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Detection of Altered T Helper 1 and T Helper 2 Cytokine Production by Peripheral Blood Mononuclear Cells in Patients with Multiple Sclerosis Utilizing Intracellular Cytokine Detection by Flow Cytometry and Surface Marker Analysis

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Production of T helper 1 and T helper 2 cytokines was investigated in peripheral blood mononuclear cells (PBMCs) from multiple sclerosis (MS) patients by a newly described technique, detection of intracellular cytokines by flow cytometry in conjunction with immunophenotype analysis. T-cell gamma interferon (IFN-γ) production and interleukin 10 (IL-10) production were examined after PBMC activation with T-cell mitogens at 5 and 24 h, and monocyte spontaneous production of IL-10 and production after PBMC activation with lipopolysaccharide (LPS) for 24 h were also examined. The data indicate that MS patients have decreased percentages of T cells capable of secreting IFN-γ compared with healthy controls, and this change is detectable at 5 and 24 h. The patients displaying decreased T-cell production of IFN-γ were essentially confined to a group being treated with the newly approved drug Betaseron (Berlex Labs, Cedar Knolls, N.J.), a recombinant form of IFN-β (rIFN-β_{1b}). By gating of the entire lymphocyte population, analysis of IFN-γ production in T cells ($CD3^+$) versus that in non-T cells ($CD3^-$) was possible. The percentage of IFN-γ-producing lymphocytes that was made up of T cells was essentially unchanged between the Betaseron-treated patients, non-Betaseron-treated patients, and controls, indicating that the suppression of IFN-γ production displayed by Betaseron-treated MS patients was a nonspecific suppression of all IFN-γ-producing lymphocytes as opposed to a suppression of T-cell production only. The data seem to indicate that treatment of MS with Betaseron corresponds to an inhibition of the lymphocyte's ability to produce IFN-γ. No changes were detected in T-cell production of IL-10 at either time point. We also observed that MS patients in general appear to have small percentages of peripheral blood monocytes spontaneously producing slight but detectable levels of IL-10. No difference was seen regarding monocyte production of IL-10 after PBMC activation with LPS between MS patients and controls. Both populations responded with high percentages of monocytes producing IL-10. The data seem to indicate that treatment of MS with Betaseron, known to decrease the exacerbation rate of relapsing-remitting MS, corresponds to a suppression of peripheral blood lymphocyte production of IFN-γ. Monocyte production of IL-10 may also play a role in regulating the disease process.

Multiple sclerosis (MS) is an autoimmune-based, demyelinating disease affecting the white matter of the central nervous system (CNS) and brain. There are over 300,000 confirmed cases of MS annually in the United States. The initial cause of MS is unknown. Clinically MS is characterized by recurrent attacks and exacerbations of neurologic dysfunction leading to progressive physical disability (10, 36). Since the disease manifests itself in CNS plaques, significant pathology in other organs is limited; however, immune abnormalities are detectable in the peripheral blood. The presence of myelin basic protein (MBP)-specific T cells has been demonstrated in the peripheral blood (9, 31), and MS patients have been found to have elevated ratios of $CD4^+/CD8^+$ cells (5, 14, 30, 38) and decreased percentages of $CD28^-$ $CD8^+$ suppressor cell precursors in the peripheral blood (5). Recent studies have indicated that cytokines play an important role in the initiation, progression, remission, and exacerbation of MS. Gamma interferon (IFN-γ) and tumor necrosis factor alpha have been found in the CNS plaque material of patients with MS and animals with experimental allergic encephalomyelitis (EAE), the best animal model of MS (25, 35). In the past, the observation that viral infections could trigger MS exacerbations led to the theory that MS may have a viral etiology. Subsequently, clinical trials of IFN-γ began in the 1970s for treatment of MS. Disappointingly, IFN-γ was found to promote MS and to increase the exacerbation rate (23, 26). These studies did, however, provide insight into the role that cytokines and the immune system play in the pathogenesis of MS. In the EAE mouse model, T-cell lines that transfer disease have been found to produce the T helper 1 (Th1) cytokines IFN-γ, interleukin 2 (IL-2), and tumor necrosis factor alpha (1, 17, 34). Immunohistochemical and RNA studies of EAE CNS tissues have shown that spontaneous recovery of mice with EAE symptoms correlates with an increase in the number of Th2-like cells, which produce elevated levels of the cytokines IL-4, IL-10, and transforming growth factor β (15, 17, 19). In addition, the induction of oral tolerance by the administration of oral MBP to EAE mice results in suppression of EAE symptoms, which

