Characterization of Human $\beta$-Interferon-binding Sites on Human Cells*

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Radioiodinated recombinant human $\beta$-interferon (rHuIFN$\beta$), with almost full (>90%) biological activity, was used to study the binding of human $\beta$-interferon to Daudi cells. Specific binding was not observed with less biologically active (<10%) radioiodinated interferon. The bound radioiodinated interferon was shown to compete with human $\beta$-interferon (HuIFN$\beta$), rHuIFN$\beta_{ser}$, human $\alpha$-interferon (HuIFNa) and with human $\gamma$-interferon (HuIFNg). Scatchard plot analyses suggest the presence of about 10,000 binding sites for HuIFN$\beta$/Daudi cell. About 6,600 of these sites can be blocked by HuIFNa and 3,700 sites can be blocked by HuIFNg. The apparent $K_d$ for HuIFN$\beta$ is 2.7 nM. The apparent $K_d$ values for HuIFNa and HuIFNg are 3.7 and 1.1 nM, respectively. It was possible to demonstrate the cross-linking of HuIFN$\beta$ to two macromolecular components of $M_s = 128,000$ and 103,000. We propose the existence of at least two binding sites for HuIFN$\beta$ in Daudi cells, one site recognizing both HuIFN$\beta$ and HuIFNg, the other site recognizing both HuIFNa and HuIFNg. Each site is capable of recognizing only HuIFNg or HuIFNa.

With the availability of increasing amounts of pure interferons from cultured cells or recombinant Escherichia coli containing the human interferon genes (1-12), it became possible to label pure HuIFNa or HuIFNg with iodine-125 and use them to characterize the binding of these interferons to human cells (13-22).

Competition studies with labeled IFNa and unlabeled HuIFN$\beta$ and HuIFNg led Branca and Baglioni (21) to postulate the existence of two kinds of receptors, recognizing types I and II (viz. IFNa, IFNg, and IFNa), respectively. Similar studies by Anderson, et al. (22) using labeled HuIFNg suggested the presence of two kinds of receptors and implied that HuIFNg can recognize both of them, a hypothesis which could be tested with $^{125}$I-labeled HuIFNg. Binding of interferons is specific for cells which are sensitive to them, as shown by Aguet and Blanchard (15) for mouse interferon, and by Joshi et al. (13) for HuIFNa, and has been demonstrated for a number of fibroblast cell lines (17, 22), lymphoid cell lines (16), and other cell types (13). Chemical cross-linking of HuIFNa to whole cells (13) or isolated cell membranes (19) has indicated the presence of a single polypeptide of $M_s = 120,000$-130,000, whereas gel filtration and sedimentation analysis of binding to solubilized receptor (19) suggest a $M_s$ closer to 95,000.

To date, it has not been possible to label HuIFNg to a sufficiently high specific activity for binding studies while still retaining high biological activity. Binding studies with this interferon are further complicated by the hydrophobic properties it displays around neutral pH, at which such studies were done, resulting in high levels of nonspecific binding, both to the cells, and to the vessels in which the experiments were performed. In this paper, we report the iodination of HuIFNg and rHuIFN$\beta_{ser}$, with little loss in biological activity, and the use of such preparations to study their bindings to Daudi cells. We report evidence for two binding sites for human interferon, one which recognizes HuIFNg and $\beta$ molecules, and another which recognizes HuIFNg and $\gamma$ molecules, the two sites being specific for HuIFN$\alpha$ or $\gamma$, while HuIFNg can recognize both.

**EXPERIMENTAL PROCEDURES**

*Interferons—HuIFNg was induced from human diploid fibroblast cultures by a previously reported procedure (23) and purified to homogeneity by immunoabsorbent column chromatography and HPLC (24, 25). Recombinant HuIFN$\beta_{ser}$ was extracted from E. coli containing the HuIFNg gene and was generously donated by Cetus Corp. (Emeryville, CA). A interferon was also purified to homogeneity as previously described (25). Samples of HuIFNa and HuIFNg were generously donated by H. Kauppinen of the Finnish Blood Transfusion Center (Helsinki, Finland). Both HuIFNg and $\gamma$ were produced from peripheral human leukocyte. Lentil lectin was used to induce HuIFNg from these cells. Human IFNg was purified by CM-Sephacore chromatography and by A&-A4 gel filtration (LKB, Sweden). Human IFNg was purified by immunoabsorbent chromatography with monoclonal anti-HuIFNg. The $M_s$ of HuIFNg, rHuIFN$\beta_{ser}$, HuIFNa, and HuIFNg was 23,000, 17,000, 18,000, and 55,000, respectively. Mouse IFNg was purchased from Lee Biologicals (La Jolla, CA). Interferon was assayed by the procedure of Armstrong (26) with minor modification. (27). HuIFNg was also assayed by a radioimmunoassay (28). Protein was assayed by the Biorad method (29) using human serum albumin as standard. The HuIFNg standard used was NIH-WHO 002-901-027. The assigned potency of the standard is 200 units/ml. The observed geometric mean titer of the standard is 474 units/ml with a standard deviation of 129 units/ml ($n = 7$). The radioimmunoassay assay of HuIFNg (28) has a coefficient of variability of 1.1% at 500 units/ml. The specific activities of the HuIFNg, rHuIFN$\beta_{ser}$, HuIFNa, and HuIFNg were $4 \times 10^8$, $2 \times 10^8$, and $1 \times 10^8$ units/mg of protein, respectively. The
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HuIFN$\alpha$ standard was NIH-SWHO G02-901-527 and the HuIFN$\gamma$ standard was G23-901-530.

