MOLECULAR AND CELLULAR BIOLOGY, Dec. 1984, p. 2745–2749
0270-7306/84/122745-05$02.00/0

Vol. 4, No. 12

Binding of ^{125}I-Labeled Recombinant β Interferon (IFN-β Ser_{17}) to Human Cells

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Received 11 May 1984/Accepted 11 September 1984

We investigated the binding of ^{125}I-labeled β interferon (IFN-β Ser_{17}), a nonglycosylated recombinant human fibroblast interferon in which cysteine at position 17 is replaced by serine by site-specific mutagenesis. An optimized chloramine T radiolabeling method produced a highly labeled, fully active ^{125}I-IFN suitable for these studies. Unlike the case with the chloramine T method, incorporation of a single mole of Bolton-Hunter reagent into a mole of IFN-β Ser_{17} led to nearly complete loss of biological activity. ^{125}I-IFN-β Ser_{17}, prepared by the chloramine T method, bound specifically to human lymphoblastoid cells (Daudi) with a dissociation constant of 0.24 nM. The number of binding sites per cell was 4,000. In competition assays, unlabeled β interferons (native, recombinant IFN-β Cys_{17}, and various preparations of IFN-β Ser_{17}) equally displaced labeled IFN-β Ser_{17} on Daudi cells. Recombinant IFN-α-1 displaced ^{125}I-IFN-β binding to Daudi cells less efficiently than did unlabeled native or recombinant β interferon. However, at the concentrations tested, native γ interferon showed no competition with ^{125}I-IFN. Our results indicate that IFN-β Ser_{17} and native IFN-β posses similar binding properties.

Interferons exert multiple effects on cells. These effects include induction of an antiviral state, inhibition of growth, modulation of the immune system, and perturbation of cell membrane characteristics. Recently it has been shown that α and γ interferons bind specifically to cells through high-affinity receptors (1, 3, 7). Because of the difficulty in obtaining sufficient quantities of pure native β interferon, few studies on the radioiodination and binding of β interferon have been published. Recent reports on the α interferon receptor have bypassed this obstacle by competing radiolabeled α interferon with unlabeled β preparations (7, 15, 25). Two of these studies have shown that α and β interferons share a common receptor that does not bind γ interferon. Others have shown that β interferon competes well with both α and γ interferons and thus may bind to either of the two receptors (3). The human fibroblast interferon gene has recently been cloned, modified by site-specific mutagenesis, and expressed in *Escherichia coli* to generate a stable, highly active β interferon termed IFN-β Ser_{17} (cysteine at position 17 in the native β interferon sequence has been replaced with serine; D. Mark, S. Lu, A. Creasey, R. Yamamoto, and L. Lin, submitted for publication). IFN-β Ser_{17}, as expressed in *E. coli*, is unglycosylated and lacks the N-terminal methionine found in native IFN-β. The availability of large amounts of recombinant IFN-β Ser_{17} has allowed us to address the following questions. (i) Can IFN-β Ser_{17} be iodinated to high specific radioactivity with retention of full biological activity? (ii) Does iodinated IFN-β Ser_{17} bind specifically to human cells? What is the affinity of IFN-β1 Ser_{17} binding; how many IFN-β binding sites are there per cell? (iii) Do native β interferon, native γ interferon, recombinant α interferon, and formulated and unformulated purified IFN-β Ser_{17} compete with radiolabeled IFN-β Ser_{17} for binding?

Besides developing methods for radioiodination of β interferon suitable for direct characterization of the IFN-β receptor, we also wished to determine whether the binding of interferon to various cells is a useful indicator of cellular response. This report represents the first step in determining whether receptor binding can be used as a screening test for patient selection before IFN-β Ser_{17} therapy. This work is part of an ongoing study of the biochemical and biological nature of IFN-β Ser_{17}, a recombinant interferon currently in the initial phases of clinical trials (18).

