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IMMUNOLOGIC DIFFERENTIATION BETWEEN *E. COLI* AND CHO CELL-DERIVED RECOMBINANT AND NATURAL HUMAN β-INTERFERONS[1]

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The products of the human IFN-β gene expressed in *E. coli*, Chinese hamster ovary (CHO) cells, and human fibroblasts appear similar when purified on a monoclonal antibody column and analyzed by reverse-phase HPLC, indicating little difference in their hydrophobic nature. SDS-PAGE differentiates *E. coli*-rHuIFN-β_{ser} (M_r = 17,000) from CHO-rHuIFN-β and HuIFN-β (M_r = 23,000), with glycosylation accounting for 26% of the apparent m.w. of the latter two proteins. CHO-rHuIFN-β is preferentially neutralized by mouse monoclonal and monospecific rabbit polyclonal anti-HuIFN-β antibodies, whereas *E. coli*-rHuIFN-β_{ser} is preferentially neutralized by goat polyclonal anti-*E. coli*-rHuIFN-β antibodies. Adsorption measurements by a sensitive radioimmunoassay indicate that the binding of the three proteins to anti-HuIFN-β antibodies is similar. The results show that all three molecules can be differentiated by the heteroclitic cross-reactivities of anti-HuIFN-β and anti-*E. coli*-rHuIFN-β antibodies to the antigens.

The HuIFN-β[4] gene has been cloned and expressed in *E. coli* (1–4). The bacterially produced *E. coli*-rHuIFN-β is unglycosylated and therefore has a smaller m.w. than the glycosylated HuIFN-β. The biologic activity of *E. coli*-rHuIFN-β is unstable, but it can be stabilized by replacing the cysteine at residue number 17 with a serine residue (5). The lack of glycosylation of *E. coli*-rHuIFN-β has raised a question concerning the role of glycosylation, if any, in the biologic actions and physiochemical properties of this molecule. Thus far, a comparison of both the biologic activity of *E. coli*-rHuIFN-β_{ser} and HuIFN-β indicate little difference between the two molecules (5). However, a key question of whether the bacterial IFN is as efficacious and as safe as HuIFN-β produced by human fibroblasts remains unanswered. This has led a number of investigators to express the HuIFN-β genes in glycosylating systems such as Chinese hamster ovary (CHO) cells, mouse cells, or insect cells (6–13). The CHO cells, transfected by the HuIFN-β gene linked to appropriate promoters, have been shown to produce 10 to 200 times more IFN than the amount produced in human fibroblasts. This recombinant HuIFN-β product is glycosylated, having a similar m.w. to the "natural" HuIFN-β produced by human fibroblasts (6).

The biologic properties of the HuIFN-β gene expressed in human fibroblast, CHO cells, and *E. coli* are remarkably similar (5, 6). We have examined the interactions between highly purified preparations of these three IFN with three different immunologic reagents: a mouse monoclonal antibody and monospecific rabbit polyclonal antibodies were raised in response to native HuIFN-β as immunogen, and goat polyclonal antibodies were prepared by using *E. coli*-rHuIFN-β as immunogen. We report that *E. coli*-rHuIFN-β_{ser}, CHO-rHuIFN-β, and native HuIFN-β differ significantly from each other in their sensitivity to neutralization with, but not in their binding to, monoclonal and polyclonal antibodies.[5]

MATERIALS AND METHODS

IFN. HuIFN-β and CHO-rHuIFN-β were produced from human diploid fibroblasts and CHO cells containing the HuIFN gene, respectively, by the procedure described (15, 16). These IFN preparations were then purified by passage through an immunosorbent column, followed by high pressure liquid chromatography (HPLC) on a Brownlee Aquapore RP-300 analytical column (0.46 x 10 cm, 30 nm pore size; 10 μm bead size; and C18 carbon chain length) according to the method of Smith-Johannsen and Tan (17), except that the immunosorbent column was made with mouse monoclonal anti-HuIFN-β immunoglobulin instead of rabbit polyclonal anti-HuIFN-β antibodies. Recombinant *E. coli*-rHuIFN-β_{ser} was produced in and extracted from *E. coli*, purified to homogeneity, and stabilized with human serum albumin. Both CHO-rHuIFN-β and *E. coli*-rHuIFN-β_{ser} were gifts from Cetus Corporation, Emeryville, CA. The purified IFN preparations were analyzed on slab SDS-PAGE according to Laemmli (18) by using 0.75 mm thick 12% acrylamide gels. Proteins were detected by silver staining (19). HuIFN-β activity was assayed by the method of Armstrong (20). All samples were assayed in triplicate except for samples in experiments comparing the binding of the various IFN with their antibodies; these were assayed in quintruplicate. All units of IFN activity given herein were referenced to a HuIFN-β standard, as described (21), which in turn is referenced to a HuIFN-β (NIH-WHO GO2-901-527) standard. The mean titer of the IFN standard is 192 IU per milliliter, with a coefficient of variation of 14.9%. Protein was assayed after the method of Sedmak and Grossberg (22) by using human serum albumin as a standard. Pure HuIFN-α was a gift from H. L. Kauppinen of the Finnish Red Cross.