Radioiodination of HuIFN$\beta$-standard—Radioiodination of rHuIFN$\beta$, or HuIFN$\beta$ was based on the method of Hunter and Greenwood (30) with further modifications by Mogensen et al. (31). The radioiodinated interferon was separated from the low-molecular weight reactants by chromatography through a 0.1 ml blue-Sepharose (Pharmacia) column (32). Radioiodination was also performed by the method of Bolton and Hunter (31) as well as by a modification of the iodogen procedure (33). The freshly labeled interferon was analyzed on SDS-PAGE (34) and further analyzed by radioautography using Cronex Lightning Plus intensifying screens and preflushed Kodak XAR film.

Binding of HuIFN to Cell and Cell Membrane—Different amounts of the radioiodinated HuIFN$\beta$ or rHuIFN$\beta$, were incubated with cell membrane (50 $\mu$g of protein prepared by the method of Thom et al. (43)) or with cells (5 x $10^6$ cells) in 100 or 500 $\mu$l of phosphate-buffered saline, respectively, for different times and in the presence or absence of unlabeled HuIFN$\beta$, HuIFN$\beta$, HuIFN$\alpha$, and HuIFN$\gamma$. The reactions were performed in 1.5-ml Eppendorf tubes. At the end of the reaction the unreacted radioiodinated and unlabeled interferon were removed by sedimenting the membranes or whole cells at 10,000 g for 15 or 3 min, respectively, through a 0.5-ml layer of dithyl phthalate-dinonyl phthalate (Manufacturing Chemist, Inc., Darmstadt, Germany) as described (35). The upper layer containing the soluble unbound reactants was removed and the interface was washed twice with 0.3 ml of phosphate-buffered saline. The liquid layer above the pellet was discarded without disturbing the pellet. The pellet at the bottom of the Eppendorf tube was separated from the rest of the tube by cutting with a hot wire. By this procedure, radioiodinated interferon which sticks to the walls of the Eppendorf tube was eliminated from the pellet count. The binding of labeled rHuIFN$\beta$, (1000 units) to Daudi cells (5 x $10^6$) was measured in the presence of increasing concentration of unlabeled HuIFN$\beta$, HuIFN$\beta$, or HuIFN$\gamma$ for 2 h at 4°C and processed as described. A Scatchard plot (38) of these results is given in Fig. 3, inset.

Cross-linking of rHuIFN$\beta$, to Daudi Cells—Radioiodinated rHuIFN$\beta$, was cross-linked to Daudi cells by the procedures described in Refs. 36 and 37 under the following conditions. A total of 5 x $10^6$ Daudi cells were mixed with 5 x $10^6$ units of radioiodinated HuIFN$\beta$, in 0.5 ml of phosphate-buffered saline containing 0.5 mg of human serum albumin in a 1.5-ml Eppendorf tube and left at 4°C for 2 h. The mixture was then layered on a 1-ml pad of 11% sucrose solution and centrifuged at 10,000 x g for 5 min. The supernatant was removed and the cells were resuspended in 150 $\mu$l of phosphate-buffered saline and diluted with either a 1 mM aqueous suspension of disuccinimidyl suberate or 0.1, 0.1, or 1.0 mM ethylene glycol bis(succinimidyl carbonate) and incubated at 4°C for 15 min. The reaction was quenched by adding 1 ml of 0.1 M Tris-HCl, pH 8.5. The cells were either stored at -20°C or, after 15 min on ice, sedimented by centrifugation at 10,000 x g for 5 min. The sedimented cells were dissolved in sample buffer for SDS-PAGE (34) under reducing conditions. The solubilized material was subjected to analyses by SDS-PAGE according to Laemmli (34) using 1.5-mm thick 7.5% gel or a gradient gel of 5–10%. Sample buffer was 62.5 mM Tris buffer, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, and 1 mm EDTA. Samples were boiled for 3 min prior to application to the gel. A mixture of proteins (Pharmacia) of the following $M_r$: 330,000, 220,000, 67,000, 60,000, 36,000, and 18,500 was also included during these SDS-PAGE experiments. At the end of the electrophoresis, the gel was stained with Coomassie blue, destained, and dried. Autoradiography of the dried gel was performed as described above.

