LETTER TO THE EDITOR

IMMUNOGENICITY OF AN INTERFERON-β1a PRODUCT

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In order to determine whether Blastoferon®, a biosimilar interferon (IFN)-β1a formulation, shares epitopes with other known IFN-β products, a series of neutralization bioassays were performed with a set of well-characterized anti-IFN-β monoclonal antibodies and human sera (World Health Organization Reference Reagents). The bioassay was the interferon-induced inhibition of virus cytopathic effect on human cells in culture (EMC virus and A-549 cells). Computer-calculated results were reported as Tenfold Reduction Units (TRU)/mL. To further assess Blastoferon® immunogenicity, in vivo production of anti-IFN β antibodies was determined in sera of patients included in the pharmacovigilance plan of Blastoferon® by the level of IFN-β1a binding antibodies (by enzyme immunoassay -EIA) and neutralizing antibodies (in the Wish-VSV system). The highly characterized neutralizing monoclonal antibodies A1 and A5 that bind to specific regions of the IFN-β molecule reacted positively with the three β1a IFNs: Blastoferon®, Rebif®, and the IFN-β WHO Second International Standard 00/572. As expected, the non-neutralizing monoclonal antibodies B4 and B7 did not neutralize any of the IFN-β preparations. The commercially available monoclonal antibody B-02 reacted essentially equally with Rebif® and Blastoferon®. The WHO Reference Reagent human serum anti-IFN-β polyclonal antibody neutralized all the IFN-β products, whereas the WHO Reference Reagent human serum anti-IFN-α polyclonal antibody G037-501-572 appropriately failed to react with any of the IFN-β products. On the basis of in vitro reactivity with known, well-characterized monoclonal and polyclonal antibody preparations, Blastoferon® shares immunological determinants with other human interferon-β products, especially IFN-β1a. In vivo antibodies were detected by EIA in 72.9% of 37 chronically treated multiple sclerosis patients, whereas neutralizing antibodies were found in 8.1% of them. Blastoferon® appears to have immunological characteristics comparable to other IFN-β1a products.

Therapeutic proteins derived from human genes by recombinant DNA technology can be immunogenic, which can adversely affect their safety and efficacy. The basis for the immunological response induced by biopharmaceutical products is an area of active current research, which has been

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recently reviewed (1). However, the precise factors influencing the development of an immune response against therapeutic proteins are largely unknown (2). Interferon-beta 1a (IFN-B1a), a recombinant glycosylated form of human IFN-β expressed in Chinese hamster ovary (CHO) cells, is structurally indistinguishable from natural IFN-β in its primary sequence and carbohydrate content. Blastoferon® is a pharmaceutical IFN-β1a product currently marketed in Argentina and several Latin America countries as a biosimilar to the innovator IFN-β1a (Rebi®) for the treatment of multiple sclerosis (3). Blastoferon®, Rebi® and the Second World Health Organization (WHO) International Standard for Human Beta Interferon, 00/572, are comparable to the First IFN-β WHO International Standard, Gb23-902-531, which is a natural fibroblast glycosylated IFN-β, but different from the IFN-β1b non-glycosylated molecule Betaseron®/Betaferon, produced in Escherichia coli. This report summarizes the results of different preclinical and clinical tests performed to investigate the immunogenicity of Blastoferon® (4).

MATERIALS AND METHODS

Interferons: three commercial products were used: Blastoferon® (IFN-B1a), which was produced by Bio Sidus S.A. (Buenos Aires, Argentina); Rebi® (IFN-B1a), by Merck-Serono (Geneva, Switzerland), and Betaseron® (IFN-β1b), by Berlex Biosciences (Emeryville, CA, USA). The WHO First International Standard for Human Interferon-β, Gb23-902-531, a natural fibroblast preparation, and the Gb23-901-531, the WHO IFN-β1b recombinant standard, having a serine-17 substitution for cysteine and lacking a terminal methionine, were obtained from the National Institutes of Health, Bethesda, MD, USA. The Second WHO International Standard for human interferon-beta, 00/572, was obtained from the National Institutes of Biological Standards and Control, Potters Bar, England. Blastoferon®, like Rebi®, and the NiBSR 00/572, are glycosylated recombinant IFN-β1a molecules produced in CHO cells transfected with the human IFN-β gene containing the native nucleotide sequence, by virtue of which they are all glycosylated. Betaseron®, on the other hand, is produced in E. coli and is therefore non-glycosylated, in addition, the IFN-β gene used has been altered and the final product lacks a terminal methionine and serine is substituted for cysteine at position 17.