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TABLE 1. Age, sex, clinical status, extended disability status scale score, and current medications for MS patients in this study

Patient	Sex[a] /age (yr)	Extended disability status[b]	Extended disability status scale score	Medication(s)[c]
1	F/54	CP	5.0	MX, AM, PB, DT, PR, CY
2	M/52	CP	6.5	DA, AM
3	F/42	CP	7.0	BS, CY
4	F/34	EX	3.0	None
5	F/26	RM	<3.0	BS, K, P
6	M/54	CP	6.0	BS, EL
7	F/31	RM	<3.0	BS, VA
8	M/37	RM	<3.0	BS
9	F/35	RM	<3.0	BS, VC, AM, CL
10	F/28	RM	<3.0	BS, B, MC
11	M/45	CP	4.0	BS, CY, HY, EL
12	M/64	CP	5.5	None
13	F/51	CP	3.5	BS, EL, A
14	F/53	EX	6.0	TM
15	F/18	CP	6.0	None
16	F/26	EX	6.5	B
17	F/49	RM	2.0	DL
18	F/53	CP	4.0	BS
19	M/48	EX	6.5	B

[a] F, female; M, male.
[b] EX, patient believed to be in current MS exacerbation; RM, patient not in current exacerbation (remission); CP, patient displaying chronic progressive MS symptoms.
[c] X, Xanax (alprazolam); PX, Paxilon (methazole); Z, Zantac (ranitidine hydrochloride); P, Prozac (fluoxetine); B, baclofen; DT, Ditropan (oxybutynin chloride); PB, phenobarbitol sodium; DL, Dalmane (flurazepam hydrochloride); EL, Elavil (amitroptyline hydrochloride); DA, Dantrium (dantrolene sodium); VA, Valium (diazepam); PR, Premarin (conjugated estrogens); HY, Hytrin (terazosin hydrochloride); AM, Amantadine hydrochloride; MX, methotrexate sodium; K, Klonopin (clonazepam); CL, Claritin (loratadine); CY, Cylert (pemoline); A, Anaprox (naproxen sodium); TM, Tranxene (clorazepate dipotassium); MC, macrobid; VC, vanconase.

correlates with the elevated production of transforming growth factor β as well as that of IL-4 and IL-10 by $CD4^+$ T-cell clones (3, 21). Since IFN-γ has been found to induce exacerbations in MS patients and Th1 cytokines are present in the CNS of animals with active EAE, it has been suggested that Th1- and Th2-type cytokines are involved in the progression and remission of MS (3, 29). T-cell clones specific for myelin antigens isolated from MS patients in remission have been shown to produce elevated levels of IL-10 in response to antigen (4), and recently MS patients have been shown to have elevated numbers of cells possessing mRNA for IL-10 in their peripheral blood and produced in response to MBP in vitro (22). We have previously demonstrated by supernatant enzyme-linked immunosorbent assay (ELISA) that cultured peripheral blood mononuclear cells (PBMCs) (without stimulus) from a higher percentage of patients with MS than from controls spontaneously produce small but detectable levels of IL-10 and that phytohemagglutinin (PHA)-stimulated PBMCs from MS patients produce significantly elevated levels of IL-10 protein and display a trend toward decreased production of IFN-γ (6).

We wished to further examine cytokine production by the PBMCs of MS patients in such a way that individual immune cell subpopulations could be examined. Conventional assays to detect cytokine production have several drawbacks. ELISA and radioimmunoassay techniques are highly specific, but they may not quantify cytokine precisely, since they reflect the net outcome of produced, absorbed, and degraded cytokine. More importantly, in mixed-cell cultures (such as PBMCs), ELISA techniques do not identify the cell population responsible for the cytokine production. In situ hybridization techniques detect cytokine RNA production in individual cells and are highly sensitive, but the presence of cytokine RNA does not guarantee translation to cytokine protein. We have utilized intracellular detection of cytokine production by flow cytometry followed by surface immunophenotyping to analyze the production of Th1 and Th2 cytokines in the peripheral blood of MS patients. In this procedure, cells are first fixed, and the membranes are permeabilized to expose the intracellular contents to cytokine detection antibodies, which is followed by staining of surface antigens by traditional staining techniques. We have examined the production of IFN-γ and IL-10 by peripheral blood T cells ($CD3^+$) in MS patients after PBMC activation with a mitogenic stimulus for 5 and 24 h and the production of IL-10 by monocytes ($CD14^+$) both spontaneously and after PBMC activation with lipopolysaccharide (LPS) for 24 h.

