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Soluble interferon-α receptor molecules are present in body fluids

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Soluble forms of the interferon-α receptor (sIFN-αR) were identified in human serum and urine by Western blotting with monoclonal antibodies (MAb) directed against IFN-αR, and by immunoprecipitation (Iptn) of a covalently cross-linked complex of IFN-αR and [^{125}I]IFN-α with anti IFN-α MAb. Elevated levels of sIFN-αR were found in sera of hairy cell leukemia patients. The soluble receptor from serum migrated as a 55 kDa protein in SDS-PAGE, and, as expected, the cross-linked product migrated as a 75 kDa protein. The soluble receptor from urine was found to be a protein of mol. wt. 45 kDa and its cross-linked complex migrated as a 65 kDa protein. The calculated mol. wt. of the entire extracellular domain of the IFN-αR prior to post-translational modifications is 47,000. Since there are 12 potential glycosylation points in this extracellular domain, its actual mol. wt. may be as high as 70,000 Da. It is therefore concluded that sIFN-αR molecules, corresponding to truncated forms of the extracellular domain of the cell surface IFN-αR, are present in human serum and in normal human urine.

Cytokine; Hairy cell leukemia; Serum; Urine

1. INTRODUCTION

Interferon (IFN)-α constitutes a family of structurally related cytokines, defined by their ability to confer resistance to viral infections. Many other biological activities of IFN-α have been reported, including inhibition of cell proliferation, induction of class I MHC antigens and several other immunoregulatory activities [1]. IFN-α is useful for the treatment of several viral diseases, including hepatitis C [2] and viral warts [3], as well as certain malignancies, such as hairy cell leukemia [4], chronic myelogenous leukemia [5] and Kaposi's sarcoma [6].

As in the case of other cytokines, IFN-α exerts its biological activities by binding to a cell surface receptor, which is common to all IFN-α subtypes, as well as IFN-β. Human IFN-α receptor was identified and cloned from Daudi cells [7]. When expressed in murine cells this receptor makes them responsive to human IFN-αB and, to a lesser extent, to other IFN-α species, indicating that additional receptors or accessory proteins may be involved in the response to various IFN-α subtypes. The cloned receptor had a single transmembrane domain, an extracellular and an intracellular domain.

Several soluble cytokine receptors which correspond to the extracellular ligand binding domains of the respective cell-associated receptors were recently identified. These include the soluble receptors of IL-6, IFN-γ [8–10], TNF [11], IL-1 [6] and IL-4 [13]. Based on these findings we suggested that the previously reported soluble IL-2 receptor [14] was not a unique case but, rather, all cytokine receptors have soluble counterparts in body fluids [8]. Here we report that a soluble form of the human IFN-α receptor is present in serum and urine.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Sera of patients were obtained from Dr. Dan Aderka (Ichilov Hospital, Tel-Aviv, Israel). Crude urinary proteins of normal individuals, concentrated 1,000-fold [8], were obtained from Serono Laboratories, Rome, Italy; anti-IFN-α monoclonal antibody (MAb) No. 74.3 immobilized on agarose hydrazide (Sigma) was prepared as described [15]; di-*N*-hydroxysuccinimidyl suberate (DSS) was from Pierce.

2.2. *Immunization of mice, cell fusion and screening (monoclonal antibodies to the IFN-αR, manuscript in preparation)*

Balb/c mice were injected with a partially purified preparation of *E. coli* IFN-αR fused to protein A (10 μg/mouse/injection). The mouse showing the highest titer by inverted sRIA (see below) was chosen for fusion. Its splenic lymphocytes were fused with an NSO/1 myeloma variant (NSO cells, kindly provided by C. Milstein, MRC, Cambridge, UK). Hybridoma supernatants were tested for the presence of anti-IFN-αR antibodies by an inverted sRIA. Microtiter plates were coated with affinity-purified goat anti-mouse antibodies followed by the addition of hybridoma supernatants and [^{125}I]IFN-αR. Hybridomas that were found to secrete anti-IFN-αR antibodies were cloned and recloned by the limiting dilution technique. Supernatant of hybridoma No. 21.4 was used in this study.