RESULTS

Biological Activity of Radioiodinated rHuIFN$\beta$,—All three methods of iodination label both natural and recombinant HuIFN$\beta$, but only the modified Hunter-Greenwood method produced relatively high incorporation (25% of theoretical), for monoiiodinated rHuIFN$\beta$, with minimal (<5%) loss in biological activity. The radiospecific activity varied from 150–450 cpm/unit, compared with 20–60 cpm/unit obtained by the Bolton-Hunter or iodogen procedures, in several experiments. On storage at -70°C both $^{125}$I-labeled HuIFN$\beta$ and rHuIFN$\beta$, retained at least 90% of their biological activity after 5 days and could be used to demonstrate specific binding. Beyond this time, however, specific binding and biological activity deteriorated rapidly, such that 95% of the biological activity was lost by 9 days after radioiodination. A small degree of radiolytic damage became quite evident by the 9th day (Fig. 1). When the biological activity has decreased to 10% of its original value, $^{125}$I-labeled rHuIFN$\beta$, cannot be displaced by unlabeled interferon, implying the loss of specific binding to Daudi cells (Table I). Similar results were obtained when natural HuIFN$\beta$ was radioiodinated (not shown).

Binding of rHuIFN$\beta$, to Cells, Cell Membranes, and Plastic—Radioiodinated rHuIFN$\beta$, with high biological activity (>90%), binds nonspecifically to Daudi cells, booted Daudi cells, human fibroblasts, human fibroblast membranes, and mouse L cells, as well as to the surface of plastic vessels in which the fibroblast cells are grown (Table I). However, in the presence of 100-fold excess of unlabeled rHuIFN$\beta$, the total amount of labeled IFN bound to live Daudi cells, human fibroblasts, and human fibroblast membranes is significantly reduced by 40–50%. This suggests that unlabeled IFN$\beta$ can compete with the labeled IFN$\beta$ for specific binding sites on human cells and human cell membranes. Two hundred- or 250-fold excess of unlabeled IFN$\beta$ did not reduce further the amount of labeled IFN$\beta$ specifically bound to human cells. Aged labeled IFN$\beta$ preparation, which has lost about 90% of its original biological activity, does not bind to specific binding sites on human cells (Table I). Similar results were obtained with $^{125}$I-labeled HuIFN$\beta$ (not shown).

The time course of binding of $^{125}$I-labeled rHuIFN$\beta$, to Daudi cells is shown in Fig. 2A. Equilibrium binding to the

![Fig. 1. Analysis of $^{125}$I-labeled rHuIFN$\beta$, by SDS-PAGE in 7% polyacrylamide. The figure shows an autoradiogram of a dried gel. Lane a, 9-day-old labeled IFN; lane b, 22-day-old labeled IFN; lane c, main fraction of a freshly labeled IFN eluted from a blue-Sepharose column, which separates labeled IFN from the low-molecular-weight reactants used during radioiodination; lanes d and e are similar to lane c, except that the labeled IFN are from fractions eluted immediately after the main fraction in lane c. The arrow indicates the band corresponding to the monomeric ($M_r = 17,000$) and tetrameric ($M_r = 68,000$) forms of labeled IFN.](image-url)
cells occurred after 2 h, both in the presence or absence of a 100-fold excess of unlabeled interferon. When Daudi cells were incubated with increasing concentrations of 125I-labeled HuIFNβ, the amount bound specifically (displaceable by unlabeled IFNβ) increased proportionately up to 1000 units/5 × 10^6 cells, whereafter the amount bound leveled off. The amount nonspecifically bound (not displaceable by unlabeled IFNβ) increases proportionately with the amount of labeled IFNβ (Fig. 2B).

