Vol. 110, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 27, 1983 Pages 537-544

DIFFERENTIAL BINDING OF HUMAN INTERFERON-α SUBTYPES TO RECEPTORS ON LYMPHOBLASTOID CELLS

Gregory E. Hannigan, Dirk R. Gewert, Eleanor N. Fish,
Stanley E. Read and Bryan R.G. Williams

The Research Institute Division of Infectious Diseases
Hospital for Sick Children
555 University Avenue, Toronto, Ontario M5G 1X8
Canada

Received December 6, 1982

Highly purified human interferon-α subtype A (HuIFNαA) was iodinated for use in direct ligand binding studies on human lymphoblastoid (Daudi) cells. Unlabelled preparations of HuIFNα subtypes A, C, D, and hybrid molecules AD (Bgl II), AD (Pvu II), and DA (Bgl II) showed different responses in competition experiments with labelled αA probe. Specifically, IFNs αD and αDA were unable to displace the probe, whereas IFNS αA, αC, and the hybrid αADs showed similar competition curves. These results support a two-idiotope model of IFN recognition by its receptor. IFN effects on [^{3}H]-thymidine incorporation and cell growth (long term effects) did not reflect the apparent affinities of HuIFNα subtypes for cell surface receptor.

The interferons (IFNs) are a family of proteins which exert multiple biological effects on cells, including induction of an antiviral state, inhibition of cell growth, modulation of immune function and alteration of surface membrane characteristics (1). Recently, direct evidence has shown that IFNs interact with IFN-sensitive cells initially through specific binding to high affinity receptors on the cell surface (2-5). Direct ligand-binding studies have suggested that IFNα and IFNβ bind with equal affinity to the same cell surface receptor in both murine (3) and human (2) systems. The absence of specific IFN receptors may account for resistance to the effects of IFN in some cells (3), but other IFN-resistant cells exhibit specific IFN binding and thus seem to express IFNα receptor (6).

The human IFNα (HuIFNα) family consists of at least five discrete molecular subtypes which may be functionally as well as structurally distinct (7-12). The different antiviral efficacies of IFNα subtypes on

0006-291X/83/020537-08$01.50/0

various cells indirectly suggest that these subtypes may possess differential affinities for the IFNα receptor (7).

In order to directly investigate the interaction of IFNα subtypes with the IFNα receptor, we have examined competition for cell surface binding of HuIFNαA by other subtypes and related hybrid IFNs. Highly purified HuIFNαA was labelled with ^{125}I for use in binding studies on human lymphoblastoid (Daudi) cells. Daudi cells are exceptionally sensitive to the growth inhibitory effect of IFNs. This extreme sensitivity coincides with greater cell-surface expression of specific IFN receptors, as compared to such expression in less sensitive cell types (4). We report here competition experiments utilizing unlabelled HuIFNαA, αC, αD and hybrid molecules HuIFNαAD (Bgl II), αAD (Pvu II) and αDA (Bgl II) which provide direct evidence that these subtypes do not recognize the IFNα receptor with equal affinity.

MATERIALS AND METHODS

Cell Culture

Daudi cells were maintained in stationary culture in RPMI 1640 supplemented to 10% with fetal calf serum (Gibco, Grand Island, NY). All experiments were carried out on cells taken from their log phase of growth i.e. $4 \times 10^5 - 8 \times 10^5$/ml.

Interferons

Recombinant human leukocyte IFN subtypes (kindly provided by Genentech, San Francisco, CA) IFNαA, IFNαC, IFNαD were used (12). The hybrid interferons IFNαAD (Bgl II) and IFNαAD (Pvu II) consist of the N-terminal 61 and 91 amino acid residues of IFNαA respectively, the remaining 104 and 74 C-terminal residues respectively are those of IFNαD. IFNαDA (Bgl II) consists of 92 N-terminal IFNαD amino acid residues and 74 C-terminal residues of INFαA (9). The unpurified IFNαC was prepared as described previously (12) and had a specific activity of approximately 10^5U/mg protein. Preparations of INFαA, αD and the hybrids αAD and αDA used in this study had specific activities in the range of $1-2 \times 10^8$U/mg protein. Human IFNα (lymphoblastoid, Electro-Nucleonics, Bethesda, MD) had a specific activity of 3×10^6U/mg protein. IFNs were stored in phosphate-buffered saline (PBS) pH 7.2, at -70°C until used. Once thawed, IFNs were stabilized in 1mg/ml bovine serum albumin (BSA) and stored at 4°C.

