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Cytokine flow cytometry differentiates the clinical status of multiple sclerosis (MS) patients

S. INOGÉS, J. MERINO, E. BANDRÉS, P. DE CASTRO*, M. L. SUBIRÁ & A. SÁNCHEZ-IBARROLA
*Departments of Immunology and *Neurology, Clínica Universitaria and School of Medicine, University of Navarra, Pamplona, Spain*

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SUMMARY

In this study we have examined intracellular cytokines in peripheral blood mononuclear cells (PBMC) of MS patients by flow cytometry (cytokine flow cytometry). MS progressive patients showed an increased number of cells producing interferon-gamma (IFN-γ) after activation with phorbol 12-myristate 13-acetate and ionomycin, compared with patients with clinically inactive forms ($P < 0001$) and with healthy controls ($P = 0001$). These cells belonged to the $CD4^+$ and $CD8^+$ subsets in similar proportions. Clinically inactive patients showed a lower level of cells producing IL-2 than controls ($P = 0{\cdot}03$) and active MS patients ($P = 0{\cdot}03$). Most IL-2-producing cells were $CD4^+$ lymphocytes, although a small part of the IL-2 was also produced by $CD8^+$ cells. The percentage of cells producing simultaneously IL-2 and IFN-γ was increased in active MS and they were mainly $CD4^+$ lymphocytes. No differences in the production of IL-4 were observed between groups. However, we found an increased IL-10 production in clinically active MS patients ($P = 0{\cdot}03$). Treatment with IFN-β of active MS patients showed lower levels of cytokines when compared with untreated MS patients. This methodological approach could help in the follow up and therapeutic monitoring of MS patients.

Keywords multiple sclerosis intracellular cytokines flow cytometry interferon-beta

INTRODUCTION

MS is a T cell-mediated chronic inflammatory disease of the central nervous system. The role of cytokines in the pathogenesis of the disease has been extensively studied, although many questions remain unresolved [1–3]. In this sense, the measurement of cytokine levels could be a useful marker of disease activity. Their detection might have diagnostic and prognostic implications as well as a putative value in monitoring clinical trials, possibly leading to new therapeutic strategies. Measurements of different cytokines in biological fluids from MS patients performed in previous studies have given conflicting results. Differences in the methodology used, specimen sampling or handling, or in patient selection criteria, can partly explain the discrepant findings [4,5].

The demonstration of cytokines at the single-cell level could help to solve the above mentioned problems [6]. The detection of intracellular cytokines by the use of anti-cytokine MoAbs and flow cytometry recently introduced by Jung *et al.* [7] ('cytokine flow cytometry' [8]), adds the possibility of defining the T cell subpopulations involved in the production of these cytokines as well as of assessing the simultaneous production of different cytokines by the same cell [9].

In this study we have developed this method in order to obtain rapid and accurate information about the immunological situation of MS patients which could help in their follow up and therapeutic monitoring.

We report an increase of proinflammatory cytokines (interferon-gamma (IFN-γ)) in clinically active forms of MS compared with inactive forms and with healthy controls, this increase being due both to $CD4^+$ and $CD8^+$ T cell subpopulations and disappearing after treatment with IFN-β. The assessment of immunoregulatory cytokines (IL-4 and IL-10) in the same patients allowed us to draw a more complete picture of their immunological situation.

MATERIAL AND METHODS

Patients

Twenty-seven patients with MS were studied. Thirteen (eight female, mean age 42 years, range 20–67 years) had chronic active progressive forms and 14 (10 female, mean age 38 years, range 20–56 years) had relapsing-remitting clinically stable forms of the disease. All these patients satisfied the diagnostic criteria of Poser *et al.* (1983). They were under no treatment at the time of

Correspondence: Alfonso Sánchez-Ibarrola, Department of Immunology, Clínica Universitaria, School of Medicine, University of Navarra, Pamplona, Spain. E-mail: asanchezib@unav.es

study. The control group consisted of 14 healthy subjects (10 female, mean age 40 years, range 19–65 years) who were age- and sex-matched to the patients.

