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CLONED HUMAN INTERFERONS α : DIFFERENTIAL AFFINITIES FOR POLYINOSINIC ACID AND RELATIONSHIP BETWEEN MOLECULAR STRUCTURE AND SPECIES SPECIFICITY

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SUMMARY : The HuIFN-αA and HuIFN-αD interferons, produced by two independent recombinant bacterial clones, have different affinities for polyinosinic acid (poly I). The monomeric form HuIFN-αA (FMM), but not the HuIFN-αD, binds to poly (I)-agarose and is protected by poly (I) from thermal inactivation. Other subtypes of HuIFN-αA including the monomer SMM and oligomers have no affinity for this polynucleotide. In addition, these interferons show different target cell preferences in agreement with our previous suggestion (23) that the polynucleotide binding domain may be responsible for species specificity. Two significant observations are 1) The fractions of HuIFN-αD and HuIFN-αA unbound on poly (I)-agarose show higher antiviral inducing activity on heterologous (MDBK) than on homologous (WISH) cells, whereas they induce about the same activity of 2'5' oligoadenylate synthetase in these two cell lines. These fractions are also active on L929 cells. 2) The bound fraction of HuIFN-αA induces almost the same antiviral and 2'5' oligoadenylate synthetase activities in MDBK and in WISH cells but neither activity in L929 cells.

The HuIFN-αA and HuIFN-αD interferons (IFNs) produced by two independent recombinant bacterial clones have been extensively studied (1-3). Their primary structures are composed of respectively 165 and 166 amino acid residues and have at least 73 % of sequence homology (4). HuIFN-αA has cysteine residues at positions 1, 29, 98, 138, and HuIFN-αD contains an additional cysteine residue at position 86 (5). In HuIFN-αA, intramolecular linkages occur between Cys 1-Cys 98 and also between Cys 29-Cys 138 ; only the latter disulfide bridge is presumably essential for biological activity (6-8). Moreover, the cysteine residues can interact to form intermolecular disulfide links and thus generate subtypes consisting of oligomers (dimers, trimers and tetramers) (9). The presence of such oligomers (10, 11), in addition to at least two monomers termed as slow moving monomer (SMM) and fast moving monomer (FMM), was recently revealed in the HuIFN-αA by non

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reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The FMM may represent a form with both S-S bonds intact and the SMM a partly reduced form with only the Cys 29-Cys 138 bond intact (11). The purified FMM was indeed homogeneous by HPLC (12) in conformity with the presence of a single band by SDS-PAGE (9). In HuIFN-αD, the arrangement of disulfides seems to be identical to that in HuIFN-αA (unpublished observations in ref. 7).

The different HuIFN-α subtypes have both different specific molecular activities and species specificities (13-16) as well as different ratios of antiviral to antiproliferative activities (17-20) ; the induction of 2'5' oligoadenylate synthetase (2'5' A synthetase) by these different forms is not related to their specific antiviral activities (3, 10, 22). Such differences could also be observed in their capacities to bind to receptors (21). Moreover, within one species, for the same antiviral activity, the HuIFN-αA dimer or trimer, compared with the monomer, are much better inducers of 2'5' A synthetase in the human than in the bovine cells (10).

We previously showed (23) that unfractionated natural human leukocyte IFN (HuLe IFN) could be separated into two fractions by affinity chromatography on polynucleotide-agarose : one of which binds to polynucleotide, while the other does not. On SDS-PAGE, the former migrates as a polypeptide of 21 000 daltons and the latter as 15 000 daltons. The IFN molecules with faster mobility display greater heterospecific antiviral activity than those with slower mobility (23). From these results, we inferred that the polypeptide domain responsible for species specificity may be closely related to the polynucleotide binding area.

In the present study, we compared the polynucleotide binding property of HuIFN-αA, HuIFN-αA (FMM) and HuIFN-αD in relation with their target cell specificities. Since they have quite different degrees of antiviral activity in human and in bovine cells, we asked whether the greater heterospecific antiviral activity of HuIFN-αD could be attributed to the absence of a polynucleotide binding domain.

