Cytokine Production and Surface Marker Expression in Acute and Stable Multiple Sclerosis: Altered IL-12 Production and Augmented Signaling Lymphocytic Activation Molecule (SLAM)-Expressing Lymphocytes in Acute Multiple Sclerosis

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Cytokine Production and Surface Marker Expression in Acute and Stable Multiple Sclerosis: Altered IL-12 Production and Augmented Signaling Lymphocytic Activation Molecule (SLAM)-Expressing Lymphocytes in Acute Multiple Sclerosis

Pasquale Ferrante, Maria Luisa Fusi,* Marina Saresella,† Domenico Caputo,‡ Mara Biasin,* Daria Trabattoni,* Antonino Salvaggio,§ Enrico Clerici,* Jan E. de Vries,‖ Gregorio Aversa,‖ Carlo L. Cazzullo,¶ and Mario Clerici2*

Ag-stimulated IL-2 production and mitogen-stimulated type 1 and type 2 cytokine production by PBMC, as well as expression of Th1- and Th2-associated phenotypical markers, of B7-1, B7-2, and CD95 (Fas) on the surface of immune cells, and the serum concentration of soluble Apo-1/Fas were evaluated in multiple sclerosis (MS) patients with either acute (AMS) or stable (SMS) disease and in healthy controls (HC). Results showed that 1) Ag-stimulated IL-2 production is reduced in MS patients compared with that in HC; 2) mitogen-stimulated type 1 cytokine production is increased, and IL-10 production is reduced in MS patients compared with those in HC, and in AMS patients compared with those in SMS; 3) whereas production of the metabolically active p70 heterodimers is comparable in SMS, AMS, and HC, production of the p70 heterodimer and the p40 chains (total IL-12) is increased in SMS compared with that in AMS and HC; 4) CD4+, CD4+SLAM+, and CD4+CD7+ lymphocytes (preferentially type 1 cytokine-producing lymphocytes) are increased in MS compared with levels in HC; 5) B7-2- as well as Fas+-expressing monocytes are augmented in MS compared with those in HC, and serum soluble Apo-1/Fas is augmented in AMS compared with SMS and HC. These results confirm that a complex imbalance in both cytokine production and the Fas system is present in MS and indicate that different cytokine profiles may be observed in patients with acute or stable disease. The data also suggest that peculiar phenotypic populations are over-represented in MS patients, and for the first time show that SLAM expression is correlated with dysregulation of type 1 and type 2 cytokine production in human pathology. The Journal of Immunology, 1998, 160: 1514–1521.

Multiple sclerosis (MS) is a chronic neurologic disease characterized by multifocal inflammation and damage involving the myelin sheath. MS patients present with a variety of clinical patterns, including acute and stable forms (1, 2). Although the etiology of MS is still unclear, an immunopathologic mechanism, mainly mediated by the activation of cell-mediated immunity (CMI), was suggested to be responsible for the destruction of the myelin sheath (3–6). CTL specific for antigenic epitopes of myelin basic protein are present, but innocuous, in peripheral blood of normal individuals (6, 7); it is postulated that because of molecular mimicry with cross-reacting and as yet undefined epitopes, these CTL would be activated, tolerance broken, and disease initiated or reactivated (7, 8).

Cytokines have been involved in the pathogenesis of MS; thus, increased levels of TNF-α and IFN-γ were detected in MS patients with acute disease (9–12), and the number of TNF-α and IFN-γ mRNA-expressing lymphocytes was suggested to be different in MS patients during different phases of disease activity (13–15). Additionally, it was observed that myelin Ag-T lymphocytes produce high quantities of IFN-γ upon restimulation with these Ags in vitro (16–18). Finally, treatment of MS with IFN-γ exacerbates the disease (19, 20), whereas therapeutic approaches based on the utilization of IFN-α and IFN-β, which are known to be effective in a number of patients (21, 22), decrease IFN-γ production by T lymphocytes, suggesting a direct pathogenetic role for this cytokine.

