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Alterations in Peripheral Blood Mononuclear Cell Cytokine Production in Response to Phytohemagglutinin in Multiple Sclerosis Patients

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Multiple sclerosis (MS) is an autoimmune demyelinating disorder of the central nervous system (CNS). The disease is characterized by inflammatory lesions in the white matter of the CNS, consisting of a specific immune response to the myelin sheath. We investigated peripheral blood mononuclear cell (PBMC) cytokine production by enzyme-linked immunosorbent assays of 21 MS patients and 19 age-matched normal controls in response to the T-cell mitogen phytohemagglutinin (PHA). Peripheral blood mononuclear cells were cultured in medium alone or in medium with 5 μg of PHA per ml for 48 h, and culture supernatants were collected for analysis. Cytokines selected for study were interleukin-10 (IL-10), gamma interferon (IFN-γ), IL-2, and IL-4. All cytokine activities described were expressed as concentrations per 500,000 cells. We found that 48% (10 of 21) of the MS patients produced small but detectable levels of IL-10 in medium alone, compared with 26% (5 of 18) of the controls. We found that the MS patients produced significantly higher quantities of IL-10 protein than the controls in response to PHA (mean supernatant concentrations of IL-10 for patients and controls, 421 and 204 pg/ml, respectively [$P < 0.05$]). No significant differences were detected in the production of IL-2, IFN-γ, and IL-4 between patients and controls in response to PHA, although patients appeared to display a trend toward decreased production of IFN-γ.

Multiple sclerosis (MS) is an inflammatory demyelinating disorder of the central nervous system (CNS) characterized by recurring periods of exacerbation leading to chronic disease (8, 30). The putative primary targets of the autoimmune response are myelin basic protein (MBP) and other myelin peptides lining the neural sheath. The autoimmune response manifests itself as lesions on the white matter of the brain and CNS which are characterized by the focal accumulation of activated T cells. There is also myelin degeneration associated with marked inflammation consisting of T cells, B cells, and macrophages (9). Inflammation in the MS lesion is likely to be triggered by specific immune recognition and activation of the immune system and suggests that MS may be an autoimmune disease. Considering that an immune response is regulated by the production of cytokines, it is probable that cytokines play an important role in the initiation, progression, remission, and exacerbation of MS. Recently, the ability to differentiate $CD4^+$ T cells into subsets on the basis of their cytokine production profiles has resulted in the Th1/Th2 paradigm, which is a model of T-cell specialization originally proposed by Mosmann and Coffman in the mid-1980s (17). Studies of the mouse model have supported their theory that, following stimulation, T-helper cells differentiate into one of two major subsets, termed Th1 and Th2. Th1 cells are reported to produce gamma interferon (IFN-γ) and interleukin-2 (IL-2) cytokines that favor cell-mediated responses such as delayed-type hypersensitivity and T-cell cytotoxicity. In contrast, Th2 cells generally are believed to secrete IL-4, IL-5, and IL-10 and are believed to regulate humorally (antibody) mediated immunity (17, 25). It is proposed that naive T-helper cells ($CD4^+$ $CD45RA^+$) first progress to a Th0 phenotype capable of secreting Th1 and Th2 cytokines before differentiating into either Th1 or Th2 cells. In addition, Th1 cells are stimulated by the production of IFN-γ and IL-12, which inhibits the development of the Th2 subset. In contrast, Th2 cells are stimulated by IL-4, and the subsequent Th2 product IL-10 inhibits development of the Th1 subset.

Cytokine regulation of MS and experimental allergic encephalomyelitis (EAE), the best animal model of MS, have recently been the focus of many studies. 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 for treatment of MS began in the 1970s. Disappointingly, IFN-γ was found to promote MS and to increase the exacerbation rate (19, 21). However, these studies did 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 Th1 cytokines IFN-γ, IL-2, and tumor necrosis factor alpha (2, 12, 28). Immunohistochemical and RNA studies of EAE CNS tissues have shown that spontaneous recovery of mice with EAE symptoms correlates with an expansion of Th2-like cells which produce elevated levels of the cytokines IL-4, IL-10, and transforming growth factor β (11, 12, 14). In addition, the administration of oral MBP to mice after the induction of EAE results in tolerance and in the suppression of EAE symptoms, which correlates with the elevated production of transforming growth factor β as well as IL-4 and IL-10 by $CD4^+$ T-cell clones (3, 16). Since IFN-γ has been found to induce exacerbations in MS patients and since Th1 cytokines are present in the CNSs of animals with active

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TABLE 1. MS patient characteristics[a]

