Human β-Interferon Incubated with Muscle Homogenate is Protected by Albumin but not by Proteinase Inhibitors (43450)

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Abstract. **The scarce bioavailability of β-interferon (IFN-β) after intramuscular administration is probably due either to the binding of IFN-β to interstitial matrix, or to lymphatic absorption and/or to local breakdown by lysosomal proteinases from muscle. In this work, we first showed that after intramuscular injection, the apparent bioavailability of natural human IFN-β is about 10% of that of recombinant IFN-α_2 and then we evaluated the effects of proteinase inhibitors and albumin on IFN-β incubated at 37°C with muscle homogenate. IFN biological activity decreased spontaneously by about 20% after incubation for 6 hr at 37°C in Hanks' solution, but it was almost completely lost after incubation with muscle homogenate. Proteinase inhibitors (α_1-antitrypsin, α_2-macroglobulin, aprotinin, soybean trypsin inhibitor, leupeptin, EP-459, and EP-475) failed to block the inactivation of IFN-β by muscle proteinases, whereas albumin exerted a partial but consistent protection.** [P.S.E.B.M. 1992, Vol 200]

Intramuscular administration of α-interferon (IFN-α) has been used frequently (1, 2) to overcome the rapid fall of plasma IFN obtained after intravenous administration. After intramuscular administration, plasma IFN-α levels are lower than those obtained after intravenous administration, because they are the result of a dynamic equilibrium between the amount of IFN entering the circulation through muscle, blood, and lymphatic capillaries and the amount of IFN leaving the plasma compartment owing to transcapillary exchange and catabolism. Unfortunately, after intramuscular injection of β-interferon, plasma levels are very low and short lived (3) and, in order to explain the scarce bioavailability of IFN-β, a credited explanation is the one envisaging its binding to interstitial matrix and/or local breakdown by muscle proteinases (4, 5). It remains uncertain whether the presence of IFN-β in the plasma is an essential requisite for antitumor efficacy, because enhancement of NK activity (6), increased expression of hormonal receptors (7), and a significant elevation of the 2′-5′-oligoadenylate synthetase in circulating leukocytes (8) are very similar after both intravenous and intramuscular injection of IFN-β. However, either cytostatic or direct antiviral activity on target cells is minimally achieved after intramuscular administration of IFN-β, and the mystery of its low bioavailability needs to be clarified. In this report, we first compared the IFN plasma concentration versus time obtained after intramuscular injection of either IFN-α_2 or IFN-β in the rabbit and then we evaluated *in vitro* the role of serine or cysteine proteinase inhibitors and human albumin on the stability of IFN-β incubated at 37°C with rabbit muscle homogenates.

Materials and Methods

Materials. Recombinant IFN-α_2 (Intron) and natural IFN-β (Naferon) were gifts from Schering (Bloomfield, NJ) and Sclavo SpA (Siena, Italy), respectively. The specific activities of the two preparations were 2×10^8 and 1.8×10^6 IU/mg protein, respectively. Aprotinin, α_1-antitrypsin, leupeptin, and soybean trypsin inhibitor were from Sigma Chemical Co. (St. Louis, MO). α_2-Macroglobulin was from Behringwerke AG (Marburg, FRG). EP 459 and EP475 are inhibitors of cysteine proteinases and were a gift from Dr. K. Hanada (Research Center, Taisho Pharmaceutical Co., Saitama, Japan). Human albumin was a gift from Sclavo SpA.

Animal Experiments. New Zealand male rabbits

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weighing 2.5 ± 0.5 kg were used throughout. For the intramuscular administration, rabbits were injected in the left thigh in two sites, first with a dose of 1 ml of saline containing 9×10^6 IU of IFN-α_2 and, 3 days later, with the same dose of IFN-β in the right thigh. This experimental scheme was used in order to minimize the variability of the plasma concentration curves between different animals. After both intramuscular administrations, small samples of blood were withdrawn at various intervals for 10-hr from the marginal ear veins and put into plastic tubes containing dry heparin (10 IU/ml of blood). Plasma samples were stored at −70°C until IFN titration was carried out.