Because different cytokines preferentially activate CMI or humoral immunity (23, 24), we analyzed IFN-γ, IL-2, and IL-12 (type 1 cytokines) as well as IL-10 (type 2 cytokine) production by PBMC of MS patients with acute (AMS) or stable (SMS) disease and compared the results with those obtained in a group of healthy donors. To extend our analysis, we also examined a family of phenotypic markers (SLAM and CD7) (25, 26) recently suggested to be expressed by T lymphocytes that preferentially produce type 1 cytokines upon restimulation in the same patients and controls. Because B7-1 and B7-2 are suggested to be differentially expressed in chronic immune responses (27, 28), we also analyzed the expression of B7-1 and B7-2 on the surface of macrophage/monocytes.
monocytes (CD14+). Finally, because 1) increased susceptibility of immune cells to apoptosis is postulated as a mechanism that is potentially protective against immune-mediated destruction of the myelin sheath in MS (29); 2) the CD95/CD95 (Ap0-1/Fas) ligand system is a key mediator of apoptosis (30); and 3) soluble Apo-1 (sApo-1)/Fas was hypothesized to protect lymphocyte against apoptosis (31), we measured the expression of Apo-1/Fas on immune cells of MS patients and controls, and the serum concentration of sApo-1/Fas in the same individuals.

Our results indicate that a profound and complex immune imbalance is present in MS. This imbalance is reflected in the impairment of cytokine production, in the overexpression of particular cytokine-producing cell populations (including Th1 markers expressing T lymphocytes), and in the dysregulation of surface Apo-1/Fas and its soluble ligand.

Materials and Methods

Patients and controls

Forty-eight patients with MS characterized by clinical and laboratory criteria and followed by the Centro Sclerosi Multipla of the Don Gnocchi Foundation (Milan, Italy) were included in the study. These patients (30 women and 18 men; median age, 30.1 year; range, 21.3–40.4 year) were affected by relapsing-remitting (RR) MS. RR MS had been clinically stable for at least 1 year before the study period in 26 patients; these patients (median age, 31.4 year; range, 20.7–40.4 year; 13 women and 13 men) were therefore classified as patients with SMS. The diagnosis of SMS was confirmed in these individuals by magnetic resonance imaging with gadolinium, which did not show areas of enhancement. The other 22 RR MS patients (median age, 30.8 year; range, 21.3–38.2 year; 17 women and 5 men) presented with clinical and laboratory features of the disease and were therefore classified as patients with AMS. AMS patients were visited and blood sampling was performed within 5 days of the onset of the acute episode and always before the onset of steroid-based therapy. Disease duration was comparable in the two groups (mean duration ± SD: SMS, 6.6 ± 6.6 year; AMS, 7.0 ± 6.1 year); none of the patients received immunosuppressive drugs in the year before the study period. Fifty-three healthy donors from the Transfusion Department of the Centro Sclerosi Multipla of the Don Gnocchi Foundation (Milan, Italy) were used as controls; median age (31.7 year; range, 22.8–40.7 year) and sex (30 women and 23 men) were comparable between RR MS patients and controls. The blood samples were collected under protocols approved by both institutions.

Blood sample collection

Whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (Becton Dickinson Co., Rutherford, NJ). PBMC were separated on lymphocyte separation medium (Organon Teknika Corp., Durham, NC) and washed twice in PBS, and the number of viable leukocytes was determined by trypan blue exclusion and a hemocytometer. The samples were kept at room temperature and used within 2 h.

In vitro Ag-stimulated IL-2 production

PBMC (3 × 10^6) were placed in flat-bottom wells of a microtiter plate (Costar; Cambridge, MA) in a final volume of 0.2 ml along with 1) no stimulation (medium background); 2) influenza virus vaccine prepared with a mixture of A/Taiwan, A/Shanghai, and B/Victoria (24 mg/ml; final dilution, 1/1000); and 3) a pool of irradiated (50 Gy) allogeneic PBMC (1 × 10^6 cells/well) from two or more unrelated healthy control volunteers (HC). Three replicate cultures were performed for each stimulation. Pooled human plasma (1/20 final dilution) and the anti-IL-2R Ab, humanized monoclonal anti-Tac (Becton Dickinson; 1 µg/ml) to prevent uptake of the produced IL-2 and allow measurement of total IL-2 production during the 7 days of culture) were added to each well 1 h after sensitization of the PBMC. Culture supernatants were harvested after 7 days, and total IL-2 produced throughout the culture period was determined by testing each supernatant for the ability to stimulate the proliferation of an IL-2-dependent cell line (CTLL). A standard curve (based on known quantities of human rIL-2) was run in all the assays. Ag-stimulated IL-2 production was measured in a bioassay, whereas mitogen-stimulated IL-2 production was quantified with ELISA methods (see below). This choice was secondary to the fact that the sensitivity of bioassays is more adequate to measure relatively small quantities of IL-2, such as those produced upon antigenic stimulation. Five successive twofold dilutions in triplicate were set up to test the supernatants for the ability to stimulate the proliferation of 8 × 10^3 CTLL/well in 96-well microtiter plates. After 24 h, the CTLL cultures were pulsed with 1 µCi of [3H]thymidine and harvested 18 h later.