MATERIALS AND METHODS

Patients. Peripheral blood specimens were drawn from 19 MS patients in various stages of clinically defined MS (33) and 15 healthy Tampa General Hospital employees who served as age-matched controls for the study. Information about the age, sex, clinical status, and current medications of the patients is presented in Table 1. Disease severity was determined according to the expanded disability status scale (18). Patient clinical status was classified as MS exacerbation, defined as the occurrence of a symptom or symptoms of neurological dysfunction not previously present and lasting at least 24 h; remission, defined as the relapsing-remitting disease type not in a current exacerbation; or possessing chronic progressive symptoms, but not of the relapsing-remitting disease type. Venipuncture was performed, and blood specimens were collected from both MS patients and normal controls in an EDTA anticoagulant blood collection tube.

PBMC separation and cell culture. Four milliliters of whole anticoagulated blood was diluted 1:1 with sterile phosphate-buffered saline (PBS) and subjected to centrifugation over a Ficoll-Hypaque density gradient (Pharmacia, Uppsala Sweden) to isolate PBMCs. The isolated cells were washed three times in sterile PBS and resuspended in RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.) containing 10% fetal bovine serum (GIBCO), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*±-2-ethanesulfonic acid) buffer (GIBCO), 50 U of penicillin per ml and 50 μg of streptomycin (GIBCO) per ml, 25 μg of Fungizone (GIBCO) per ml, and 10 μg of gentamicin (GIBCO) per ml. The concentration of cells was determined with an automated hematology analyzer (Cell-Dyn 3000; Abbott, Chicago, Ill.). Cell concentrations ranging from 0.7×10^6 to 1.3×10^6/ml were used in all experiments. Cells were cultured either in medium alone for 5 h; in medium with 5 μg of PHA, 10 ng of phorbol myristate acetate (PMA), and 1 μg of ionomycin per ml for 5 and 24 h; or medium with 5 μg of LPS per ml for 24 h. In all cases, 3 μM monensin (Sigma) was added to the PBMC cultures for the final 5 h to shut down extracellular transport of cytokines and to allow intracellular accumulation.

Intracellular detection of cytokines by flow cytometry. An antibody configuration was selected which assessed T-cell production of IFN-γ and IL-10 in response to PBMC stimulation with PHA, phorbol myristate acetate, and ionomycin as well as monocyte production of IL-10 both spontaneously and after PBMC activation with LPS. For cytokine analysis, PBMCs were first washed in PBS and fixed in 200 μl of 4.0% paraformaldehyde in PBS for 10 min and then washed again in PBS. To detect intracellular production of IFN-γ and IL-10 in conjunction with surface CD3 in T cells, fixed PBMCs were resuspended in 200 μl of permeabilization buffer (PB), consisting of 5.0% nonfat dry milk and 0.5% saponin in PBS, to which 0.5 μg of unlabeled mouse antibody to human IFN-γ (Genzyme) was added. Cells were incubated at room temperature for 25 min. The cells were then washed in PBS containing saponin (PBSS), resuspended in 200 μl of PB with 0.5 μg of (fluorescein isothiocyanate-labeled [Southern Biotechnology Associated, Inc., Birmingham, Ala.]) goat anti-mouse $F(ab')_2$ immunoglobulin G (heavy and light chains), and then incubated at room temperature for 10 min. Cells were then washed in PBSS and resuspended in 200 μl of PB (with 2 μl of heat-inactivated mouse serum serving to block unbound sites on the anti-mouse conjugate), incubated at room temperature for 10 min, and washed in PBSS. The cells were then resuspended in 200 μl of PB with 0.5 μg of rat antibody to human or viral phycoerythrin (PE)-labeled (Pharmingen) IL-10, incubated for 25 min at room temperature, and washed with PBSS. To detect surface CD3, the cells were resuspended in 200 μl of PBS without saponin, to which 1.0 μg of PE/Cy5 labeled mouse monoclonal antibody to CD3 (Sigma) was added, and then the cells were incubated at room temperature for 10 min and then washed with PBS. To detect intracellular production of IL-10 in conjunction with surface CD14 in monocytes, fixed PBMCs were resuspended in 200 μl of PB with 0.5 μg of rat antibody to human or viral IL-10 (PE labeled [Pharmingen]), incubated for 25 min at room temperature, and washed with PBSS. To detect surface CD14, the cells were resuspended in 200 μl of PBS without saponin, to which 1.0 μg of fluorescein isothiocyanate-labeled mouse monoclonal antibody