To determine whether the treatment could influence intracellular cytokine production, we also studied a group of 16 patients (eight active progressive forms, mean age 42 years, range 32–52 years, and eight relapsing-remitting stable forms, mean age 33 years, range 22–48 years) treated with IFN-β (Betaseron) at a dose of 8×10^6 U subcutaneously every other day.

Peripheral blood mononuclear cell isolation and processing

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation on Ficoll–Hypaque. Blood sampling was done early in the morning and managed blind with respect to the clinical status of the patient. PBMC at the interface were collected and washed three times with PBS. After washing, the cells were adjusted to a concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine and cells were stimulated for cytokine production for 4 h at 37°C with 10 ng/ml phorbol 12-myristate 13-acetate in combination with 1 μg/ml ionomycin (PMA + I; (Sigma, St Louis, MO) in the presence of 10 μg/ml of brefeldin A (Sigma), which disrupts intracellular protein transport and causes cytokines to accumulate in the Golgi apparatus, yielding an enhanced cytokine signal that can be detected by flow cytometry. Preliminary experiments indicated that maximal accumulation of intracellular cytokine occurred after 4 h of PMA + I stimulation in the presence of brefeldin A.

To determine the T cell subpopulation responsible for the cytokine production, a two- or three-colour staining technique was performed. The activated cells were pooled and washed in PBS with bovine serum albumin (BSA; 0·5%) and sodium azide (0·01%) (PBS–BSA–azide). Then 100 μl of the cellular suspension were stained with a fluorochrome-conjugated antibody specific for a cell surface antigen (CD4 or CD8 PerCP; Becton Dickinson, San José, CA) for 15 min at room temperature. Thereafter, cells were fixed (Fix and Perm; Caltag, San Francisco, CA) according to the manufacturer's instructions. After washing in PBS–BSA–azide, cells were resuspended in 100 μl of PBS and an optimal dose of a fluorochrome-conjugated antibody for intracellular staining (double-labelling was performed with anti-human IL-2–FITC–anti-human IFN-γ–PE, and anti-human IFN-γ–FITC–anti-human IL-4–PE; Becton Dickinson; for single-labelling, anti-human IL-4–FITC and anti-human IL-10–FITC (Caltag, Ingelheim, Germany) were used) was added, together with 100 μl of permeabilizing solution (Fix and Perm; Caltag). Cells were incubated for 30 min at 4°C and washed once in PBS–BSA–azide. The supernatants were aspirated and the pellets resuspended in 500 μl of 0·5% paraformaldehyde to be analysed by flow cytometry.

As controls we included cells labelled with isotype-matched irrelevant MoAbs and non-activated lymphocytes labelled with anti-cytokine antibodies.

Acquisition and analysis of flow cytometry data

Samples were analysed on a FACSORT flow cytometer (Becton Dickinson) using Cell Quest software (Becton Dickinson). Ten thousand events per lymphocyte population were acquired and analysed. Analysis gates were set on lymphocytes according to forward and side scatter properties. Results are expressed as the percentage of cytokine-producing cells in a population of specified cells (see Results).

Statistical analysis

Differences between groups were tested with the Mann–Whitney non-parametric *U*-test when data were not normally distributed, and with Student's paired *t*-test when the data were normally distributed. Reported *P* values are two-tailed and $P<0\cdot05$ was considered statistically significant.

RESULTS

The production of cytokines by unstimulated or PMA + I-activated lymphocytes was evaluated in 13 chronic active progressive MS patients, 14 stable relapsing-remitting MS patients and 14 controls. Results are expressed as the percentage of cytokine-producing cells in the total lymphocyte population.

Spontaneous cytokine production by PBMC

Freshly isolated lymphocytes from MS patients and healthy controls did not constitutively express proinflammatory cytokines (IL-2, IFN-γ) (Table 1). No differences were found between the three groups.