In vitro cytokine production

PBMCs resuspended at 3 × 10^6/ml in RPMI 1640 were either unstimulated or stimulated for 2 days with PHA (Life Technologies, Grand Island, NY) diluted 1/100 (final concentration) at 37°C in a moist, 7% CO2 atmosphere. Supernatants were harvested after 48 h, because kinetic studies previously indicated that 48 h was the optimal time for assessing lymphokine production after PHA stimulation (32, 33). Production of IL-2, IFN-γ, and IL-10 by PBMCs was evaluated with commercially available ELISA kits as follows: IL-2, human IL-2 Interest-2 (Genzyme, Cambridge, MA); IFN-γ, human IFN-γ Interest γ (Genzyme); and IL-10, human IL-10 Interest 10 (Genzyme). To evaluate IL-12 production, PBMC (3 × 10^6/ml) were stimulated with Staphylococcus aureus (SAC) for 48 h. Two commercially available kits measuring either only the p70 heterodimer (Quantikine IL-12 Immunoassay, R&D Systems, Minneapolis, MN) or both the p70 heterodimer and the 40-kDa chains (total IL-12; Genzyme) were used to measure IL-12 in the supernatants. All test kits were used following the procedures suggested by the manufacturer. Cytokine production was calculated from a standard curve of the corresponding recombinant human cytokine in each case.

FACS analyses

Leukocyte differentiation was performed using a Coulter counter (Coulter Electronics, Inc., Miami Lakes, FL). Lymphocyte and monocyte subsets were evaluated by flow cytometric analysis, using 50 µl of EDTA peripheral blood incubated for 30 min at 4°C with fluorochrome-labeled mAbs. After incubation, erythrocyte lysis and fixation of marked cells were performed using the Immuno-Prep EPICS Kit (Coulter Electronics) and Q-Prep Work Station (Coulter Electronics). Monitor Ab

The following mAbs were used: anti-CD4 (T4) energy-coupled dye (ECD phycoerythrin (PE)-Texas Red; IgG1 isotype), anti-CD8 (ECD; T8; IgG1), anti-CD4 FITC (IgG1), anti-CD19 (B4) PE (RD1; IgG1), anti-CD14 (Mo2) RD1 (IgM), anti-CD7 (3A1) FITC (IgG2b), and their isotype controls MsIgG1-FITC, MsIgG1-RD1, MsIgG2b-FITC, MsIgM RD1, MsIgG1 ECD (Cyto-stat Coulter Clone, Coulter Corp.). The specific mAb for CD95 (Fas Ag) PE (IgG1), CD80 (B7-1) FITC (IgM), CD86 (B7-2) FITC (IgG1), isotype controls MsIgG1 FITC, MsIgG1 PE, MsIgM FITC (PharMingen San Diego, CA), and SLAM (A12) PE (IgG1; DXN Research Institute, Palo Alto, CA; 1 µg of SLAM was incubated with 50 µl of EDTA peripheral blood) were also used.

Immunophenotypic analyses

The flow cytometric analyses of lymphocyte and monocyte subsets were performed using an EPICS XL flow cytometer (Coulter Electronics) equipped with a single 15-nW argon ion laser operating at 488 nm interfaced with a 486 DX2 IBM computer (IBM United Kingdom, U.K.). Multiparametric data were acquired for 5000 events and analyzed using Coulter System II software (Coulter Electronic). Orange-red fluorescence from PE and RD1 (FL2) was collected through a 575-nm longpass filter, green fluorescence from FITC (FL1) was collected through a 525-nm bandpass filter, and finally, fluorescence from ECD (FL3) was measured through a 620-nm bandpass filter. Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1, FL2, and FL3. Samples were first run using single fluorochrome-stained preparations for color compensation. The lymphocytes were selectively analyzed using forward and side scatter properties; the monocytes were identified using the specific mAb Mo1 (CD14). The absolute number of labeled cells was estimated by multiplying the absolute number of cells per microliter by the percentage of labeled cells.

