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The immunoregulatory abilities of polymorphonuclear neutrophils in the course of multiple sclerosis

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THE polymorphonuclear neutrophils (PMN) possess sufficient potential to affect both immune response and inflammation, however it has not been yet described in the course of multiple sclerosis (MS). We have studied binding of fluorescein isothiocyanate (FITC)- stained TNF-α by PMN, the expression of CD11a, CD11b, and CD18 molecules of β2-integrines and the expression of CD10 (neutral endopeptidase-NEP) and of CD13 (aminopeptidase N; APN) antigens on PMN in three different groups of MS patients. The control group included neurological patients (OND) with noninflammatory diseases. The obtained results have proved that during MS exacerbation and in the course of chronic progressive MS, PMN reveal several forms of preactivation, including significantly higher stained-TNF-α binding, higher expression of CD11b and CD18, as well as CD10 and CD13 antigens, in comparison with MS remission or OND. We suggest that the increased expression of these molecules on PMN of MS patients in exacerbation of the disease and to a lower degree in the course of CP-MS is a result of PMN priming, and directly prove the PMN involvement in the disease pathogenesis.

Key words: Multiple sclerosis, Neutrophils, Tumour necrosis factor, Tumour necrosis factor receptors, Integrins, Proteases

Introduction

Multiple sclerosis (MS) is a disease in which multifocal inflammation and damage of the blood–brain barrier and myelin sheath are salient pathologic features. Overwhelming evidence demonstrates that MS is a predominantly T-cell and monocyte/macrophage-mediated autoimmune disorder.[1,2] Polymorphonuclear neutrophils (PMN) have not been considered as a cell population participating in it.[3] PMN can however express immunoregulatory abilities, that has not been yet described in the course of MS. Activated *in vitro* PMN produce a number of immune mediators including cytokines like IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, TNF, TGF-β1.[4,5] Therefore the regulatory functions of these cells may be postulated in the course of MS.

PMN in the peripheral blood (PB) of MS patients can be primed mainly by inflammatory cytokines like IL-1, INF-γ, or by TNF-α secreted by mononuclear cells. Hypothetically PMN priming or activation in PB of MS patients may depend also on complement (especially C5a) immunological complexes, or certain metabolites of the arachidonic acid (LTB_4, PAF).

Priming results in the enhanced expression on PMN of receptors for chemokines and other chemotactic peptides,[6] priming also enhances the expression of CD11b/CD18 molecules on PMN cell surface[7,8] which results in the indirect activation of PMN.[9,10] In the presented paper we have suggested that PMN priming can be observed in MS patients peripheral blood.

Patients and methods

Patients (34 total; 19 women and 15 men, aged 22–56 years) were selected with a clinically definitive diagnosis of MS[11] and with Kurtzke Expanded Disability Status Scale[12] scores 5 or fewer and categorized as having the relapsing-remitting MS (RR-MS) course for at least 5 years but currently in remission (REM) ($n=13$), as having either secondary CP-MS for at least 2 years ($n=13$) or RR-MS and currently experiencing clinical exacerbation (REL) of the disease ($n=12$). Exacerbations were defined as the appearance of new symptoms or significant worsening of the old ones, attributable to MS, for at least 24 hours without any fever.

The control group consisted of patients with other neurological diseases (OND) (14 total; nine women and five men, aged from 24 to 37 years), including those with vasomotor headache ($n=8$) and ischialgia ($n=6$).

Sample collection

The studies were performed on PMN of the peripheral venous blood, collected into heparin-containing tubes (10 U/ml).

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TNF labelling with fluorescein isothiocyanate (FITC)

The synthesis and biological analysis of the TNF molecules were performed at the Department of Bioorganic Chemistry, Lódź, Poland (Patent No. 168858). TNF staining was done with FITC (Serva), according to Shirakawa *et al.*[13] 100 mg of FITC and 100 µl of 0.2 M carbonate buffer (pH = 9.2) were added to 400 µg of TNF, diluted in 400 µl of PBS and then, incubated for 6 h at 4°C. The labelled TNF was separated from the non-labelled with FITC by gel filtration on a 3.5 cm^3 column, filled with Sephadex G-25. TNF labelled FITC (TNF-FITC) was sterilized by filtration, using a 0.22 µm filter and then, stored at 4°C. The staining efficiency was measured, taking into account the absorbency at 280 nm, in comparison with the absorbency at 495 nm. The investigations were performed within 10 days after the TNF labelling due to relatively low stability of the labelling.

