

## A New Cell Enzyme-Linked Immunosorbent Assay Demonstrates Gamma Interferon Suppression by Beta Interferon in Multiple Sclerosis

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**Multiple sclerosis (MS) is a demyelinating disorder of the central nervous system of unknown etiology. Immune mechanisms involving the proinflammatory cytokine gamma interferon (IFN- $\gamma$ ) are believed to play an important role in the pathogenesis of MS. IFN- $\beta$ -1b has been introduced as a treatment for MS and was found to reduce the number and severity of clinical exacerbations. To examine the influence of IFN- $\beta$ -1b on myelin basic protein (MBP)-specific and phytohemagglutinin-induced IFN- $\gamma$  production, we developed a cell-released capturing enzyme-linked immunosorbent assay (CRC-ELISA), which rapidly measures spontaneous and antigen- or mitogen-induced cellular IFN- $\gamma$  production. CRC-ELISA documented a significant MBP-specific T-cell response in the blood of untreated MS patients, as assessed by IFN- $\gamma$  production. This response was suppressed in MS patients treated with IFN- $\beta$ -1b. The present work confirms in vivo the in vitro suppressive effects of IFN- $\beta$ -1b on IFN- $\gamma$  production in MS. Moreover, it provides a powerful new technique for detection of cytokines.**

Multiple sclerosis (MS) is a demyelinating disorder of the central nervous system (CNS). Myelin basic protein (MBP) is a major component of myelin that is affected in MS. Fragments of MBP and anti-MBP antibodies are found in the CNS lesions and in the cerebrospinal fluid of MS patients (2, 4, 23).

The presence of activated T cells in the blood, brains, and cerebrospinal fluid of MS patients suggests that the disease is immune mediated (11). T cells recognizing myelin antigens, including MBP and myelin proteolipid protein, are constituents of the normal T-cell repertoire (15, 16, 24). In MS, T cells reactive to self-antigens, including myelin proteins, become activated. Such T cells are able to cross the blood-brain barrier. Upon encountering myelin antigens inside the blood-brain barrier, infiltrating T cells become reactivated and release cytokines that are capable of amplifying immune responses. The interplay between T helper 1 (Th1) and Th2 cells and the balance of their respective activities are of crucial importance in determining which type of immune response will ensue (26). Gamma interferon (IFN- $\gamma$ ), produced by activated Th1 cells, plays a key role in the induction of immunopathogenetic features in MS lesions, such as astrogliosis (25), macrophage activation (1, 8), induction of major histocompatibility complex (MHC) (22) and T-cell homing into the CNS by inducing cell adhesion molecules on endothelial cells and enhancing their adhesiveness for T cells (21), and upregulation of MHC class II molecules on endothelial cells and astrocytes, rendering them capable of antigen presentation (7).

Acute exacerbations of MS occur more frequently after viral infections and after administration of IFN- $\gamma$  (17, 18). Therefore, downregulation of activated T cells and, particularly, IFN- $\gamma$  production could be advantageous in MS. One molecule with this potential is IFN- $\beta$  (14). There is substantial evidence that MHC class II expression can be downregulated by IFN- $\beta$ ,