Concerning immunoregulatory cytokines (IL-4, IL-10) (Table 1), IL-4 was not detected in unstimulated cells in any of the three groups. However, both in control and MS patients we detected spontaneous IL-10 production. Moreover, the percentage of cells that spontaneously produced IL-10 was significantly higher in active forms of MS than in inactive forms of the disease (12·59% *versus* 6·77%; $P=0\cdot03$). No differences were found between controls and MS patients.

Cytokine production after stimulation with PMA + I

After activation with PMA + I (Table 2), the percentage of IL-2-secreting cells in inactive MS patients was significantly decreased when compared with active MS patients (17·24% *versus* 26·75%; $P=0\cdot03$) and with healthy controls (17·24% *versus* 25·08%; $P=0\cdot03$). No differences were found between active MS patients and healthy controls.

Concerning IFN-γ-secreting cells (Table 2), we found a higher percentage in active MS patients than in inactive patients (19·10% *versus* 11·51%; $P<0001$) and healthy controls (19·10% *versus* 14·57%; $P=0\cdot001$).

When we studied the simultaneous expression of IFN-γ and IL-2 in peripheral blood lymphocytes, we found that active MS patients showed a higher percentage of double-positive cells than inactive MS patients (7·87% *versus* 4·28%; $P=0\cdot007$). Controls (5·62% ± 1·52%) were not different from MS patients.

Table 1. Percentage (mean ± s.d.) of non-activated peripheral blood lymphocytes producing cytokines in non-treated clinically different groups of MS patients and controls.

	Controls ($n=14$)	Inactive MS ($n=14$)	Active MS ($n=13$)
IL-2	0·64 ± 0·46	0·77 ± 0·29	0·51 ± 0·37
IFN-γ	0·67 ± 0·46	0·78 ± 0·60	0·59 ± 0·40
IL-4	0·72 ± 0·47	1·54 ± 2·31	1·06 ± 1·01
IL-10	6·78 ± 5·17	6·77 ± 7·84*	12·59 ± 9·94*

*$P=0\cdot03$.

Table 2. Percentage (mean ± s.d.) of phorbol 12-myristate 13-acetate + ionomycin (PMA + I)-activated peripheral blood lymphocytes cytokine-producing cells in non-treated and IFN-β-treated clinically different groups of MS patients and controls.

	Controls ($n=14$)	Non-treated inactive MS ($n=14$)	Non-treated active MS ($n=13$)	IFN-β-treated active MS ($n=8$)
IL-2	25·08 ± 5·68†	17·24 ± 11·28*†	26·75 ± 10·41*†††	17·95 ± 7·65†††
IFN-γ	14·57 ± 3·79**	11·51 ± 4·61††	19·10 ± 5·54**††***	11·98 ± 3·79***
IL-4	2·78 ± 1·92	2·56 ± 1·68	1·72 ± 1·09	1·97 ± 1·29
IL-10	2·51 ± 1·92	2·38 ± 1·58	3·82 ± 3·09	3·29 ± 3·80

*†$P=0·03$; **$P=0·001$; ***$P=0·002$; ††$P<0·001$; †††$P=0·003$.

No differences were found in the production of IL-4 or IL-10 between groups.

Phenotypic characterization of the cytokine-producing subpopulation

To determine the lymphocyte subpopulation responsible for the production of cytokines, we performed three-colour flow cytometry by staining lymphocytes with anti-CD4 or anti-CD8 and two anti-cytokine antibodies with different fluorochromes.

Our results show that IL-2 was mainly produced by $CD4^+$ cells, although $CD8^+$ lymphocytes contributed in some proportion (Fig. 1). Both $CD4^+$ IL-2^+ and $CD8^+$ IL-2^+ subsets were decreased in inactive MS patients with respect to active patients ($P=0·05$ and $P=0·01$, respectively) and controls ($P=0·05$ and $P=0·01$, respectively).

By contrast, IFN-γ was produced in similar proportions by $CD4^+$ and $CD8^+$ T cells (Fig. 2). Both $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ subsets were increased in active MS patients compared with inactive patients ($P=0·001$ and $P<0·001$, respectively). When compared with controls, active MS patients showed higher values of the $CD4^+$ IFN-γ^+ subset ($P=0·01$) and inactive MS patients show a decrease in the $CD8^+$ IFN-γ^+ T cell subset ($P=0·01$).