to CD14 (Caltag) was added, and then the cells were incubated at room temperature for 10 min and then washed with PBS. The cells were then subjected to flow cytometric analysis with LYSIS II software on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

RESULTS

Intracellular detection of activated T-cell IFN-γ and IL-10. Production of Th1 and Th2 cytokines in the peripheral blood of 19 MS patients and 15 healthy controls was investigated by intracellular detection of cytokines in activated PBMCs by flow cytometry. Patient data regarding disease state, clinical disability, and current medications are shown in Table 1. The production of IFN-γ and IL-10 by T cells was investigated after 5 and 24 h of activation. Purified PBMCs from MS patients and healthy controls were cultured in medium containing PHA (5 μg/ml), phorbol myristate acetate (10 ng/ml), and ionomycin (1 μg/ml) to stimulate lymphocyte cytokine production. The addition of 3 μM monensin to all cell cultures for the final 5 h abrogated the extracellular transport of secreted protein without altering the de novo synthesis of proteins and allowed the accumulation of cytokine to detectable levels. Intracellular production of IFN-γ and IL-10 as well as surface detection of CD3 was determined by staining according to the antibody configuration described above, and the cells were analyzed by three-color flow cytometry. The T-cell gate was drawn by immunoscatter (CD3 versus side scatter) as opposed to scatter properties alone to ensure the absence of $CD3^-$ debris in the T-cell gate and to ensure accurate calculation of T-cell percentages (Fig. 1A to C). We found that as a whole, MS patients had significantly decreased percentages of T cells capable of secreting IFN-γ compared with controls. This decrease was detectable after PBMC activation for both 5 h (mean percentage of T cells producing IFN-γ: MS patients, 12.8%, controls, 19.3%; $P < 0.05$) and 24 h (MS patients, 8.4%; controls, 14.2%; $P < 0.05$) of activation (Fig. 2A and D), although the percentages of T cells secreting IFN-γ were greatly decreased at 24 h for both MS patients and controls. This decreased production at 24 h follows the expected kinetics regarding IFN-γ production detectable with this method according to Sander et al. (32). Of the 19 MS patients in our study, 10 were currently being treated with Betaseron (BS [Berlex Labs, Cedar Knolls, N.J.]), a recombinant form of IFN-β (rIFN-β_{16}) approved for treatment of MS. We examined the decreased percentages of peripheral blood T cells producing IFN-γ in MS patients in the context of BS treatment and found that after 5 h of activation, the decrease was essentially exclusive to BS-treated (BS+) patients (mean percentage of T cells producing IFN-γ: BS+ MS patients, 9.9%; BS− MS patients, 15.9%; controls, 19.3%; $P < 0.05$ for both BS+ MS patients versus controls and BS+ patients versus BS− MS patients) (Fig. 2B). After 24 h of activation, the difference in IFN-γ-producing T cells between the BS+ MS patients and the controls was not statistically significant, probably because the percentages of T cells producing IFN-γ were smaller after 24 h of PBMC activation.