**Competition between Various Interferons for Binding to Daudi Cells**—Once it was established that 1000 units/5 × 10^6 cells approached saturation for specific binding, competition between 125I-labeled rHuIFNβser and unlabeled rHuIFNβser, HuIFNα, or HuIFNγ for specific binding to Daudi cells was studied. HuIFNα or HuIFNγ in addition to rHuIFNβser can compete with 125I-labeled rHuIFNβser for binding to Daudi cells. The amount of labeled IFNβ bound specifically to Daudi cells at different concentrations of excess amount of unlabeled IFNβ, α, and γ is given in Fig. 3. These binding data were analyzed by Scatchard plot (Fig. 3, inset), which shows a linear correlation between binding of 125I-labeled rHuIFNβser and competition from either HuIFNβser, HuIFNα, or HuIFNγ. In all cases, correlation coefficients were better than 0.95. The analysis reveals the presence of about 17,000, 34,000, and 565 sites/cell, with an apparent Kd of 3.7 nM, which recognize HuIFNα, and 0.77 sites/cell, with an apparent Kd of 1.1 nM, which recognize HuIFNγ.

**Cross-linking of Bound 125I-labeled rHuIFNβser to Daudi Cells**—Saturation levels of 125I-labeled rHuIFNβser were cross-linked to Daudi cells after incubation for 2 h at 4 °C, and analyzed by SDS-PAGE under reducing conditions. When 1 mM disuccinimidyl suberate was used as the cross-linker, 125I-labeled β interferon was found associated with a macromolecular complex of Mr = 125,000—145,000 (Fig. 4A, lane a). Unlinked monomeric, dimeric, and tetrameric forms of 125I-labeled rHuIFNβser, with Mr values of 17,000, 34,000, and 68,000, respectively, are also observed on the autoradiogram. Attempts were also made to cross-link the bound labeled-IFNβ to Daudi cells by a reversible cross-linker such as ethylene glycol bis(succinimidyl succinate). When 0.1 or 1.0 mM ethylene glycol bis(succinimidyl succinate) was used as the cross-linker, 125I-labeled rHuIFNβser was found in association with a complex of Mr = 145,000 (Fig. 4B, lanes b and c). In addition, a second component, Mr = 120,000, was also seen on all occasions when 0.1 mM of the cross-linker was used (Fig. 4B, lane b). No cross-linked complex, except excessively cross-linked aggregates, were seen at 0.01 mM ethylene glycol bis(succinimidyl succinate) (Fig. 4B, lane a). Again, monomeric, dimeric, and tetrameric forms, in addition to the excessively cross-linked aggregates, are also seen. It appears that 0.1 mM ethylene glycol bis(succinimidyl succinate), is the optimal concentration of cross-linker to cross-link IFNβ to its binding sites on Daudi cells. When unlabeled rHuIFNβser is incubated, the amount of cross-linked complexes are reduced or not present (Fig. 4A, lanes b, c, and d; Fig. 4B, lanes d, e, and f).

**DISCUSSION**

Although we have previously reported that chloramine-T and tetranitromethane rapidly inactivate HuIFNβ (39), implying the presence of tyrosine residues essential for biological
activity, we have found it possible, by applying the modification of Mogensen et al. (16), to obtain moderate levels of incorporation of 125I into the protein with minimal loss in biological activity. This suggests that at least one available tyrosine (or possibly histidine) residue is not essential for biological activity. Since biological activity is lost rapidly after radiolysis, one likely explanation is that protein in solution is denatured by free radicals generated by high-energy emitting isotopes. The correlation between specific binding and biological activity supports the previous explanation that the native conformation of the molecule is essential for its specific binding (15).

Radioiodinated rHuIFNβsa, binds to human cells, human cell membranes, boiled Daudi cells, mouse L cells, and to plastic. However, only the IFNβ bound to human cell and human cell membrane is displaced by unlabeled IFNβ (Table I). The bound IFNβ could also be cross-linked to Daudi cells and the amount of labeled IFN cross-linked is reduced by adding unlabeled IFNβ during binding. These observations suggest that the labeled IFNβ binds to specific binding sites. Treatment of Daudi cells with a low concentration of Pronase (10 μg/ml) for 30 min at 37°C, which does not cause cell death, eliminated the specific binding of labeled IFNβ to these cells (not shown). This suggests that the binding sites are protein(s) and, as shown, are denaturable by heat (Table I). Unlabeled mouse IFNβ (100-fold excess) did not displace the bound labeled IFNβ from Daudi cells, suggesting the binding sites are species specific.

