Treatment of multiple sclerosis patients with interferon-β primes monocyte-derived macrophages for apoptotic cell death

Johan Van Weyenbergh,* Juana Wietzerbin,* Dany Rouillard,† Manoel Barral-Netto,‡ and Roland Liblau§

*U365 INSERM, †Section de Recherche, Institut Curie, and §U546 INSERM, Hôpital La Pitié-Salpêtrière, Paris, France; and ‡Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Salvador-Bahia, Brazil

Abstract: Although interferon (IFN)-β has shown a significant clinical benefit in multiple sclerosis (MS), its mechanism of action remains unclear. We found that IFN-β treatment of patients with MS resulted in a significant increase in apoptotic cell death (measured by annexin V staining and nuclear fragmentation) of monocyte-derived macrophages, as compared with cells derived from patients before treatment. Stimulation of the cells with IFN-β in vitro resulted in an even further increase of annexin V binding, as well as increased Fas (CD 95, APO-1) expression. However, no increased Fas expression, apoptotic monocytes, or monocytopenia were observed upon in vivo treatment. This indicates that IFN-β does not deliver a death signal to monocytes but rather primes for subsequent macrophage apoptosis upon activation or differentiation. *J. Leukoc. Biol.* 70: 745–748; 2001.

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS), debilitating mostly young adults. The immunomodulatory and antiviral cytokine interferon (IFN)-β is the first drug to show therapeutic effect in relapsing-remitting and secondary-progressive MS [1–3], but its mechanism of action remains as enigmatic as the etiology of MS itself [4, 5]. In an animal model of MS, experimental autoimmune encephalomyelitis (EAE), myelin-specific autoreactive T cells have been shown to initiate an autoimmune attack leading to demyelination. It has been proposed that apoptosis of T cells in EAE is a mechanism to control inflammation in the brain [6]. In MS patients, incubation with IFN-β in vitro has been shown to augment activation-induced T cell death [7]. However, in later stages of tissue pathology, T cells are greatly outnumbered by macrophages, which in fact execute the final step in demyelination, i.e., myelin phagocytosis [8, 9]. Therefore, we investigated the effect of IFN-β treatment in vivo and in vitro upon monocyte/macrophage apoptosis in patients with MS.

Seven patients (four women, all relapsing-remitting) with clinically definite MS were selected for this study. Informed consent was obtained, and blood was drawn before and at 1, 2, and 3 months of treatment with IFN-β1a, 72 h after injection (Avonex™, 30 μg intramuscularly once a week). All of the patients were free of exacerbations and had not received corticosteroids or immunosuppressive drugs during the 3 months preceding treatment. All patients had a clinically satisfactory response to IFN-β treatment, because none of them manifested progression of handicap after 1 year of treatment. Mononuclear cells were isolated by density-gradient centrifugation (Ficoll-Paque, Pharmacia, Sweden) and cryopreserved at each time point. The four samples from each patient were defrosted and processed simultaneously after verifying cell viability, which was identical to defrosted cells from healthy controls processed in parallel. Cells were stained with annexin V-fluorescein isothiocyanate (FITC), anti-CD 14-FITC, anti-CD 64-FITC, anti-Fas (CD 95, APO-1)-phycoerythrin (PE), or isotype-matched control antibodies (all from Immunotech-Coulter, Marseilles, France) before and after in vitro culture. Cells were cultured for 40 h in RPMI medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin, and 10% fetal calf serum (all from Gibco-BRL, Cergy Pontoise, France), in the absence or presence of IFN-β (1000 U/ml). Cells were analyzed in a cytofluorometer (FACScan, Becton-Dickinson, San Jose, CA) using Lysis II software. Monocytes were identified as CD 14$^+$, CD 64$^+$ cells with characteristic forward- and side-scatter. Nuclear fragmentation was demonstrated by fluorescence microscopy following Hoechst 33258 staining. Statistical analysis was performed using Prism-Graph Pad software; two-tailed paired Student's t-test was used to compare patients before and after treatment; and two-way analysis of variance (ANOVA) was used to examine interaction between in vivo and in vitro treatment. A P-value < 0.05 was considered significant.