Drawing a gate around the entire lymphocyte population (instead of only the T-cell population) provided the ability to look at the production of IFN-γ in both T cells ($CD3^+$) and non-T cells ($CD3^-$) and then compare the relative percentages (Fig. 1D and E). It was found that in examining all IFN-γ-producing lymphocytes after 5 h of activation (when IFN-γ production was maximal) the percentage of cells that were T cells was essentially unchanged between the BS+ patients (mean, 74.5%), BS− patients (mean, 72.1%), and controls (mean, 70.0%) (Fig. 2C). These data indicate that the suppression of IFN-γ production displayed by BS+ MS patients was a generalized suppression of all IFN-γ-producing lymphocytes, as opposed to a suppression of T-cell production only. It is also of note that T-cell ($CD3^+$) producers of IFN-γ were much brighter than non-T-cell ($CD3^-$) producers (Fig. 1E). Although this method of cytokine detection does not allow quantitation, a relative comparison of production between two populations can be achieved by comparison of fluorescence intensities.

Regarding the T-cell and general lymphocyte production of IL-10, minimal percentages of lymphocytes (T cell or otherwise) were detected in all classes of MS patients and controls, and no significant differences between patients and controls were seen (data not shown). In most patients and controls, a population of IL-10-producing T cells was detectable only after 24 h of activation (distinct from the IFN-γ-producing T cells), but the percentages were generally below 3.0% (Fig. 1C). Virtually no T cells producing both IFN-γ and IL-10 were seen after activation. These data seem to indicate that the cytokine bias of the majority of peripheral blood T cells after activation is toward IFN-γ production and not production of IL-10.

Intracellular detection of spontaneous and activated monocyte IL-10. Production of IL-10 from peripheral blood monocytes both spontaneously and in response to activation was also investigated. To detect spontaneous IL-10 secretion by this method, purified PBMCs from MS patients and controls were isolated and cultured for 5 h in medium with 3 μM monensin to allow accumulation of cytokine. No mitogens were added to these cell cultures. The monocyte gate was drawn with immunoscatter (CD14 versus side scatter), again to eliminate $CD14^-$ debris from the gate and to allow for the accurate calculation of percentages (Fig. 1F to H). We found that 12 of 16 MS patients assayed had small percentages of monocytes in the peripheral blood that were spontaneously secreting slight but detectable levels of IL-10. In 12 of 13 healthy controls, absolutely no spontaneous monocyte production of IL-10 was detected (mean percentage of monocytes spontaneously producing IL-10: patients, 2.5%; controls 0.06%; $P < 0.05$) (Fig. 3A). To assess the production of IL-10 by peripheral blood monocytes in response to activation, patient and control PBMCs were cultured as described above for 24 h in medium containing 5 μg of LPS per ml, with 3 μM monensin added for the final 5 h to allow accumulation of cytokine. Gates were drawn and cells were analyzed as described above (Fig. 1F and I). We found that all MS patients and controls responded with high percentages of peripheral blood monocytes producing detectable IL-10, and there was no significant difference between the two populations (mean percentage of monocytes producing IL-10 in response to LPS: MS patients, 50.3%; controls, 52.4%) (Fig. 3B).

DISCUSSION

T-cell and general lymphocyte production of IFN-γ and IL-10 in response to mitogenic stimuli and monocyte production of IL-10 both spontaneously and in response to activation were investigated with MS patients with the newly described method of intracellular detection of cytokines by flow cytometry with surface marker analysis (2, 8, 13, 16, 32). The advantages of this method are the detection of cytokine protein, as opposed to cytokine RNA, and the ability to examine the production of cytokines by individual cell populations while culturing mixed populations of cells to retain the various cell-cell interactions. This method of cytokine analysis requires the culture of cytokine-producing cells (usually including mitogenic activation) with the carboxylic ionophore monensin (37). Monensin disrupts the extracellular transport of secreted pro-