2.3. *Interferons*

Recombinant IFN-α2 (2×10^8 U/mg) was kindly provided by Dr. C. Weismann, University of Zurich. IFN-αB (2×10^8 U/mg) was kindly provided by Dr. Gruter from Ciba-Geigy. IFN-αB was labeled by a modification of the chloramine T method [16]. Briefly, 7 μg of IFN-αB was labeled with 1 mCi of Na[^{125}I] in the presence of 1 mg/ml of chloramine T (20 s on ice), to a specific activity of 4×10^7 cpm/μg.

2.4. *Western Blotting*

Samples were subjected to SDS-PAGE under non-reducing conditions and electroblotted (in 25 mM Tris, 1.92 mM glycine, 20% methanol) onto nitrocellulose sheets (Schleicher and Schuell, 0.45 μm). Following electroblotting the sheet was incubated with a Blocking buffer (10% non-fat milk in PBS containing 0.05% Tween-20 and 0.02% sodium azide) and then for 2 h at room temperature with the anti-IFN-αR antibody. The nitrocellulose sheet was washed with 0.05% Tween-20 in PBS and incubated overnight at 4°C with [^{125}I]goat anti-mouse antibodies (0.7×10^6 cpm/ml, in the Blocking buffer). The blot was then washed, dried and autoradiographed.

2.5. *Cross-linking and immunoprecipitation (Iptn)*

Samples of serum or urine were incubated (1 h at 4°C) with [^{125}I]IFN-αB (300,000 cpm) in the absence or in the presence of a 100-fold excess of unlabeled IFN-αB or IFN-α2. DSS dissolved in dimethyl sulfoxide (Me_2SO) was then added to a final concentration of 1 mM and the mixture was left for 20 min at 4°C. The reaction was stopped by the addition of 1 M Tris-HCl, pH 7.5, and 1 M NaCl to a final concentration of 100 mM. The samples were immunoprecipitated by the addition of anti-IFN-α MAb immobilized on agarose hydrazide (25 μl, 7 mg/ml) [15]. Following incubation (overnight, 4°C), the beads were washed 3 times with PBS, suspended in a sample buffer containing 2% mercaptoethanol and the supernatants were analysed by SDS-PAGE (7.5% or 10% acrylamide gels [17]) followed by autoradiography.

3. RESULTS

Sera from two hairy cell leukemia (HCL) patients and normal human sera (NHS) were subjected to SDS-PAGE under non-reducing conditions and Western blotting with anti-IFN-αR MAb. The HCL serum exhibited a band of mol. wt. 55 kDa (Fig. 1, lanes C and D), while no such band was observed in NHS (Fig. 1, lane B). The high mol. wt. protein seen in lanes B–D was most probably serum immunoglobulin which cross-reacted with the second antibody used in the Western blotting. In order to characterize the 55 kDa protein and to check its ligand binding ability, aliquots of the various sera were covalently cross linked to [^{125}I]IFN-αB. A specific but weak band of mol. wt. 75 kDa could be observed (lanes F). The cross-linked products were enriched by Iptn with immobilized anti-IFN-α MAb. Indeed a broad band centering around 75 kDa, probably consisting of the 55 kDa protein cross-linked to [^{125}I]IFN-α, was clearly observed in the HCL sera (lanes H and J) but not in the NHS (lane L). The specificity of this binding was verified by cross-linking experiments in the presence of an excess of either unlabelled IFN-α2 or IFN-αB. Indeed the 75 kDa band was significantly reduced (lanes I and K). In addition to the specific (displacable) 75 kDa band, some non-displacable bands (50, 80, 97 and >100 kDa) were observed. The non-specific 80 kDa band (clearly seen in lanes H–M) could not be completely resolved from the 75 kDa band. It should be noted that in some experiments traces of slFN-αR were detected in NHS by cross-linking and Iptn (not shown).

A sample of crude urinary proteins obtained from normal individuals (1 μl 1,000-fold concentrated) was subjected to SDS-PAGE under non-reducing conditions, followed by Western blotting with anti-IFN-αR MAb. A protein band of mol. wt. 45 kDa was observed (Fig. 2a). The high mol. wt. protein seen in the same lane was identified by protein microsequencing as human immunoglobulin that cross-reacted with [^{125}I]goat anti-mouse antibodies. The ligand binding capacity of the 45 kDa protein was checked by incubating a crude urine sample with [^{125}I]IFN-αB in the presence