Serum concentration of sApo-1/Fas

The serum concentration of sApo-1/Fas was evaluated using a commercially available ELISA assay (Bender MedSystems, Vienna, Austria) following the procedures suggested by the manufacturer. The serum concentration (units per milliliter) was calculated from standard curves according to the instruction of the manufacturer.

Statistical analyses

Data have been summarized according to standard statistical tests; nonparametric descriptive statistics (median, interquartile range) were preferred to avoid assuming a definite theoretical distribution for each dataset.
Nonparametric tests were performed to evaluate differences between MS patients, divided according to their clinical status (SMS or AMS), and HC. In particular, for every variable, a Kruskal-Wallis (KW) one-way analysis of variance was performed; if this analysis showed significant differences between the different groups of individuals, nonparametric subtests (Mann-Whitney U tests) were used to examine differences between MS patients as a whole and HC, between MS patients classified according to clinical status, and between AMS or SMS and HC. Possible relationships between different immunologic parameters were evaluated using nonparametric Kendall’s rank correlation.

Results

Ag-stimulated IL-2 production
PBMC from 48 MS patients (26 SMS and 22 AMS) and from 16 HC were stimulated with either influenza virus (Flu) or a pool of allogeneic PBMC (Allo), and IL-2 production was measured in a bioassay. These two different stimuli were used because we previously demonstrated that they activated different Th APC pathways (34), thereby allowing a better evaluation of subtle defects in the immune response. Flu and all soluble Ags are recognized by CD4 T lymphocytes upon presentation by autologous APC after Ag processing (34). Th function in response to alloantigens can use the same pathway as soluble Ags; alternatively, CD4 and CD8 T lymphocytes can be stimulated either by allogeneic peptides processed and presented in association with self MHC molecules or by direct recognition of non-self MHC molecules on the surface of the allogeneic lymphocytes (34). Because soluble and alloantigens were used to analyze Th defects in HIV infection, we used these same Ags in MS patients to allow comparison of these results with those obtained in previous studies.

The results are shown in Figure 1, A (Flu-stimulated IL-2 production; by KW, \( p = 0.03 \)) and B (Allo-stimulated IL-2 production; by KW, \( p = 0.01 \)). IL-2 production in response to both Ags was statistically reduced in MS patients compared with that in HC (IL-2, \( p < 0.01 \); Flu, \( p < 0.01 \); Allo, \( p < 0.01 \)). When data from MS patients were divided according to the two clinical subsets, the differences between AMS and SMS and those between AMS and HC did not reach statistical significance. Nevertheless, SMS patients produced significantly reduced amounts of IL-2 upon Flu and Allo stimulation compared with HC (Flu, \( p = 0.01 \); Allo, \( p < 0.01 \)).

Mitogen-stimulated type 1 and type 2 cytokine production
PBMC of 48 MS patients (26 SMS and 22 AMS) and of 53 HC were stimulated in vitro with PHA for 48 h, and IFN-\( \gamma \), IL-2, and IL-10 production was measured with ELISA assays. The median values and the interquartile ranges for each cytokine are shown in Figure 2 for both groups of MS patients and for the HC (by KW: IL-2, \( p < 0.01 \); IFN-\( \gamma \), \( p < 0.01 \); IL-10, \( p < 0.01 \)). The production of the type 1 cytokines IL-2 and IFN-\( \gamma \) was significantly increased in MS individuals compared with that in HC (IL-2, \( p < 0.01 \); IFN-\( \gamma \), \( p < 0.01 \)), whereas IL-10 production was reduced in MS patients compared with that in HC (\( p < 0.01 \)). Data were subsequently analyzed by dividing the MS patients according to the clinical classification, and the following results (median production) were observed: IL-2: SMS, 986 pg/ml; AMS, 1417 pg/ml; HC, 658 pg/ml (SMS vs AMS, \( p < 0.01 \); SMS vs HC, \( p < 0.01 \); AMS vs HC, \( p < 0.01 \); Fig. 2A); IFN-\( \gamma \): SMS, 697 pg/ml; AMS, 1098 pg/ml; HC, 315 pg/ml (SMS vs AMS, \( p < 0.01 \); SMS vs HC, \( p < 0.01 \); AMS vs HC, \( p < 0.01 \); Fig. 2B); and IL-10: SMS, 537 pg/ml; AMS, 290 pg/ml; HC, 266 pg/ml (SMS vs AMS, \( p < 0.01 \); SMS vs HC, \( p < 0.01 \); AMS vs HC, not significant; Fig. 2C).