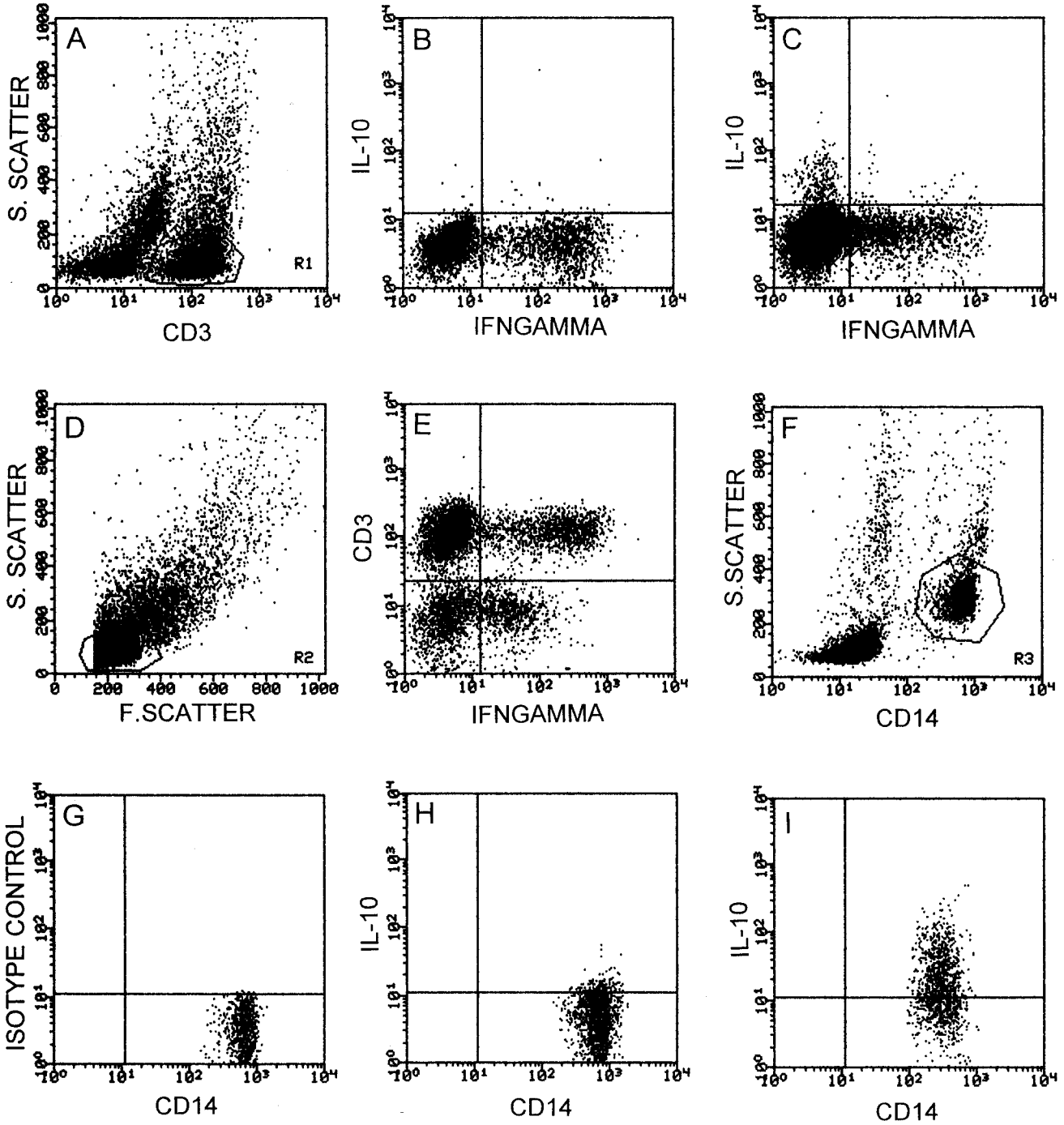

FIG. 1. Representative scatter plots derived from analysis of intracellular cytokines by flow cytometry. (A) T-cell gate. (B) T-cell intracellular IFN-γ and IL-10 after 5 h of activation. (C) T-cell intracellular IFN-γ and IL-10 after 24 h of activation. (D) Lymphocyte gate. (E) IFN-γ detection in T cells versus that in non-T cells. (F) Monocyte gate. (G) Monocyte gate isotype control. (H) Monocyte spontaneous production of IL-10 from representative MS patient. (I) Monocyte IL-10 production from LPS-activated PBMCs. Scatter: S., side; F., forward.

teins without affecting their de novo synthesis, thus allowing the accumulation of intracellular cytokines to levels detectable by flow cytometry. The cultured cells must then be fixed with an agent that will not disrupt the antigenic structure of the target proteins and permeabilized to expose the intracellular contents to the battery of detection antibodies. After the detection of intracellular contents, surface markers can be detected by conventional staining techniques, provided they are not damaged by the fixative agent. The use of three-color flow cytometry allows three channels for the analysis of dual cytokine production in a population of cells positively identified by a surface antigen (with the third channel).