Evaluation of TNF-FITC binding to PMN

One hundred µl of the whole blood was incubated with TNF-FITC at the concentration of 1000 ng/ml for 2 h at 4°C. The erythrocytes were lysed, using 1 ml of FACS Lysing Solution (Becton Dickinson) for 15 min at room temperature. Cells were centrifuged for 5 min at 400 × ***g***, then washed with 3 ml of PBS and suspended in 100 µl of PBS. The fluorescence intensity of PMN was measured using a flow cytometry FACScan (Becton Dickinson) and Lysis II software. Each measurement was repeated four times and presented here as a mean fluorescence intensity (MFI). Specificity of the binding of TNF-FITC to PMN was evaluated in blocking experiment by preincubation of the PMN with non-stained TNF in excess for 45 min before TNF-FITC binding (Fig. 1).

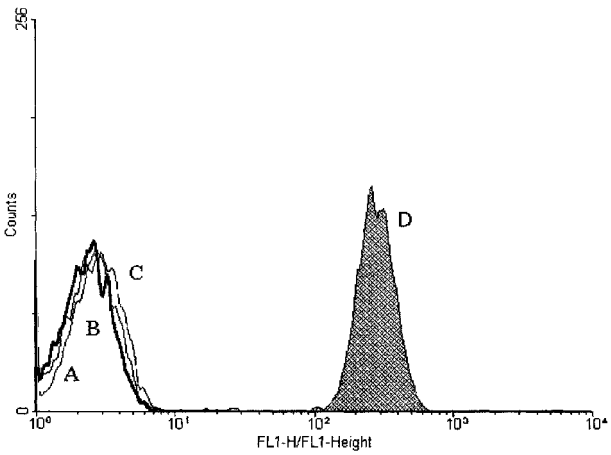

FIG. 1. Flow cytometric analysis of the TNF-FITC binding to PMN. Non-stained TNF added in 1000 times excess (C), significantly diminished MIF corresponding to nonspecific binding of control IgG-FITC (A) and IgG-FITC + non-stained TNF (B). The result (D) proves specificity of the TNF-FITC binding to TNF-R on PMN.

Evaluation of the expression of CD11a, CD11b and CD18 molecules of LFA-1 and Mac-1 intregine and CD10 and CD13 Ag of neutral endopeptidase (NEP) and aminopeptidase N (APN) on PMN

The determination of molecule expressions on the surface of PMN was performed in the whole blood. Techniques, generally used for immunofluorescent labelling of the cells in whole blood collected on heparin, were applied. One hundred µl of blood was mixed and incubated at room temperature with appropriate quantities of monoclonal antibodies, provided by Dako (Denmark). CD11a, CD11b and CD18 antibodies were used. A double-step staining procedure was used for the evaluation of CD10 and CD13 Ag expression. Mouse immunoglobulins anti-CD10 and CD13 (Dako) were used as a first step. Rabbit anti-mouse IgG polyclonal immunoglobulins G stained with R-phycoerythrin were used as a secondary antibody. Mouse IgG2a, stained with RPE, was used as a negative control. Erythrocytes were eliminated by an addition of lysing solution (Becton Dickinson) into the blood samples. After a short time of incubation and rinsing, the cells were suspended in physiological buffered saline (PBS). FACscan flow cytometer with a 488 nm argon laser (Becton Dickinson) and Lysis II software were used. The results were expressed as the values of MFI of the labelled surface antigens.

Results

The MFI of the PMN incubated with FITC-TNF was highest in the group with exacerbation of RR-MS (REL) (Table 1) and the value was significantly higher ($P<0.01$) compared with the other groups of MS patients and to OND. In the course of CP-MS and in MS remission (REM) the tested values were in the range observed in the control group (OND).

Expression of CD10, CD13 Ag and CD11b/CD18 molecules on PMN was significantly increased in REL ($P<0.05$ and $P<0.01$) compared with that examined in CP-MS, REM and OND (Table 1). CD11a expression on PMN in REL was the same as in CP-MS patients and in OND. In the course of CP-MS only expressions of CD11b and CD18 but not CD10 and CD13 were significantly increased ($P<0.01$ and $P<0.05$) compared with OND. Expressions of CD11a, CD11b and CD10 were markedly diminished ($P<0.01$ and $P<0.05$) during remission of MS compared with the controls (Table 1).

Discussion

It has been proved in previous studies that there is a more intensive inflammatory response in acute exacerbations of RR-MS patients than in the course of

Table 1. Mean fluorescence intensity (MFI) of fluorescein isothiocyanate (FITC) labelled TNF binding to polymorphonuclear neutrophils (PMN) and the CD10, CD13 Ag, and CD11a, CD11b, CD18 molecule expression on PMN in patients with relapsing-remitting MS during exacerbation (REL) (1), in chronic progressive MS (CP-MS) (2) during remission (REM) of the disease (3) and in the other neurological diseases (OND) (4)

