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Two recombinant human interferon-beta 1a pharmaceutical preparations produce a similar transcriptional response determined using whole genome microarray analysis

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Key words
biosimilars – interferon-beta – microarrays – multiple sclerosis – lymphocytes

Abstract. Objectives: Recombinant human interferon-beta (IFN-) is a well-established treatment for multiple sclerosis (MS). The regulatory process for marketing authorization of biosimilars is currently under debate in certain countries. In the EU, EMEA has clearly defined the process including overarching and product-specific guidelines, which includes clinical testing. Biosimilarity needs to be based on comparability criteria, including at least molecular characterization, biological activity relevant for the therapeutic effect and relative bioavailability ("bioequivalence"). In the case of such complex diseases as MS, where the effect of treatment is not so directly measurable, in vitro tools can provide additional data to support comparability. Genomic microarrays assays might be useful to compare multisource biopharmaceuticals. The aim of the present study was to compare the pharmacodynamic genomic effects (in terms of transcriptional regulation) of two recombinant human IFN- -1a preparations on lymphocytes of multiple sclerosis patients using a whole genome microarray assay. Methods: We performed an ex vivo whole genome expression profiling of the effect of two preparations of IFN- 1a on non-adherent mononuclears from five relapsing-remitting MS patients analyzing microarrays (CodeLink™ Human Whole Genome). Patients blood was drawn, PBMCs isolated and cultured in three different conditions: culture medium (control), 1,000 U/ml of IFN- 1a (BLA-(STOFERON™, Bio Sidus) and 1,000 U/ml of IFN- 1a (REBIF™, Serono) RNA was purified from non-adherent cells (mostly lymphocytes), amplified and hybridized. Raw data were generated by CodeLink™ proprietary software. Data normalization, quality control and analysis of differential gene expression between treatments were done using linear model for microarray data. Functional annotation analysis of IFN- 1a MS treatment transcription was done using DAVID. Results: Out of the approximately 45,000 human sequences examined, no evidence of differential regulation was found when both treatments were compared (minimum adjusted p-value > 0.999). The IFN- 1a effect differentially regulated the expression of 868 genes. The expression of standard markers such as GTP cyclohidrolase, MxA, and OAS isoenzymes A and B changed as a consequence of the action of IFN- 1a. Conclusions: This exhaustive and highly sensitive assay did not show differences in the genomic expression profile of these two products under the assayed experimental conditions. These results suggest that this technology might be useful for the initial comparison of biosimilars, being part of a comprehensive comparability program that includes clinical testing.

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Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease that affects the central nervous system (CNS) [Hauser and Oksenberg 2006]. After empirical research with clinical trials, IFN- has become a well-established treatment for MS [Sibley and Group 1993]. This cytokine essentially acts through transcriptional regulation; however, the relevant genes involved in its therapeutic effects

have not been fully elucidated yet. The regulatory process for marketing authorization of biosimilars is currently under debate. Biosimilarity needs to be based on comparability criteria, including at least molecular characterization, biological activity relevant for the therapeutic effect and relative bioavailability ("bioequivalence"). In the case of such complex diseases as MS, where the effect of treatment is not so directly measurable, in vitro tools can provide additional data to support comparability.

Guidelines have addressed this topic suggesting that new technologies might be useful to compare multisource biopharmaceuticals and multidisciplinary studies are underway in order to standardize criteria and platforms. As an example, recent guidelines from the European Medicines Agency (EMEA, London) address this issue and state that the use of genomic microarray deserves consideration for the assessment of pharmacodynamic actions of this kind of products because of its ability to detect minor changes in the biological response to active substances. Also the USA Food and Drug Administration (FDA) is carrying out a multidisciplinary project called MAQC (Micro Array Quality Control) to standardize the use of this methodology as a regulatory tool [Ji and Davis 2006]. This study was performed to compare the genomic response at gene expression levels induced by two similar IFN- 1a formulations: Blastoferon® (Bio Sidus SA, Buenos Aires, Argentina) and Rebif® (Serono S.p.A, Bari, Italy).

