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In vitro correction of the interleukin-2 and interferon-gamma defect in multiple sclerosis

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SUMMARY

The effect of phytohaemagglutinin (PHA) and/or phorbol myristic acetate (PMA) on human interferon-gamma (HuIFN-γ) and interleukin 2 (IL-2) production was measured in peripheral blood leucocytes (PBL) from multiple sclerosis (MS) patients. Seven out of 12 MS patients studied had PBLs which were unable to produce any detectable HuIFN-γ after stimulation with PHA. The PBL cultures of the same seven patients were also defective for IL-2 production after PHA stimulation. Addition of PMA before and during PHA stimulation resulted in restored IL-2 and HuIFN-γ production in all otherwise non-responsive cultures. In MS cultures responsive to PHA alone, the addition of PMA resulted in a 10-fold increase of HuIFN-γ and IL-2 production. Analysis of interleukin 1 (IL-1) production by peripheral blood monocytes (PBM) isolated from the same cultures, revealed that both spontaneous and PMA-induced IL-1 yield was the same for all cultures tested, regardless whether they produced IL-2 and HuIFN-γ after PHA stimulation or not.

Keywords interferon-gamma interleukin 1 interleukin 2 multiple sclerosis phorbol myristic acetate

INTRODUCTION

In recent years several studies from different laboratories have evidenced a defective interferon-gamma (HuIFN-γ) production by peripheral blood leucocytes (PBL) derived from multiple sclerosis (MS) patients after stimulation with certain mitogens (Neighbour, Miller & Bloom, 1981; Salonen *et al.*, 1982; Vervliet *et al.*, 1983, 1984). The causal factors underlying this defective responsiveness have not yet been elucidated. One approach to get more insight into this problem is to consider the HuIFN-γ system in the context of the immunoregulatory network in which it functions. In particular, the interleukin 1 (IL-1) and interleukin 2 (IL-2) have a strong impact in the interferon system. IL-1 is produced by macrophages activated either directly (with LPS, PMA) or through T-cells (PHA, Con A) (Palacios, 1982). IL-1 functions as an essential activating signal in all T-cell dependent, antigen-specific immune responses. Underlying the stimulatory effect of IL-1 is its participation in the induction of IL-2 (Farrar *et al.*, 1980). IL-2, in turn, regulates the synthesis of HuIFN-γ (Farrar *et al.*, 1981). A defect in the HuIFN-γ production might therefore be connected to abnormalities in the IL-1 and IL-2 system.

In this study the production of IL-2 and HuIFN-γ by MS-derived PBLs, after stimulation with the T-cell mitogen PHA was investigated, whereas synthesis of IL-1 by isolated peripheral blood

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monocytes (PBM) of the same cultures was studied by induction with the classical IL-1 inducer PMA (Mizel, Rosenstreich & Oppenheim, 1978). So far, no study evaluating IL-1 and IL-2 in MS has been reported. We also investigated the effect of PMA on HuIFN-γ and IL-2 production by MS-derived PBL cultures after stimulation with PHA. Phorbol myristic acetate (PMA) has been shown to act as a potent co-mitogen for IL-2 production by peripheral blood lymphocytes and can replace the monocyte requirement in mitogen-induced IL-2 production (Farrar *et al.*, 1982).

MATERIALS AND METHODS

MS-patients. The criteria used in the diagnosis of MS were those of Rose *et al.* (1976). The degree of neurological impairment according to the disability status scale of Kurtzke (1970) varied between 3 and 8. All patients were in the stable phase of the disease. None of them received corticosteroids or immunosuppressive therapy.

IL-2 and HuIFN-γ induction. Isolation of the mononuclear cells by Ficoll density gradient centrifugation was as described in a previous paper (Vervliet *et al.*, 1983). 10 μg/ml of phytohaemagglutinin (PHA, type Y, Sigma, Saint Louis, Missouri, USA), 5 ng/ml of phorbol myristic acetate (PMA, Sigma, Saint Louis, Missouri, USA), 10 μg/ml of PHA + 5 ng/ml of PMA; or medium was added in duplicate to 200 μl of 2×10^6 cells/ml of PBL cultures in 96-well microtitre plates (Nunc). After 24 h culture supernatants from individual wells were harvested, pooled, centrifuged at 600 ***g*** for 10 min and stored at −20°C until assayed for IL-2 and IFN-γ activity.

Induction of IL-1. Adherent cells were prepared as described by Linker-Israeli *et al.* (1983). Briefly, 200 μl of PBL cell suspension/well at 4×10^6 cells/ml in 20% fetal bovine serum (FBS) complete RPMI were dispensed in flat-bottom, 96-well microtrays at 37°C for 1 h. Non-adherent cells were removed and adherent cells were washed five times with prewarmed medium. After the washes the cell monolayers were controlled for cell monolayer integrity. The adherent cells were incubated with 10, 1 and 0 ng/ml PMA in complete RPMI at 37°C in 5% CO_2. After 24 h the supernatants were harvested for IL-1 assay.