With this technique, a significant decrease in the percentages of peripheral blood T cells that can be stimulated to produce IFN-γ in MS patients was identified, which was detectable after both 5 and 24 h of PBMC activation with T-cell

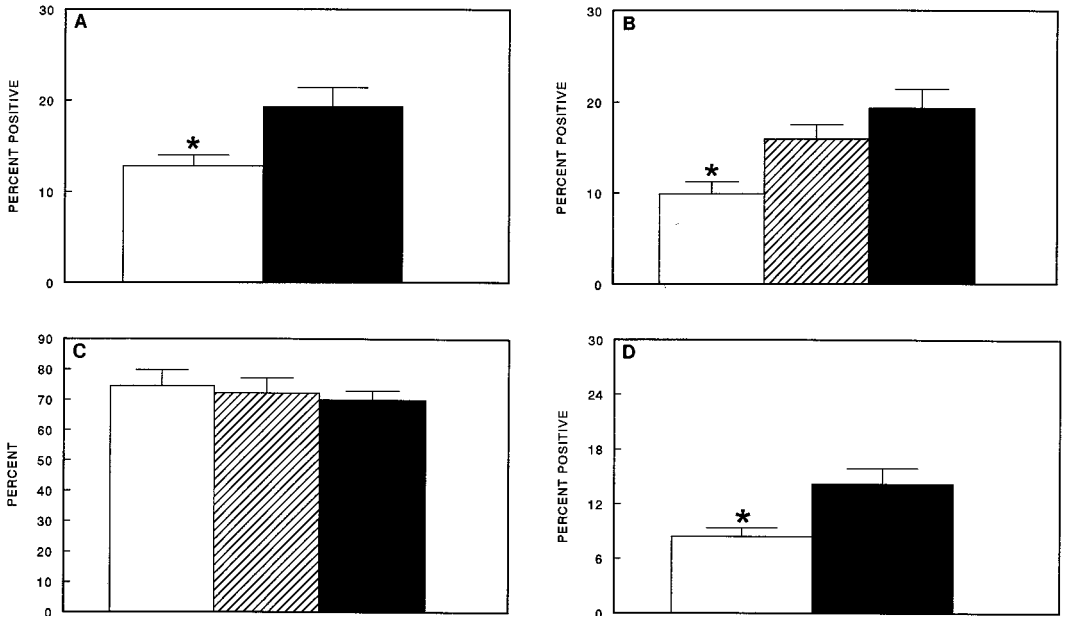

FIG. 2. Percentage of IFN-γ-producing T cells after 5 h of activation for all MS patients (open bar) and controls (solid bar) (A) and BS+ MS patients (open bar), BS− MS patients (hatched bar), and controls (solid bar) (B). PBMCs were cultured in medium with 5.0 μg of PHA per ml, 10 ng of phorbol myristate acetate per ml, 1 μg of ionomycin per ml, and 3 μM monensin for 5 h. For analysis, the gate was drawn around $CD3^+$ lymphocytes only. (C) Percentage of all IFN-γ-producing lymphocytes that are T cells ($CD3^+$). For analysis, the gate was drawn around all lymphocytes by scatter, and IFN-γ lymphocytes were plotted versus CD3 expression. Open bar, BS+ patients; hatched bar, BS− patients; solid bar, controls. (D) Percentage of IFN-γ-producing T cells after 24 h of activation for all MS patients (open bar) and controls (solid bar). PBMCs were cultured in medium with 5.0 μg of PHA per ml, 10 ng of phorbol myristate acetate per ml, and 1 μg of ionomycin per ml for 24 h with monensin added for the final 5 h. Statistical analysis was performed with Student's *t* test, and for all graphs, significant differences are indicated by asterisks ($P < 0.05$).