Thus, different cytokine profiles were observed in MS patient compared with controls. These changes (increased type 1 cytokine and decreased IL-10 production) were exacerbated in patients suffering from an acute MS episode.

Because of the augmented production of IL-2 and IFN-\( \gamma \) observed in AMS and because IL-12 is a pivotal mediator of the effector phase of CMI, we measured SAC-stimulated IL-12 production in the same patients and controls. Because the metabolically active form of IL-12 is the p70 heterodimer, but both the p70 and the metabolically inactive p40 chains can be secreted by cells (35), we used two different kits: one measuring only p70 and the second measuring both the p70 heterodimer plus the p40 chains (total IL-12). The results are shown in Figure 3 and indicate that SAC-stimulated p70 production was comparable in MS and HC.
and in AMS and SMS patients (Fig. 3A; not significant by KW). In contrast to these results a more complex pattern was obtained when SAC-stimulated production of total IL-12 was examined (by KW, p < 0.01). To rule out the possibility that these results stemmed from differences in the relative percentage of CD14 cells in the samples, we analyzed the percentage of CD14 monocytes in all AMS and SMS patients and in HC. Results showed the percentages of these cells to be comparable in the three groups (data not shown). Thus, total IL-12 production was 1) significantly augmented in SMS compared with that in AMS patients (p < 0.01), and 2) significantly reduced in AMS compared with HC (p = 0.02; Fig. 3B).

**Phenotypic analyses**

**Lymphocytes.** The total number of lymphocytes, the percentage of CD4 and CD8 T lymphocytes, and the percentage of CD4 T lymphocyte coexpressing SLAM or CD7 (two cell surface markers suggested to be expressed by T lymphocytes that preferentially produce type 1 cytokines upon restimulation) were analyzed in PBMC of MS patients and HC. Results are shown in Table I and indicate that whereas absolute lymphocyte count and the percentage of CD8 T lymphocyte did not differ between MS patients and controls, CD4 T lymphocytes were significantly increased in MS patients compared with controls (p = 0.03). More interestingly, the percentages of both CD4 SLAM T lymphocyte and CD4 CD7 T lymphocytes were increased in MS compared with those in HC (CD4 SLAM, p = 0.01; CD4 CD7, p < 0.01). No differences were observed when CD4 SLAM T lymphocytes were compared in AMS and SMS patients (Table I). The percentages of CD4 SLAM T lymphocytes were also examined. The results suggested a trend such that the percentage of both lymphocyte subsets was decreased in MS patients compared with controls, although this trend did not reach statistical significance (data not shown).
We also verified that the presence of CD4\(^+\)SLAM\(^+\) and CD4\(^+\)CD7\(^+\) T lymphocytes was statistically correlated. Thus, a positive correlation between expression of the two Th1-defining subsets (Spearman correlation coefficient (SCC) = 0.0394; \(p < 0.01\)) was detected. Additionally, CD4\(^+\)SLAM\(^+\) lymphocytes were negatively correlated with both CD4\(^+\)SLAM\(^-\) (SCC = −0.0349; \(p < 0.01\)) and CD4\(^+\)CD7\(^-\) (SCC = −0.0332; \(p < 0.02\)) lymphocytes (data not shown).

**Monocytes.** The percentage of B7-1- and B7-2-expressing monocytes (CD14\(^+\) cells) was analyzed in the same MS patients and HC. Whereas no differences were detected in the expression of B7-1 (data not shown), the results indicated that a significantly increased percentage of monocytes from MS patients expresses B7-2 compared with those from HC (\(p = 0.02\)). The significance was maintained when data from MS patients were broken down according to the clinical classification (AMS vs HC, \(p = 0.02\); SMS vs HC, \(p = 0.04\)). A similar phenomenon was observed when the percentage of Apo-1/Fas (CD95)-expressing monocytes was compared between MS patients and controls (\(p < 0.01\)).

**Serum concentration of sApo-1/Fas**

The serum concentration of sApo-1/Fas was measured in MS patients and controls. The median values and the interquartile ranges for each cytokine are shown in Figure 4 for both groups of MS patients and for the HC (by KW, \(p = 0.01\)).