Patient no.	Sex[b]/ age (yr)	IL-10 (pg/ml)[c]	Status[d]	EDSS score[e]	Medication(s)[f]
1	M/53	810	CP	6.0	BS, SY
2	F/58	715	CP	6.5	P, X
3	F/44	695	CP	5.5	BS, PR
4	M/56	687	EX	8.0	B
5	F/45	614	CP	4.0	P
6	F/30	598	EX	6.5	B, BS
7	F/20	563	CP	5.0	BS, LX, T
8	M/69	499	CP	8.0	B, DT
9	M/56	499	EX	4.5	BS, P
10	F/38	440	EX	5.0	M
11	M/39	402	CP	5.0	S, H, CU, L, V, TH
12	F/53	338	CP	3.5	BS, ST, HY
13	F/49	333	CP	6.0	PX, DS, AT
14	M/50	309	CP	7.5	B, AM, P, NX, MB
15	F/32	305	EX	5.5	BS, EL
16	F/55	279	CP	2.0	BS, E, PG, PR
17	F/66	198	EX	6.5	X, BS, PR
18	M/30	192	EX	5.0	M
19	M/76	187	CP	7.5	DT, LV, LZ, TP, MV, DB
20	F/35	152	CP	3.0	BS, AM
21	F/32	37	EX	5.0	SP, B

[a] Patients are listed in decreasing order of PBMC IL-10 production in response to PHA.

[b] F, female; M, male.

[c] IL-10 production by PBMCs in response to 48-h culture with 5 μg of PHA per ml normalized to 5×10^5 PBMCs.

[d] EX, patients believed to be undergoing current MS exacerbation; CP, patients displaying chronic progressive MS symptoms.

[e] EDSS, expanded disability status scale.

[f] Key to medications: X, Xanax (alprazolam); PX, Paxilon (methazole); Z, Zantac (ranitidine hydrochloride); P, Prozac (fluoxetine); B, Baclofen; DT, Ditropan (oxybutynin chloride); DN, Donnatal; PB, Phenobarb; M, methylmednisolone; T, Tenuate (diethylpropion hydrochloride); BS, Betaseron ($beta_{Ser}$ interferon); Q, vitamin B_{12}; DL, Dalmane (flurazepam hydrochloride); TH, Theophyline; HC, hydrocortisone; EL, Elavil (amitroptyline hydrochloride); DA, Dantrium (dantrolene sodium); SY, Symmetral (amantadine hydrochloride); VA, Valium (diazepam); PR, Premarin (conjugated estrogens); E, estrogen; PG, progesterone; LB, Lithobid (lithium carbonate); C, Cogard; ST, Synthroid (levothyroxine sodium); HY, Hytrin (terazosin hydrochloride); MV, Mevacor (lovastatin); DB, Diaβeta (glyburide); SP, Septra (trimethoprim and sulfamethoxazole); AM, amantidine hydrochloride; LX, Lasix (furosemide); NX, noroxin (norfloxacin); MB, Mestinon bromide (pyridostigmine bromide); S, Solumedrol (methylprednisolone sodium succinate); H, heparin; CU, Coumadin; L, Lanoxicap (Digoxin); V, Ventolin (albuterol sulfate).

EAE, it has been proposed that Th1- and Th2-type cytokines are involved in the progression and remission of MS (3, 23). 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 MS patients have recently 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 (18). We have investigated peripheral blood mononuclear cell (PBMC) cytokine production by 21 MS patients at the protein level using a culture supernatant enzyme-linked immunosorbent assay (ELISA) of cell response to the polyclonal T-cell mitogen phytohemagglutinin (PHA). The Th2-type cytokines IL-10 and IL-4 and the Th1-type cytokine IFN-γ, as well as IL-2, were selected for analysis. Patient responses were compared with those of 19 age-matched healthy controls.

Peripheral blood specimens were drawn from 21 multiple sclerosis patients in various stages of clinically defined MS (27) and 19 healthy Tampa General Hospital employees to serve as age matched controls for the study. Patient information on age, sex, clinical status, and current medications is presented in Table 1. Disease severity was determined according to the expanded disability status scale (13). Patient clinical status was classified as MS exacerbation, which was defined as the occurrence of a symptom or symptoms of neurological dysfunction not previously present and lasting at least 24 h, or as possession of chronic progressive symptoms which had been previously present but which were 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.

To isolate PBMC, 2 ml of whole anticoagulated blood was diluted 1:1 with sterile phosphate-buffered saline (PBS) and was subjected to centrifugation over a Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden). The isolated cells were washed three times in sterile PBS and resuspended in Cellgro complete medium (Mediatech, Herndon, Va.) containing 5% fetal bovine serum (Gibco BRL, Grand Island, N.Y.), 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (GIBCO), 10^5 μg of penicillin and streptomycin (GIBCO) per ml, 25 μg of fungizole (GIBCO) per ml, and 10 μg of gentamicin (GIBCO) per ml. The medium and all additives were determined by the manufacturers to contain less than 1.0 EU of endotoxin per ml. The concentration of cells was determined by using an automated hematology analyzer (CelDyn 3000; Abbott, Chicago, Ill.). Cell concentrations ranging from 0.7×10^6 to 1.3×10^6 cells per ml were used in all experiments. The appropriate concentration of MBP (30 μg/ml; Sigma, St. Louis, Mo.) or PHA (5 μg/ml; Sigma) was then added. Cells were cultured at 37°C in 5% CO_2 for 48 h.