MATERIALS AND METHODS

Materials

[α-^{32}P] ATP (410 Ci/mmol) was obtained from the Radiochemical Centre Amersham, England. Poly (I) : poly (C), poly (I) : poly (C) -agarose and poly (I) -agarose were from Choay, France. Polyethylene imine was a gift from BASF, France. Cellulose MN 300 was from Macherey Nagel and Co. The polyethylene-imine (PEI) cellulose thin-layer plates were prepared in the laboratory according to the procedure of Randerath and Randerath (24). Eagle's medium, Dulbecco's medium and foetal calf serum were from Eurobio, France. N-2-Hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES) was from Sigma Chemical Co, St Louis, MO., USA.

Cells, Virus and Interferons

Cells used were mouse fibroblasts (L929), Madin-Darby bovine kidney fibroblasts (MDBK) and spontaneously transformed cells from human placental tissue (WISH). L929 and MDBK cells were grown in Eagle's medium ; WISH cells in Dulbecco's medium. Both media were supplemented with 10 % foetal calf serum. Vesicular stomatitis virus (VSV) of the Indiana Strain was grown in the L929 cells. Stock virus samples were usually 10^8 plaque forming units/ml (PFU/ml). Recombinant IFNs, HuIFN-αA and HuIFN-αD and HuIFN-αA_M (FMM) monomeric form of HuIFN-αA (9) that can be attributed to the FMM form according to Felix et al. (12), were a generous gift from Dr. S. Pestka.

Determination of IFN titers

Cloned HuIFN-αA and HuIFN-αD were titrated on MDBK or WISH cells. Estimation of IFN titers was based on the amounts required to give 50 % inhibition of the cytopathic effect. HuIFN-αA and HuIFN-αD titers were expressed in relation to the HuLe reference IFN (NIH cat. no. G023-901-527), which titrates 20 000 international units/ml, and under our conditions, i.e. on MDBK cells, yielded 12 800 units/ml. The specific activities of HuIFN-αA and HuIFN-αD in MDBK cells were respectively 2.3×10^8 and 5×10^7 units/mg protein.

Assay of VSV yield

L929 cells treated during 18 h at 37°C with HuIFN-αA or HuIFN-αD were infected with VSV at a multiplicity of infection of 1. After 1 h at 37°C, non-adsorbed virus was removed and cells were incubated in serum-free medium for 18 h at 37°C. Cultures were frozen and thawed three times and cell debris were removed by centrifugation. The virus titers were measured by serial dilutions of the supernatant and then by a plaque assay in L929 cells and expressed as PFU/ml of supernatant. VSV yield in control untreated cultures is usually 10^8 PFU/ml.

Affinity chromatography

Polynucleotide-agarose column : agarose beads with covalently linked poly (I) were packed into a sterile polystyrene syringe. The column (volume 0,5 ml) was extensively washed with 1M NaCl, and equilibrated with 50mM Tris-HCl, pH 7.5. 3×10^6 units of HuIFN-αA or 2.5×10^6 units of HuIFN-αD (0.1 ml in each case) were diluted in 0.5 ml of 10mM Tris-HCl, pH 7.5 and loaded onto a poly (I)-agarose column in 10mM Tris-HCl, pH 7.5. The column was then successively washed with ten 0.5 ml fractions of 10 mM Tris-HCl, and eluted with ten 0.5 ml fractions of 1 M NaCl in the same buffer.

Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

Polyacrylamide slab gels (15 %) were prepared according to Laemmli (25) except that 2-mercaptoethanol was omitted. The fractions corresponding to the peak of the elution profile from the poly (I)-agarose column as shown in Figure 1C were first dialysed against electrophoresis buffer for 5 h and then deposited on the gels. At the end of electrophoresis, each track was cut into 2 mm slices. IFN activities was eluted at 4°C for 18 h in 0.2 ml culture medium containing 10 % foetal calf serum. The mixed prestained proteins (from BRL) used as molecular weight markers were Ovalbumin (43 000 daltons), α-Chymotrypsinogen (25 700 daltons), β Lactoglobulin (18 400 daltons), Lysozyme (14 300 daltons) and Cytochrome C (12 300 daltons).

Assay of 2'5' A synthetase

4×10^5 cells (either MDBK, WISH or L929) were treated with the different forms of IFN in medium containing 10 % foetal calf serum. After 18 h at 37°C, the cells were washed in 140 mM sodium chloride, 3 mM magnesium chloride and 35 mM HEPES pH 7.5 then lysed for 5 min at 0°C in 10 mM potassium chloride, 2 mM magnesium acetate, 7 mM 2-mercaptoethanol, 10 mM HEPES pH7.6 and 0.5 % Nonidet P-40 (26).The lysates were centrifuged for 6 min in an Eppendorf centrifuge and the supernatants were stored at - 80°C. Seventy five µl of each cell extract was mixed with 15 µl poly (I) : poly (C)-agarose (10 to 15 mg/ml). After 15 min incubation at room temperature, the matrix was washed twice with 500 µl of 5 mM magnesium acetate, 1 mM dithiothreitol, 25 mM potassium chloride, 10 % glycerol and 20 mM Tris-HCl pH8 and resuspended in 10 µl of the same buffer containing 7 mM [α-^{32}P] ATP (0.05 µCi per assay), 25 mM magnesium acetate, 12 µg/ml poly (I) : poly

(C), 0.25 mg/ml bovine serum albumin, 0.25 mg/ml creatine kinase and 10 mM creatine phosphate. After 2 h at 37°C the reaction was stopped by addition of 20 µl of 50 mM EDTA ; 6 µl of each incubation mixture were then spotted on a PEI cellulose plate and 2'5' oligoadenylates were separated from ATP by ascending chromatography in 2 M Tris-HCl pH 8.6 (27). After autoradiography, the spots were cut out and their radioactivity was determined according to Spector (28). Conversion of ATP into oligoadenylates was calculated and specific 2'5' A synthetase activity expressed as nmol of 2'5' oligoadenylates synthesized/h/mg of crude extract protein.

RESULTS AND DISCUSSION

Differential poly (I)-binding capacities of HuIFN-αD, HuIFN-αA (FMM) and HuIFN-αA

When a solution of HuIFN-αD was chromatographed on a poly (I)-agarose column in the presence of low salt, most of the molecules with antiviral activity passed through the column (Figure 1A) and no significant antiviral activity was recovered by further elution with high salt. The elution profiles of the IFN-induced 2'5' A synthetase activity (Figure 1B) also resembled those of antiviral activity. On the other hand, when a purified monomeric form