MATERIALS AND METHODS

Interferons. IFN-β Ser_{17} was purified from *E. coli* by selective solvent extraction and size exclusion chromatography (M. W. Konrad and L. S. Lin, PCT Int. Appl. WO 83 03,103). All IFN-β Ser_{17} samples used in this study were greater than 98% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17), Fast Green staining (12), and quantitative densitometry with a Shimadzu CS-930 densitometer. Specific activity of the purified protein was 1×10^8 to 2×10^8 U/mg. Formulated IFN-β Ser_{17} samples contained 0.25 mg of IFN-β Ser_{17} per ml, 12.5 mg of human serum albumin per ml, and 1.25% dextrose (pH 7.0 to 7.5). Unformulated IFN-β Ser_{17} contained 0.1% SDS in 0.1 M Tris buffer (pH 7.5). Partially purified preparations of recombinant IFN-α-1 (1.5×10^8 U/mg; Cetus Corp., Emeryville, Calif.) and recombinant IFN-β Cys_{17} (4×10^6 U/mg; Cetus Corp.), native human fibroblast interferon β (3×10^5 U/mg, a kind gift from Y. C. Tan, University of Calgary, Alberta, Canada), and native human γ interferon (5×10^7 U/mg, Meloy Laboratories Inc., Springfield, Va.) were also used in this study.

Iodination. IFN-β Ser_{17} was radiolabeled by either the method of Bolton and Hunter (6) or a modification of the chloramine T method of Hunter and Greenwood (13). Optimal radiolabeling protocols were developed by using low-specific-radioactivity compounds. $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.) was diluted with unlabeled NaI to a specific activity of ca. 1 μCi/μmol. Low-specific-radioactivity diiodo-Bolton-Hunter reagent (ca. 8 μCi/μmol) was prepared from *N*-succinimidyl 3-(4-hydroxyphenyl) propion-

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ate (Pierce Chemical Co., Rockford, Ill.) and was purified by the procedure of Bolton and Hunter (6).

Iodination of IFN-β Ser_{17} (0.01 μmol of protein per reaction) with diiodo-Bolton-Hunter reagent (0 to 0.4 μmol) was performed in 145 μl of 50 mM sodium phosphate (pH 7.5), 0.1% in SDS and 20% in dimethylformamide. After a 30-min incubation at 23°C, reactions were quenched by diluting them 100-fold into a Tris-glycine buffer (a 10-fold concentrate of the SDS-PAGE running buffer of Laemmli [17]). Samples of the quenched reaction mixture were subjected to SDS-PAGE analysis (17) to separate covalently coupled ^{125}I from unbound radioactive by-products. Unfixed, unstained gels were dried after electrophoresis. Labeled protein bands were located by autoradiography, bands were excised from the dried gel, and incorporation of ^{125}I was quantitated by gamma counting (76% efficiency). Specific radioactivities were calculated from the amount of coupled radioisotope and the known amount of interferon employed in the labeling reaction. (Based on the determination of mass by quantitative amino acid analysis, a 1% solution of IFN-β Ser_{17} has an absorbance at 280 nm of 17.) Biological activities of iodinated samples were determined by a cytopathic effect assay (see below).

Low or high specific radioactivity iodinations of IFN-β Ser_{17} by the chloramine T method were performed in 0.1 M Tris-hydrochloride (pH 7.5), 0.1% in SDS (Tris-SDS). For high-level iodinations, carrier-free $Na^{125}I$ (1.5 to 2.5 mCi, and 0.6 to 1.0 nmol) and 5 μg of IFN-β Ser_{17} (0.25 nmol) per reaction were employed. Various amounts (0 to 10 nmol) of chloramine T (Sigma Chemical Co., St. Louis, Mo.) were used to obtain proteins having different specific radioactivities. Total reaction volumes were no more than 25 μl. To minimize oxidative inactivation of interferon, the order of addition of reagents was $Na^{125}I$, chloramine T, protein. No more than 15 s elapsed between the additions of sodium iodide and protein. Reactions were allowed to proceed for 10 min at 23°C, at which time 200 μl of tyrosine-saturated Tris-SDS buffer was added. To prevent loss of biological activity, reducing agents were not used to quench reactions. After an additional 5-min incubation, 200 μl of bovine serum albumin solution (1 mg/ml in Tris-SDS) was added, a sample of the reaction mixture was removed for SDS-PAGE analysis and bioassay, and the remainder of the reaction mixture was passed through a calibrated 3-ml column of Sephadex G-10 equilibrated with Tris-SDS. This column effectively separated covalently labeled protein from ^{125}I-containing by-products. Protein recovery after G-10 chromatography was routinely greater than 90%. Samples of the labeled protein pools recovered from G-10 chromatography were also analyzed by SDS-PAGE and bioassay. Specific radioactivity of labeled protein was determined in a manner identical to that used for Bolton-Hunter-labeled protein. Radiolabeled proteins were stored at 23°C in Tris-SDS buffer and were used for receptor binding studies within 5 days of preparation. Significant losses in biological activity could be observed after 7 days of storage under the conditions described above.