Antiviral titers were determined using a cytopathic effect reduction assay utilizing encephalomyocarditis virus and T98G, a human neuroblastoma cell line (13). In this assay, one unit of NIH human reference standard G-023-901-527 was equivalent to 2.8 ± 1.5 laboratory IFNα standard units.

Iodination of HuIFNαA

Labelling with ^{125}I was carried out using a solid-phase lacto-peroxidase method (14,2). A 100 µl volume containing 10 µl 3% β-D-glucose, 10 µl hydrated Enzymo-beads (Bio Rad, CA), 2mCi Na ^{125}I, and 10^6U HuIFNαA in PBS, pH 7.2, was reacted overnight at 4°C. Free ^{125}I was separated from IFN-bound ^{125}I on a 12 ml Sephadex G-75 column equilibrated in PBS containing 1 mg/ml BSA. Iodination caused no detected loss of antiviral activity. Fractions containing maximum antiviral activity were pooled, and contained 91-95% 10% trichloroacetic acid (TCA) precipitable radioactivity. ^{125}I-IFNαA was purified to homogeneity on an NK2 monoclonal antibody column (Celltech, Berkshire) as revealed by SDS-PAGE analysis (15), shown in Fig. 1. ^{125}I-IFNαA was stabilized in 1 mg/ml BSA and stored at 4°C throughout use in binding experiments.

Binding of ^{125}I-IFNαA to Daudi Cells

10^6 Daudi cells were incubated in a final reaction volume of 0.5 ml medium containing appropriate amounts of ^{125}I-IFNαA. After 60 min at 37°C, duplicate samples were layered on 200µℓ of a 2:1 (v/v) mixture of di-n-butyl-phthalate: dinonyl phthalate (BDH, Toronto, Ontario), specific gravity = 1.012 g/ml (16) and centrifuged in a Eppendorf microcentrifuge for 4 sec at 8800xg. Medium containing unbound ^{125}I-IFNαA was aspirated and cell-associated radioactivity determined on a Gamma counter (Nuclear-Chicago). For competition experiments, indicated amounts of unlabelled HuIFNα subtypes and hybrid IFNs were included in the 0.5 ml reaction volume.

Inhibition of [^3H]-Thymidine Incorporation and Cell Growth

Duplicate 1 ml samples of control and interferon-treated Daudi cells were seeded into 24-well cluster plates (Nunclon, Gibco) at a density of 3×10^5/ml growth medium. After 19 or 48 hours (indicated in Figure legends) incubation at 37°C these samples were pulse-labelled for 30 min with 1 µCi/ml [methyl-^3H]-thymidine (63Ci/mmol, ICN, Irvine, CA). Three aliquots of 300 µl of each treatment were collected on glass-fibre filters (Whatman GF/C) and washed twice with 10 ml ice-cold 10% TCA and once with 10 ml ethanol. After drying the filters were added to 5 ml toluene-based scintillant and counted on a Beckman LS8100 liquid scintillation counter.

For growth inhibition experiments, 1ml of cells (3×10^5/ml) were seeded as above and interferon treated as indicated. After incubation at 37°C for 48 hours the cells were counted in a Coulter counter.