The effect of treatment with IFN-β on the production of cytokines

The effect of the treatment of MS patients (chronic progressive forms, $n=8$) with IFN-β was evaluated in terms of the production of intracellular cytokines by PBMC.

We found that the levels of proinflammatory cytokines, namely IL-2 (26·75% *versus* 17·95%; $P=0·003$) and IFN-γ (19·10% *versus* 11·98%; $P=0·002$), were lower in active MS patients under IFN-β treatment compared with untreated patients. However, the percentage of IL-4- or IL-10-producing cells was not altered by this type of treatment (Table 2).

DISCUSSION

Quantification of intracellular cytokines at the single-cell level constitutes a valuable approach to evaluate the immunological status of patients with MS. We have shown in this study a good correlation between the cytokine profile and the clinical activity of patients.

In order to detect cytokine production we induced non-specific lymphocyte activation. As few resting lymphocytes express cytokines, we took the fact that only activated lymphocytes express cytokines as evidence of the specificity of the labelling. We used PMA + I to activate PBMC of MS patients (activation was confirmed by induction of CD69, data not shown). We decided to use the same activator (PMA + I) for the induction of all the cytokines

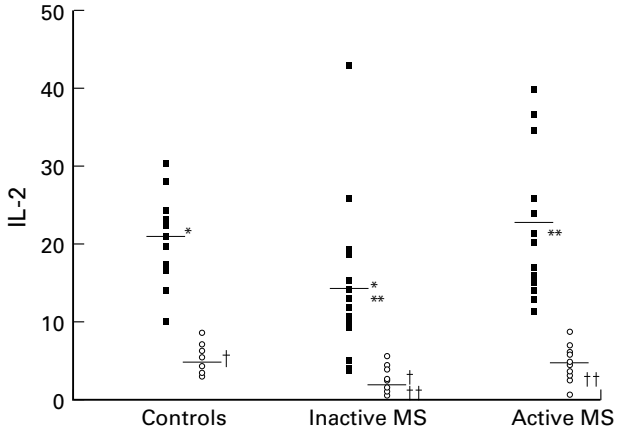

Fig. 1. IL-2 production by peripheral blood phorbol 12-myristate 13-acetate + ionomycin (PMA + I)-activated lymphocytes in CD4 and CD8 T cells in non-treated clinically different groups of MS patients and controls. ○, $CD8^+$ IL-2^+; ■, $CD4^+$ IL-2^+. *,**$P=0·05$; †,††$P=0·01$.

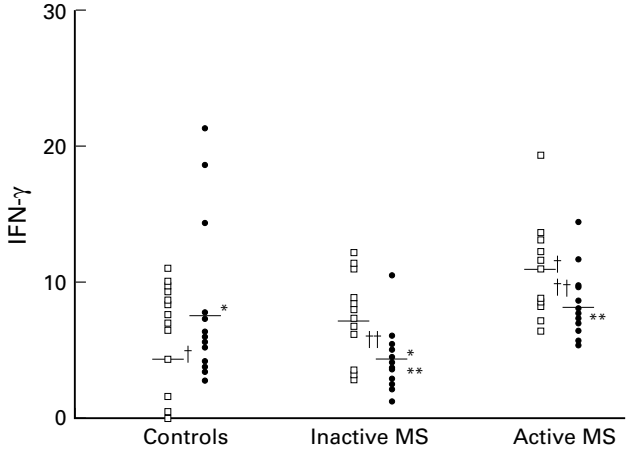

Fig. 2. IFN-γ production by peripheral blood phorbol 12-myristate 13-acetate + ionomycin (PMA + I)-activated lymphocytes in CD4 and CD8 T cells in non-treated clinically different groups of MS patients and controls. ●, $CD8^+$ IFN-γ^+; □, $CD4^+$ IFN-γ^+. *$P=0·01$; **$P<0·001$; †$P=0·01$; ††$P=0·001$.

for more general applicability in clinical practice. This type of activation has been criticised as not being a physiologic stimulus. However, different studies show that the cytokine phenotypes derived upon PMA + I activation represent the physiologic potential of cellular cytokine production [8,10,11].