We found that treatment of patients with IFN-β in vivo had no effect on annexin V-binding or Fas surface expression in freshly isolated monocytes (unpublished results); i.e., apoptotic features were absent in peripheral blood monocytes. Accord-

TABLE 1. Clinical and Hematological Features of the MS Patients

Sex	Age	Disease duration (yr)	Lymphocyte count before treatment	Lymphocyte count at 3 months	Monocyte count before treatment	Monocyte count at 3 months
M	33	4	2187/μl	1832/μl	162/μl	421/μl
F	38	5	1485/μl	1404/μl	495/μl	288/μl
F	45	4	1602/μl	1630/μl	524/μl	371/μl
F	36	4	1374/μl	1971/μl	430/μl	691/μl
M	44	1	2116/μl	1914/μl	570/μl	406/μl
M	38	5	3100/μl	2213/μl	673/μl	564/μl
F	30	7	2756/μl	3578/μl	345/μl	580/μl

ingly, as shown in **Table 1**, no monocytopenia was detected in any of the patients. However, when comparing monocytes after 40 h of stimulation by plastic adherence (i.e., after differentiation into macrophages), we observed an increase in annexin V staining induced by IFN-β treatment in vivo, as compared with cells derived before treatment. The percentage of annexin V-positive macrophages rose from 16.7 (±4.7)% before treatment to 57.2 (± 18.4)% after 3 months of treatment (**Fig. 1A**, open bars, $P=0.02$, paired t-test). In addition, nuclear fragmentation, the hallmark of apoptotic cell death, was observed almost exclusively in annexin V-positive monocytes derived from patients after IFN-β treatment (**Fig. 2A**), consistent with the annexin V-staining results. Also, stimulation of the cells with IFN-β in vitro resulted in an even further increase of annexin V binding, up to 43.1 (±7.8)% in untreated patients and 67.5 (±4.1)% at 3 months of treatment (Fig. 1A, solid bars). Although a trend for time-dependent increase can be observed for in vitro- and in vivo-induced apoptosis (Fig. 1A), a statistically significant increase in apoptosis induced by IFN-β in vitro was observed only at 0 and 2 months, whereas in vivo treatment led to significant apoptosis at 1 and 3 months only. Analysis by two-way ANOVA revealed that the in vivo and in vitro effects of IFN-β on apoptosis occurred independently ($P=0.84$ for interaction between in vitro and in vivo). This is somewhat unexpected, because in vivo treatment by IFN-a2 has been shown to down-regulate the IFN-α/b receptor [10], thereby probably precluding in vitro restimulation by IFN-α or -β. The apparent absence of in vitro unresponsiveness induced by previous in vivo treatment might rely in differences between IFN species, dose or route of administration.

Because the importance of apoptotic cell death in MS and EAE pathology has been become clear in recent years [5–7], a central role of the Fas/FasL pathway has been proposed [11–14], although clear data about participating cell types are lacking. To our surprise, we observed no significant increase in Fas expression on macrophages after in vivo treatment with IFN-β (Fig. 1B, open bars, $P>0.1$ at all time points). However, Fas expression was strongly increased by IFN-β stimulation in vitro (Fig. 1B, solid bars), reaching statistical significance at 0 and 2 months of treatment. It is interesting that IFN-β stimulation in vitro resulted in a significant increase in macrophage apoptosis at these same time points, as described above. Again, no interaction was observed between in vivo and in vitro treatment, as determined by two-way ANOVA ($P=0.63$). Addition and cross-linking of an agonist anti-Fas monoclonal

antibody had no effect on spontaneous or IFN-β-induced macrophage apoptosis in three patients tested (unpublished results). A similar strong resistance to Fas-induced cell death has been described recently in T cell lines derived from
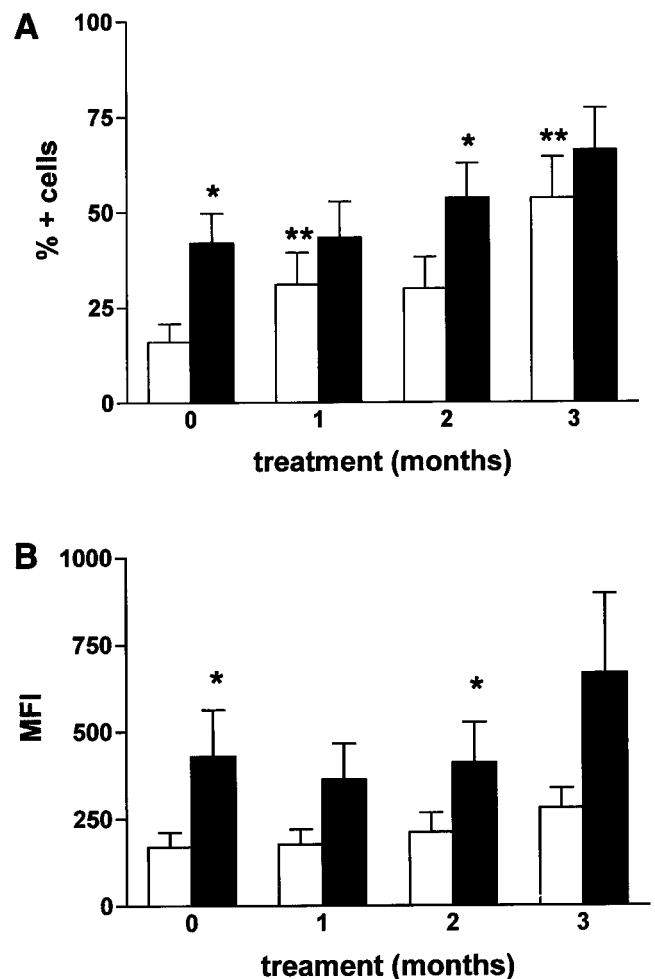