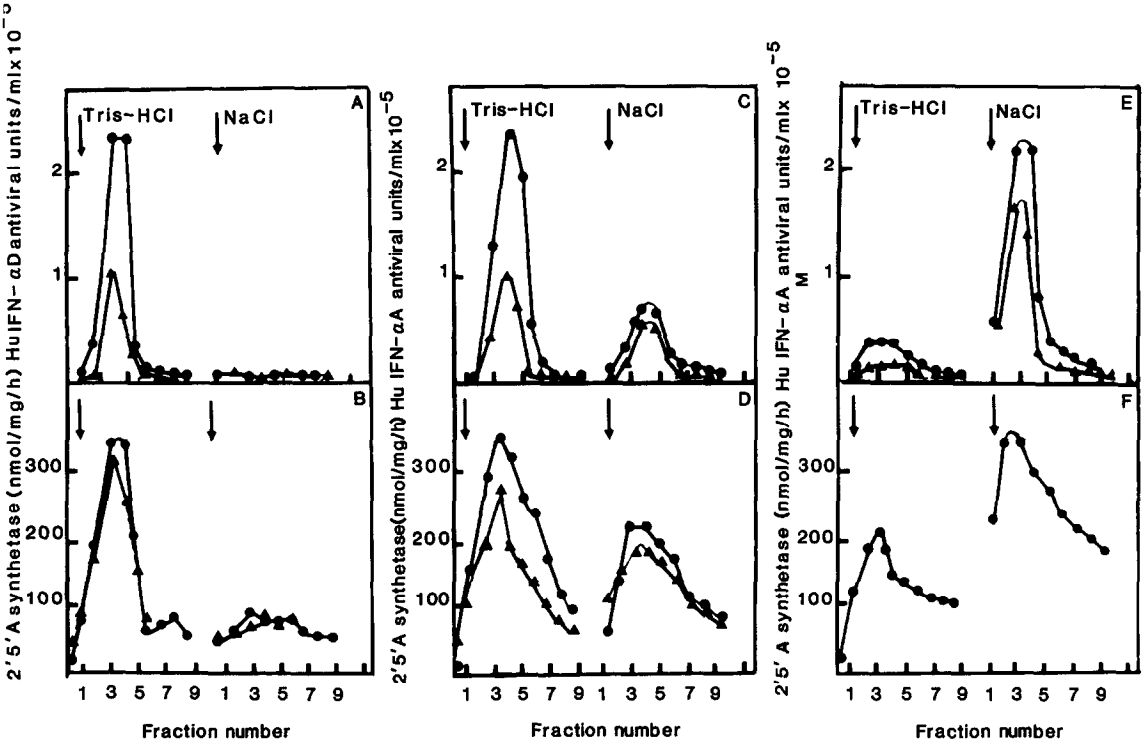

FIGURE 1 . Chromatography of HuIFN-αD, HuIFN-αA and HuIFN-αA_M (FMM) on poly (I)-agarose

$2.5x10^5$ units of HuIFN-αD (A and B), $3x10^5$ units of HuIFN-αA (C and D) or $3x10^5$ units of HuIFN-αA_M (FMM) (E and F) (as assayed on MDBK cells) were loaded onto the column as described in Methods. A, C and E show the antiviral activities of each fraction measured on MDBK (●——●) and WISH (▲——▲) cells. B, D and F show the 2'5' A synthetase specific activity induced in MDBK (●——●) and in WISH (▲——▲) cells ($4x10^5$ in each case) by each fraction diluted 1 000 fold in medium containing 10 % foetal calf serum.

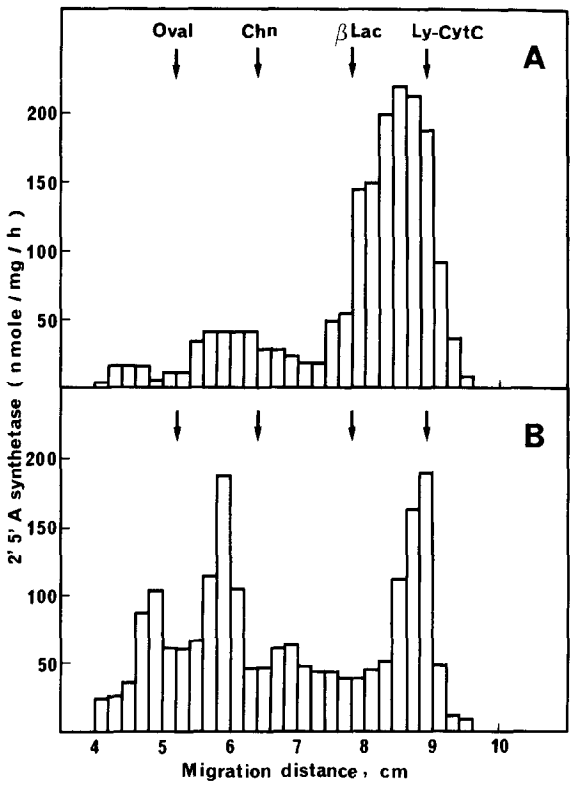

FIGURE 2 . SDS-polyacrylamide gel electrophoresis of HuIFN-αA unbound and bound fractions from poly (I)-agarose column.