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**Table I. T lymphocytic subpopulations in multiple sclerosis (MS) patients and control**

<table>
<thead>
<tr>
<th></th>
<th>MS (n = 45)</th>
<th>AMS (n = 22)</th>
<th>SMS (n = 23)</th>
<th>Controls (n = 16)</th>
<th>(p^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte(^c)</td>
<td></td>
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</tr>
<tr>
<td>Median</td>
<td>2200</td>
<td>2250</td>
<td>2100</td>
<td>2250</td>
<td>NS</td>
</tr>
<tr>
<td>i.q. range</td>
<td>1700–2850</td>
<td>1700–2700</td>
<td>1800–2875</td>
<td>1900–2850</td>
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<tr>
<td>CD4(^d)</td>
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<td>0.02</td>
</tr>
<tr>
<td>Median</td>
<td>47.1</td>
<td>45.3</td>
<td>48.6</td>
<td>41.9</td>
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</tr>
<tr>
<td>i.q. range</td>
<td>40.0–52.4</td>
<td>40.0–49.0</td>
<td>43.3–53.0</td>
<td>36.4–46.5</td>
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</tr>
<tr>
<td>CD8(^e)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Median</td>
<td>25.1</td>
<td>25.1</td>
<td>25.1</td>
<td>27.3</td>
<td></td>
</tr>
<tr>
<td>Lq. range</td>
<td>20.8–29.0</td>
<td>22.5–28.0</td>
<td>21.0–28.0</td>
<td>22.2–32.0</td>
<td></td>
</tr>
</tbody>
</table>

- \(^a\) MS patients were divided into patients with either acute multiple sclerosis (AMS) or stable multiple sclerosis (SMS).
- \(^b\) KW tests, \(p\) value.
- \(^c\) Cells/μl.
- \(^d\) Percentage.
- \(^e\) i.q., interquartile.

**Table II. SLAM- and CD7-expressing CD4\(^+\) T cells in MS patients and controls**

<table>
<thead>
<tr>
<th></th>
<th>MS (n = 39)</th>
<th>AMS (n = 18)</th>
<th>SMS (n = 21)</th>
<th>Controls (n = 16)</th>
<th>(p^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4(^+)SLAM(^+)</td>
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<tr>
<td>Median</td>
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<td>15.1</td>
<td>15.6</td>
<td>11.2</td>
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</tr>
<tr>
<td>i.q. range</td>
<td>10.1–20.2</td>
<td>9.9–18.5</td>
<td>13.1–20.0</td>
<td>8.5–13.5</td>
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</tr>
<tr>
<td>CD4(^+)CD7(^+)</td>
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<td></td>
<td></td>
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<td>&lt; 0.01</td>
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<tr>
<td>Median</td>
<td>40.7</td>
<td>44.2</td>
<td>37.6</td>
<td>32.6</td>
<td></td>
</tr>
<tr>
<td>i.q. range</td>
<td>32.2–48.0</td>
<td>38.5–48.0</td>
<td>33.0–44.0</td>
<td>28.4–37.1</td>
<td></td>
</tr>
</tbody>
</table>

- \(^a\) MS patients were divided into patients with either acute multiple sclerosis (AMS) or stable multiple sclerosis (SMS).
- \(^b\) KW tests, \(p\) value.
- \(^c\) Percentage.
- \(^d\) i.q., interquartile.

**Discussion**

We analyzed a number of immunologic parameters in MS patients with either stable or acute disease to better define the immunopathology underlying this condition. Parameters examined included Th function by evaluation of Ag-stimulated IL-2 production, mitogen-stimulated type 1 and type 2 cytokine production, expression of a series of surface markers, and the Apo-1/Fas system by analyzing both monocyte expression and serum concentration of the soluble receptor.

We observed that 1) Ag-stimulated TH function was defective as assessed by IL-2 production, and 2) mitogen-stimulated type 1 cytokine production was increased and type 2 cytokine production was reduced in MS patients compared with those in HC. Mitogen-stimulated IFN-\(\gamma\) and IL-2 production was increased and IL-10 production was decreased in MS patients compared with those in SMS patients, suggesting that different cytokine profiles are associated with acute (higher type 1 cytokine) or stable (higher IL-10) disease. These results are remarkably similar to those obtained by Correale et al. (36), who analyzed cytokine production by proteolipid protein-specific T cell clones obtained from patients either during acute episodes of disease or during the remission phase, and from controls.