Materials and methods

IFN- 1a pharmaceutical preparation

IFN- 1a is a recombinant glycosylated form of IFN- structurally indistinguishable from natural IFN- in its primary sequence and carbohydrate content, expressed in Chinese hamster ovary (CHO) cells [Knight and Fahey 1982, Utsumi et al. 1989]. The two preparations compared of this active ingredient were Rebif® (Serono S.p.A, Bari, Italy; imported by Serono Argentina SA) and Blastoferon® (Bio Sidus SA, Buenos Aires, Argentina), which was designed as Rebif® biosimilar. Blastoferon® has been approved for commercial use by the Argentinean National Regulatory Agency (ANMAT) on April 2005 and has demonstrated equivalent bioavailability to Rebif® after subcutaneous injection (Di Girolamo et al., manuscript in preparation). Pre-filled syringes of 12 MUI of both products were used in the experiment (Blastoferon® lot number F12-JE05-C and Rebif® lot number L6784001B RBS 308049-B).

Patients

In vitro whole genome expression profiling of the effect of two preparations of IFN- 1a was performed on non-adherent mononuclear cells from 5 relapsing-remitting multiple sclerosis patients (RRMS, 2 males, 3 females, aged from 15 to 54 years) using microarray analysis (CodeLink™ Human Whole Genome, GE Healthcare Biosciences Corp., Sunnyvale, CA, USA).

RRMS diagnosis was made according to established criteria [McDonald et al. 2001]. Their expanded disability status scale (EDSS) score was between 0 and 8. Among the five patients, two were receiving IFN- 1a at the time of blood sample collection, experiencing good clinical response; one had received IFN- 1a for 3 years until one year before blood sample collection, when immunomodulatory treatment was discontinued because of lack of efficacy; and the other two had never received IFN- 1a. No patient had received corticosteroid or immunosuppressive therapy during at least one month before blood sample collection. Informed consent was obtained from all the patients.

Non-adherent mononuclear cells isolation and IFN- 1a treatment

Peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll-Hypaque density gradient. Cells from each subject were aliquoted into four fractions:

– Fraction 1 "BASAL"; this sample was processed immediately to obtain total RNA.

The other three fractions were suspended at 10^6 cells/ml and incubated for 24 h in RPMI 1640 medium with glutamine and 10% fetal bovine serum, at 37 ºC in 5% CO_2 humidified atmosphere, under three conditions:

- Fraction 2 "CONTROL"; sample without IFN- .
- Fraction 3 "BLASTOFERON"; sample with 1,000 IU/ml of Blastoferon® preparation.
- Fraction 4 "REBIF", sample with 1,000 IU/ml of Rebif® preparation.

RNA amplification, labeling and microarray hybridization

After 24 hours of culture, non-adherent cells (mostly lymphocytes) were recovered, centrifuged, lysed and homogenized. For each sample, total RNA was isolated with the RNAeasy® Mini Kit extraction and purification system (QIAGEN Inc., Valencia, CA, USA) and analyzed for quality, integrity and DNA contamination by spectrophotometry and agarose gel electrophoresis prior to cDNA synthesis. CodeLink™ Expression Bioarray System was used for sample preparation and hybridization. Total RNA (0.5 g) from each sample was primed for reverse transcription with oligo-$d(T)_{24}$ containing the T7 RNA polymerase promoter site. After second-strand cDNA synthesis, the dscDNA served as the template for an in vitro transcription (IVT) reaction to produce the target cRNA. IVT was performed by the linear amplification method in the presence of biotin-11-UTP to label the target cRNA (Perkin Elmer, Boston, MA, USA). After fragmentation, 8 g of cRNA and CodeLink™ Human Whole Genome Bioarray were hybridized overnight at 37 ºC. The prearrayed 30-mer oligonucleotide slides contained over 45,000 well characterized human gene and transcripts. Labeling with Cy5-streptavidin was performed and slides were scanned at 635 nm for 3.5 s with 4.5 m pixel size resolution in the arrayWoRx™ "e" scanner (Applied Precision, LLC, Issaquah, WA, USA). Image analysis and raw data were assessed with CodeLink™ Expression Analysis Software version 4.1. Values obtained were globally normalized to the median expression value of the whole array spots.