RESULTS AND DISCUSSION

Specific binding of ^{125}I-IFNαA to Daudi cells was saturated at 2000U/ml (Fig. 2a) and Scatchard analysis of the saturation isotherm resulted in a linear plot indicating a single component binding (Fig. 2a). This is in agreement with results previously reported for binding of ^{125}I-IFNαA to Daudi cells (2). Incorporation of [^3H]-thymidine into acid-insoluble material was inhibited to a level of 50% of untreated control cells by 5U/ml of IFNαA (Fig. 2b). Since binding sites were saturated at 2000U/ml, this suggests that a large proportion of IFN receptors are unoccupied when [^3H]-thymidine incorporation is maximally inhibited by

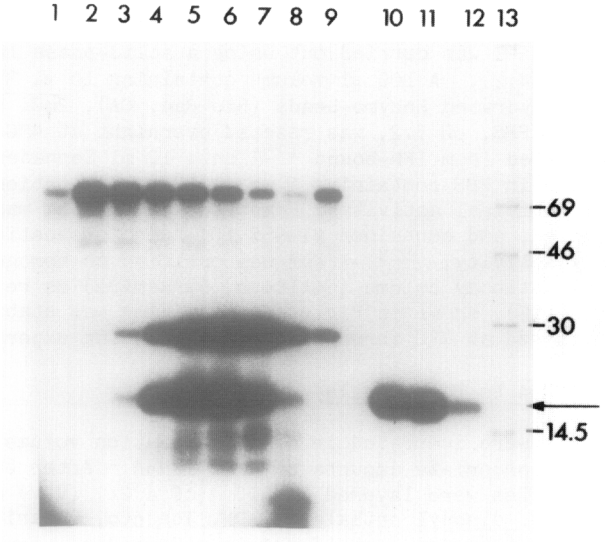

Figure 1 SDS-PAGE analysis of ^{125}I-IFNαA preparations. HuIFNαA was iodinated according to Materials and Methods. Fig. 1 represents an autoradiogram of a 15% SDS-polyacrylamide gel. Lanes 1-9 represent eluates from Sephadex G-75 chromatography of ^{125}I-IFNαA (500 µl each collected at 6 ml/hr). Approximately 10,000 counts per minute (cpm) were loaded on each lane. Lanes 10 and 11 represent NK2 monoclonal antibody purified ^{125}I-IFNαA. The arrow identifies an 18,800 dalton (18.8K) protein which exhibited antiviral activity. A major 28K contaminant was not retained on the NK2 column, and did not exhibit antiviral activity. The 69K contaminant probably represents BSA non-specifically labelled during chromatography. Lane 12 shows the molecular weight markers. Fractions represented by lanes 6 and 10 showed identical Daudi cell-surface binding characteristics.

IFNαA. This condition of excess receptors has also been reported with respect to the antiproliferative effect of HuIFNα (Namalwa) on Daudi cells (4).

Competitive displacement of ^{125}I-IFNαA was obtained with unlabelled preparations of IFNαA, and IFNαC (Fig. 3). In addition, the hybrids IFNαAD (Bgl II) and αAD (Pvu II) were able to fully displace the probe. In contrast, IFNαD and hybrid αDA (Bgl II) were unable to displace ^{125}I-IFNαA across a five and four log range of biologically active concentrations (Fig. 3). These data suggest that either IFNαD and IFNαDA possess a much lower affinity for receptor sites occupied by ^{125}I-IFNαA, or that these two IFNs recognize a different receptor.