Fig. 1. Samples from seven MS patients treated in vivo with IFN-β were obtained before (0 months) and at 1, 2, and 3 months of treatment. Mononuclear cells were cultured for 40 h in the absence (open bars) or presence of 1000 U/ml IFN-β (solid bars) and gated on monocytes/macrophages in a cytofluorometer. Data are expressed as: (A) % annexin V-positive cells (SE); (B) mean fluorescence intensity (MFI, SE) for anti-Fas antibody staining, all corrected for background fluorescence. Statistical analysis (paired t-test) was performed comparing unstimulated cells at each time point with unstimulated cells before treatment (**$P<0.05$) and comparing IFN-β-stimulated cells with unstimulated cells at the same time point (*$P<0.05$).
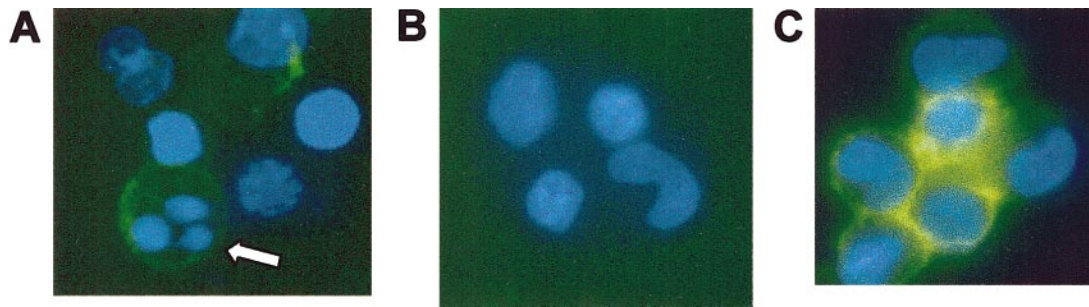
Fig. 2. Mononuclear cells were cultured for 40 h, stained with annexinV-FITC, fixed, stained with Hoechst 33258, and analyzed by fluorescence microscopy (Zeiss). (A) Nuclear fragmentation was evident almost exclusively in macrophages derived from patients after treatment and correlated with annexin V staining (arrow). (B) In cells derived from patients before treatment, annexin V staining and nuclear fragmentation were virtually absent. (C) Anti-HLA-ABC-FITC staining is shown as a positive control. Paired samples from a representative patient are shown.

MS patients [15]. Taken together, our results demonstrate that IFN-β can prime macrophage apoptosis in vivo as well as in vitro, possibly through a Fas-independent mechanism. A recent study describes the ability of IFN-β to induce apoptosis in melanoma cell lines through a TRAIL-dependent mechanism [16]. Clearly, further studies are warranted to investigate the molecular mechanism(s) of IFN-β-induced apoptosis in MS.

Finally, our findings extend those of Kaser et al. [7] to cells of the monocytic lineage and to the in vivo level; i.e., monocytes primed by IFN-β treatment of MS patients undergo apoptotic cell death upon subsequent activation and differentiation, which is mimicked by adherence to plastic in vitro. This phenomenon is likely to occur in the CNS of MS patients, because monocytes inevitably become activated and differentiate into macrophages when crossing the blood-brain barrier. It should be stated, however, that no apoptotic features were observed in freshly isolated peripheral blood monocytes before or after treatment. IFN-β-induced apoptosis in circulating monocytes, leading to monocytopenia and eventually immunosuppression, would indeed represent a highly undesirable side effect, which was not observed in our patients (see Table 1) nor in any of the large clinical trials [1–3]. Rather, IFN-β-induced apoptosis seems to require prior activation of T lymphocytes [7] or macrophages (this study) and hence to selectively affect cells with potentially demyelinating capacities. Similarly, elimination by apoptosis of T lymphocytes and macrophages has been shown to correlate with clinical recovery in the EAE model [14]. Therefore, triggering programmed cell death in effector cells in situ might be one possible mechanism through which IFN-β reduces active lesions, as determined by magnetic resonance imaging, and exerts its beneficial effect in MS. We have shown previously that the high-affinity immunoglobulin G receptor (CD 64), preferentially expressed on activated macrophages, is a target for antagonistic regulation by IFN-β and IFN-γ in MS patients [18], which might incriminate CD 64 as an effector molecule in demyelination. Based on the results of this study, we are led to believe that selective triggering of apoptosis in activated macrophages, which can be achieved by a CD 64-directed immunotoxin—recently demonstrated in a transgenic mouse model [19], might be considered as a future adjunctive or alternative to IFN-β therapy in MS.

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