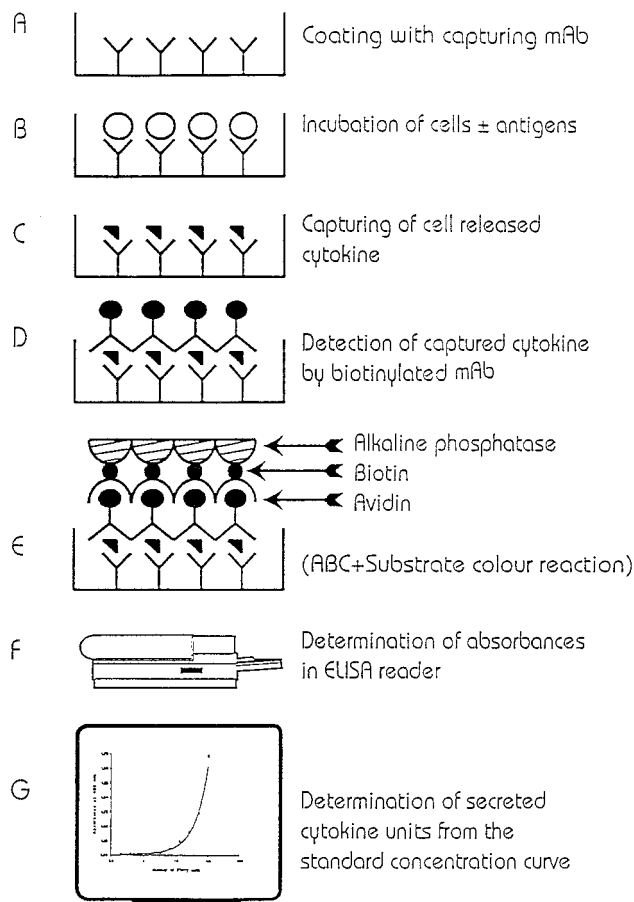


FIG. 1. Scheme of CRC-ELISA for detection of cell-released cytokines (for example, IFN- $\gamma$ , as in the present study). The method can detect cell-released cytokines produced in response to specific antigens or mitogen or produced spontaneously. ABC, avidin-biotin alkaline phosphatase-complex.

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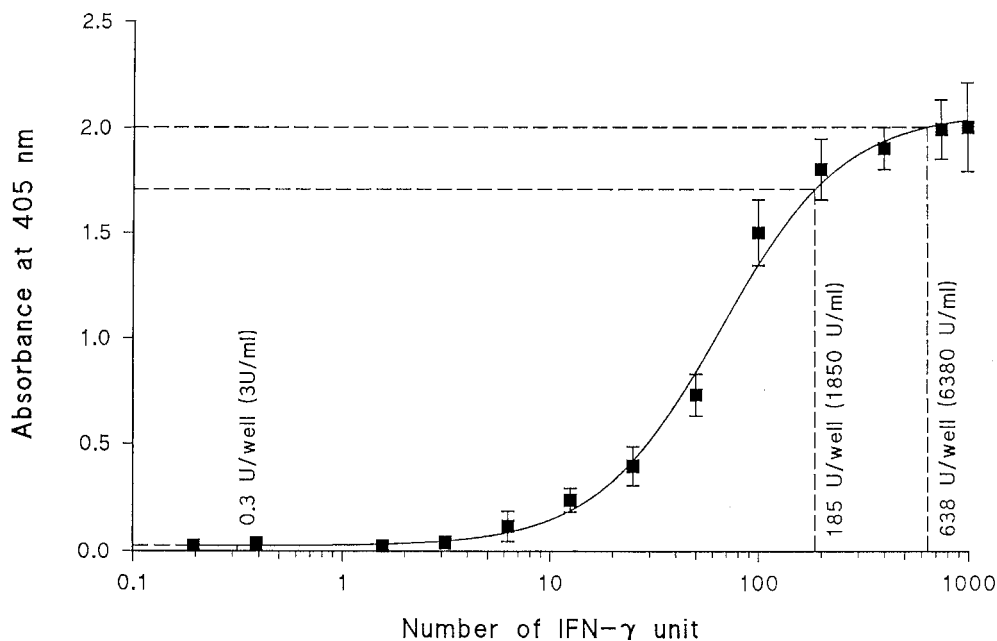


FIG. 2. rIFN- $\gamma$  standard curve. The curve was designed from the absorbencies, obtained after capturing and detecting different known concentrations of rIFN- $\gamma$  (see Materials and Methods), to high-binding microtiter plate wells precoated with anti-IFN- $\gamma$  MAb. The absorbencies of the known IFN- $\gamma$  concentrations were entered into a data set of a graphical computer software program as y-axis data with a linear scale. The IFN- $\gamma$  units were entered into the same data set as x-axis data with a logarithmic scale. Into another data set, the absorbencies of the specimens were entered as y-axis data. Units corresponding to these absorbencies were measured from the standard curve and are shown as x-axis values. Dashed lines indicate the minimum and maximum units that the computer can detect from the assay's standard curve. During the study more than 10 standard curves were made, and there was no significant variation among the curves.

which acts by interfering with the transcription of class II-specific mRNA (10). In vitro, IFN- $\beta$  suppresses the ability of peripheral blood lymphocytes (PBL) to produce IFN- $\gamma$  in response to mitogen and antigen stimulation (9, 14). Clinical studies have shown a significant decrease in the number and severity of exacerbations in patients treated with IFN- $\beta$ 1b compared to those in patients treated with a placebo (8a). In the present work we demonstrated the in vivo effects of IFN- $\beta$ 1b treatment of MS patients on MBP-specific as well as phytohemagglutinin (PHA)-induced IFN- $\gamma$  production. This was made possible by the development of a cell-released capturing enzyme-linked immunosorbent assay (CRC-ELISA), a new, rapid, objective, and sensitive technique capable of measuring cellular production of cytokines.

