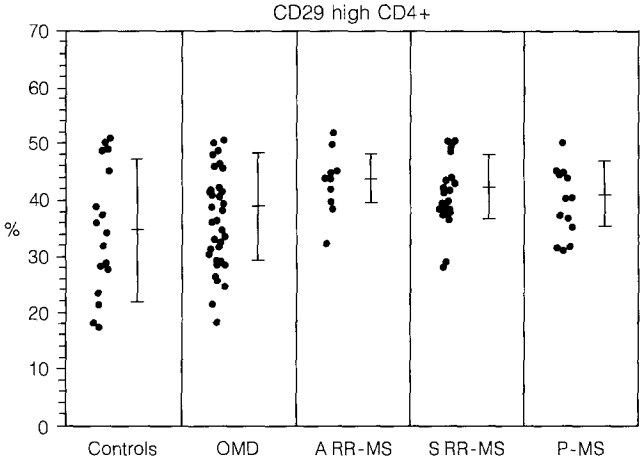

Fig. 1. Percentage values of CD29 high CD4+ T-lymphocytes in the peripheral blood of patients with attack *(A RR-MS)* and stable *(S RR-MS)* relapsing-remitting multiple sclerosis, progressive multiple sclerosis *(P-MS)*, other medical diseases (OMD) and healthy controls

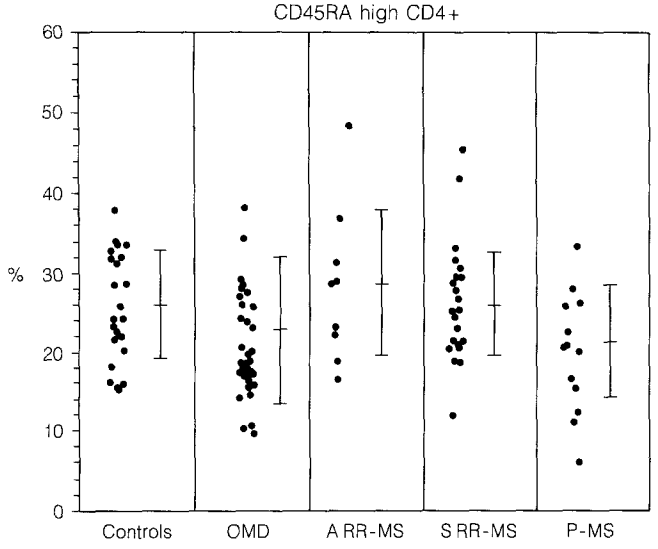

Fig. 2. Percentage values of CD45RA high CD4+ T-lymphocytes in the peripheral blood of patients with attack *(A RR-MS)* and stable *(S RR-MS)* relapsing-remitting multiple sclerosis, progressive multiple sclerosis *(P-MS)*, other medical diseases (OMD) and healthy controls

23 (26%) RR-MS patients in the stable phase showed more than 44% memory lymphocytes (HC mean value + 1 SD). Similar figures were observed in 6 of 10 (60%) patients during a relapse. Three out of 14 (21.4%) secondary progressive (P-MS) patients had less than 14% naive cells (HC mean value − 1 SD). No significant correlations were found between CD45RA high CD4 + and CD8+ cell levels.

Discussion

In the present study we found increased levels of CD29 high CD4+ and CD25 cells in RR-MS patients. The increase of CD29 high CD4+ and CD25 cells was more significant in RR-MS patients during relapse.

T-cell surface antigens are used to differentiate T-cell subpopulations. Their expression represents different functional statuses of lymphocytes. CD29 and CD45R are two of these T-cell markers and the significance of their presence on T-cells has been well characterized [15, 16, 25, 26]. CD29 high CD4+ cells provide help for Ig production by pokeweed mitogen (PWM)-stimulated B-lymphocytes [15] and are considered cells with helper-inducer functions. CD45RA (high-molecular-weight LCA isoform) CD4+ bearing cells are believed to have suppressor inducer functions because they regulate CD8+ mediated inhibition of B-cell Ig production [16, 26]. The expression of CD45RO/CD29 was also observed on antigen-stimulated T-cells [1, 4, 7, 13, 15, 24, 25], while CD45RA antigen was present on non-antigen stimulated T-cells [21, 24]. For this reason the terms "memory" (CD45RO high/CD29 high) and "naive" (CD45RA high) T-cells are now currently used to differentiate these subpopulations further [21]. That CD45RO/CD29 high T-cells function as memory cells is also suggested by the presence of only 5% of these in cord blood; their percentage increases with age and they represent 40% of circulating T-cells in adults [21].

It has been hypothesized that memory T-cells (CD45RO/CD29 high) can be triggered to increase the rate of replication by cross-reactive high-affinity antigens [3].

We found increased levels of memory T-cells predominantly during the attack phase of RR-MS. It is well known that infectious diseases can worsen the course of MS and trigger an attack [12, 30, 31]. Since infectious antigens are released into the circulation during infectious diseases, the increase of memory T-cells in RR-MS could be caused by viral or bacterial antigen(s) able to cross-stimulate pre-existing memory T-cells. The clearance of these transient antigens could lead to the end of the attack phase.

In P-MS patients, we observed a reduction of naive cells (CD45RA high cells). This reduction has been reported previously and it was interpreted as a switch from naive to memory cells [32]. However, this hypothesis is not confirmed by our data, as we did not find increased levels of memory T-cells in these patients. In our opinion this observation could be better explained by impaired cellular responsiveness during the course of P-MS to permanently present antigens (anergy; anti-idiotypic modulation).

Activation of T-cells is also characterized by transient expression of markers such as IL-2 receptor [6, 11] or transferrin receptor [29]. The increase of CD25+ cells has been also reported in MS [2, 8, 9, 23]. In our study RR-MS patients either in stable or in attack phase showed a statistically significant increase of CD25+ cells compared with healthy controls. In contrast, no significant differences of CD25+ cell levels were observed between RR-MS patients in stable and in attack phase of disease. Our results confirm previous reports [2, 14, 19, 20, 23, 32] and stress the occurrence of an immune-system activation in RR-MS.

In conclusion, our results indicate that RR-MS (predominantly in the acute phase) is characterized by expression of high levels of pre-existing T-cell markers and by transient appearance of newly synthesized membrane antigen(s). This finding suggests that different T-cell responsiveness to constantly or transiently present antigen(s) could account for the different findings observed in the two forms of MS. However, further studies are necessary to confirm that our results reflect different biological events.

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