IL-1 assay. Serial two-fold dilutions (100 μl/well; 1:8 to 1:128 final dilution) of each sample were made in triplicate in 96-well microtitre plates using medium as diluent. To each well 100 μl of 2×10^5 human thymocytes (Maizel *et al.*, 1981) and 20 μl PHA at a final concentration of 0·2 μg/ml were added and the cultures were incubated for 48 h at 37°C in 5% CO_2, pulsed, 16 h before harvesting, with 2·5 μCi/well of [^{3}H]thymidine (25 Ci/mmol specific activity). The results are expressed as a stimulation index (SI) obtained with the following formula:

$$\text{SI} = \frac{\text{c.p.m. PHA, thymocytes with IL-1}}{\text{c.p.m. PHA, thymocytes without IL-1}}.$$

The SI in our results are obtained at a final dilution of 1:8. PMA by itself did not stimulate the thymocytes.

IL-2 assay. The IL-2 assay was performed as described by Linker-Israeli *et al.* (1983). Briefly, each sample was processed in two-fold dilutions in complete RPMI in volumes of 100 μl/well in 96-well Nunc tissue culture trays. To these, 100 μl/well of 4×10^3 CTLL cells (an IL-2 dependent murine line of cytotoxic T lymphocytes; kindly provided by Dr G. Degiovanni, University of Liège, Belgium) were added. The trays were incubated for 24 h at 37°C in 5% CO_2, pulsed with 0·5 μCi [^{3}H]thymidine (45 Ci/mmol specific activity) for 4 h, harvested with a multiple-cell culture harvester on glass-wool filters (Titertek, Flow Laboratories) and counted on a Beckman liquid scintillation counter. An international IL-2 standard not being available, the experimental probit data were compared with an internal laboratory standard. PMA or PHA by themselves did not stimulate the CTLL cells.

Interferon assay. Interferon assays were carried out as described in a previous paper (Vervliet *et al.*, 1983). The assay was carried out in Hep-2 cells (ATCC CCL23), with vesicular stomatitis virus, New Jersey strain, as challenge virus. Yields of HuIFN-γ were expressed as laboratory units; 1 laboratory unit corresponds to the quantity necessary to cause 50% inhibition of the viral cytopathic effect (CPE). IFN produced after PHA or PHA + PMA stimulation was of the γ-type as

Table 1. HuIFN-γ and IL-2 production by MS-derived PBL cultures after PHA stimulation alone, and after stimulation with PHA and PMA simultaneously

	Production after PHA stimulation							
	Without PMA				With PMA			
Type of lymphokine		Number of cultures	Average yield (U/ml)	Responders (%)		Number of cultures	Average yield (U/ml)	Responders (%)
HuIFN-γ	Responder*	5	216(63)†	42	Responder	5	1728(608)	92
					Responder	6	718(492)	
	Nonresponder	7	<32		Nonresponder	1	<32	
IL-2	Responder‡	4	23(5)	33	Responder	4	218(93)	100
					Responder	8	140(26)	
	Nonresponder	8	<8		Nonresponder	0	<8	

* HuIFN-γ titre $\geqslant$32 U/ml.
† Mean (s.e.).
‡ IL-2 titre $\geqslant$8 U/ml.

shown by characterization with specific antisera. The interferon samples were neutralized only by specific anti-HuIFN-γ antiserum, not by anti-α or anti-β.

RESULTS

Interferon-gamma and IL-2 production by PBLs of MS patients after stimulation with PHA, PMA, or both PHA + PMA

PBLs from MS patients were cultured without added inducer, with PHA (10 μg/ml), with PMA (5 ng/ml), or with PHA (10 μg/ml) + PMA (5 ng/ml). After 24 h the supernatant fluids were harvested for interferon and IL-2 titration. When PHA was used alone, only 42% of the cultures produced HuIFN-γ (Table 1). In concurrence, only 33% of the cultures produced IL-2. There is a remarkable concordancy in responsiveness for both lymphokines: four out of 12 PBL cultures tested produced both whereas seven cultures produced neither of them. In only one case the culture produced HuIFN-γ but no IL-2. When PMA was used alone no HuIFN-γ (< 32 U/ml) or IL-2 (< 8 U/ml) could be detected in the supernatant fluids. When PHA and PMA were added simultaneously, response frequency as well as average yield of both HuIFN-γ and IL-2 production increased drastically (Table 1). Response frequency mounted to nearly 100%. The increase in average yields can better by evaluated when comparison is made within the group of initially 'responder' cultures, between yields after PHA stimulation alone and stimulation with PHA and PMA simultaneously (Table 1). Both HuIFN-γ and IL-2 production were increased about 10 times by PMA addition.