#### MATERIALS AND METHODS

**Patients.** Forty-four patients had clinically definite MS (19). The MS patients were divided into two groups: (i) 29 untreated MS patients (22 females) with an age range of 24 to 68 years (mean, 46), none of whom had ever received any immunomodulatory treatment, and (ii) 15 IFN- $\beta$ 1b-treated MS patients (14 females) with an age range of 25 to 53 years (mean, 42). The untreated MS patients were selected because they displayed the same disease characteristics as the treated patients had displayed prior to being treated with IFN- $\beta$ 1b. The patients in group ii had all been treated with 8 MIU of IFN- $\beta$ 1b administered via subcutaneous injection every second day for at least 3 months. Samples from these patients were taken 10 to 14 h after the drug had been given. Nineteen control patients (5 females) had other neurological diseases (OND) of the noninflammatory type. Their age range was 23 to 77 years (mean, 61). Eight patients had muscular tension headache; 2 patients each had Alzheimer's-type dementia, cerebrovascular disease, and chronic pain syndrome; and one patient each had amyotrophic lateral sclerosis, myelopathy of unknown cause, polyneuropathy, cervical radiculopathy, and radial nerve palsy. Fifteen healthy control subjects (7 females) of the staff of the department ranged between the ages of 22 and 46 years (mean, 32).

**Preparation of human PBL suspensions.** Peripheral blood was taken into heparinized tubes and diluted with the same volume of tissue culture medium (Dulbecco's medium; Flow Laboratories, Irvine, United Kingdom) with antibiotics. PBL were separated by density gradient centrifugation on Lymphoprep (Nyegaard, Oslo, Norway). The cells in the interphase were collected, washed

three times with medium, and suspended in complete medium supplemented with 5% fetal calf serum (GIBCO, Paisley, United Kingdom), 1% minimal essential medium (Flow), 2 mM glutamine (Flow), 50  $\mu$ g of penicillin per ml, and 60  $\mu$ l of streptomycin per ml. The cells were counted in a Bürker chamber, and their viability was assessed by trypan blue exclusion. Cell viability always exceeded 95%.

**CRC-ELISA for detection of IFN- $\gamma$ .** To detect cellular production of IFN- $\gamma$ , a new CRC-ELISA was introduced. The assay is based on capturing the cytokine at the time of its release from the cells with a specific capturing monoclonal antibody (MAb). In order to detect the secreted cytokine in this assay, enzyme immunoassay/radioimmunoassay flat-bottom, high-binding plates (Costar) were coated with 100  $\mu$ l of anti-IFN- $\gamma$  (1-D1K) MAb (5  $\mu$ g/ml; Mabtech, Stockholm, Sweden) diluted in carbonate bicarbonate buffer (pH 9.6) at 4°C overnight. After four washes with 0.05 M phosphate-buffered saline (PBS), the wells were blocked with 100  $\mu$ l of 5% bovine serum albumin per well for 90 min at room temperature. After the wells had again been washed four times with PBS, suspensions of PBL were applied in triplicate to individual wells in 200- $\mu$ l amounts to obtain a final concentration of  $2 \times 10^5$  cells per well. This cell number was selected after we performed titration experiments to attain the optimal cell number for the assay. Cultures either were not stimulated or received either purified human MBP (6) at a final concentration of 10  $\mu$ g/ml or PHA (Difco, Detroit, Mich.) at a final concentration of 0.5  $\mu$ g/ml. After 48 h of incubation at 37°C in a humidified atmosphere of 7% CO<sub>2</sub>, the cells were removed by flicking the plates, which were then washed five times with Tween-PBS. To detect any captured IFN- $\gamma$ , 100  $\mu$ l of biotinylated anti-IFN- $\gamma$  (7B-B6-1) MAb (0.5  $\mu$ g/ml; Mabtech) diluted in PBS containing 0.5% Tween 20 and 2% bovine serum albumin was added to the wells. After another 60 min of incubation at 37°C and five washes, 100  $\mu$ l of avidin-biotin alkaline phosphatase complex (Vector Laboratories, Burlingame, Calif.) diluted 1:100 in PBS was added for 45 min. Unbound avidin-biotin alkaline phosphatase complex was removed by five consecutive washings with Tween-PBS, and 100  $\mu$ l of freshly prepared enzyme substrate solution was added to each well. Absorbance was measured after 15 min of incubation in the dark in a 405 Multiscan photometer (mcc/340; Labsystem, Helsinki, Finland). In order to quantify the IFN- $\gamma$  secreted by the cells cultured in the plate, the IFN- $\gamma$  standard curve was obtained by simultaneously incubating different known concentrations (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1,024 U/well) of recombinant human IFN- $\gamma$  (rIFN- $\gamma$ ; gift from P. van der Meide, TNO Primate Center, Rijswijk, The Netherlands) for 60 min at room temperature in wells precoated with anti-IFN- $\gamma$  MAb. The procedure for developing the plate was continued as described above, and the absorbencies measured from the standard concentration of IFN- $\gamma$  were used to plot the IFN- $\gamma$  standard curve. Thereafter, the absorbencies obtained from the cultures, which correspond to the secreted IFN- $\gamma$ , were automatically converted by the computer to units per well, based on