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[4] Abbreviations used in this paper: HuIFN, human interferon; rHuIFN, recombinant human interferon; HuIFN-α, human leukocyte interferon; HuIFN-β, human fibroblast interferon; CHO-rHuIFN-β, recombinant human fibroblast interferon-β expressed in Chinese hamster ovary cells; CHO, Chinese hamster ovary; *E. coli*-rHuIFN-β_{ser}, recombinant human fibroblast interferon-β substituted in cys-17 by ser-17; *E. coli*-rHuIFN-β, recombinant human interferon-β expressed in *E. coli*; HPLC, high pressure liquid chromatography.

[5] During the preparation of this manuscript, Le *et al.* (14) reported a monoclonal antibody, TIF-1, that was found to neutralize natural HuIFN (IFN-γ) but not *E. coli*-derived rHuIFN-γ.

(Helsinki, Finland).

Antibodies to IFN. Rabbit polyclonal and mouse monoclonal anti-HuIFN-β produced in this laboratory as described (23, 24) were purified by an HuIFN-β affinity column (21). Goat anti-*E. coli*-rHuIFN-β raised in the goat by using purified *E. coli*-rHuIFN-β was a gift from Dr. Leo Lin (Cetus Corp.).

Immunoglobins purified by affinity chromatography were incubated with each preparation of pure HuIFN, derived from the different cell types, to neutralize the anti-viral activity of each IFN. The neutralization procedure consisted of i) serially diluting a given concentration of pure IFN twofold in Microtest II wells, ii) adding and mixing an equal volume of growth medium (minimal Eagle's medium with 5% fetal calf serum) containing a fixed concentration of anti-IFN immunoglobulin(s) with the serially diluted IFN and incubating at 37°C in a CO_2 incubator for 1 hr, iii) transferring each of the antibody/IFN mixtures to a monolayer of diploid human fibroblast cultures grown in Microtest II wells and incubating for 16 hr, iv) and thereafter, challenging the human fibroblast cultures in the Microtest II wells with virus and assaying for anti-viral activity. The anti-viral titers of the antibody/IFN mixture and a control in which anti-IFN immunoglobulin was omitted were compared. The difference between the two represents the amount of IFN activity neutralized during incubation by the fixed amount of anti-HuIFN-β. Each assay was done in triplicate.

The binding of IFN to its immunoglobulins was assayed by a sensitive double-sandwiched radioimmunoassay (25) modified after the method of Inoue and Tan (21). Briefly, the purified mouse monoclonal immunoglobulin was immobilized to the walls of a polystyrene cuvette. The IFN and monospecific rabbit anti-HuIFN-β was incubated with the polystyrene matrix containing the immobilized monoclonal antibody molecules. The complex was then incubated with I^{125}-labeled donkey anti-rabbit immunoglobulin, which bind to rabbit anti-HuIFN-β on the IFN/polystyrene complex. The coefficient of variation of this binding assay is <5% from 10 to 1000 U of HuIFN-β. It is instructive to note the background count in the control of this immunoprecipitation is low, in the range of 140 to 180 cpm.

RESULTS

Comparison of E. coli-rHuIFN-β$_{ser}$, CHO-rHuIFN-β, and HuIFN-β by immunosorbent column chromatography and HPLC. When HuIFN-β, CHO-rHuIFN-β, and *E. coli*-rHuIFN-β$_{ser}$ are purified separately by immunosorbent column chromatography, followed by chromatography on a reverse phase column, these IFN have similar purification patterns. For example, all three IFN are retained on the immunosorbent column and are eluted by glycine-HCl buffer (pH 2.0). Each of the three IFN have almost identical migration rates on the reverse phase HPLC column (Fig. 1).