Immunoassay kits for the human cytokines IL-10, IL-2, IL-4, and IFN-γ (Biosource International, Camarillo, Calif.) for cytokine protein determinations and assays were used according to the manufacturer's protocol. The detection limits for the cytokine ELISA were as follows: IL-10, 5 pg/ml; IFN-γ, 4 pg/ml; IL-2, 8 pg/ml; and IL-4, 2 pg/ml. Interassay variability was controlled by testing a minimum of four standard curve points concurrently with patient or control specimens for each assay. Culture supernatants from patient and control PBMCs incubated for 48 h in either medium alone or medium containing PHA or MBP were used in all experiments. ELISA results were expressed as cytokine concentrations (in picograms per milliliter) adjusted to 500,000 PBMCs.

Cytokine ELISA analysis of culture supernatants from patient and control PBMCs incubated for 48 h in medium alone revealed that 48% (10 of 21 [data not shown]) of the MS patients produced a small but detectable IL-10 response (detection limit, 5 pg/ml; range, 10 to 114 pg/ml) following culture in medium alone for 48 h. In contrast, only 26% (5 of 18) of the controls produced detectable concentrations of IL-10 following culture in medium alone (range, 13 to 71 pg/ml) (mean supernatant IL-10 concentrations for all patients and for all controls, 20 and 8 pg/ml, respectively [Fig. 1]). Detectable levels of IL-2, IL-4, and IFN-γ were not produced by PBMCs following culture in medium alone for patients or controls (data not shown). Following culture in medium with 5 μg of PHA per ml serving as a polyclonal T-cell stimulus, we found that MS patient PBMCs produced a significantly greater IL-10 response than control PBMCs (mean supernatant IL-10 concentrations for patient and controls, 421 and 204 pg/ml, respectively [$P < 0.05$] [Fig. 2A]). MS patients were also found to exhibit a trend toward decreased PBMC IFN-γ production in response to PHA, although the difference was not statistically significant in our study (mean supernatant IFN-γ concentrations for patients and controls, 248 and 370 pg/ml, respectively [Fig. 2B]). There were no significant differences in PBMC IL-4 and IL-2 protein production levels between MS patients and

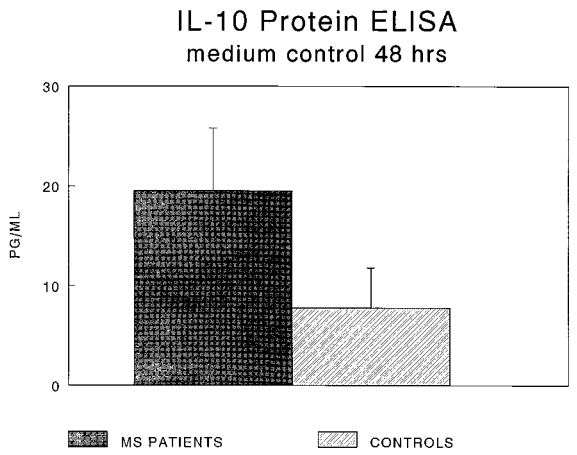

FIG. 1. IL-10 production following culture in medium alone. PBMCs from patients and controls were isolated and cultured in medium alone for 48 h. Supernatants were assayed for IL-10 activity by ELISA. All measurements are normalized to 500,000 cells per ml. The difference was not statistically significant following testing by pooled Student's t test ($P < 0.05$).

controls in response to PHA (mean supernatant IL-4 concentrations for patients and controls, 88 and 72 pg/ml, respectively; mean supernatant IL-2 concentrations for patients and controls, 208 and 217 pg/ml, respectively) (data not shown). For all assays, no differences were seen among the patient population when variables such as age, sex, or treatment medications were considered.

In addition, we investigated PBMC cytokine production as described above in a similar culture response to 30 μg of MBP (Sigma) per ml for five MS patients and five controls. IL-10 was not produced in levels higher than those seen in medium alone, and IFN-γ, IL-2, and IL-4 were not detected in these cultures for both patients and controls (data not shown).