**Table III. B7-2- and Fas-expressing monocytes in multiple sclerosis (MS) patients and controls**

<table>
<thead>
<tr>
<th></th>
<th>MS (n = 39)</th>
<th>AMS (n = 18)</th>
<th>SMS (n = 21)</th>
<th>Controls (n = 15)</th>
<th>(p^b)</th>
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<tr>
<td>CD14(^+)B7-2(^+)</td>
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<td>Median</td>
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<td>68.5</td>
<td>52.6</td>
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<tr>
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<td>60.0–82.5</td>
<td>60.3–80.5</td>
<td>28.9–72.7</td>
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</tr>
<tr>
<td>CD14(^+)Fas(^+)</td>
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<td></td>
<td></td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Median</td>
<td>92.5</td>
<td>93.5</td>
<td>91.1</td>
<td>83.1</td>
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<tr>
<td>i.q. range</td>
<td>89.1–98.2</td>
<td>92.4–98.0</td>
<td>89.0–96.0</td>
<td>80.0–86.0</td>
<td></td>
</tr>
</tbody>
</table>

- \(^a\) MS patients were divided into patients with either acute multiple sclerosis (AMS) or stable multiple sclerosis (SMS).
- \(^b\) KW tests, \(p\) value.
- \(^c\) Percentage.
- \(^d\) i.q., interquartile.
Although SAC-stimulated production of the metabolically active IL-12 p70 heterodimer was similar in AMS, SMS, and controls, the production of total IL-12 was significantly increased in SMS compared with AMS patients and significantly reduced in AMS patients compared with HC, suggesting an alteration in the production of the metabolically inactive p40 chains in MS patients. The p40 homodimer subunit of IL-12 has been shown to be an antagonist of the biologic effects of IL-12 in vitro (37) and was recently demonstrated to elicit a potent immunosuppressive effect on Th1-mediated immune functions (rejection of allografts) in vivo (38) and to directly antagonize IL-12-dependent IFN-γ responses in murine endotoxin shock (37). We have not directly quantified the presence of p40 homodimers in the supernatants; it is nevertheless tempting to speculate that the augmented production of total IL-12 observed in SMS patients could include increased quantities of p40 homodimers, allowing for a regulatory mechanism controlling type 1 cytokine production and activation of CMI. Increased IL-12 p40 chain was reported by others in acute MS plaques; these findings were interpreted as supporting a direct involvement of IL-12 in MS (39). We consider the pathogenic role of IL-12 in MS to be secondary to the ratio of IL-12 p70 to p40. Thus, overproduction of the nonfunctional IL-12 p40 homodimer could be present and involved in dampening cell-mediated immunity in SMS patients, whereas reduced production of this chain could be involved in increasing potentially pathologic responses in acute episodes of MS. In this regard it is important to underline that a therapeutic use of the p40 homodimer of IL-12 in acute MS was recently hypothesized (40).

The percentage of CD4-expressing T lymphocytes was significantly increased in MS patients compared with controls, and as previously reported, CD8 cells tended to be reduced in the same patients (41–44). Because type 1 cytokine secretion was also augmented in MS patients and because markers suggested to be associated with the preferential production of type 1 cytokines upon restimulation (25, 45) are now available, we tested whether the increased percentage of CD4 could be secondary to the expansion of lymphocytes expressing such markers. Phenotypical analysis confirmed the cytokine data, as the percentage of SLAM-expressing lymphocyte was increased in MS patients compared with that in HC. Engagement of SLAM up-regulates IFN-γ production by CD4 T lymphocyte upon restimulation (25, 45). Because IFN-γ production in MS patients was augmented compared with that in controls, we hypothesize that this increase was due to CD4+ SLAM+ T lymphocytes. That an imbalance in the percentage of lymphocyte-expressing markers, which was suggested to be associated with Th1 and Th2, is present in MS was further underlined by the observations that 1) optimally proliferating and IL-2-secreting CD4+CD7- lymphocytes (26) were augmented in MS compared with control values; and 2) SLAM- and CD4+CD7+ T lymphocytes were reduced in MS patients compared with those in controls. The strength of these data was underlined by the observation that the detection of SLAM+ T lymphocytes was correlated positively to that of CD4+CD7- lymphocyte and negatively to the detection of SLAM- and CD4+CD7- cells. Thus, clusters of differentiation suggested to correlate with different functional abilities of immune cells are expressed in a discreet and characteristic way in MS patients. On the other hand, although increased production of IL-2 and IFN-γ was observed in AMS compared with SMS patients, this augmented cytokine production was not reflected in statistical differences in the percentage of T lymphocytes expressing either SLAM or CD7+. This discrepancy could be due to a number of reasons, currently under investigation, including 1) the presence in AMS patients of one or more type 1 cytokine-secreting populations not expressing either SLAM or CD7, 2) an increased secretion of type 1 cytokines on a per cell basis in AMS patients, and 3) the expansion in these patients of a type 1 cytokine-secreting γδ T lymphocyte population.