Antibody Preparations. Murine monoclonal antibodies (mAbs) A1, A5, and A7 are anti-IFN-β neutralizing antibodies; mAbs B4 and B7 are non-neutralizing antibodies against Betaseron® that have been produced in Balb/c x DBA f, mice following injection of IFN-β1b (5-6). The mAb A1 preparation was hybridoma culture supernatant. The mAbs A5, A7, B4, and B7 were used as mouse ascites fluid. The isotypes of these mAbs are IgA (for A5), IgG1 (A1), IgG2a (A7 and B7) and IgG2b (B4). The murine mAb B-02 is an anti-IFN-β neutralizing antibody purchased from Yamasa Corporation, Japan. The human polyclonal antisera G037-501-572 and G038-501-572 are both World Health Organization (WHO) International Reference Reagents (NIH Reference Reagent Notes #44 and #45); the G037 is a human serum specific for human IFN-α with a WHO-assigned titer of 8,000, and the G038 is a human serum anti-human IFN-β with a WHO-assigned titer of 1,500 (7).

Characterization by monoclonal and polyclonal antibodies

Each IFN preparation was subjected to epitope detection by the different antibodies/antisera and when reactivity was detected, titration was performed to assess whether detection resulted in a quantitatively similar decrease of antiviral activity.

IFN Neutralizing Antibody Bioassay. For neutralization, the bioassay employed was the inhibition by IFN of the cytopathic effect of encephalomyocarditis (EMC) virus, on human lung carcinoma A549 cells, objectively evaluated by the naphthol blue-black dye-uptake procedure (8). Antibody samples serially diluted two-fold were mixed with an equal volume of 20 laboratory units per milliliter (LU/ml) of each IFN-β product. The mixtures were incubated for two hours at 36°C and added to A549 cell monolayers in 96-well plates. After overnight incubation at 36°C, cells were washed and challenged with EMC virus in a concentration intended to cause 80-90% cytopathic effect (CPE) by the following day. The extent of CPE was determined by naphthol blue-black dye uptake by cells, measured at 620 nm in a plate spectrophotometer (MultiScan MS Reader, Labsystems, Helsinki, Finland). Data were processed by a computer program that provides sigmoidal dose-response curves generated from the absorbance values and 50% endpoints were determined. The titer of neutralizing antibodies was expressed in TRU (Tenfold Reduction Units)/ml. 1 TRU/ml being the serum dilution able to reduce IFN-β titer from 10 to 1 LU/ml. The formula applied for TRU calculation was TRU/ml = (f x N-1)/10, where f = the reciprocal of the serum dilution at 50% endpoint and N = IFN concentration (in LU/ml) measured in the same titration. A sample was considered positive if the Nab titer was at least 10 TRU (9-10). Controls on each plate included uninfected cells, EMC virus-infected
cells, cells treated with antibody alone, and a simultaneous IFN titration to measure directly the interferon activity in LU/ml employed in that day’s test.

**Anti-interferon-beta binding antibodies in patients**

Serial samples from 37 MS patients in treatment with Blastoferon for a median of 22.7 months were assayed for IFN-β binding antibodies of the IgG isotype (Babs) by enzyme immunoassay (EIA). EIA microtiter plates were coated overnight at 4°C with 2 μg/ml of the anti-IFN-β monoclonal antibody B02 (Yamasa, Tokyo, Japan) in phosphate-buffered saline (PBS), washed 4 times with PBS and blocked with 0.5% non-fat dry milk in PBS for 1 h at room temperature. The plates were washed 4 times with PBS containing 0.05% Tween-20 (PBS-T) and then loaded with IFN by incubation (1 h at room temperature) with 100 μl/well IFNβ1a (150 ng/ml) in PBS-T containing 0.5% non-fat dry milk (MILK/PBS-T). Plates were then washed 4 times with PBS-T.

For testing, each serum sample was diluted 1/10 in MILK/PBS-T and 100 μl added to each well. As controls, samples were also mixed, in duplicate, with IFN-β and diluents. After 2 h incubation at room temperature, the plates were washed with PBS-T, and bound antibody was detected by adding 100 μl HRP-conjugated goat anti-human IgG (Fc specific, Sigma, St Louis, MO, USA, cat #A170) diluted 1/50000 in MILK/PBS-T and incubated for 1 h at room temperature. The plates were washed with PBS-T and developed by adding hydrogen peroxide and o-phenylenediamine; reaction was stopped by adding 100 μl of 4N H2SO4. Plates were read at 450 nm. Signal intensities for a given test sample are reported as the mean of the absorbance measured in the duplicate wells, after subtraction of the mean background absorbance observed for the same test sample in the duplicate control wells with diluent on the same plate. The cutoff was calculated as the mean + 3 SD of the average net absorbance of 8 individual measurements of a pooled normal human serum.

**Interferon-beta neutralizing antibody in patients’ sera**

Bab-positive samples were tested for neutralizing antibodies (Nab) by a modification (11) of the CPE assay described above: Wish cells were used instead of A549 cells and vesicular stomatitis virus (VSV) was used instead of EMC virus.