Cells and culture conditions. Daudi cells (Naval Biosciences Laboratory, Oakland, Calif.) were grown in Dulbecco modified eagle medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 mg of amphotericin B per ml (GIBCO Laboratories, Grand Island, N.Y.). Cells were maintained in log phase at concentrations between 2×10^5 and 8×10^5 cells per ml. For binding studies, cells were collected by centrifugation and were suspended in fresh medium at 2×10^6 or 5×10^6 cells per ml.

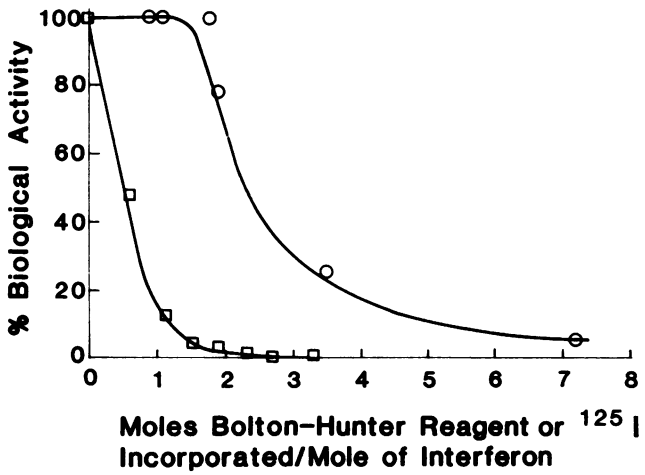

FIG. 1. Comparison of chloramine T and Bolton-Hunter methods for iodination of recombinant IFN-β Ser_{17}. Low-level iodination of IFN-β Ser_{17} by the chloramine T (○) or the Bolton-Hunter (□) method was performed as described in the text. Values for biological activity are the means of duplicate cytopathic effect assays.

Binding studies. Only ^{125}I-IFN-β Ser_{17} prepared by the chloramine T method was used in the binding studies. Specific saturable binding at 0°C was determined by using 10^6 Daudi cells in 0.225 ml of medium containing various concentrations of ^{125}I-IFN-β Ser_{17} with or without a 100-fold excess of unlabeled IFN-β Ser_{17}. After 90 min, cells were washed at 0 to 4°C with two 1-ml volumes of phosphate-buffered saline; each time, cells were collected by centrifugation at $8,000 \times g$ for 2 min. The bound tracer in the cell pellet was counted in a Beckman model 5500 gamma counter. To correct for nonspecific binding of ^{125}I-IFN-β Ser_{17} to the polypropylene tubes, cells were transferred to clean tubes during the washing procedure or values for binding to the tubes were subtracted from assay values. Binding data were analyzed according to Scatchard (21). For competition studies, 10^6 Daudi cells in 0.55 ml of medium containing 175 U of ^{125}I-IFN-β Ser_{17} per ml and various amounts of unlabeled interferons were incubated for 1 h at 37°C, washed, and counted as described above.

Interferon assays. Specific activity determinations and antiviral titers of the interferons used in this study were determined in a virus-yield reduction assay and a cytopathic effect assay, respectively, using GM2504 human trisomic fibroblasts and vesicular stomatitis virus according to Stewart (22). Interferon assays were calibrated with National Institutes of Health reference α (G-023-901-527) and β (G-023-902-527) interferon standards and an in-house IFN-γ laboratory standard. A National Institutes of Health reference for IFN-γ standard had not been established at the time of this work. One laboratory IFN-γ unit was defined as that amount eliciting a response equivalent to 1 U of IFN-α in the assays described above.

RESULTS

Iodination. Two methods of iodination were compared that would give sufficiently high levels of radioisotope incorporation (average of 1 mol of ^{125}I per mol of IFN-β Ser_{17}) for possible use in receptor binding studies. Although up to 3 mol of Bolton-Hunter reagent could be incorporated into 1

mol of IFN-β Ser_{17}, a 1:1 relationship existed between protein modification by this reagent and the loss of biological activity (Fig. 1). An effort to preferentially label either the alpha or epsilon amino groups led to iodinations at both pH 7.5 and pH 9.5. The stoichiometry of inactivation was similar under both conditions (data not shown).