Because the interaction between CD28 and B7-1 was suggested to preferentially stimulate type 1 cytokine production, whereas CD28 triggering of B7-2 should mainly activate the generation of type 2 cytokines (46, 47), we examined B7-1 and B7-2 expression in monocytes of our patients. It is nevertheless important to underline that the expression of B7-2 is induced earlier than that of B7-1 in most acute and chronic immune responses, with the noticeable exception of murine allergic encephalomyelitis (48, 49), a classic animal model for human MS (50). A significant increase in the percentage of B7-2-expressing monocyte was detected in MS patients compared with HC, whereas no differences were observed in the expression of B7-1 despite the fact that both type 1 cytokine production and the percentage of Th1 markers expressing lymphocytes were augmented in such patients. The increased expression of B7-2 observed in our patients is apparently in contrast with these findings and could be explained as a regulatory loop with which the hyperactivated immune response is attempting to down-modulate itself. Other experiments will nevertheless be necessary to clarify this result.

The interaction between membrane-expressed Apo-1/Fas and its ligand is known to trigger apoptosis (30, 51–53), and the secretion of a soluble form of Fas receptor (sApo-1/Fas) was recently suggested to be a mechanism by which cells escape apoptosis (31, 54). Alterations of this system resulting in accelerated/augmented apoptosis are known to occur in HIV infection (55, 56) and in drug-induced apoptosis of leukemia cells (57). In particular, in HIV infection both overexpression of Apo-1/Fas (58–60) and a reduced serum concentration of sApo-1/Fas were observed and were proposed as prognostic markers of disease progression. Apoptosis of Ag T cells was hypothesized as a mechanism leading to death of autoreactive T lymphocytes, thus limiting the duration of acute episodes of disease. We analyzed the expression of Apo-1/Fas on immune cells and the serum concentration of sApo-1/Fas in MS patients and observed that the percentage of Apo-1/Fas-expressing immune cells is increased in MS patients compared with that in controls and in AMS compared with that in SMS patients and that augmented expression of Apo-1/Fas is mostly evident on the surface of macrophages. Because macrophage-induced, Fas-dependent apoptosis in HIV infection is an important mechanism leading to apoptosis of CD4+ and CD8+ T lymphocytes (61, 62), the up-regulation of the Apo-1/Fas receptor present in MS patients (and most evident in AMS) could lead to the activation of self-regulatory, apoptosis-based loops in the attempt to eliminate autoreactive lymphocytes. An increment in the serum concentration of sApo-1/Fas, a mediator suggested to be capable of preventing apoptosis, was observed in AMS patients. This confirms the dysregulation of the nerve growth factor/TNF receptor family we observed in MS and could, at least theoretically, be involved in disease relapse by preventing apoptosis of autoreactive T cell clones. More detailed analyses of susceptibility to apoptotic death in different phases of the disease and of possible correlations with the various components of the TNF receptor family will be necessary to clarify these findings.

In conclusion, a complex alteration of the immune response is present in MS. This alteration includes two phenomena that to our knowledge are reported here for the first time in human pathology: 1) the augmented percentage of SLAM-expressing lymphocytes,
and 2) the dissociation between the production of the p70 and p40 components of IL-12. Immune defects in MS patients can be detected both functionally (impaired cytokine production) and phenotypically, and could be summarized as an attempt on the part of an hyperactivated immune system to reduce its normal activation either by producing factors known to suppress immune responses (IL-10 and p40 heterodimers) or by modulating the susceptibility of immune cells to apoptosis. Other detailed and possibly longitudinal analyses will be needed to ascertain the importance of these observations within a clinical and therapeutical setting.

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References