Electrophoresis was performed as described in Methods. 2'5' A synthetase activity induced by each fraction eluted from gel was assayed on MDBK cells. A and B represent respectively the 2'5' A synthetase activity induced in MDBK cells by the bound fraction of HuIFN-αA from figure 1C (A) and by the unbound fraction of HuIFN-αA from figure 1C (B). 0,4 x 10^5 units of unbound fraction and 0,15 x 10^5 units of bound fraction were applied on gels. The recovery in each case was 100 %. Each fraction eluted from gel was diluted, 20-fold for poly (I)-bound fraction, and 100-fold for poly (I)-unbound fraction and used to induce 2'5' A synthetase activity in 4 x 10^5 MDBK cells. The migration positions of prestained protein markers are indicated by arrows : Oval, Ovalbumin (43 000 daltons) ; Chn, α-Chymotrypsinogen (25 700 daltons) ; β-lac, β-latoglobulin (18 400 daltons) ; Ly-Cytc, Lysosyme and Cytochrome C (14 300 daltons and 12 300 daltons).

HuIFN-αA (FMM) was chromatographed, only a small percentage of molecules having antiviral activity was washed out of the column with 10 mM Tris (Figure 1E) while most of the active molecules were retained on the polynucleotide ligand and subsequently eluted with IM NaCl (Figure 1F). Thus, HuIFN-αD and HuIFN-αA (FMM) have different affinities for a single-stranded polynucleotide. However, when a HuIFN-αA preparation, that had not been enriched in monomer FMM, was similarly analysed, approximately two-thirds of the antiviral and 2'5' A synthetase inducing activities (Figures IC and 1D) were found in the unbound fractions, the remaining one-third was retained on the column and could be eluted

with IM NaCl. Analysis by SDS-PAGE in the absence of 2-mercaptoethanol showed that the bound fraction contained mostly the monomer (Figure 2A) and that the unbound fraction was composed of a mixture of monomer and oligomers (Figure 2B).

It was clear from this analysis that the monomers in the bound and unbound fractions should have different conformations. Since HuIFN-αA (FMM) binds to poly (I)-agarose, the monomer in NaCl eluted fractions should correspond to this molecular form, and the monomer in the unbound fraction could be the monomer SMM. We have recently shown that in the HuIFN-αA, monomer, dimer and trimer were present in the proportion of 73 : 26 : 1 according to the antiviral profile upon SDS-PAGE analysis (10) ; it may be inferred that the monomeric fraction which accounts for 73 % of HuIFN-αA includes FMM and SMM forms reported by others (11). These results indicate that, in HuIFN-αA, only the FMM but neither the SMM nor the oligomeric forms has an accessible poly (I) binding domain and also hint that this poly (I) binding domain may be masked by oligomerisation. The same experiments were carried out with HuIFN-α1 and HuIFN-α2. When chromatographed on poly (I)-agarose, HuIFN-α1, is not retained and is comparable to HuIFN-αD, whereas HuIFN-α2, like HuIFN-αA, is separated into two fractions, one of which binds to poly (I) (20-30 %), and the remaining 70-80 % constitutes the unbound fraction.

Uncoupling of the induction of antiviral and 2'5' A synthetase activities

For HuIFN-αD and the unbound fraction of HuIFN-αA, the antiviral activity is much higher in bovine than in human cells (Figures 1A, 1C), whereas the degree of 2'5' A synthetase induction was similar in these two cell lines. Thus, the antiviral activity is not quantitatively correlated with the induction of the 2'5' A synthetase. These observations lead to the conclusion, in agreement with the results reported by Goren et al. (22), that HuIFN-αD for the same antiviral inducting activity, (Figures 1A, 1B) is a much better inducer of 2'5' A synthetase activity in WISH than in MDBK cells. This conclusion is also valid for the oligomeric form of HuIFN-αA which has no affinity for poly (I) (Figures 1C, 1D).