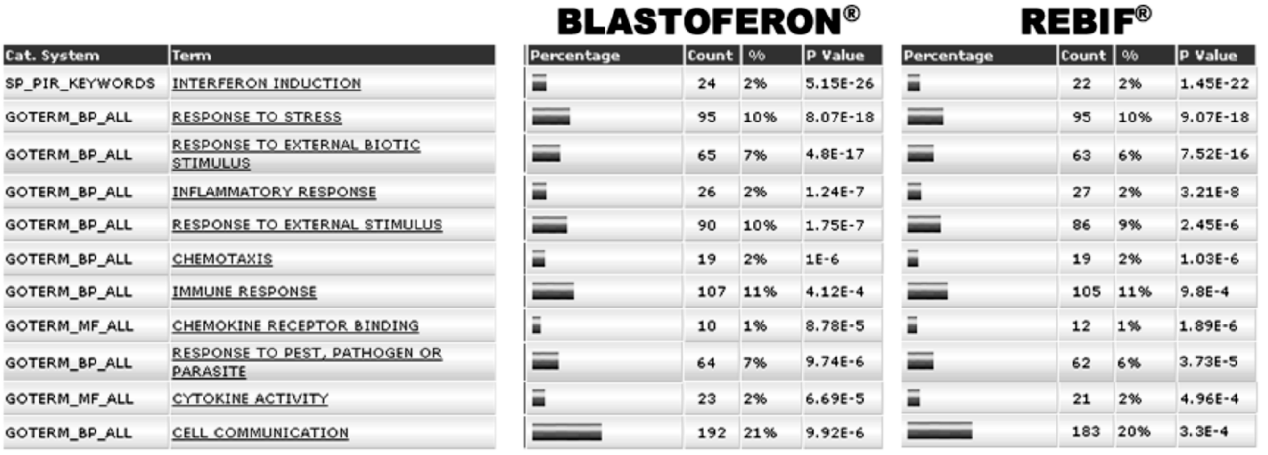
Bioinformatics and statistical data analysis

Raw data were imported into R statistical programming language environment, version 2.3.01. Graphic and statistical analysis was performed using CodeLink and Linear Models for Microarray Data (LIMMA, 2.6.2) [Smyth 2005] packages included in Bioconductor Project [Dudoit et al. 2003]. The preprocessing of the data sets to filter spots flagged as "bad", background correction and between microarrays normalization was done with CodeLink package.

Log_2 Fold change and p-value of differential expression for each gene were obtained performing the following comparisons:

- Blastoferon vs. Rebif = BLASTOFERON minus REBIF, i.e. differential gene expression between both products.
- IFN- 1a Effect = (BLASTOFERON plus REBIF) minus CONTROL minus BASAL, i.e. differential gene expression due to IFN- 1a effect, irrespective of pharmaceutical preparation used.
- Blastoferon = BLASTOFERON minus CONTROL/2 minus BASAL/2, i.e. differential gene expression due to Blastoferon® preparation.
- Rebif = REBIF minus CONTROL/2 minus BASAL/2, i.e. differential gene expression due to Rebif® preparation.

Differentially expressed genes were identified using an empirical Bayes moderated t-test and ranked in order of evidence for differential expression with LIMMA. The empirical Bayes procedure provides an effective framework for studying the relative changes in gene expression for a large number of genes. It uses a simple non-parametric mixture prior to model the population of affected and unaffected genes, thereby avoiding parametric assumptions about gene expression [Efron and Tibshirani 2002]. The p-values associated with the t-test were adjusted for multiple testing by using the Hochberg and Benjamini [1990] method. Thus, genes with an adjusted p-value lower than 0.05 were identified as differentially expressed.