If iodine incorporation was kept to 1 to 2 mol of ^{125}I per mol of IFN-β Ser_{17}, nearly quantitative recovery of biological activity could be obtained by a modified chloramine T protocol (Fig. 1). Radiolabeled protein having a specific radioactivity of 100 to 150 μCi/μg (monoiodination of IFN-β Ser_{17} with carrier-free ^{125}I yields a specific radioactivity of 120 μCi/μg) and a biological activity of 1×10^8 to 2×10^8 U/mg could be routinely prepared. SDS-PAGE analysis under reducing conditions of the chloramine T-labeled samples demonstrated them to be over 95% monomeric. Carrier protein (bovine serum albumin) was not labeled by this protocol. Because IFN-β Ser_{17} is partially inactivated by reducing agents (R. Drummond, unpublished observation), iodination must not be quenched by reagents capable of reducing disulfide bridges.

Unlabeled IFN-β Ser_{17} bioactivity is stable for months at 23°C if stored in buffers containing SDS. Because buffered SDS precipitates at 4°C, we normally store iodinated IFN-β Ser_{17} at room temperature and use the labeled protein within a few days of its preparation. Although we have observed significant losses in biological activity of iodinated IFN-β Ser_{17} after only 7 days of storage, SDS-PAGE analysis of these low-activity samples suggests that this inactivation is not a result of peptide bond cleavage.

Binding studies. To determine whether IFN-β Ser_{17} binds specifically to human cells, Daudi cells were incubated with various amounts of chloramine T-labeled ^{125}I-IFN-β Ser_{17} in the absence or presence of a 100-fold excess of unlabeled IFN-β Ser_{17}. These experiments were performed at 0°C to ensure that metabolic events that might occur after binding would be inhibited, and that only initial binding would be measured. Results of binding experiments were highly reproducible with different batches of ^{125}I-IFN-β Ser_{17}. Figure 2A shows the results of a typical binding experiment. Binding in the absence of unlabeled interferon was saturable. In the presence of a 100-fold excess of unlabeled interferon, binding of ^{125}I-IFN-β Ser_{17} was reduced and exhibited a linear relationship with the amount of ^{125}I-IFN-β Ser_{17} added to the cells. The extent of nonspecific binding in these experiments was high, ranging from 25 to 50% of the total binding at saturation. In addition to the high nonspecific binding observed with Daudi cells, high nonspecific binding of ^{125}I-IFN-β Ser_{17} to the polypropylene tubes used in these experiments was also observed. This was corrected for either by subtracting tube backgrounds or by transferring the cells to fresh tubes during the wash procedure.

The Scatchard plot (Fig. 2B) of the specific binding data approaches linearity. The linear portion of the plot yields a dissociation constant, K_d, of 0.24 nM. The number of binding sites per cell, based on extrapolation of the plot, is 4,000.

A variety of human interferons were tested for their ability to complete with ^{125}I-IFN-β Ser_{17} for binding to Daudi cells (Fig. 3). All β interferons, including native IFN-β, various formulations of IFN-β Ser_{17}, and IFN-β Cys_{17}, were found to be equally effective at competing with IFN-β Ser_{17} for the IFN-β receptor. Recombinant IFN-α-1 also displaced ^{125}I-IFN-β Ser_{17}, but 30- to 100-fold higher concentrations of IFN-α-1 (on the basis of antiviral activity) were required to achieve the levels of displacement obtained with the β interferons. Native IFN-γ showed little competition at the concentrations used.

DISCUSSION

A number of studies have been published that describe methods for preparing iodinated interferons for radiorecep-