the standard curve. In parallel wells, another IFN- $\gamma$  standard curve was established by adding rIFN- $\gamma$  to cell cultures so that similar conditions to those used for the standard curve were used for the cultures of the specimens. The curve values of these wells did not show variations from the standard curve values obtained by direct application of the rIFN- $\gamma$  to the wells without subsequent cell culture. In this assay, background absorbencies (wells without coating MAb) were very low, and they were subtracted from the absorbencies of the specimens.

**Measurement of cytokine levels by conventional ELISA.** The same principle as used for the CRC-ELISA was employed to detect IFN- $\gamma$  in the supernatant from the cultures, except that the cells were not cultured in the plate coated with capturing antibody. Instead, the cells were cultured in a separate plate and stimulated as described above. Supernatants were collected and transferred to the plate precoated with the anti-IFN- $\gamma$  MAb and incubated for 4 h at room temperature. After several washings, the biotinylated detecting antibody was added, and the procedure was continued as described above. All plates and reagents were the same as those used for the CRC-ELISA.

**Enumeration of IFN- $\gamma$ -secreting cells.** To compare the CRC-ELISA to the enzyme-linked immunospot (ELISPOT) assay, we ran the CRC-ELISA as described above in parallel with a modified (12) ELISPOT assay, described by Czerkinsky et al. (5), for 10 randomly selected patients with MS. PBL were applied in duplicate to individual wells in 200- $\mu$ l amounts to obtain a final concentration of  $2 \times 10^5$  cells per well.

**Statistics.** The Mann-Whitney test was used for statistical analysis.

## RESULTS

**Standard curve for rIFN- $\gamma$ .** To estimate the amount of IFN- $\gamma$  produced by a certain number of cells in each well after culture termination, a standard curve was plotted by using the absorbency values obtained after the incubation of rIFN- $\gamma$  at different concentrations (Fig. 1 and 2). Units corresponding to the absorbencies of the specimens were obtained from the standard curve. The CRC-ELISA measured accurately as little as 1 U of IFN- $\gamma$  per well (i.e., 10 U/ml). The upper value that could be measured by the CRC-ELISA was 638 U of IFN- $\gamma$  per well (i.e., 6,380 U/ml). Thereafter, a plateau was reached (Fig. 2).

**Influence of cell number on IFN- $\gamma$  production in response to MBP, PHA, and no stimulation.** In the present study we titrated different cell numbers to determine the optimal number of cells for examining IFN- $\gamma$  production by the CRC-ELISA without stimulation or after stimulation with MBP or PHA. For this purpose, the IFN- $\gamma$  production in different numbers of PBL from five healthy controls, five untreated MS patients, and five MS patients treated with IFN- $\beta$ -1b was measured. The highest IFN- $\gamma$  production was detected in all cultures at a cell number of  $2 \times 10^5$ /well, and hence this number was selected for the study (titration is not shown).