		BLASTOFERON®				REBIF®			
Cat. System	Term	Percentage	Count	%	P Value	Percentage	Count	%	P Value
SP_PIR_KEYWORDS	INTERFERON INDUCTION		24	2%	5.15E-26		22	2%	1.45E-22
GOTERM_BP_ALL	RESPONSE TO STRESS		95	10%	8.07E-18		95	10%	9.07E-18
GOTERM_BP_ALL	RESPONSE TO EXTERNAL BIOTIC STIMULUS		65	7%	4.8E-17		63	6%	7.52E-16
GOTERM_BP_ALL	INFLAMMATORY RESPONSE		26	2%	1.24E-7		27	2%	3.21E-8
GOTERM_BP_ALL	RESPONSE TO EXTERNAL STIMULUS		90	10%	1.75E-7		86	9%	2.45E-6
GOTERM_BP_ALL	CHEMOTAXIS		19	2%	1E-6		19	2%	1.03E-6
GOTERM_BP_ALL	IMMUNE RESPONSE		107	11%	4.12E-4		105	11%	9.8E-4
GOTERM_MF_ALL	CHEMOKINE RECEPTOR BINDING		10	1%	8.78E-5		12	1%	1.89E-6
GOTERM_BP_ALL	RESPONSE TO PEST, PATHOGEN OR PARASITE		64	7%	9.74E-6		62	6%	3.73E-5
GOTERM_MF_ALL	CYTOKINE ACTIVITY		23	2%	6.69E-5		21	2%	4.96E-4
GOTERM_BP_ALL	CELL COMMUNICATION		192	21%	9.92E-6		183	20%	3.3E-4

Figure 1. Functional classification of the genes most significantly regulated by IFN- 1a preparation. Two independent DAVID (Database for Annotation, Visualization and Integrated Discovery) analyses were performed (one for Blastoferon and another for Rebif) and the outputs of some of the most up- or down-regulated functional classes were extracted and composed in a single figure.

Table 1. Quantitative distribution of the genes differentially expressed by IFN- 1a.

P value < 0.05 (false discovery rate < 5%)	**IFN-ß1a effect** (Blastoferon + Rebif) – C – B	**Blastoferon vs. Rebif** Blastoferon - Rebif
Non differential genes	53,009	53,877
Down-regulated genes	323	0
Up-regulated genes	545	0
Total differential genes	868	0

(C = Control, B = Basal).

Functional classification of differentially expressed genes

The ranking of functional categories based on co-occurrence with sets of genes included in a list (the differentially expressed genes in our case) was done using DAVID (Database for Annotation, Visualization and Integrated Discovery) tool [Dennis et al. 2003].

Confirmation by real time RT-PCR

The expression of MX2, OAS2, GBP1 genes were independently quantified by RT2 Real-Time® RT-PCR Assays (SuperArray Bioscience Corp., Frederick, MD, USA) using the SYBR® Green (Molecular Probes Inc., Eugene, OR, USA) detection method with high qualitiy gene-specific PCR primer sets and master mixes for microarray data validation.

Results

Table 1 shows that both products ("IFN-Effect") differentially regulated the expression of 868 genes (p-value < 0.05). When Blastoferon® and Rebif® were compared, none of the 45,000 well characterized human genes and transcripts examined, was differentially expressed between them (minimum p-value > 0.999). The expression levels of selected genes (MX2, OAS2, and GBP1) were confirmed by means of real time RT-PCR assays (data not shown).

The functional annotation analysis of the IFN- 1a transcriptome showed an over-representation of the following classes: interferon response, stress response, inflammatory response, signal transduction, chemotaxis and cellular communication. To further compare Blastoferon® and Rebif®, an independent functional annotation analysis was done for the genes up- and down-regulated by each product, resulting in an almost identical clustering for Blastoferon® and Rebif® (Figure 1).

As an additional illustration of this similarity, the fold change response of the 100 most significant up- and down-regulated genes by both IFN- 1a pharmaceutical preparations is shown in Figure 2: both products have almost superimposable gene stimulation patterns.