Our study used ELISAs to detect secreted cytokine protein in supernatants following PBMC culture in medium and in medium with the T-cell mitogen PHA. Cytokine production levels from MS patients were compared with production levels from healthy age-matched controls. We found that a greater percentage of MS patients spontaneously produced small but detectable levels of IL-10 following culture in medium alone compared with controls. This seems to agree with the recent finding that the peripheral blood of MS patients contains higher numbers of cells expressing mRNA for IL-10 than that of controls (18). IFN-γ, IL-2, and IL-4 were not detectable following culture in medium alone. Since PHA is a non-T-cell receptor-specific, polyclonal T-cell mitogen and since T cells have been implicated in the initiation and progression of MS (1, 20, 26, 29, 32), we investigated PBMC cytokine production in response to PHA. We found that the PBMCs of MS patients produced significantly higher levels of IL-10 in response to PHA than those of healthy age-matched controls (Fig. 2A) and displayed a trend toward decreased production of IFN-γ (Fig. 2B). These results suggest that there is a more generalized bias of the PBMC population from MS patients toward IL-10 production. The mononuclear phagocyte is also known to be a producer of IL-10, and, considering the evidence for monocyte activation in vivo in MS (6, 10, 15), we cannot exclude the possibility that monocytes in our PBMC preparations contribute to the increases in IL-10 that we detected in samples from MS patients, even though there was not a significant difference in the percentages of peripheral blood monocytes between patients and controls (data not shown).

It has been shown that $CD4^+$ $CD45RA^-$ memory T cells produce greater quantities of IL-10 upon stimulation than $CD4^+$ $CD45RA^+$ naive T cells (31). We have previously shown (in agreement with other investigators) that MS patients have a significant increase in memory T cells in their peripheral blood (5, 7, 22, 24). It is possible that the increase in IL-10 production is contributed to by the stimulation of the increased percentages of memory T cells found in these patients. Since MS exacerbation and plaque inflammation are suggested to be Th1-mediated phenomena, with accumulation of activated Th1 cytokine pattern-expressing lymphocytes in the CNS, it is also possible that sequestration of Th1 pattern lymphocytes in the CNSs of MS patients leads to relative increase in the peripheral blood of Th2 pattern lymphocytes. Additionally, a more general peripheral blood bias toward Th2-type responses may develop over time in MS patients as a result of the chronic nature of the disease. The trend toward decreased production of the Th1-type cytokine IFN-γ, although not statistically significant in our study, also supports this theory. Studies monitoring the same individual patients over time, tracking their cytokine production profiles, and correlating these patterns with disease activity (exacerbation versus remission) will be necessary to develop a better understanding of the significance of the alteration in IL-10 activity that we observed. Similar

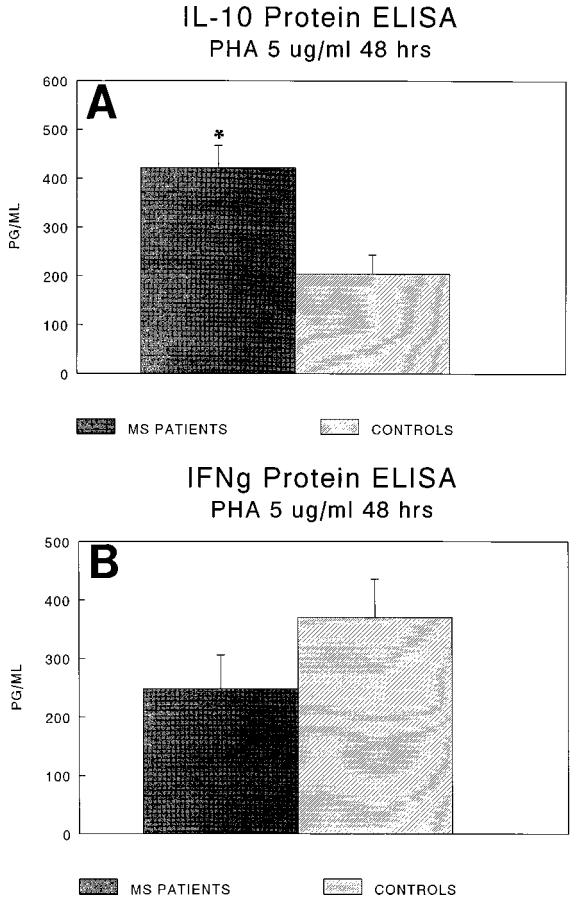

FIG. 2. IL-10 (A) and IFN-γ (B) production in response to PHA. PBMCs from patients and controls were isolated and cultured in medium containing 5 μg of PHA per ml for 48 h. Supernatants were assayed by ELISAs for the indicated cytokines. The average cytokine concentrations ± the standard errors of the means for MS patients and controls are indicated. All measurements were normalized to 500,000 cells per ml. A significant difference ($P < 0.05$ by pooled Student's t test) is indicated by the asterisk.

cytokine studies of blood leukocytes from patients with other inflammatory-type autoimmune diseases will be necessary to determine whether the described increase in PBMC IL-10 production is unique to MS or whether it is found in other such diseases.

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