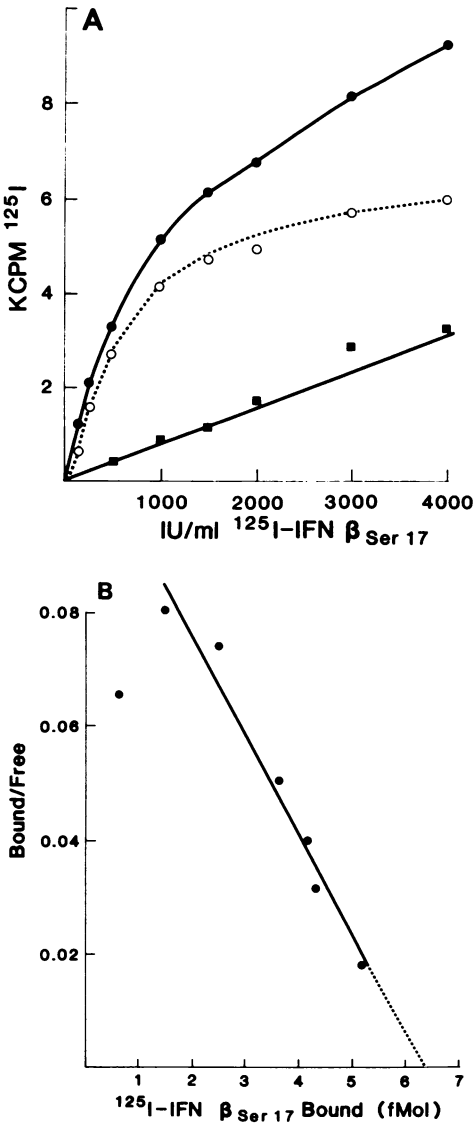

FIG. 2. Binding of ^{125}I-IFN-β Ser_{17} to Daudi cells. (A) Specified amounts of ^{125}I-IFN-β Ser_{17} were added, with or without a 100-fold excess of unlabeled interferon, to 10^6 Daudi cells in a final volume of 0.225 ml of medium. After incubation for 90 min at 0°C, the cells were washed by centrifugation and suspension in phosphate-buffered saline. The ^{125}I associated with cell pellets was quantitated by gamma counting. Symbols: ●, total binding of ^{125}I-IFN-β Ser_{17} in the absence of unlabeled IFN-β Ser_{17}; ■, binding of ^{125}I-IFN-β Ser_{17} in the presence of 100-fold excess unlabeled IFN-β Ser_{17}; ○, specific binding (difference between total binding and nonspecific binding). (B) Scatchard analysis of the specific binding curve from A. The interferon bound is expressed as femtomoles. The following values were used in calculations: 1,130 cpm of ^{125}I equals 1 fmol; the specific biological activity of ^{125}I-IFN-β Ser_{17} is 2×10^8 U/mg, and the M_r of IFN-β Ser_{17} is 20,000. The K_d determined from the slope of this line is 0.24 nM. The abcissa intercept, 6.4 fmol, represents 4,000 binding sites per cell.

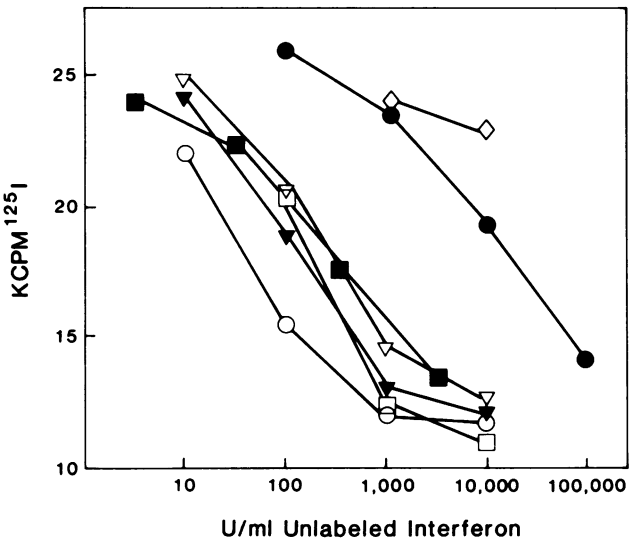

FIG. 3. Competition of interferons with ^{125}I-IFN-β Ser_{17} for binding sites on Daudi cells. ^{125}I-IFN-β Ser_{17}, 175 U/ml, and various concentrations of unlabeled interferons were added simultaneously to 10^6 Daudi cells in 0.55 ml of medium. After incubation at 37°C for 1 h, the cells were washed and the ^{125}I associated with the cells was quantitated by gamma counting. The interferons were as described in the text. Symbols: ○, IFN-β Ser_{17} (used for iodination); ▼ and ▽, two formulations of IFN-β Ser_{17}; ■, IFN-β Cys_{17}; □, native IFN-β; ●, IFN-α-1; ◇, native IFN-γ.

tor assays (1, 3, 7, 11, 16, 20, 26; F. J. Ruzicka, M. J. Hawkins, and E. C. Borden, Fed. Proc. **43**:690, 1984). Most of these studies report 25 to 50% recovery of biological activity after iodination. Unfortunately, because of the imprecise nature of the interferon biological assay commonly used to measure recovery (22), the significance of the reported values is often uncertain. The ambiguity in recovery of biological activity arising from imprecision in bioassay is greatest when levels of radioiodine incorporation are low. Optimally, all tracer molecules should be monoiodinated. As this level is experimentally approached, possible losses in interferon biological activity are more readily detected.