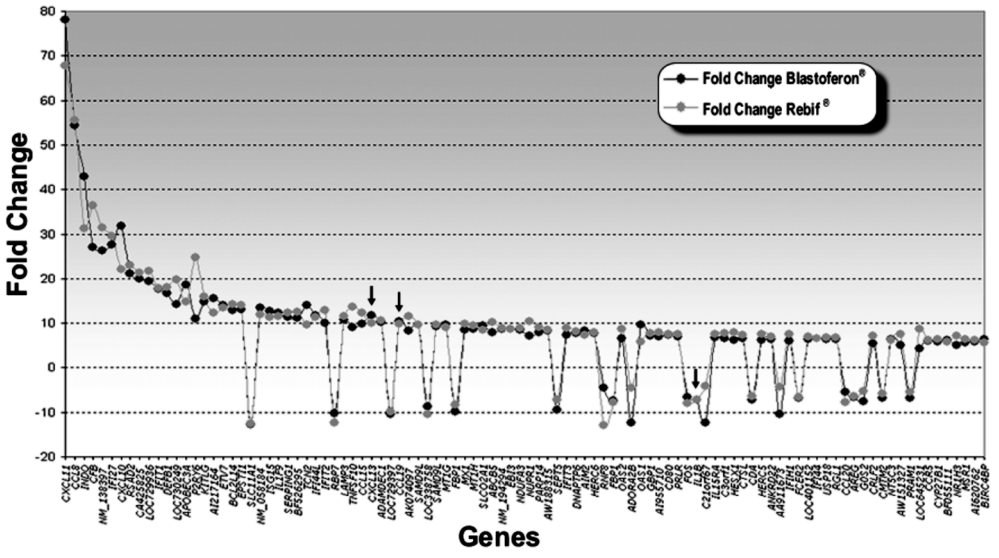


Figure 2. Response of the 100 genes that changed most upon stimulation with IFN- 1a. Each point represents individual fold change induced by either Blastoferon or Rebif. Arrows indicate genes that are further discussed in the text.

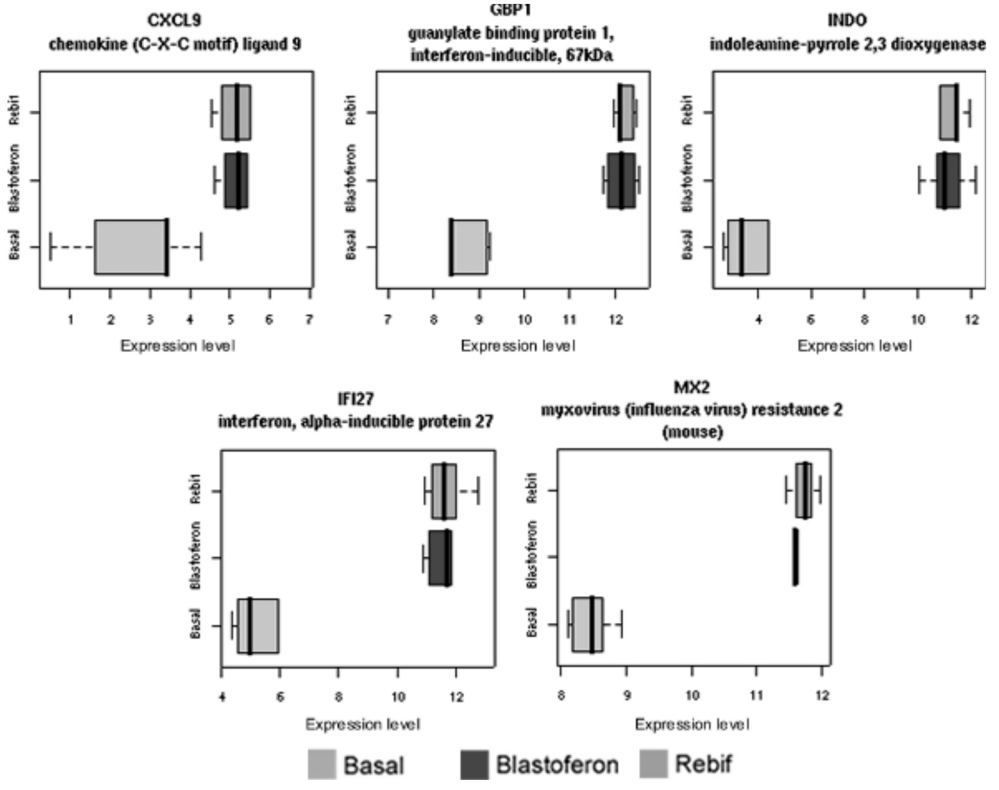


Figure 3. Comparison by box and whiskers plot of the expression levels of some genes specifically regulated by IFN- 1a. The analysis of CXCL9, GBP1, INDO, IFI27 and MX2 expression shows the similar response induced by both commercial preparations.