The new observations from this work, compared with what is published on HuIFNα and HuIFNγ receptor binding sites (13–22), is that both IFNα and IFNγ compete with rHuIFNβsa for specific binding to Daudi cells (Fig. 3). In consideration of the previous reports on IFNα and IFNγ receptors and the data herein, we propose the existence of two types of HuIFNβ receptor sites on Daudi cells. One type of receptor site binds IFNα and IFNβ. Another type of receptor binds IFNγ and IFNβ. Both receptors will bind only IFNα or IFNγ but not both. To test this hypothesis, it will be useful to study the effects of monoclonal antibodies raised against

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**Fig. 3.** The binding of 125I-labeled rHuIFNβsa, to Daudi cells in the presence of increasing amounts of unlabeled rHuIFNβsa, (○), HuIFNα (●), HuIFNγ (□). See "Experimental Procedures" for details. Each point is the mean of triplicate experiments. Scatchard analysis of these binding data is given in the inset.

**Fig. 4.** A, cross-linking of 125I-labeled rHuIFNβsa, to Daudi cells by disuccinimydyl suberate and analysis of the cross-linked complex by SDS-PAGE in 5–10% polyacrylamide. For details, see "Experimental Procedures." The figure shows an autoradiogram of a dried gel. Lane a shows radioiodinated interferon cross-linked to Daudi cells. The other lanes show radioiodinated interferon cross-linked to Daudi cells in the presence of 1-fold excess (lane b), 10-fold excess (lane c), and 100-fold excess (lane d) of unlabeled HuIFNβsa. The Mr markers: thyroglobulin (330,000); ferritin (220,000); albumin (67,000); catalase (60,000); lactate dehydrogenase (36,000). The arrow indicates the position of the interferon-receptor binding complex, M = 125,000 to 145,000. B, cross-linking of 125I-labeled rHuIFNβsa, bound to Daudi cells by different concentration of ethylene glycol bistuccinimydyl succinate and analysis of the cross-linked complex by SDS-PAGE in 7% polyacrylamide. For details, see "Experimental Procedures." The figure shows an autoradiogram of a dried gel. Lanes a–c show radioiodinated rHuIFNβsa, cross-linked to Daudi cells at 0.01, 0.1, and 1 mM of the cross-linker, respectively. Lanes d–f are radioiodinated interferon cross-linked to Daudi cells by 0.01, 0.1, and 1 mM of the cross-linker in the presence of a 100-fold excess of unlabeled rHuIFNβsa. The molecular weight markers are similar to the ones used in A. The position of the two arrows indicates the interferon-receptor binding complex of M = 145,000 and 120,000.
different epitopes on the IFNβ molecule on the inhibition of IFNα binding by IFNα and IFNγ, or, alternatively, to study the displacement of IFNβ by constructing (40) IFNa(IFN-γ) hybrid molecules. Furthermore, the inability to produce completely neutralizing monoclonal antibodies to HuIFNP (25, 42) is consistent with the concept of the two types of IFNβ binding sites.

Although the Scatchard analysis of the binding data in Fig. 3 (inset) provided only one Kd value for rHuIFNαβ, it is possible that the apparent Kd represents a median of two closely related Kd values for rHuIFNαβ. Bearing in mind that these are apparent Kd values, hence only relative values, it is surprising to note that the total number of IFNβ sites (10,000) is equal to the sum of the sites (10,300) displaceable by IFNα and IFNγ (Fig. 3, inset). Such an agreement, albeit relative, is quantitatively consistent with the given hypothesis.

Validation of this hypothesis would be strengthened by the demonstration of two binding sites for HuIFNP. To date, only two putative binding sites should have M values of 103,000 and 128,000. The observation of Pestka et al. (41) that the HuIFNβ functions as a dimer may at first suggest that the two complexes are derived from a single receptor cross-linked to a monomeric or a dimeric form of the interferon molecule. It is also unlikely that the M value of 103,000 component is a degradation product of the M value of 128,000 component since it was not observed when 0.01 and 1.0 mM ethylene glycol bis(succinimyld succinate) were used, instead of 0.1 mM. However, we have not fully demonstrated differential displacement from either of these components by HuIFNα or HuIFNγ. Further detailed studies are in progress to show the quantitative displacement of labeled rHuIFNαβ from the receptors by unlabelled HuIFNα and HuIFNγ. Gel analysis of the cross-linked receptor-interferon complexes performed under non-reducing conditions shows the same bands as seen during reducing conditions. Thus, it is likely that the two receptor binding components are not held together by disulfide bond(s).

The results herein suggest the presence of two HuIFNβ binding sites, one recognizing HuIFNα and -β and the other IFNγ and -β. Similar results were also obtained with 125I-labeled natural HuIFNβ, suggesting that both natural HuIFNβ and recombinant HuIFNαβ behave similarly with respect to their binding to Daudi cells.

REFERENCES