The availability of large amounts of a purified, modified recombinant interferon, IFN-β Ser_{17}, has afforded us the opportunity to evaluate two radioiodination methods, one employing ^{125}I Bolton-Hunter reagent, the other employing chloramine T and Na^{125}I. Previous reports have described the iodination of both α and β interferons with Bolton-Hunter reagent. In the case of the α interferons, 30 to 100% recovery of biological activity has been observed (5, 11). For native β interferon, greater than 50% of the biological activity was recovered after iodination (16). A consistent observation in our laboratory has been an almost quantitative inactivation of IFN-β Ser_{17} by Bolton-Hunter reagent. The stoichiometry of the inactivation suggests a site-specific modification, perhaps a result of interaction between the hydrophobic reagent and a hydrophobic site on the protein (10, 14, 23, 24). Although Bolton-Hunter-labeled α and β interferons have been reported to bind to receptors (11, 27; Ruzicka et al., Fed. Proc. **43**:690, 1984), our Bolton-Hunter labeled IFN-β Ser_{17} lost biological activity, and therefore we did not analyze its binding properties. We are currently investigating the site of the modification of IFN-β Ser_{17} by Bolton-Hunter reagent.

Unlike the Bolton-Hunter method, the traditional chloramine T method could be optimized to yield active, iodinated IFN-β Ser_{17} suitable for receptor binding studies. The inherent hydrophobicity of recombinant IFN-β Ser_{17} has greatly complicated our work. This protein is poorly soluble in the absence of detergents, and tends to aggregate and stick to vessel walls. The intrinsic hydrophobicity of the protein was compensated for by special sample manipulation and experimental protocols during receptor binding studies.

Although the specific binding of α and γ interferons to cultured mammalian cells has been reported previously (2, 3, 7, 20, 26), we present direct evidence for specific binding of IFN-β Ser_{17}. Our values for the affinity of binding of IFN-β Ser_{17} and the sites per cell are close to those reported by others for binding of IFN-α under similar conditions (15). Our competition results with labeled β and unlabeled α interferon support the concept of a common binding site for α and β interferon, a concept previously suggested by competition experiments between labeled α and unlabeled β interferon (7, 15). Because we wished to measure only the initial interaction of interferon and its binding site, our binding studies were performed at 0°C. At 37°C, additional processes such as internalization (4, 9), down regulation (8), and activation of secondary sites (19) also occur. These secondary processes may distinguish α from β interferon. Competition studies by others have demonstrated that β interferon binds to a γ interferon binding site on human fibroblasts, as well as to a common α and β binding site (3). We did not observe displacement of ^{125}I-IFN-β Ser_{17} by γ interferon in our competition experiments on Daudi cells. However, because different cell lines were used in these two studies, our results do not necessarily contradict those previously reported (3). The number of α interferon binding sites has been shown to vary greatly in different cell lines (20, 25). Preliminary studies from our laboratory on two additional human cell lines indicate that the number of β interferon binding sites also varies greatly. A similar situation may exist for γ interferon sites as well. It is possible that Daudi cells have many fewer γ than β binding sites, a condition that would make competition difficult to observe.

To date we have detected insignificant differences between the binding of a modified β interferon, IFN-β Ser_{17}, and native fibroblast interferon; nor is there a difference in binding for diverse formulations of this product. These are particularly important observations for a lymphokine intended for clinical use, especially when the desire is to employ a molecule that mimics the behavior of the native molecule as much as possible.

The availability of a suitably labeled tracer, iodinated IFN-β Ser_{17}, should prove useful in evaluating receptor binding as a screening test for interferon therapy and in the study of interferon pharmacokinetics. This information may eventually help define therapeutic uses for IFN-β Ser_{17}.

ACKNOWLEDGMENTS

We thank Charles Vitt and his staff for performing interferon assays, D. Nitecki for helpful discussions on iodination procedures, M. Konrad and T. White for their support and encouragement, W. Bloch, M. Doyle, and K. Koths for comments on the manuscript, and E. Jarvis for editing the manuscript.

This work was partly funded by Shell Oil Company.

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