The high degree of similarity (minimum adjusted p-value > 0.999) is also shown by the box and whiskers plot of the expression levels of some specific genes related to interferon action (Figure 3). Moreover, when genes differentially expressed by IFN- were analyzed using hierarchical clustering, both products were clustered together in each patient (Figure 4).

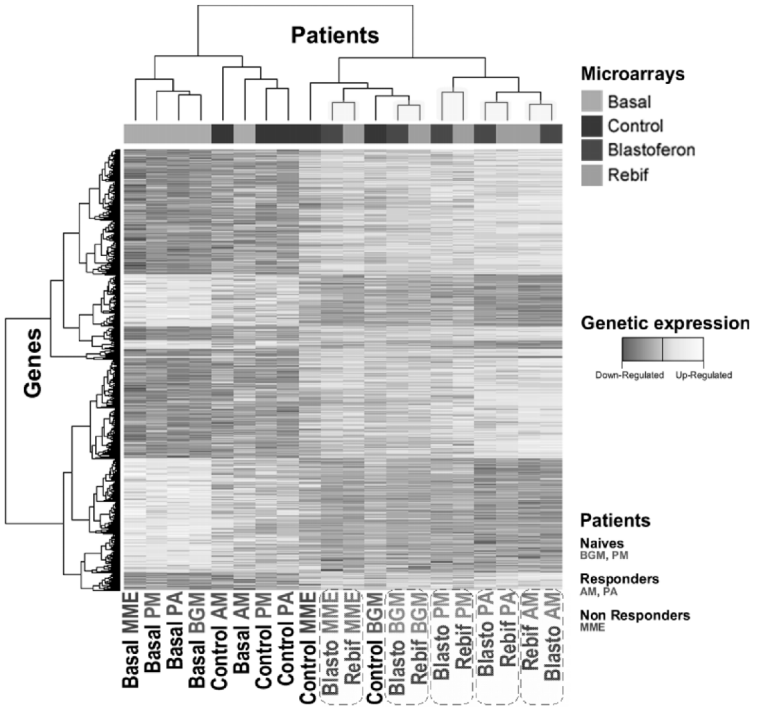


Figure 4. Hierarchical clustering of differentially expressed genes due to the interferon effect. The graph shows a very clear association pattern for both products in each patient.

Discussion

Data herein show that, under these experimental conditions, the two assessed pharmaceutical preparations of IFN- 1a resulted in a similar transcriptional regulation in mononuclear leukocytes in patients with MS. This finding was made possible by using the powerful whole genome microarray analysis.

Although there are several shortcomings in this study (some related to the method we selected and some to the design), none of them hinders an appropriate comparison between both products. The most recent evaluation from the MicroArray Quality Control project revealed that the bioarray platform that we have used has higher levels of sensitivity relative to the other microarray platforms assessed [Patterson et al. 2006, Shi et al. 2006]. It detects nearly 30% more genes, including as much as 60% more of the lowest expressed genes, with concentrations as low as 50 fM, when compared to other commercial microarray platforms. Detection sensitivity of microarrays is a key performance attribute to support expansion of genomics into clinical applications. A previous comparison of 6 different microarray platforms concluded that high-quality arrays and appropriate analysis determine biological rather than technological variance [Yauk et al. 2004]. Microarrays have been used for the study of MS pathophysiology and response to treatment [Comabella and Martin 2007] and are considered among the most powerful techniques for drug discovery [Stoughton and Friend 2005].