Effect of poly (I) on the thermostability of HuIFN-αD and HuIFN-αA (FMM)

In order to confirm that HuIFN-αA (FMM) but not HuIFN-αD interacts with poly (I) ligand, heat inactivation of these two species was tested in the presence or absence of poly (I), since poly (I) binding could increase thermal stability. Figure 3 shows that at 65°C, both IFN

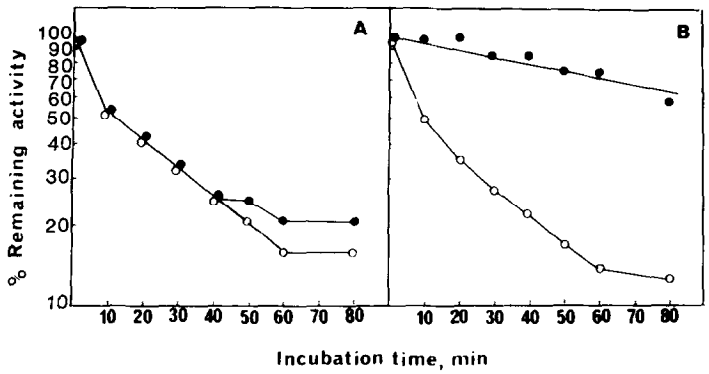

FIGURE 3 . Effect of poly (I) on the thermostability of HuIFN-αA_M (FMM) and HuIFN-αD

Antiviral activity of HuIFN-αA_M (FMM) and HuIFN-αD after heating at 65°C for various times in the absence (O——O) or presence of poly (I) (●——●). Aliquots (0,2ml) containing $5x10^5$ units of HuIFN-αD (A) or $6x10^5$ units HuIFN-αA_M (FMM) (B) in 10mM Tris buffer at pH 7.5 were incubated in a water bath at 65°C. The concentration of the poly (I) was 30 µg/ml (6 µg per aliquot). At the indicated times, 20 µl aliquots were removed, diluted 1 : 100 in culture medium containing 10 % foetal calf serum and cooled in a ice bath. IFN titrations were carried out on MDBK cells immediately after incubation at 65°C.

species have a half life of 10 min ; and, after 60 min at this temperature, only 15 % of the initial antiviral activity remains. This is in agreement with a previous report that the antiviral activity of HuIFN-αA is not stable at 65°C (29). The presence of poly (I) at a concentration of 30 µg/ml, however, totally protected HuIFN-αA (FMM) from inactivation during 20 min ; and 57 % of the initial antiviral activity remained even after 80 min of heating (Figure 3B), while no such protection was observed for HuIFN-αD (Figure 3A) under identical conditions.

Species specificity

An interesting feature of the poly (I)-agarose chromatography profile of HuIFN-αA (Figure 1C) and HuIFN-αA (FMM) (Figure 1E) was that the bound fractions had about the same antiviral inducing activities in both human and bovine cells. In the unbound fractions, the antiviral activity was much lower in human than in bovine cells, as it was observed with the non-binding HuIFN-αD (Figure 1A). The higher heterospecific activity, which seems to correlate with the absence of a polynucleotide binding domain, was further demonstrated in murine L929 cells (Figure 4). In these cells, both the unbound fractions of HuIFN-αA and HuIFN-αD displayed appreciable antiviral and 2'5' A synthetase inducing activities. The bound fraction of HuIFN-αA, which constitutes the monomeric form (Figure 2A), even at high IFN concentrations, clearly had no detectable antiviral or 2'5' A synthetase inducing