SDS-PAGE comparison of HuIFN-β, CHO-rHuIFN-β, and E. coli-rHuIFN-β$_{ser}$. Analysis of the HPLC-purified proteins by SDS-PAGE indicates that they consist of a single protein staining band (Fig. 2). Both HuIFN-β and CHO-rHuIFN-β migrate with an apparent m.w. of 23,000, whereas *E. coli*-rHuIFN-β$_{ser}$ migrates with an apparent m.w. of 17,000, a difference that was expected because *E. coli*-rHuIFN-β is not glycosylated. The pure IFN preparations are used in the neutralization and binding studies reported herein.

Neutralization of HuIFN-β, CHO-rHuIFN-β, and E. coli-rHuIFN-β$_{ser}$ by antibodies to IFN. Mouse monoclonal anti-HuIFN-β was found to neutralize the anti-viral activity of CHO-rHuIFN-β 12 times more effectively than HuIFN-β or *E. coli*-rHuIFN-β$_{ser}$ (Table I). Similarly, rabbit anti-HuIFN-β was found to neutralize the anti-viral activity on CHO-rHuIFN-β more effectively than HuIFN-β or *E. coli*-rHuIFN-β$_{ser}$ by threefold to fourfold (Table I). Goat anti-*E. coli*-rHuIFN-β neutralized the anti-viral activity of *E. coli*-rHuIFN-β$_{ser}$ 10 and six times more than HuIFN-β or CHO-rHuIFN-β, respectively (Table I). The antibodies do not cross-react with HuIFN-α. These values were reproducible by repeated assays at different times.

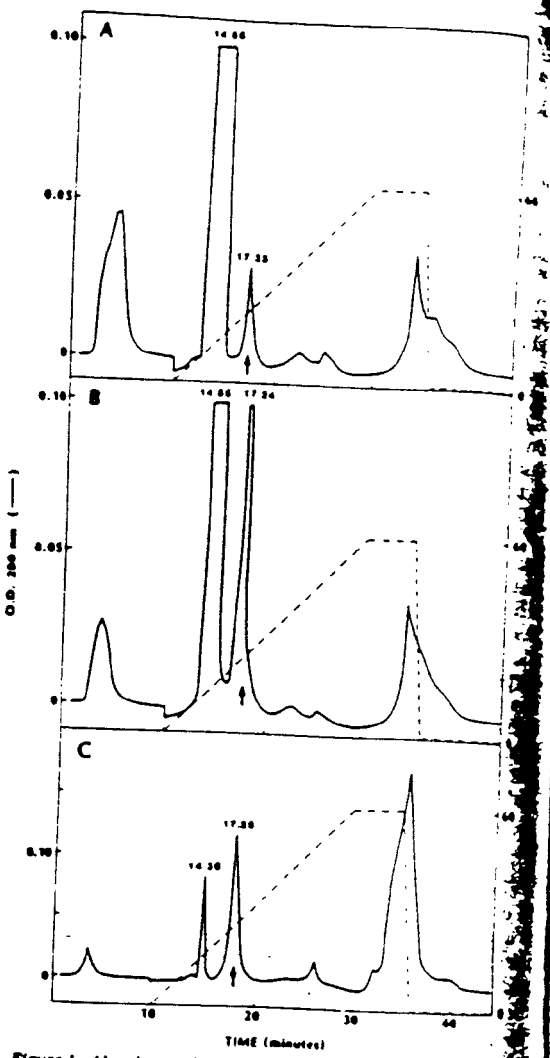

Figure 1. Absorbance (280 nm) profile of immunosorbent-purified IFN on a Brownlee Aquapore RP-300 column. HPLC was carried out as described in *Materials and Methods*. Arrow indicates peak fraction of IFN. A, CHO-rHuIFN-β; B, *E. coli*-rHuIFN-β$_{ser}$, and C, HuIFN-β. The numbers above the IFN peak indicate the time in minutes when the IFN was eluted.

Binding of HuIFN-β, CHO-rHuIFN-β, and E. coli-rHuIFN-β$_{ser}$ to anti-HuIFN-β immunoglobulins. Although all three types of human IFN were retained on the immunosorbent column made with monoclonal anti-HuIFN-β immunoglobulin, small variations in the recovery and retention of human IFN in this chromatographic step makes it difficult to assess small differences in the binding of the three types of IFN to their immunoglobulins. A sensitive and precise radioimmunoassay was recently developed (24) and was used to compare the binding of HuIFN-β, CHO-rHuIFN-β, and *E. coli*-rHuIFN-β$_{ser}$ with anti-HuIFN-β antibodies. The assay measures the amount of each type of IFN bound to the antibody molecules. The results of this radioimmunoassay show that the three types of IFN have similar titration curves (Fig. 3).