***In Vitro* Experiments.** For these experiments, rabbits were sacrificed with an overdose of Nembutal (Serva, Heidelberg, FRG) dissolved in saline and injected into an auricular vein. The quadriceps were isolated, collected, weighed, and homogenized 1/10 with Hanks' balanced salt solution containing 0.2 mg/ml of streptomycin and 200 units of penicillin G with a Dounce homogenizer. To 10 ml of homogenate containing an average of 140 mg/ml of proteins, appropriate amounts of either proteinase inhibitors or human albumin and IFN-β (10^4 IU/ml) were added. At predetermined intervals, 2-ml samples were collected and, after centrifugation at 105,000*g* at 4°C for 1 hr, supernatants were stored at −70°C for IFN titration. Controls were performed by incubating both IFN-β and IFN-α_2 for similar periods in either Hanks' balanced salt solution or in muscle homogenate. Proteins were measured

IFN Determinations. IFN were measured as described by Langford *et al.* (10) using human amnion (WISH) cells and vesicular stomatitis virus (Indiana strain) as a challenge virus. All samples were assayed at least twice in triplicate. International standards of human IFN-β (G-023-902-527) and human IFN-α (G-023-901-527) were obtained from National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, and laboratory values were normalized to standard values.

Results

Comparison of Plasma Concentration Curves after Intramuscular Injection of IFN-α_2 and IFN-β. As shown in Figure 1, serum levels of both IFN increased rapidly after intramuscular administration and reach the maximum level within 1 hr. The maximum IFN concentrations in plasma (C_{max}) were 2070 ± 310 and 286 ± 80 IU/ml for IFN-α_2 and IFN-β, respectively. However, the plasma concentration profiles were quite different. In fact, IFN-α_2 declined biexponentially and rapidly during the following 8 hr, whereas IFN-β decreased slowly and a little activity was still present 8–10 hr after IFN-β injection. Calculation of the areas under the curve allowed us to conclude that bioavail-

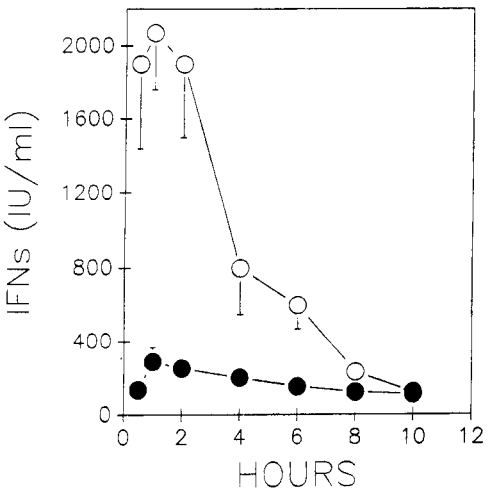

Figure 1. Plasma kinetic curves obtained after intramuscular injection in five rabbits of 9×10^6 IU of IFN-α_2 (○) or IFN-β (●). Values are expressed as mean ± SD.

ability of IFN-β after intramuscular administration was only about 10% in comparison to IFN-α_2.

Incubation of IFN with Muscle Homogenate. Biological activity of IFN-α_2 incubated at 37°C with either Hanks' balanced salt solution or with muscle homogenate (140 mg proteins/ml) remained constant for 6 hr (data not shown). On the other hand, after incubation of IFN-β in Hanks' solution, biological activity spontaneously decreased by about 20%, whereas, in the presence of muscle homogenate, it was almost completely lost during the 6 hr of incubation (Fig. 2).

The Role of Proteinase Inhibitors and Albumin. In order to prevent or reduce the inactivation of IFN-β by muscle homogenate, IFN-β was incubated at pH 7.4 with muscle homogenate containing various inhibitors at physiological concentrations or at concentrations known to be effective against specific proteinases. Inhibitors were as follows: α_2-macroglobulin (2 mg/ml), leupeptin (5 and 50 m*M*), aprotinin (20×10^3 units), soybean trypsin inhibitor (0.5 mg/ml), α_2-antitrypsin (5 mg/ml), and EP 459, and EP 475 (50 μM and 0.5 m*M*). The last two are cysteine proteinase inhibitors, inhibiting particularly papain and catepsins B and L. As shown in Figure 2, all proteinase inhibitors tested so far were unable to inhibit the IFN-β inactivation. Only the addition of human albumin to the homogenate protected the IFN-β in a dose-dependent fashion, and the protection was almost complete at concentrations of albumin ranging from 5% to 10% (Fig. 3).

