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Diminished production of type-I interferons and interleukin-2 in patients with multiple sclerosis

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Abstract

Several lines of evidence have supported the role of immunological mechanisms in the pathogenesis of multiple sclerosis (MS) and new immunomodulatory strategies for its treatment, e.g. subcutaneous application of interferon (IFN)-\textit{\textbeta}. have emerged. We investigated the ability of peripheral blood mononuclear cells (PBMC) in 21 consecutive patients with clinically definite MS to produce interferons and lymphokines in response to viral or mitogenic stimulation. Ten patients showed clinical signs of disease activity (acute relapse) and 11 patients were in a stable condition. Additionally, white blood count, leukocyte differentiation and lymphocyte subtyping were performed. A group of age-related healthy blood donors served as control (\(n=20\)). There was no difference between patients and controls in the production of IFN-\textgamma, tumor necrosis factor (TNF)-\textalpha, and soluble interleukin (IL)-2 receptor. IFN-\textalpha and IFN-\textbeta responsiveness, however, was significantly lower in patients with stable disease than in patients with active disease and controls (\(p<0.001\)). Furthermore, secretion of IL-2 after stimulation was significantly diminished in both patient groups as compared to the control group (\(p<0.01\)). Analysis of T-cell subsets revealed a significantly lower amount of CD8\textsuperscript{+} T-cells in patients with stable disease, leading to a significantly higher CD4/CD8 ratio in this group as compared to patients with active disease. Our study depicted an IL-2 deficiency in MS patients which is shared with other autoimmune diseases. In addition, our findings suggest that the ability to produce type-I IFNs, IFN-\textalpha and IFN-\textbeta, is primarily impaired in MS patients and changes in correlation to the course of disease activity. © 1997 Elsevier Science B.V.

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1. Introduction

Although the etiology of multiple sclerosis is still unknown, there has been a considerable expansion in understanding the pathogenesis of the disease process during the last few years (Poser, 1994). Immunological mechanisms, both cellular and humoral, seem to play a central role in the inflammatory process which leads to demyelination and destruction of oligodendrocytes in the central nervous system (CNS) (Raine, 1994; McFarland, 1995).

Some studies have focused on the presence of autoreactive T-lymphocytes in MS patients, and in particular on the usage of specific T-cell receptor elements for the recognition of immunodominant regions of autoantigens, such as myelin basic protein (Wucherpfennig et al., 1990; Lodge et al., 1994). Others have assessed the role of cytokines, peptidic or glycopeptidic mediators of the immune system, during the course of the disease. Beck et al. (1988) were the first to show that PBMC of individual MS patients produced increased amounts of IFN-\textgamma and TNF-\textalpha at an early stage of an exacerbation of the disease, thus preceding the clinical manifestation of a relapse. These findings were later confirmed for TNF-\textalpha on the mRNA level (Rieckmann et al., 1995). TNF-\textalpha is understood to play a crucial role in the upregulation of adhesion molecules on endothelial cells and leukocytes, therefore being essentially
involved in T-cell migration to the sites of inflammation (Hartung et al., 1995). Furthermore, high levels of expression of proinflammatory as well as immunosuppressive cytokines were detected in MS lesions of postmortem CNS tissue by means of immunocytochemistry (Cannella and Raine, 1995).

The understanding of the role of cytokines in multiple sclerosis has become increasingly important since new treatment strategies for multiple sclerosis have emerged (McDonald, 1995). Interferon beta-1b was reported to reduce exacerbation rates and the severity of exacerbations in patients with relapsing–remitting MS (The IFNB Multiple Sclerosis Study Group, 1993). However, reports on the ability to release type-I interferons upon viral stimulation in MS patients are rare.

Interferon responses of PBMC in general were shown to be diminished in patients with MS (Neighbour et al., 1981; Salonen et al., 1982), but the amount of IFN production could not be correlated with the clinical assessments of disease activity. Other authors revealed completely normal IFN-responses upon viral stimulation in MS patients (Tovell et al., 1983; Haahr et al., 1986). Altogether, the results were largely inconclusive, since different methods have been used for IFN-induction and detection.

The aim of this study was to establish a whole blood assay in order to investigate the ability of MS patients in different stages of disease activity to produce interferons and lymphokines in response to viral or mitogenic stimulation.