DISCUSSION

Because HuIFN-β, CHO-rHuIFN-β, and *E. coli*-rHuIFN-β are products of the HuIFN-β gene and have similar biologic activities (6), all three IFN might be expected to cross-react with antibodies against the polypeptide backbone of any one of the three IFN. As anticipated, all of the IFN are found to cross-react with polyclonal rabbit anti-HuIFN-β (Table I). However, the extent to which these antibodies neutralize the three human IFN is significantly different (Table I).

Figure 2. SDS-PAGE analysis of purified HuIFN-β. Samples were heated for 3 min in buffer containing 100 mM thioglycolic acid shortly before application in the gel slots. Proteins were stained by silver staining. Lane *A*, protein markers (Pharmacia low m.w. standards): phosphorylase B (M_r = 94,000), albumin (M_r = 67,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), soyabean trypsin inhibitor (M_r = 20,000), and α-lactalbumin (M_r = 14,400). Lane *B*, HuIFN-β. Lane *C*, *E. coli*-rHuIFN-β. Lane *D*, CHO-rHuIFN-β.

The binding of the three proteins of anti-HuIFN-β antibodies was compared by immunosorbent column chromatography and by two immunoprecipitation procedures. It was not possible to distinguish small differences between the binding of the three proteins to an anti-HuIFN-β column because the reproducibility in the recovery and retention of HuIFN-β in this chromatographic step is too low to confidently permit quantitative comparisons. In one immunoprecipitation procedure (21), CHO-rHuIFN-β and HuIFN-β were found to immunoprecipitate I^{125}-labeled polyclonal rabbit anti-HuIFN-β antibodies equally well (not shown). This suggests that overall, both types of IFN have similar binding affinities for the polyclonal monospecific rabbit anti-HuIFN-β antibodies. In another immunoprecipitation procedure (25), CHO-rHuIFN-β appeared to bind slightly better to monoclonal and polyclonal anti-HuIFN-β antibodies as did HuIFN-β (Fig. 3). However, these differences fall within the variation of the biologic assay.

Thus, the HuIFN-β gene, when expressed in different host systems, led to the production of protein molecules that are antigenically distinct. A similar situation exists for HuIFN-γ. A monoclonal antibody capable of neutralizing native HuIFN-γ neither neutralizes nor binds to *E. coli*-derived rHuIFN-γ (14). Furthermore, these antigenic differences appear to be due to different conformations of the two HuIFN-γ, rather than the absence of glycosylation per se (14). We found that CHO-rHuIFN-β is neutralized more effectively than HuIFN-β by both immunoreagents. The mouse monoclonal anti-HuIFN-β antibody is 10-fold more effective and the rabbit polyclonal anti-HuIFN-β antibodies are 3.3-fold more effective (see Table I).

It seems unlikely that these differences in neutralization are due to differences in the specific activity of the IFN molecules, because CHO-rHuIFN-β and HuIFN-β have identical biologic specific activities, and the specific activity of *E. coli*-rHuIFN-$β_{ser}$ is only slightly lower (Table I). It also seems unlikely that the differences could be attributed to impurities, because the IFN used were found to be pure by two criteria (Figs. 1 and 2), and the same ranges of concentrations of IFN were used in these neu-

TABLE I

The neutralization of CHO-rHuIFN-β, E. coli-rHuIFN-$β_{ser}$, and HuIFN-β by antibodies to HuIFN-β[1]

IFN	Specific Activity (IU/mg)	IU of HuIFN-β Activity Neutralized by		
		Rabbit anti-HuIFN-β	Mouse monoclonal anti-HuIFN-β	Goat anti-*E. coli*-HuIFN-β
CHO-rHuIFN-β	2.3×10^8	108 +/− 23[a]	60 +/− 13[d]	346 +/− 73[g]
Natural HuIFN-β	2.4×10^8	33 +/− 7[a]	6 +/− 1[e]	192 +/− 41[h]
E. coli rHuIFN-$β_{ser}$	1.5×10^8	25 +/− 5[b]	6 +/− 1[f]	2000 +/− 424[i]
HuIFN-α	2.0×10^8	None	None	None

[1] Each value represents the mean and standard deviation, n = 3. Significance test between *a* and *b* ($p < 0.01$); *a* and *c*, *d* and *e*, *d* and *f*, *g* and *i*, *h* and *i* ($p < 0.005$); *b* and *c* ($p < 0.1$); *g* and *h* ($p < 0.02$). Rabbit polyclonal, mouse monoclonal, and goat polyclonal antiserum was diluted 2000-, 15-, and 800-fold, respectively, before incubating with preparations of CHO-rHuIFN-β, HuIFN-β and *E. coli*-rHuIFN-$β_{ser}$, which were serially diluted in microtest plates. The concentrations of all three IFN preparations were adjusted to 10,000 IU/ml before serial dilution. Details of the neutralization assay are given in *Materials and Methods*. The amount of IFN neutralized is determined by the following formula:

$$\text{IFN (IU) neutralized} = \frac{f^c}{f^s} - 1$$

in which f = serial dilution factor of IFN, $c = c^{50}$ well in which the end point of the IFN preparation without antibody is determined, $s = s^{50}$ well in which the end point of IFN/antibody mixture is determined. IFN activities are standardized as described in *Materials and Methods*.