Discussion

We evaluated the pharmacological behavior of both IFN-α_2 and IFN-β after intramuscular administration in rabbits and observed that IFN-β hardly appeared in the plasma.

Our results agree with other studies either in humans (11) or in animal models (3, 12), and the minimal

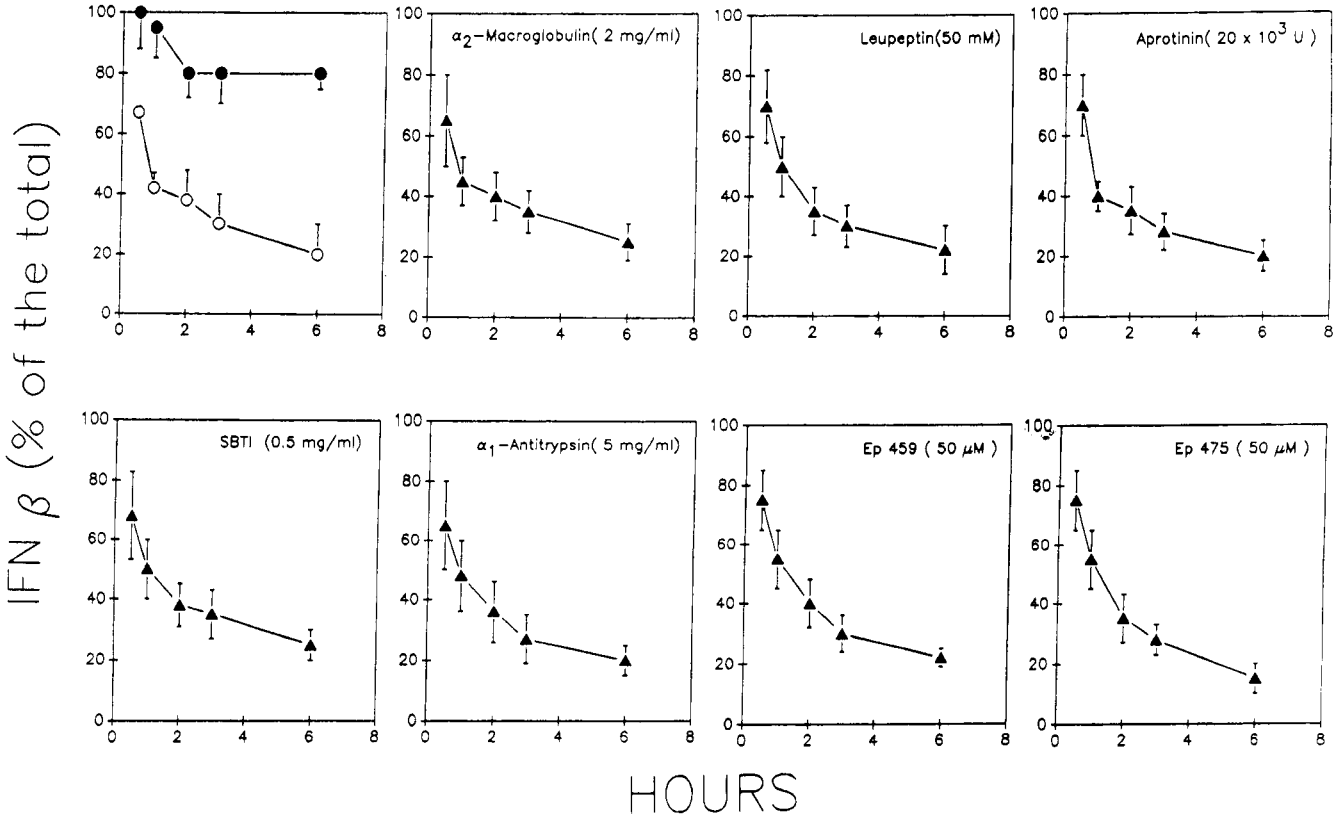

Figure 2. Kinetics of inactivation of IFN-β incubated at 37°C in Hanks' balanced salt solution (●), in muscle homogenate (○), or in muscle homogenate containing proteinase inhibitors (▲). Values are expressed as mean ± SD of percentages of initial values; $n = 6$.