IL-1 production by isolated PBM of MS patients after stimulation with PMA

PBMs isolated from the same MS-derived PBL cultures which were used for HuIFN-γ and IL-2 induction, were stimulated with PMA at doses of 0, 1, and 10 ng/ml. After 24 h the supernatant fluids were harvested for IL-1 assay. The control PBMs without added PMA spontaneously produced IL-1 at low level (Table 2). Stimulation with 1 ng/ml was insufficient to raise the IL-1 response above the background level. Addition of 10 ng/ml PMA resulted in a five-fold increase in IL-1 production in all PBM cultures. Hence, it seemed possible that PBL cultures defective for HuIFN-γ and IL-2 production might contain PBM with a lowered IL-1 production capacity. Therefore, IL-1 production was analysed as a function of HuIFN-γ and IL-2 production. As can be seen from Table 2, spontaneous and PMA-induced IL-1 production was the same either when PBM were derived from 'responder' or from 'non-responder' PBL cultures, i.e. whether producing HuIFN-γ and IL-2 or not.

Table 2. IL-1 production by isolated monocytes of MS-patients after stimulation with PMA at different concentrations. The same data are also shown as a function of the HuIFN-γ producing (+) or non-producing (−) capacity of the PBL culture

Donor group	IL-1 (SI) after stimulation with PMA (ng/ml)					
	0		1		10	
	n	Average yield	*n*	Average yield	*n*	Average yield
All cultures tested	8	3·7(0·3)	8	4·2(0·5)	9	25·0(1·7)
HuIFN-γ (+)	3	4·1(0·9)	3	4·6(1·4)	3	25·0(3·7)
HuIFN-γ (−)	5	3·5(0·2)	5	4·0(0·4)	6	24·9(2·0)

DISCUSSION

In previous studies (Vervliet *et al.*, 1983, 1984) we demonstrated that the major percentage of MS-patient derived PBL cultures did not produce any detectable HuIFN-γ after stimulation with PHA or Con A, in contrast with normal control cultures which all gave good IFN yields. However, MS-derived PBL cultures which did respond to Con A or PHA yielded the same amount of HuIFN-γ as did control cultures. Thus the MS-PBL cultures could be divided into two groups: a 'responder' group producing HuIFN-γ after PHA stimulation in the same way as normal control cultures, and a 'non-responder' group. Therefore, it seemed reasonable in this particular study to substitute the 'responder' MS group for normal control donors as internal positive control.

The present study clearly demonstrates that MS-derived PBL cultures defective for HuIFN-γ production also failed to produce IL-2 after PHA stimulation. The apparent deficiency in PHA-stimulated IL-2 and HyIFN-γ production by MS-derived PBL cultures was repaired by the addition of phorbol myristic acetate (PMA). PMA by itself was unable to induce IL-2 and HuIFN-γ production. Direct evidence for a deficiency in IL-1 in the PBL-PHA system is lacking, because with our thymocyte IL-1 assay it was impossible to discriminate between IL-1 and IL-2. When peripheral blood monocytes (PBM)—isolated from the same PBL cultures tested for IL-2 and IFN production—were stimulated directly with PMA, all PBM cultures produced the same amount of IL-1. This means that all MS derived PBL cultures, whether they produced IL-2 and HuIFN-γ after PHA stimulation or not, contained monocytes which were able to produce IL-1 when stimulated directly with PMA. When Con A or PHA are used to induce IL-1 production in macrophages, the presence of OKT4$^+$ T cells is required (Palacios, 1982). The T4$^+$ subpopulation was also claimed to produce both IL-2 (Kung *et al.*, 1979) and HuIFN-γ (Palacios *et al.*, 1983) after Con A and PHA stimulation. Hence the same cell that produces IL-2 and HuIFN-γ assists macrophages to synthesize IL-1. These data and the results of our experiments suggest some (functional) aberrations in the T4$^+$ subpopulation in MS. Indeed, our data suggest that the IL-2 and HuIFN-γ defect in MS is not due to the absence of the production machinery, but is rather an aberration in the interaction with PHA; i.e. some refractoriness of the T cells to the signal provided by PHA. PMA might overcome this deficiency by acting as a comitogen in the stimulation of IL-2, as shown in other studies (Santoro *et al.*, 1983; Farrar *et al.*, 1980.) The observation that PMA enhances capping of ConA (Kwong & Mueller, 1979) could be meaningful in this context, PMA-facilitating interaction (triggering) or PHA or Con A with the cell. In our experimental system, PMA by itself was unable to trigger IL-2 and HuIFN-γ response and thus only acts as co-mitogen. PMA seems to activate T cells via different receptors than those used by Con A and PHA (Palacios, 1982). There are indications that the same cell is the immediate target of both Con A and PMA and that combination of the two stimulants induces the T cells to produce IL-2 (Farrar *et al.*, 1980).

In cultures responding to PHA, IL-2 and HuIFN-γ were drastically increased by PMA. Different mechanisms are possible to explain this increased production level, such as prolongation of the production phase (G1) of the cell cycle (Stadtler *et al.*, 1981); inhibition of suppression and/or absorption of IL-2; stimulation of the synthesis of a specific protein or group of proteins involved in IL-2 production (see Farrar *et al.*, 1982).

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