2. Materials and methods

2.1. Patients

We investigated 21 consecutive patients (11 males and 10 females, ages 20–57, mean age ± standard deviation 37 ± 10) fulfilling the diagnostic criteria of multiple sclerosis (Poser et al., 1983). Eleven patients had the relapsing–remitting course of MS, 8 patients were suffering from the secondary-progressive subtype and 2 patients had the progressive-relapsing course of the disease according to the standardized definitions (Lublin et al., 1996). All patients were examined and classified independently by two clinical neurologists and informed consent was obtained. 10 patients were seen during an acute episode of new disease activity (acute relapse) and 11 patients were in a stable condition. An acute relapse was defined as significant worsening of pre-existing symptoms as measured by the Expanded Disability Status Scale (Kurtzke, 1983) or appearance of new neurological deficits lasting for at least 24 h. None of the patients had signs of systemic infections and plasma levels of CRP, α1-macroglobulin and neopterin were within normal limits. The patients had not been treated with immunosuppressive drugs or steroids during the preceding 3 months before blood sampling. As a control group, we examined 20 age-matched healthy blood donors who fulfilled the criteria of the German regulations for whole blood donation.

2.2. Leukocyte count and lymphocyte subsets

Peripheral venous blood samples were tested for whole blood and differential blood count by means of an automatic analyzer (Coulter STKS flow cytometer). Lymphocyte subpopulation analysis was performed by flow cytometry on a Coulter Epics XL/MCL flow cytometer (Coulter Electronics, Hileah, FL, USA). Following the instructions of the manufacturer, preparation and immunofluorescence staining of EDTA-anticoagulated blood samples were assisted by the Multi-Q-Prep Epics Immunology work station (Coulter, Krefeld, Germany). For gating of mononuclear lymphatic cells, we used forward and side light scatter and immunostaining with anti-CD45 (CD45-FITC/anti-CD45R1/anti-CD11b/anti-CD34) and anti-CD14 (CD14-PE). For the determination of lymphocyte subpopulations such as T and B lymphocytes, CD4+ and CD8+ T-cells, activated T-cells, as well as NK cells, the following antibodies were used: CD3-ECD/CD4-PE/CD8-FITC, CD45RO-PE/Cd11a-PE/Cd3-PE and CD25-PE. MsIgG2b-ECD/MsIgG1-PE/MsIgG2a-PE antibodies served as controls (all antibodies were obtained from Coulter).

2.3. Whole blood assay

Heparinized blood was drawn by vein puncture from patients and controls, stored at 4 °C and cultured in a whole blood assay within 4 h of collection according to a technique described previously (Kirchner et al., 1982). For mitogenic induction of IL-2, sIL-2R and IFN-γ, we used the plant lectin phytohemagglutinin (PHA, Murex Diagnostic/Burroughs Wellcome, Dartford, UK) in a final concentration of 5 μg/ml. IFN-α and IFN-β production was stimulated by Newcastle disease virus (NDV, strain Kumarov, inactivated by UV-irradiation at 366 nm for 90 min, kindly provided by R. Zawatzky, German Cancer Research Center, Heidelberg) in a final concentration of 0.8 hemagglutinating units/ml. Lipopolysaccharide (LPS) (Sigma, Deisenhofen, FRG), consisting of purified Escherichia coli OIII:B4 endotoxin, was used for the induction of TNF-α at a final concentration of 1 μg/ml. 100 μl whole blood was mixed with 850 μl culture medium (Seromed RPMI 1640, 20 mM Hepes; Biochrom, Berlin, Germany) supplemented with 2 mM l-glutamine, 100 U of penicillin per ml and 100 μl of streptomycin per ml (Gibco, Karlsruhe, Germany) in 5 ml PPN test tubes (Greiner, Nütingen, Germany). 50 μl of the appropriate mitogen solution was added for the induction of cytokine production. As negative control, we tested the cytokine production in unstimulated cultures. Tubes were covered and incubated in a humidified atmosphere of 5% CO₂ at
37 °C. Supernates were harvested after 48 h (IL-2, TNF-α, IFN-α, IFN-β) and 96 h (IFN-γ and sIL-2R) and stored frozen at −80 °C until assayed.

2.4. Cytokine measurement

The concentrations of cytokines in the supernates of cell cultures were determined by means of quantitative enzyme-linked immunosorbent assay (ELISA). IFN-α was additionally determined in sera samples of the individual patients. ELISA kits for IFN-α (intraassay variance 3.7%, interassay variance 5.3%), IFN-γ (intraassay variance 5.2%, interassay variance 6.0%), TNF-α (intraassay variance 3.5%, interassay variance 4.0%) and IL-2 (intraassay variance 3.9%, interassay variance 5.5%) were obtained from Bio Source International (Camarillo, CA, USA). The ELISA kit for detection of sIL-2R (intraassay variance 3.6%, interassay variance 2.0%) was obtained from R&D Systems (Minneapolis, MN, USA) and for IFN-β (intraassay variance 4.1%, interassay variance 3.7%) from Medgenix Diagnostics (Ratingen, Germany). Serial dilutions of samples and standards were incubated in microtiter wells and the assays were performed as described in the manufacturer’s protocol. The color intensity was measured photometrically in an ELISA reader (Anthos Labtec, Salzburg, Austria). Individual concentrations could be determined from a standard curve. Minimal detectable doses were 5 pg/ml (TNF-α, IFN-α, IFN-γ, IL-2, sIL-2R) and 3 units/ml (IFN-β), respectively.