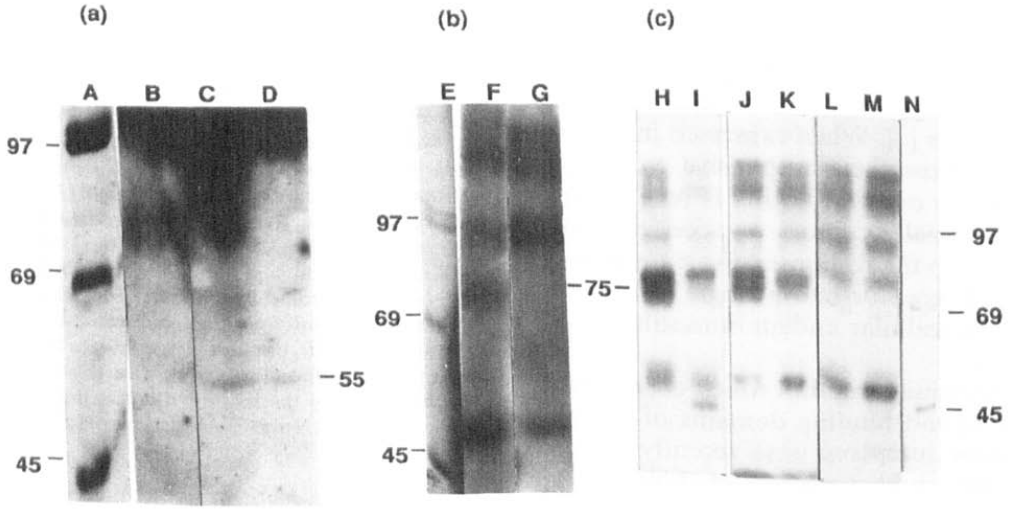

Fig. 1. (a) Western blotting of human sera with anti-IFN-αR MAb. Lanes: A, mol. wt. markers (kDa); B, normal human serum (NHS, 5 μl); C and D, serum from a hairy cell leukemia (HCL) patient (5 and 1 μl, respectively). (b) Autoradiogram of cross-linked complexes. Lanes: E, mol. wt. markers (kDa); F, serum (5 μl) from an HCL patient cross-linked to [^{125}I]IFN-αB; G, NHS (5 μl) cross-linked to [^{125}I]IFN-αB. (c) Autoradiogram of cross-linked complexes following Iptn with anti-IFN-α MAb. Lanes: H–K, serum (50 μl) from an HCL patient cross-linked to [^{125}I]IFN-αB in the absence (H and J) or in the presence of an excess of unlabelled IFN-α2 (I) or IFN-αB (K); L and M, NHS (50 μl) cross-linked to [^{125}I]IFN-αB in the absence (L) or in the presence (M) of unlabelled IFN-α2; N, mol. wt. markers (kDa).

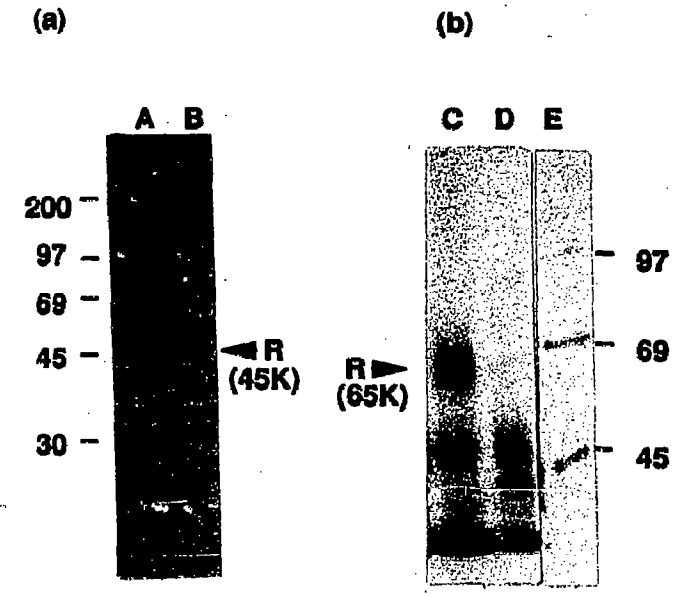

Fig. 2. (a) Western blotting of sIFN-αR from urine. Lanes: A, mol. wt. markers (kDa); B, crude urine (1 μl). (b) Autoradiogram of a cross-linked complex of urinary sIFN-αR and [^{125}I]IFN-α following Iptn. Lanes: C, urinary proteins, 1,000-fold concentrated (100 μl) cross-linked to [^{125}I]IFN-αB and immunoprecipitated with anti-IFN-α MAb; D, same as C except that excess unlabeled IFN-αB was added prior to cross-linking; E, mol. wt. markers (kDa).