Shortcomings of the design include the size and cell type of the sample, the dose of IFN- 1a and the duration of the cell culture. Though the number of patients included in the study is small, each individual is acting as its own control, thereby reducing heterogeneity and maximizing power. Even though the evaluation of lymphocyte transcription levels might provide incomplete genome screening results in terms of such a complex CNS pathology as MS, this cell type stands as the only ethically available for in vitro studies and has been thoroughly used as a common model for MS studies [Satoh et al. 2006a].

As to the duration of the culture, 24 hours was selected since it is a very common incubation time in most previous studies with microarray analyses of IFN- effect [Weinstock-Guttman et al. 2003]. Finally, the dose was selected to detect most IFN- related effect. We cannot exclude differences at lower levels of IFN- or incubation times, although this possibility does not seem very probable.

The study was designed to compare two similar IFN- 1a formulations. Since this cytokine is a transcriptional regulator and is used to treat MS patients, a critical issue was the selection of the genes to be tested. Currently, no clear explanation for IFN- effect in MS is available, so, whole genome assay was selected. In addition, genetic factors are involved in the pathophysiology of MS [Hauser and Oksenberg 2006]; to decrease the risk of loosing significant genes present or regulated differentially between healthy subjects and MS patients, we performed the study on leukocytes of patients in vitro.

For conventional generic products, the rationale for comparison with innovator products is that if equivalent concentrations of the same molecule are achieved with both products, then similar responses should be expected. For biosimilars, regulatory authorities, such as EMEA, have produced specific

guidelines to deal with differences originated in the cell lines from which the biosimilar is obtained or in the manufacturing process. Since for IFN- a direct measure of response is difficult to obtain and implies exposure of large numbers of people to experimental conditions, an alternative approach can be the detection of the response by relevant effector cells, combined with a formal study of relative bioavailability. In this report we have shown that mononuclear leukocytes of MS patients are unable to differentiate between Blastoferon® and Rebif® under the experimental conditions used.

As a consequence of the recent expiration of some patents, there is a growing need of reliable tests in order to address comparability of biological response to biosimilar pharmaceutical products [Roger 2006]. Previous studies reporting the genomic effects of IFN- in the treatment of MS patients have been performed, but none of them compared the effects of two biosimilar products. Indeed the only comparative studies with IFN- 1a were based on pharmacokinetic parameters [Munafo et al. 1998] or clinical endpoints [Panitch et al. 2002]. While both designs are classic and pertinent, they probably lack the ability to detect minor differences and their results might be partial and controversial [Rogge et al. 1999]. Our study is an example that microarray technology could become a useful tool for comparability studies.

Although a detailed analysis of IFN- 1a effect in this experiment is under way (Kauffman et al., manuscript in preparation), well recognized IFN- related genes were identified among the top fold-change ranked genes. In agreement with previous studies [Baranzini et al. 2005, Buttmann et al. 2006, Satoh et al. 2006b, Sturzebecher et al. 2003] our results show the up-regulation of TRAIL (Tumor Necrosis Factor Superfamily, Member 10), Mx1 (Myxovirus Resistance 1), Mx2 (Myxovirus Resistance 2), CXCL10 and 11 (Chemokine C-X-C-Motif Ligands 10 and 11), STAT1 (Signal Transducer and Activator of Transcription 1), IRF-7 (Interferon Regulatory Factor 7), AIM2 (Absent in Melanoma 2), OAS2 (2'-5'-oligoadenylate synthetase 2, 69/71 kD) and GBP1 (Guanylate Binding Protein 1) and the down-regulation of ITGAM (Integrin alpha M), IL1B (Interleukin-1) and NRG1 (Neuroregulin 1).

In conclusion, we have investigated the in vitro pharmacodynamic genomic effects of IFN- 1a treatment on a whole genome microarray assay on lymphocytes from MS patients comparing the similarity between two biological products manufactured by two pharmaceutical companies. This exhaustive and highly sensitive assay did not show differences in the genomic expression profile of these two products under the assayed experimental conditions. Since no difference in genomic expression under experimental conditions does not necessarily result in clinical safety and efficacy, further studies are required to assess these issues.

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