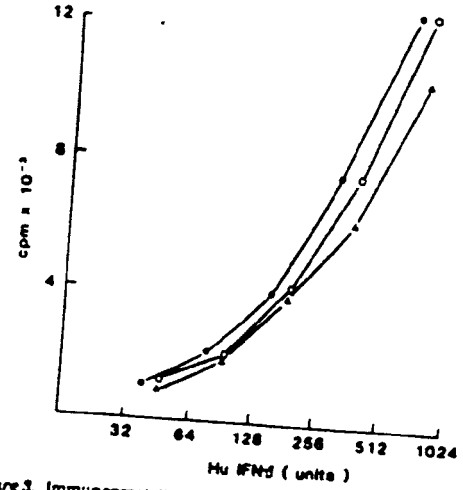

Figure 3. Immunoprecipitation of CHO-rHuIFN-β (●), *E. coli*-rHuIFN-β_{ser} (○), and HuIFN-β (△) polyclonal and monoclonal antibody complex. The background counts in the controls ranged from 140 to 180 cpm.

tralization reactions (Table I). The heterolitic nature of antibody cross-reactivity has also been reported for a monoclonal antibody to beef myoglobin (I. East, D. Dorrow, and S. J. Leach, personal communication) and between mutant and original H-2 antigen (26).

Because the HuIFN-β gene is expressed in a hamster cell environment, it is possible that the CHO cells glycosylate HuIFN-β differently than do human fibroblast cells. Such differences in glycosylation may result in an unmasking of the anti-viral site on the CHO cell-produced IFN molecule, with the site becoming more accessible to the anti-HuIFN-β antibody while the overall conformation of the protein molecule remains unchanged. Alternatively, differences in glycosylation could produce overall conformational differences between the molecules such that the anti-viral site on the IFN molecule cross-reacts with higher affinity with the neutralizing antibody. In either case, CHO-rHuIFN-β would be preferentially neutralized by anti-HuIFN-β, as reported in Table I.

Although anti-HuIFN-β immunoglobulins neutralize CHO-rHuIFN-β more effectively than their own antigen, the polyclonal goat anti-*E. coli*-rHuIFN-β was, as expected, found to cross-react with *E. coli*-rHuIFN-β_{ser} more than with IFN produced from CHO or human fibroblast cells (Table I), suggesting that the bacterial IFN is also immunologically different from mammalian cell-produced HuIFN-β. It is known that the smaller m.w. *E. coli*-produced IFN is not glycosylated (Fig. 2), and that strong denaturant (1% SDS) is required to extract the IFN from recombinant bacteria, whereas both CHO and human fibroblasts secrete HuIFN-β into the culture medium, thus eliminating the need of an extraction step. This difference could very well contribute to an *E. coli*-rHuIFN-β conformation, which is different from the mammalian cell-produced IFN. By this approach, it is possible to differentiate HuIFN-β, CHO-rHuIFN-β, and *E. coli*-rHuIFN-β_{ser} on the basis of the different extents to which the anti-viral activities of each IFN are neutralized by the three anti-IFN antibodies used in this study. Additionally, the degree of cross-neutralization is different in each case.

In view of the immunologic nonidentity of the IFN, it is important to know whether these *in vitro* immunologic differences are significant enough for the host's immune system to perceive the recombinant HuIFN-β as foreign. If so, the recombinant HuIFN-β could elicit an antigenic response *in vivo*. Recently, it was reported that recombinant HuIFN-α was antigenic in several human cancer patients treated i.v. with recombinant HuIFN-α (27), whereas an antigenic response to IFN is rarely observed in human cancer patients treated with either natural HuIFN-α, HuIFN-β, or both (T. A. McPherson, Y. H. Tan, and H. L. Kauppinen, personal communication). Hence, it would be instructive to know whether recombinant HuIFN-α is also immunologically nonidentical to natural HuIFN-α *in vitro*. If so, this could explain the antigenic response elicited *in vivo* by recombinant HuIFN-α.

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