2.5. Statistics

Statistical analysis was performed using commercially available software for personal computers (SPSS for Windows 5.01, SPSS GmbH Software, Munich, Germany). The Mann-Whitney-U test was used to compare non-parametrically distributed data between patient and control groups. p values < 0.05 were considered to be significant.

3. Results

3.1. Differential blood cell count and lymphocyte subpopulations

Differential blood cell counts showed no significant differences between the two patient groups (Table 1) and were all within normal ranges. With regard to the lymphocyte subpopulations, we found a significantly lower amount of CD8+ T-cells in patients with stable disease, leading to a significantly higher CD4/CD8 ratio in this patient group as compared to MS patients undergoing an acute exacerbation (p < 0.03).

3.2. Cytokine production after mitogenic stimulation

Measurement of IFN-γ, TNF-α and sIL-2R in the supernates of mitogen-stimulated whole-blood cultures revealed no significant differences in mean values between the two patient groups. Patients with stable disease (156.6 pg/ml) produced significantly (p < 0.0001) lower amounts of IFN-α than controls (924.1 pg/ml) and patients with disease activity (773.6 pg/ml). No influence of age and sex was observed. Results are shown in Fig. 2. No IFN-α was detectable in sera samples of the patients.

Detection of NDV induced IFN-β secretion revealed

| Differential blood cell counts (cells/µl) | Acute exacerbation (n=10) | Stable disease (n=11) |
|----------------------------------------|--------------------------|-----------------------|
| Leukocytes                             | 7140±2000                | 7800±3600             |
| Monocytes                              | 530±150                  | 470±190               |
| Neutrophils                            | 4330±1600                | 5490±2870             |
| Lymphocytes                            | 1980±620                 | 1600±600              |
| Lymphocyte subpopulation (lymphocytes/µl) |                         |                      |
| CD3 T cells                            | 1536±642                 | 1198±443              |
| CD3, CD25 activated T cells (%)         | 5.3±2.1                  | 5.6±2.8               |
| CD4 T cells                            | 948±372                  | 870±362               |
| CD8 T cells                            | 409±109                  | 276±134*              |
| CD4:CD8 cell ratio                     | 2.3±0.5                  | 3.4±1.1*              |
| CD19 B cells                           | 268±150                  | 209±149               |
| CD16, CD56 NK cells                    | 196±101                  | 169±106               |

Values are given as means ± standard deviations. * p<0.03.
Table 2
Cytokine production in a whole-blood assay in MS patients with acute exacerbation (n=10), with stable disease (n=11) and controls (n=20)

| Cytokine   | MS (acute exacerbation) | MS (stable) | Controls |
|------------|-------------------------|-------------|----------|
| TNF-α (pg/ml) | 122.6±76.3              | 100.1±50.2  | 128.1±82.4 |
| IFN-γ (pg/ml) | 3755±3488.1             | 2358.5±1760.7 | 4494.8±3207.9 |
| sIL-2R (pg/ml) | 5492.7±2302             | 5318.4±2374 | 4691±1673  |

Values are given as means±standard deviations.

Fig. 1. Interleukin-2 production in patients (n=10) with acute exacerbation (A), patients (n=11) with stable disease (B) and control (n=20) group (C). Each open figure represents the average of duplicate determination of interleukin-2 in the supernates of (PHA)-stimulated cell cultures. The horizontal bars represent mean interleukin-2 levels in each patient group.

Fig. 2. Interferon-α production in patients (n=10) with acute exacerbation (A), patients (n=11) with stable disease (B) and control (n=20) group (C).

Fig. 3. Interferon-β production in patients (n=10) with acute exacerbation (A), patients (n=11) with stable disease (B) and control (n=20) group (C).

4. Discussion

The present study revealed a remarkable decrease in production of IFN-α and IFN-β upon viral stimulation in PBMC of MS patients with stable disease and an impaired ability to secrete IL-2 in all MS patients irrespective of disease activity. Production of IFN-γ and TNF-α did not differ from values observed in the control group, and titers of sIL-2R were not increased in the patient group either. Additionally, MS patients with stable disease showed a significantly lower number of CD8+ T-cells.