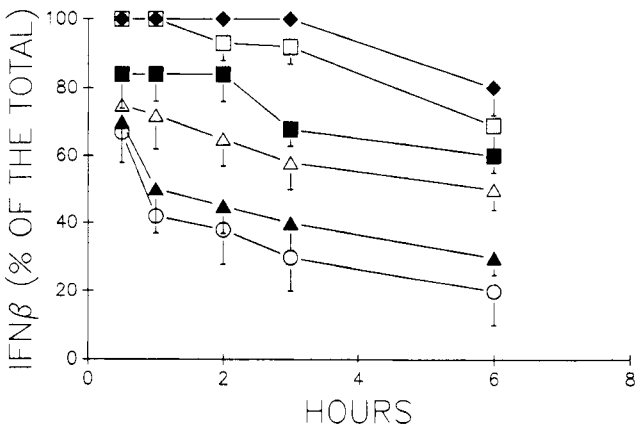

Figure 3. Kinetics of inactivation of IFN-β incubated at 37°C in muscle homogenate (○) or in muscle homogenate containing 0.1% (▲), 1% (△), 2.5% (■), 5% (□), and 10% (◆) human albumin. Values are expressed as mean ± SD of percentages of initial values; $n = 6$.

bioavailability of IFN-β is probably multifactorial in the sense that local binding to interstitial matrix, local catabolism or inactivation, and a preferential absorption of IFN-β through the lymphatics (13) may explain in part the finding. It is possible that while IFN-β moves along the lymphatic vessels and nodes, it activates lymphocytes, and because the daily lymph flow is about 2500-fold slower than plasma flow, the little amount of IFN-β emerging in the venous blood per unit of time is further diluted and catabolized. This interpretation would explain why, even in the presence of hardly detectable IFN-β plasma levels, a marked increase of typical IFN markers in blood mononuclear cells and other immunomodulatory effects has been noted (6–8).

Moreover the very low concentration peak and the profile of the elimination phase observed after intramuscular injection of IFN-β suggest that the patterns of absorption, distribution, and elimination of this IFN are quite different from those followed by IFN-α (14, 15).

In order to ascertain whether IFN-β administered via the intramuscular route could be hydrolyzed by muscle proteinases, we performed *in vitro* experiments incubating IFN-β with muscle homogenates. Previously, Ramamurthy and Fleischmann (16) showed that IFN-β titers are significantly reduced upon 48 hr of incubation at 37°C with fibroblasts. However, by considering that IFN has a very rapid turnover and that after intramuscular administration IFN-β causes some local swelling and pain, i.e., symptoms of a sterile inflammation (3), we suspect the local release of lysosomal enzymes.

We tested a series of protease inhibitors, because, first, a skeletal muscle proteinase is a serine proteinase (17) and, second, because incubation was carried out at pH 7.4, which is the optimum pH for these proteinases.

Unexpectedly, neither cysteine nor serine proteinase inhibitors were able to prevent the inactivation of IFN-β. So, at the present, it remains uncertain how much IFN-β is really hydrolized locally or is bound instead to intercellular matrix. IFN-α_2 is not inactivated by muscle homogenates, and this agrees well with the fact that IFN-α_2 diffuses well from the injection site in the plasma pool so that the C_{max} are higher than those obtained after injection of IFN-β.

Finally, we observed an important protective role of human albumin on IFN-β incubated with muscle homogenate. This is not entirely surprising, because 0.1% albumin is known to reduce adsorption of IFN to glass and, moreover, albumin may interact hydrophobically with IFN-β (18) so that the enzymatic actions of proteases could be reduced or, alternatively, it may prevent it from binding to the matrix lectins (19). A final possibility that remains to be explored is that human albumin currently used for therapeutic purposes contains some potent proteinase inhibitors as contaminants of a type not evaluated in this work. Indeed, the highest protection of IFN has been obtained by 10% albumin, a 100-fold higher concentration than that currently used by the pharmaceutical industry.

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