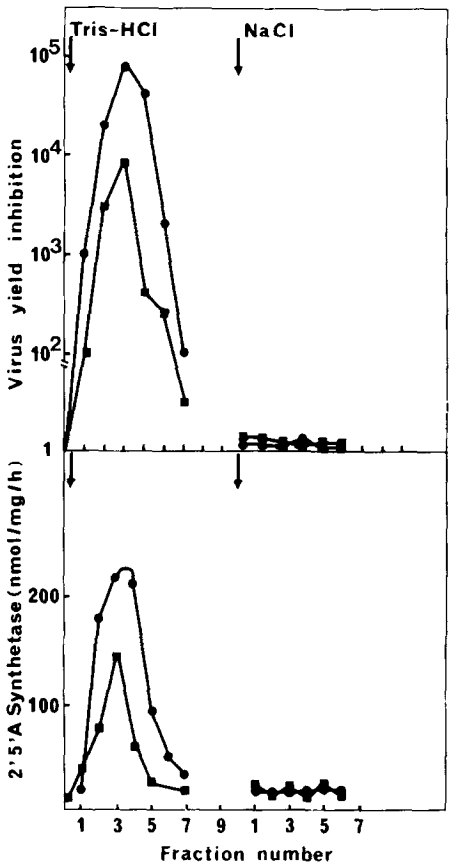

FIGURE 4 . Antiviral and 2'5' A synthetase activities induced in L 929 cells by the HuIFN-αD fractions and the HuIFN-αA fractions.

The HuIFN-αD and HuIFN-αA fractions obtained after poly (I)-agarose chromatography (Figs. 1A and 1C) were diluted 10-fold and incubated with L 929 cells for 18 h. Samples were prepared in duplicate. One sample was used to inhibit the VSV yield (A) and the other to determine the 2'5' A synthetase specific activity (B) induced by the HuIFN-αD fractions (●—●) and HuIFN-αA fractions (■—■) obtained after poly (I)-agarose chromatography (Figs. 1 A and 1C). The reported virus yield inhibitions are the ratio of the virus titers obtained in untreated control cells to those in treated cells. The VSV yield in untreated control cultures was $2x10^8$ PFU/ml.

activities in L929 cells. This is in agreement with our recent results that the monomeric form of HuIFN-αA is equally active on MDBK and WISH cells, whereas the dimeric and trimeric forms have more antiviral activity on heterologous than homologous cells (10).

We previously suggested that the absence or presence of a polynucleotide binding domain in fractionated human leukocyte IFNs is correlated with the degree of heterospecific antiviral activity (23). In the present work, we show with recombinant HuIFN-αD and HuIFN-αA that the absence of the polynucleotide binding domain in HuIFN-αD is correlated with the higher heterospecific antiviral activity. Similarly, such correlation between the absence of the

binding site in monomer SMM or in oligomers of HuIFN-αA and their reduced species specificity, compared with HuIFN-αA (FMM), was also observed. The polynucleotide binding domain of HuIFN-αA (FMM), defined by its ability to bind to single-stranded polynucleotide might be a strongly cationic domain of the protein that we tentatively call the C site. This positively charged site may be masked in HuIFN-αA (SMM) due to a conformational change resulting from the opening of the S-S bond between Cyst 1 and Cyst 98. It may also be hidden in the dimer or trimer. Such a model has been suggested for diphteria toxin, in which a positively charged site, presumed to be the nucleotide binding site (30) is masked in its dimeric form (31).

It is tempting to postulate that the HuIFNα receptors on human cell surface may contain a negatively charged region with which the interaction of the C site of HuIFN-αA may ensure stability and high affinity. Consequently, masking of the C site in HuIFNα would lead to a diminished affinity, hence to a lower antiviral activity. Finally, for structure/function study or for clinical trials of IFNs, the differential binding of IFNs to poly (I)-agarose afford a simple tool to purify the "native" form such as HuIFN-αA (FMM), free from oligomers, with low antiviral activities, supposed to be formed during storage.

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