In previous publications, low IFN responses to various viral antigens have occasionally been described for MS patients (Neighbour et al., 1981; Salonen et al., 1982). However, the findings were not related to the clinical stage of the disease activity. Abb et al. (1983) showed an
association of low IFN-\(\alpha\) responsiveness with the MHC allele HLA-DR2, which is over-represented in MS patients (Hillert, 1994). The defective IFN response could therefore possibly be related to the genetic background of MS patients. In the present study, however, diminished production of IFN-\(\alpha\) and IFN-\(\beta\) was exclusively observed in MS patients with stable disease. Patients admitted to hospital during an acute attack revealed normal IFN-\(\alpha\) and IFN-\(\beta\) values.

In response to viral stimulation, PBMC produce mainly IFN-\(\alpha\) and a small proportion of IFN-\(\beta\) (Berg et al., 1975). The two interferons have similar biological and physico-chemical properties, are structurally related (Kirchner, 1986), and are, therefore, classified as type-I interferons. However, there are striking homologies within the coding as well as flanking regions of the IFN-\(\alpha\) and IFN-\(\beta\) genes, both located on chromosome 9 of the human genome. Our findings suggest a primary deficit in type-I IFN response in MS patients which may render them susceptible to factors causing exacerbations of the disease.

Since interferons are the only cytokines with clear antiviral potency, viruses might be responsible for triggering the pathophysiological processes leading to an exacerbation in MS. One possible mechanism could be initiated by a virus with tropism to the central nervous system, leading to destruction of myelin sheaths and triggering off an immune response to released autoantigens, such as myelin basic protein (MBP). In this regard, coronavirus and human herpesvirus-6 are currently of interest on the basis of polymerase chain reaction and stimulated cell cultures could also result from increased central nervous functions. Haugen and Letourneau, 1990; Merrill, 1990). Interleukin-2 further acts as an endogenous brain neurokine which is able to influence central nervous functions (Haugen and Letourneau, 1990; Merrill, 1990). Interleukin-2 mRNA has recently been found to be present in human oligodendrocytes (Otero and Merrill, 1995), implicating a role for IL-2 in the development of oligodendrocytes, their MBP content and reconstitution (Benveniste and Merrill, 1986; Saneto et al., 1986; Benveniste et al., 1987).

Studies on cytokine patterns within postmortem CNS specimens, however, revealed high levels of IL-2 expression in acute MS-lesions (Cannella and Raine, 1995). Thus, decreased amounts of IL-2 in the supernates of stimulated cell cultures could also result from increased binding of the cytokine to its receptor on activated T-cells, indicating higher in vitro proliferation upon mitogenic stimulation in MS patients as compared to healthy controls.

Decreased numbers of CD8\(^+\) T-cells in MS patients have repeatedly been observed by others (Crucian et al., 1995). Our results support the hypothesis that CD8\(^+\) T-cell mediated suppressor function is lost before and during MS attacks and recovers with remission (Antel et al., 1979).

Interestingly, we did not find significant differences in the production of TNF-\(\alpha\) and IFN-\(\gamma\) between the two patient groups in this cross-sectional study. Since IFN-\(\gamma\) and IL-2 are both produced by activated T-lymphocytes, the impaired IL-2 production may implicate a selective inhibitory mechanism that may downregulate IL-2 but not IFN-\(\gamma\) secretion of this T-cell subpopulation in MS patients. However, an involvement of NK-cells, being known as IL-2 producers, can not be excluded.

Investigating CNS specimens from MS patients, Cannella and Raine (1995) found high expression of TNF-\(\alpha\) in acute and chronic plaques, thus stressing its role in the pathogenesis of the disease. In their study, however, TNF-\(\alpha\) was mainly produced by microglial cells and by foamy macrophages within the lesions, i.e. cells which are not detectable in the peripheral blood.

In conclusion, we found abnormalities in the IFN-\(\alpha\) and Columbia MS/MRI Analysis Group, 1995) could then possibly be regarded as substitution of a preexisting deficit.
IFN-β response of MS patients in relation to disease activity and in their ability to produce IL-2. The etiology of these immunological deficits is unknown. We conclude that the selective immunological abnormalities in MS patients provide the background for disease susceptibility, progression and exacerbation, possibly triggered by diverse exogenous factors, e.g. viruses. The individual production of endogenous type-I IFNs in MS patients may be a useful tool for monitoring disease activity and for studying the underlying pathophysiology.

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