**CRC-ELISA compared to conventional ELISA of supernatants of mononuclear cell suspensions and to ELISPOT assay.** Samples from 10 MS patients were used to compare the number of IFN- $\gamma$  units recorded spontaneously or after stimulation with MBP or PHA. The levels of production of MBP-reactive IFN- $\gamma$  recorded by the CRC-ELISA were significantly higher than those detected in supernatants of mononuclear cells from the same patients by the conventional ELISA ( $P < 0.05$ ). The total number detected by the CRC-ELISA was about 140 U/ml, compared to 80 U/ml detected by the conventional ELISA. After PHA stimulation, a similar difference between the CRC-ELISA and the conventional ELISA was observed ( $P < 0.05$ ). Only seven cells secreting IFN- $\gamma$  in response to MBP were recorded by the ELISPOT assay (Fig. 3). However, the difference in the spontaneous IFN- $\gamma$  production recorded by the CRC-ELISA and that recorded by the conventional ELISA was not significant. Few spontaneous IFN- $\gamma$ -secreting cells were detected by the ELISPOT assay, while about 400 IFN- $\gamma$ -secreting cells were detected after PHA stimulation (Fig. 3). To study the inter- and intra-assay variations, we repeated the experiments five times. Furthermore, we ran the assay for the same patients several times or incubated the cells

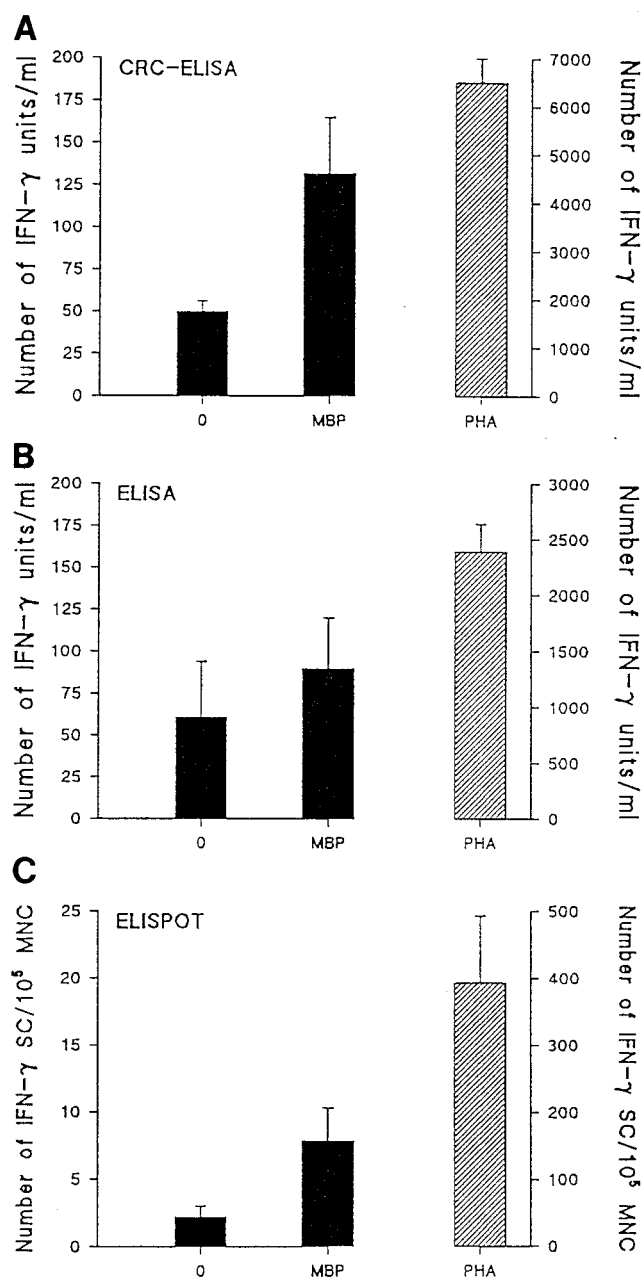


FIG. 3. Comparison of the CRC-ELISA with a conventional ELISA of supernatants of mononuclear cell suspensions and with the ELISPOT assay. Suspensions of PBL from 10 MS patients were plated at  $2 \times 10^5$  cells per well and cultured for 48 h. Triplicate cultures were exposed to the optimal dilution of MBP or PHA. Control cultures received no stimulation. Data are the results of five different experiments. Error bars indicate standard deviations. MNC, mononuclear cells; SC, secreting cells.

from the same patients in different plates. The variations in the assay were very minor. This was also the case for two other cytokines (interleukin-4 [IL-4] and IL-10) that were used to test the specificity of the assay. Adding secondary antibodies to these cytokines did not give a signal above the background level.