**RESULTS**

**Characterization by monoclonal and polyclonal antibodies**

In order to determine whether Blastoferon® shares the same epitopes as other known IFN-β products, a series of neutralization bioassays were performed with a battery of well-characterized anti-IFN-β monoclonal antibodies and World Health Organization Reference Reagent human sera. Table 1 summarizes the neutralization results obtained with

<table>
<thead>
<tr>
<th>Table 1. Neutralization of Blastoferon® and other Interferon-β Products by various Monoclonal Antibody (mAb) and Polyclonal Antibody (pAb) Preparations.</th>
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</thead>
<tbody>
<tr>
<td><strong>Neutralizing Antibody Titer (TRU/ml) vs. different interferon-β preparations</strong></td>
</tr>
<tr>
<td>Antibody preparation</td>
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<tr>
<td>pAb G038 (anti-IFN-β)</td>
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<tr>
<td>pAb G037 (anti-IFN-α)</td>
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<tr>
<td>mAb A1 (anti-IFN-β)</td>
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<td>mAb A5 (anti-IFN-β)</td>
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<td>mAb A7 (anti-IFN-β)</td>
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<td>mAb B4 (anti-IFN-β)</td>
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<td>mAb B7 (anti-IFN-β)</td>
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<td>mAb B-02 (anti-IFN-β)</td>
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Table II. Antibodies induced by Blastoferon® treatment in vivo.

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<tr>
<th>Assay</th>
<th>Positive</th>
<th>Negative</th>
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<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Binding Antibodies</td>
<td>27 (72.9)</td>
<td>8 (27.1)</td>
</tr>
<tr>
<td>Neutralizing Antibodies</td>
<td>3 (8.1)</td>
<td>34 (91.9)</td>
</tr>
</tbody>
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Blastoferon® in comparison with other commercial interferon-β products and WHO interferon standards. As expected, the WHO International Reference Reagent human serum G037-502-572 against IFN-α failed to neutralize any of the IFN-β, whereas the WHO International Reference Reagent G038-501-572 (human serum containing antibodies against glycosylated IFN-β) neutralized to similar levels the three glycosylated IFN-β1a preparations produced in CHO cells, namely Blastoferon®, Rebi®, and the NIBSC 00/572 (Second WHO International Standard for Human IFNβ1a). As previously reported (NIH Reference Reagent Note #45), a lower antibody titer was obtained with the IFN-β1b Betaseron® preparation compared to the titer observed with the Gb23-902-531 fibroblast IFN-β (the First WHO International Standard for Human IFN-β). The mAb A1 neutralized all five IFN-β products, whereas mAb A7 reacted only with Betaseron®, as previously reported (5). The mAb A5, an IgA, reacted with the three recombinant IFN-β1a products and Betaseron®. As expected, the binding, non-neutralizing mAbs B4 and B7 failed to neutralize any of the IFN-β products. The mAb B-02 neutralized the two IFN-β1a commercial products (Blastoferon® and Rebi®), and to a much lesser extent, also IFN-β1b.

Antibodies generated in the course of in vivo treatment

Quarterly obtained samples from 37 multiple sclerosis patients treated with Blastoferon® for a median time of 22.7 months were assessed for the presence of binding and neutralizing antibodies against IFN-β. As shown in Table II, binding antibodies were detected in 72.9% of patients, whereas neutralizing antibodies were detected in 8.1% of the cases.

DISCUSSION

On the basis of neutralizing antibody reactivity in vitro with known, well-characterized antibody preparations, the results taken together clearly indicate that Blastoferon® shares immunological determinants with other human IFN-β products, especially other IFN-β1a products. Of the three functional epitopes of huIFNβ identified with the mAb A-series, two were associated with both antiviral and anti-proliferative activities (5); the A-series neutralizing mAbs recognized a linear epitope within the IFN-β molecule. An analysis by synthetic peptide mapping showed that IFN-β residues 39–48 were bound by the A-series, whereas the non-neutralizing B-series mAbs bound less specifically at multiple sites near the aminoterminus, demonstrating that amino-acid residues 32–56 represent the major region responsible for the expression of biological activity of human IFN-β (12).

There are some shortcomings that have to be mentioned here. The results presented came from humoral immunogenicity assays; however, the inclusion of future in vivo studies in experimental animal models could show a more thorough picture including cellular immunogenicity assays. Furthermore, the use of monoclonal antibodies coming from unpurified mouse ascite fluid, albeit common practice, might include non-specific immunoglobulins able to cause unwanted cross-
reactivity in characterization assays.

The detection of antibodies in serum samples of patients confirms that IFN-β1a is immunogenic in vivo at a lower rate than generally reported, though longer follow-up and a larger number of patients are required to estimate the precise incidence of antibody production. Since neutralizing antibodies might result in a lack of the effectiveness of IFN-β, we assessed the clinical response in the patients and we did not detect any sign of lack of effectiveness (35 patients had remained relapse-free since the active registry began). Preclinical and clinical testing of immunogenicity is part of the comparability analysis of a biosimilar pharmaceutical. Data presented here are consistent with what has previously been reported for other formulations of IFN-β used in the treatment of multiple sclerosis (13-15). Further studies in the long-term follow-up should confirm this conclusion.

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