	MFI of TNF-FITC binding	CD10 expression	CD13 expression	CD11a expression	CD11b expression	CD18 expression
1. REL ($n = 12$)	134.6 ± 17.3 range: 115.8–151.6 1 vs. 2 $P < 0.01$ 1 vs. 3 $P < 0.01$ 1 vs. 4 $P < 0.01$	52.7 ± 10.7 range: 38.7–69.8 1 vs. 2 $P < 0.05$ 1 vs. 3 $P < 0.01$ 1 vs. 4 $P < 0.01$	138.6 ± 20.8 range: 126.4–168.8 1 vs. 2 $P < 0.05$ 1 vs. 3 $P < 0.01$ 1 vs. 4 $P < 0.01$	70.7 ± 12.0 range: 51.5–94.7 1 vs. 3 $P < 0.01$	942 ± 56 range: 827–1010 1 vs. 2 $P < 0.01$ 1 vs. 3 $P < 0.01$ 1 vs. 4 $P < 0.01$	280.9 ± 39.9 range: 210.1–347.1 1 vs. 3 $P < 0.01$ 1 vs. 4 $P < 0.01$
2. CP-MS ($n = 12$)	119.1 ± 14.5 range: 98.6–144.2	42.9 ± 13.9 range: 29.4–76.2 2 vs. 3 $P < 0.01$	124.1 ± 29.6 range: 87.2–174.2	70.4 ± 9.1 range: 58.2–81.2 2 vs. 3 $P < 0.05$	743 ± 114 range: 622–968 2 vs. 3 $P < 0.01$ 2 vs. 4 $P < 0.05$	277.2 ± 30.9 range: 221.2–308.7 2 vs. 3 $P < 0.01$ 2 vs. 4 $P < 0.01$
3. REM ($n = 13$)	112.0 ± 15.2 range: 89.4–141.2	23.8 ± 2.6 range: 20.4–27.6 3 vs. 4 $P < 0.05$	115.2 ± 13.6 range: 89.4–140.8	56.5 ± 13.3 range: 31.2–84.1 3 vs. 4 $P < 0.05$	483 ± 166 range: 278–760 3 vs. 4 $P < 0.01$	197.5 ± 42.1 range: 162.1–286.4
4. OND ($n = 14$)	110.4 ± 7.5 range: 94.5–121.6	37.8 ± 13.6 range: 20.6–67.2	106.9 ± 17.1 range: 80.2–148.2	69.7 ± 14.7 range: 52.7–94.6	610 ± 100 range: 460–728	184.5 ± 45.7 range: 127.4–274.2

CP-MS.[2] The obtained data suggest that relapsing-remitting MS follows cycles of immunological activation as a result of Th-1 response (exacerbation), which is then followed by a suppressor response that downregulates inflammation (remission).[1,2] In CP-MS there is continuous low-grade inflammation with no obvious exacerbations or remissions.

The involvement of TNF in immunopathological processes in MS has already been known for quite some time.[14] This cytokine is also one of the most potent priming factors for PMN *in vitro*.[7,15] We suggest that serum TNF of patients with acute exacerbations of RR-MS or in lower degree in the course of CP-MS can be the main priming factor of PMN.

TNF *in vitro* stimulates the expression of many of the PMN surface molecules. Simultaneously TNF stimulates its own receptors expression (TNF-R) on PMN.[16] This has been confirmed in our study as the increased TNF binding to PMN in the course of acute exacerbations of MS. We have observed also the significant increase of the CD10, CD13 Ag and CD11b/CD18 molecule expression on PMN of MS patients, mainly in the course of exacerbation compared with MS remission and with OND groups. This may be a sign of priming of PMN in MS exacerbation and to a lower degree in the course of CP-MS.

Podikoglou *et al.*[3] have shown that the typical functions of PMN, like adherence, chemotaxis, phagocytosis or bactericidal action have been significantly diminished in PB of MS patients. The results that we have obtained in our study correspond with Goto *et al.*.[17] In some earlier observations Aoki *et al.*[18] and Guarnieri *et al.*[19] have shown that the increased intracellular neutral proteinase and medullasine concentrations can be the symptom of PMN activation in the blood of patients with acute exacerbation of MS.

PMN contain large amounts of proteinases, like NEP and APN, in their intracellular granules.[20,21] The increased expression on *in vitro* stimulated PMN is a result of rapid translocation of an intracellular pool to the cell surface.[22,23] The increased expression of CD11b/CD18–Mac-1 molecules probably results from their rapid shift from internal granules to the surface of primed PMN. Such process has not been observed in CD11a/CD18 molecules of LFA-1 integrine expression of *in vitro* primed PMN.[24,25] In our studies we have not noticed the increased expression of CD11a molecule on PMN of MS patients with acute exacerbations or in the course of CP-MS. In our opinion, these can suggest PMN priming in patients with active MS. Activated PMN can produce oxygen and nitrogen species, some matrix degrading metalloproteinases and some cytokines.[5,26] Priming is a preliminary event before receptor-induced stimulation of PMN. It is then a sign of these cells' hyperreactivity. PMN priming features observed in the PB of patients with active MS suggest that these cells may participate in the MS immunopathology.

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