or absence of excess of unlabeled IFN-α, and then cross-linking with DSS. Following Iptn with anti-IFN-α MAb and SDS-PAGE, a specific cross-linked product of mol. wt. 65 kDa was detected (Fig. 2b). This complex was smaller by 10 kDa than the cross-linked complex from serum.

In conclusion, two forms of sIFN-αR were found in body fluids, a 55 kDa protein in serum and a 45 kDa protein in urine.

4. DISCUSSION

sIFN-αR forms were identified in human serum and urine with the aid of a newly developed anti-IFN-αR MAb. High levels of sIFN-αR were found in sera of HCL patients that were undergoing IFN-α therapy, whereas the levels in NHS were very low. It is possible that IFN-α increases shedding or release of the sIFN-αR in vivo. We observed that IFN-α treatment of Daudi cells in vitro resulted in a significant down-regulation of a 90 kDa membrane-bound IFN-αR. Such down-regulation could stem from shedding of the extracellular domain of the receptor. Furthermore, we obtained indications that a sIFn-αR of the same size as that of the receptor in serum (55 kDa) is present constitutively in culture medium of Daudi cells (manuscript in preparation).

The calculated mol. wt. of the entire extracellular domain of the IFN-αR prior to post-translational modifications is 47,000 Da. Since there are 12 potential *N*-glycosylation sites in this extracellular domain, its actual mol. wt. may be as high as 70,000 Da. Thus it appears that the naturally occurring soluble receptors are truncated forms of the extracellular portion of the IFN-α receptor. The truncation could occur at either termini of the extracellular portion of the receptor and may consist of both a polypeptide and a polysaccharide. Interestingly, the two soluble receptors for human TNF were found to be C- and N-terminally truncated [18]. The urinary IFN-αR has a mol. wt. which is lower by 10 kDa than that of the soluble receptor found in serum and cell culture medium. This 45 kDa urinary protein is probably generated by further truncation of the soluble 55 kDa receptor.

Both forms of the sIFN-αR retained the ability to bind their ligand, as demonstrated by cross-linking experiments which yielded complexes of mol. wt. 65 kDa (urine) and 75 kDa (serum). In both cases the mol. wt. corresponded to a 1:1 complex of the respective soluble receptor and the 20 kDa IFN-α. An excess of either IFN-αB or IFN-α2 added to the cross-linking reactions significantly reduced the signal, thereby proving the specificity of the interaction between these soluble receptors and IFN-α.

Based on the sensitivity of the Western blotting we estimate that the concentration of the soluble receptor in normal human urine is in the range of 0.1–1 ng/ml, while the level in serum from HCL patients is in the range of 10–100 ng/ml. Similar levels (1 ng/ml for urine, 20–40 ng/ml for NHS and 150–250 ng/ml, in sera of HIV-seropositive patients) were found in the case of the soluble IL-6 receptor [19,20].

The sIFN-αR is a newly discovered member of the family of soluble cytokine receptors. So far the physiological role of the soluble cytokine receptors has not been established. Two mechanisms have been proposed for the formation of these receptors; (i) proteolytic cleavage of the membrane anchored receptor, as shown with IL-2R [21] and TNF-RI [22], and (ii) alternative splicing of mRNA as in the case of IL-4 [23], IL-6 [24] and IL-7 [25]. The soluble receptors bind their specific ligands and modulate their activity. In some cases they inhibit the biological activity, as was shown in the TNF system [26,27], while in some other cases they enhance the activity of the cytokine, as demonstrated with the IL-6 system [28]. The recombinant soluble TNF receptor was found to prevent septic shock in animal models [29], and soluble forms of the IL-1 receptor were found to have profound inhibitory effects on the development of in vivo alloreactivity in mouse allograft recipients [30]. Similarly, the soluble forms of IFN-αR may find use as modulators of IFN-α activity in autoimmune disease in which aberrant expression of IFN-α was reported, e.g. systemic lupus erythematosus [31].

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