With this approach we have shown that clinically active patients show a higher percentage of IFN-γ-producing lymphocytes after stimulation with PMA + I than healthy controls or inactive MS patients. The quantification of IFN-γ production by PBMC of MS patients has offered conflicting results when using ELISA for detection of this cytokine in plasma [12,13], cerebrospinal fluid (CSF) [14] or culture supernatants [15–20]. In these cases, no correlation with clinical activity or magnetic resonance imaging (MRI) findings was found. Only investigators using detection of cytokine mRNA [21,22] showed a good correlation with disease activity, as supported by our results.

Our study shows that the level of production of IL-2 is lower in inactive forms of MS (remission) than in active MS, in agreement with other published data [23–25]. Importantly, however, as previously noted by Merrill *et al*. [26], the production of IL-2 is lower in remission than in controls, probably indicating an immunosuppressed state after the acute relapse recovery.

IL-10 was the only cytokine which we could detect in resting lymphocytes, especially in patients with active MS. An immunosuppressive function has been postulated for IL-10 in MS, as it is present mainly in non-active MS forms [27,28]. Activated lymphocytes produced less IL-10 than resting cells, a fact which can be attributed to the stimulus used (PMA + I). It has been described that the calcium ionophore A23187 inhibits IL-10 production induced by PMA [29].

We detected very small amounts of IL-4, with no differences between groups. Published data about IL-4 levels in MS patients are not conclusive [21,25,30]. In some cases a small increment has been demonstrated in inactive forms by the use of highly sensitive techniques. It is probable that our method lacked the sensitivity to detect these subtle changes.

Our results suggest that the balance between the different cytokines is more important than the absolute levels of each. Thus, we have shown that that clinically active forms of the disease present the highest levels of proinflammatory cytokines, such as IFN-γ, and at the same time the highest amounts of IL-10, which has been implicated as an immunosuppressive cytokine [27,28]. Perhaps the latter represents an early or weak attempt to regulate an overactive inflammatory process. Indeed, Diab *et al*. found IL-10-producing cells in experimental allergic encephalomyelitis (EAE) lesions at the end of the acute phase [31]. In remission, on the other hand, IFN-γ is below normal levels, as lower levels of IL-10 are sufficient to maintain a non-inflammatory situation without clinical activity of the disease.

The simultaneous detection of cytokines and cell surface antigens allowed us to define the pattern of cytokines in different T cell subpopulations. While IL-2 is produced almost exclusively by $CD4^+$ cells, $CD8^+$ lymphocytes contribute almost as much IFN-γ as the CD4 in active MS (Tc1). Some evidence points to a possible role of $CD8^+$ T cells in the pathogenesis of MS: (i) its presence in central nervous system (CNS) lesions of MS; (ii) they are the only T cells able to recognize antigens locally in CNS; and (iii) an immunoregulatory effect on the course of experimental EAE has been attributed to these cells [32]. Our data could add new evidence to the pathogenic role of $CD8^+$ cells in MS patients.

PBMC activation with PMA + I led to the production either of IL-2 or of IFN-γ. Only a small percentage of cells produced both cytokines simultaneously, as already reported [7,9]. This modest percentage of mainly $CD4^+$ cells was nevertheless higher in clinically active MS. These data are in concordance with the Th1 pattern of cytokines described in MS patients [33,34], including the study of T cell clones derived from MS patients in different clinical situations [35].

Finally, we found lower frequencies of IL-2- and IFN-γ-producing cells in patients with progressive MS who were treated with IFN-β than in untreated cases. This type of treatment is being used in the relapsing-remitting form of the disease, although some clinical trials are trying to extend this application to other clinical forms [36]. This result supports the use of IFN-β in progressive forms of the disease, given the biological response to the treatment we have observed in these patients.

In conclusion, we believe that this method of cytokine detection could help in the follow up of MS patients, offering valuable information about their immunological situation.

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