mitogens (Fig. 2A and D). This decrease was found to be exclusive to the MS patients being treated with the newly approved drug BS (rIFN-β_{1b}) (Fig. 2B). Since IFN-γ has been shown to precipitate the MS attack (23, 26), it was hoped that the down-regulatory effects of rIFN-β_{1b} on IFN-γ production and function would decrease the MS exacerbation rate. Although BS has indeed been shown to be effective in decreasing the MS exacerbation rate (12, 27), the mechanism of this reduction is currently not well understood. Recently, rIFN-β_{1b} was shown to stimulate monocyte production of IL-10 in vitro (28) and to suppress the secretion of IFN-γ when administered to cultured lymphocytes in vitro (24). The data indicate that one of the effects of in vivo rIFN-β_{1b} treatment appears to be the suppression of the ability of T cells to produce IFN-γ. This would seem to be expected, considering the knowledge that IFN-γ is implicated in the initiation of an MS attack and that rIFN-β_{1b} treatment has been shown to decrease the exacerbation rate for relapsing-remitting MS (12, 27). Regarding the analysis of all lymphocytes, the data show that the percentage of IFN-γ-producing lymphocytes that are T cells remains unchanged in BS+ patients, BS− patients, and controls (Fig. 2C). This would indicate that the suppression of the production of IFN-γ is common to all IFN-γ-producing lymphocytes and not just the T-cell component. The decrease in percentages of T cells producing IFN-γ was significant after both 5 and 24 h of PBMC activation, but the percentages of cells were greatly reduced after 24 h of activation. This was expected considering the kinetics of IFN-γ production described by Sander et al. (32) with this method. The difference between the BS+ patients was not significant at 24 h, probably because of the smaller percentages seen.

A percentage of peripheral blood monocytes was also identified that spontaneously produced detectable levels of IL-10

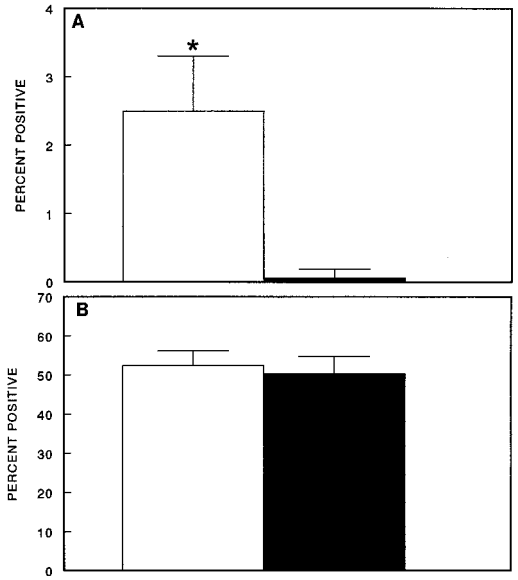

FIG. 3. (A) Percentage of monocytes spontaneously producing IL-10. PBMCs were cultured in media containing only 3 μM monensin for 5 h to allow accumulation of cytokine. Open bar, MS patients; solid bar, controls. (B) Percentage of monocytes producing IL-10 in response to activation. PBMCs were cultured in media containing 5 μg of LPS per ml for 24 h with 3 μM monensin added for the final 5 h to allow accumulation of cytokine. For analysis, the gate was drawn around $CD14^+$ PBMCs only. Open bar, MS patients; solid bar, controls. Statistical analysis was performed with Student's *t* test, and for all graphs, significant differences are indicated by an asterisk ($P < 0.05$).

(Fig. 3A). Since these cells were cultured for 5 h in monensin only, and no activating mitogens were present, only spontaneous cytokine was detected, and the levels were very low compared with those of activated cells. In the healthy control population, virtually no monocytes were spontaneously producing IL-10. Although BS treatment has been shown to stimulate monocyte IL-10 production in vitro (28), in our assay, monocyte IL-10 was not restricted to the BS+ group. Since MS patients have been shown to have activated monocytes in their peripheral blood in vivo producing cytokines (7, 11, 20), this finding is not unexpected. It may represent some role for IL-10 in the regulation of MS. When PBMCs were stimulated with LPS for 24 h to activate monocytes, both MS patients and controls responded with high percentages of bright IL-10-producing monocytes (Fig. 3B).

This study supports the theory that the exacerbation of MS is mediated by Th1 cytokines such as IFN-γ. Successful treatment of MS will probably include agents that will down-regulate the expression of Th1 cytokines and up-regulate the expression of Th2 cytokines such as IL-10, as is currently being seen with rIFN-β_{1b}.

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