**Effects of IFN- $\beta$ -1b treatment on MBP-specific and PHA-induced IFN- $\gamma$  production.** To study the effects of IFN- $\beta$ -1b treatment on MS patients, the CRC-ELISA was used to com-

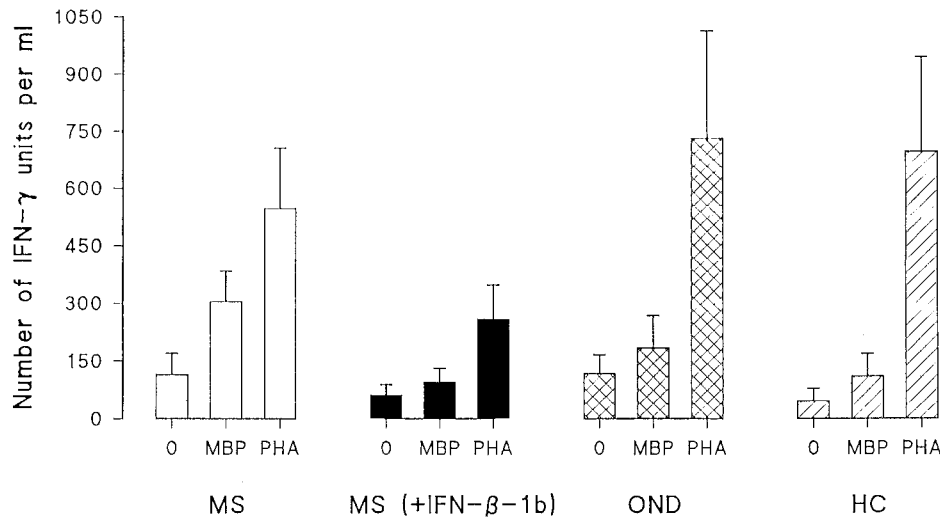


FIG. 4. Effects of IFN- $\beta$ -1b treatment on MBP-specific and PHA-induced IFN- $\gamma$  production. Suspensions of PBL from 15 IFN- $\beta$ -1b-treated and 29 untreated MS patients, 17 patients with OND, and 15 healthy controls (HC) were plated at a cell number of  $2 \times 10^5$  per well and cultured for 48 h. Triplicate cultures were exposed to the optimal dilution of MBP or PHA or left without stimulation. Note the lower IFN- $\gamma$  production in response to MBP and PHA in IFN- $\beta$ -1b-treated MS patients compared to production in untreated MS patients. Error bars indicate standard deviations.

pare MBP-induced IFN- $\gamma$  production in PBL from MS patients treated with IFN- $\beta$ -1b and PBL from untreated patients. The PHA response reflected by IFN- $\gamma$  production was also studied in both groups. For untreated MS patients, MBP stimulation of PBL induced higher IFN- $\gamma$  production than no stimulation ( $P < 0.03$ ) did. This MBP-reactive IFN- $\gamma$  production was evidently suppressed in MS patients who received IFN- $\beta$ -1b treatment; there was no significant difference in IFN- $\gamma$  production after MBP stimulation compared to IFN- $\gamma$  production after culture in the absence of the antigen. Furthermore, the MBP-specific IFN- $\gamma$  production in the IFN- $\beta$ -1b-treated MS patient group was significantly reduced compared to that in untreated MS patients ( $P < 0.05$ ). PHA stimulation of IFN- $\gamma$  induction in the IFN- $\beta$ -1b-treated patients was found to be lower than PHA stimulation of IFN- $\gamma$  induction in the untreated MS patients. However, in both groups, it was still higher than the IFN- $\gamma$  induction with no stimulation (Fig. 4). OND patients and healthy controls did not show a significant difference in induction of IFN- $\gamma$  in response to MBP compared to induction with no stimulation. Nevertheless, a very high induction of IFN- $\gamma$  was recorded for both OND patients and healthy controls after stimulation with PHA (Fig. 4).