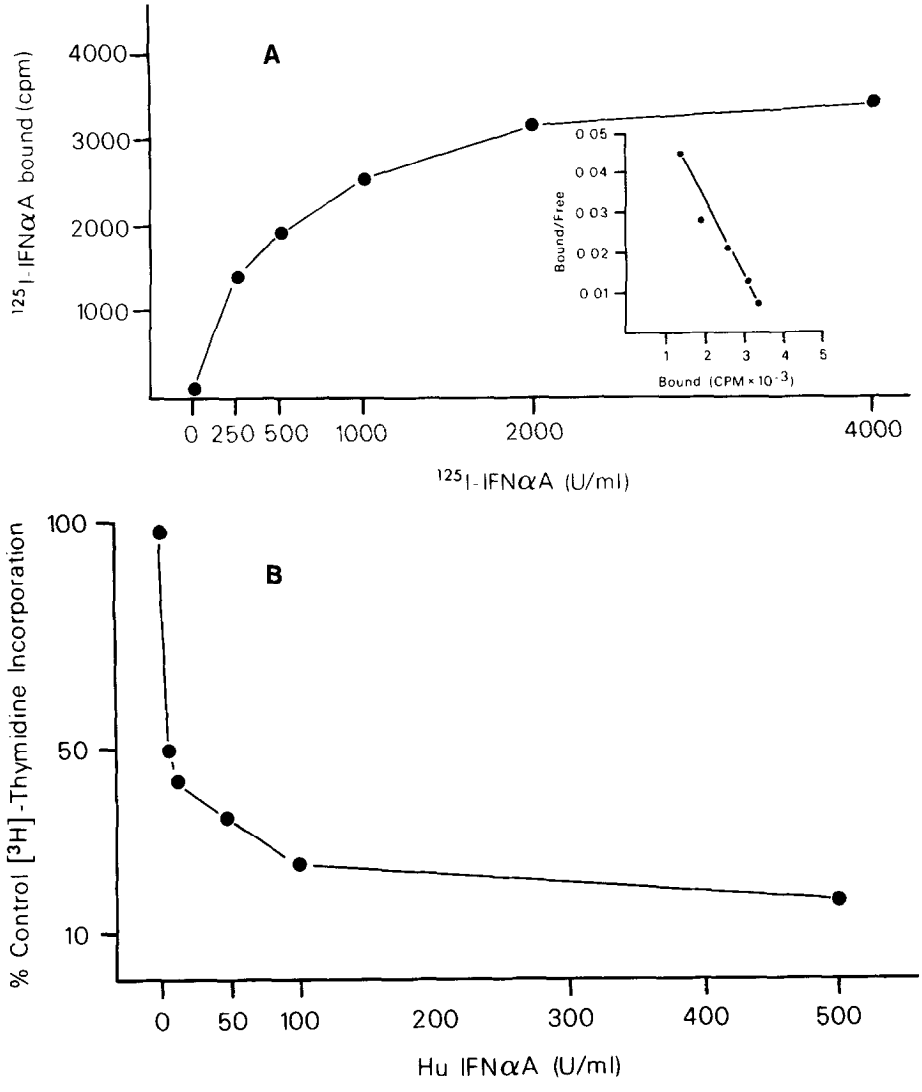

Figure 2 (a) Specific saturation of ^{125}I-IFNαA binding sites. Binding of ^{125}I-IFNαA was as described in Materials and Methods with indicated concentrations of ^{125}I-IFNαA. Non-specific binding was determined in the presence of a 20-fold excess of unlabelled HuIFNαA and represented a maximum of 20% total cpm bound. Points are mean values of duplicate determinations. Inset is the Scatchard plot of this data. (b) [^{3}H]-thymidine incorporation in IFNαA-treated Daudi cells. Daudi cells were seeded as described in Materials and Methods and treated with indicated concentrations of IFNαA for 19 hrs. Points are means of triplicate determinations.

Recently Streuli et al. (7) have postulated a two-idiotope model for IFNα binding to target cells, based on variable antiviral efficacies of HuIFNαA and IFNαD, and hybrids IFNαAD (Bgl II) and IFNαDA (Bgl II) in the protection of human WISH cells against challenge with vesicular