## DISCUSSION

The present work has established a new method to detect cytokine production and has adopted that method to monitor the effects of IFN- $\beta$ -1b treatment of MS patients. Many studies have emphasized the role of cytokines in modulating immune responses during infections and autoimmunity. However, such studies have focused mainly on the determination of cytokine levels in bodily fluids. Since cytokines act autocrinely or paracrinely, with very short half-lives, and have high affinity for nearby receptors, cellular induction of cytokines has been detected, rather than cytokine levels in bodily fluids. To bypass this problem, we used the CRC-ELISA to detect the cytokines immediately after they were released. This was clearly shown in this study, where significant detection of IFN- $\gamma$  was registered by the CRC-ELISA compared to detection by a conventional ELISA. Other principles used for cellular cytokine detection include enumeration of cytokine mRNA-expressing cells by

the in situ hybridization technique (13) and evaluation of cellular production of cytokines by ELISPOT assays, which detect single cells secreting cytokines (15). However, even though both methods can give actual numbers of T cells with a certain functional ability, they are based on subjective analysis. The in situ hybridization technique enables detection of mRNA of a broad range of cytokines. However, mRNA expression does not always correlate to the actual production of cytokines (20). The CRC-ELISA described in this work is an objective assay that rapidly quantifies the amount of produced cytokine. The assay is based on detection of cytokines by the capturing MAb before they are utilized or destroyed by the in vitro conditions of the culture. The CRC-ELISA is also useful in limiting-dilution analysis to measure frequencies of antigen-reactive T cells. In this context, we have initiated studies in an experimental allergic encephalomyelitis animal model for MS to compare the number of the MBP-specific IFN- $\gamma$ -secreting cells detected by the classical ELISPOT assay with the frequency of the MBP-specific IFN- $\gamma$ -producing cells detected by the CRC-ELISA. Our preliminary data showed that the CRC-ELISA is more sensitive in the assessment of cell frequencies (1a). The data of the present work also support the above notion, since the levels of IFN- $\gamma$  detected by CRC-ELISA after MBP stimulation (about 140 U/ml) were higher than the number of IFN- $\gamma$ -secreting cells detected by the ELISPOT assay (seven cells). Recording concentrations of a cytokine may be more essential than assessing the number of producing cells in certain situations. In the present study, only seven cells secreted 140 U of IFN- $\gamma$  per ml, suggesting that the number of cells found does not indicate how much of a cytokine is produced. Although CRC-ELISA enables studies of selected cytokines with defined effector or immunoregulatory roles, the inter- and intracellular regulation accomplished by mutual cytokine effects may affect the final cellular production of certain cytokines.

Using the CRC-ELISA, we examined the in vivo effects of IFN- $\beta$ -1b on the production of IFN- $\gamma$  in patients who had received IFN- $\beta$ -1b treatment and compared the results with those from untreated MS patients, as well as OND patients and healthy controls. The significant suppression of MBP-in-

duced IFN- $\gamma$  production in the IFN- $\beta$ -1b-treated MS patients reflects the *in vivo* effects of IFN- $\beta$ -1b on IFN- $\gamma$  production. These effects were previously shown *in vitro* (9, 14). However, this inhibitory effect is not specific for MBP, since IFN- $\gamma$  production after PHA stimulation was also reduced in the IFN- $\beta$ -1b-treated patients, although to a lesser level. The viability of the PBL, as assessed by trypan blue exclusion both at the onset and at the end of the culture, has ruled out the possibility that PBL from IFN- $\beta$ -1b-treated patients might be less viable and that fewer cells had therefore survived the 48-h incubation period. In support of our results, Brod et al. (3) have recently shown that the capacity of PBL to produce tumor necrosis factor alpha, IFN- $\gamma$ , and IL-4 in response to CD3 MAb was reduced in IFN- $\beta$ -1b-treated patients, and the capacity of PBL to produce IL-6 was increased. Whether the inhibitory effect of *in vivo* treatment with IFN- $\beta$ -1b on IFN- $\gamma$  production, in response to MBP, is related to the reduced number and severity of exacerbations in individual MS patients treated with IFN- $\beta$ -1b is presently under investigation. Furthermore, we are also investigating the mechanism of action of IFN- $\beta$ -1b and its effects on other cytokines in MS.

In conclusion, our study has achieved two goals: (i) establishing a new method to detect cytokine production and (ii) using that method to monitor the effects of IFN- $\beta$ -1b treatment of MS patients. These effects were assessed by examination of MBP-specific and PHA-induced IFN- $\gamma$  production in treated versus untreated MS patients, compared to patients with OND and healthy subjects. The assay represents a new, objective, sensitive, and rapid approach to detecting cellular production of cytokines and may provide an advantage in cytokine detection in the biomedical field.

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