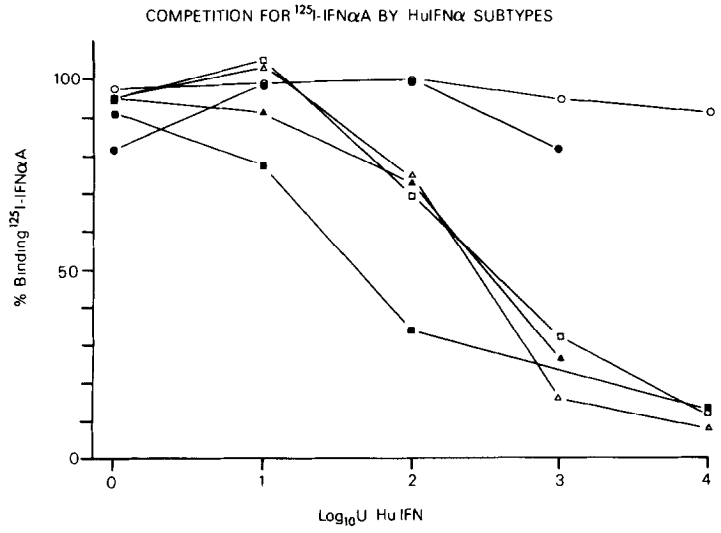

Figure 3 Competition for ^{125}I-IFNαA binding sites by unlabelled HuIFNα subtypes. 10^6 Daudi cells were incubated at 37°C for 60 min with 250U ^{125}I-IFNαA in a final volume of 0.5 ml RPMI containing no unlabelled competitor (100% bound) or the indicated concentrations of HuIFNα subtypes. IFNα subtypes were the same preparations as used in growth and [^{3}H]-thymidine inhibition studies.
▲ HuIFNαA; Δ HuIFNαC; □ HuIFNαAD (Pvu II);
■ HuIFNαAD (Bgl II); ○ HuIFNαD; ● HuIFNαDA (Bgl II).

stomatitis virus. In this model the N-terminal region of IFNαA would be responsible for high affinity binding of the molecule, whereas the C-terminal region would contribute toward a low affinity binding. On the other hand, both the N-terminal and C-terminal regions of IFNαD contribute toward lower affinity binding of this IFNα subtype to the same receptor on human target cells. The lack of ^{125}I-IFNαA displacement from Daudi cell receptors by unlabelled IFNαD and IFNαDA is entirely in accord with such a model (Fig. 3). Furthermore the high affinity of IFNαAD (Bgl II) and IFNαAD (Pvu II) implicit in this model, due to N-terminal IFNαA region, is directly evident as effective displacement of ^{125}I-IFNαA by these two hybrids (Fig.3). Thus the observed binding pattern of IFNα subtypes on lymphoid cells is consistent with the two idiotope model and provides direct ligand binding evidence for such a scheme.

The effects of IFNα subtypes on cell growth and [^{3}H]-thymidine incorporation do not parallel each other (Table 1), thus suggesting that

TABLE 1

INHIBITION OF CELL GROWTH AND [^{3}H]-THYMIDINE INCORPORATION BY HuIFNα SUBTYPES ON DAUDI CELLS

Interferon (10U/ml)	% Inhibition	
	Cell Growth	[^{3}H]-Thymidine Incorporation
Lymphoblastoid	76	81 ± 2.6
A	52	78 ± 2.5
C	63	51 ± 0.6
D	68	85 ± 4.5
AD (Bgl II)	48	53 ± 2.1
AD (Pvu II)	43	61 ± 6.0
DA	45	30 ± 5.6

Exponentially growing Daudi cells were seeded at 3 x 10^5 cells/ml into 24-well cluster dishes and incubated for 48 hrs in the presence of 10U/ml of various interferon α subtypes. Cells were counted in a Coulter counter and [^{3}H]-thymidine incorporation followed as described in Materials & Methods. Results are expressed as percentage of values obtained in control cultures, and represent means of duplicate determinations for cell growth, and means of triplicate determinations for [^{3}H]-thymidine incorporation, ± standard deviations.

these two IFN-mediated responses are not coupled. This has also been shown by Gewert et al. (16). In addition, the magnitudes of these responses do not reflect the apparent relative affinities of IFNα subtypes for surface receptor. This lack of correlation may be due to the relatively long incubation times involved in measuring the inhibition of [^{3}H]-thymidine incorporation (19 - 48hrs) and cell growth (48hrs) relative to binding data which is obtained after 1 hr incubation. These long-term responses appear to be inadequate in describing subtle receptor-ligand interactions as, with time, all the IFNα subtypes tend to exert similar end point effects on Daudi cells (data not shown).

Measurement of a more immediate response to IFN, such as the elevation of intracellular guanylate cyclase levels (17), will allow a better understanding of the relationship between IFN binding and the resulting

biological effects. Iodination of HuIFNαD for use in ligand binding studies will further clarify the status of IFN receptor(s) in mediation of biological effects by HuIFNα on both IFN-sensitive and IFN-resistant (18) Daudi cells.

ACKNOWLEDGEMENTS

This work was supported by grants from the Canadian National Cancer Institute and the Medical Research Council of Canada. D.R.G. is an MRC Fellow and B.R.G.W. is an MRC Scholar.

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