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Purification of soluble cytokine receptors from normal human urine by ligand-affinity and immunoaffinity chromatography

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ABSTRACT

Affinity chromatography of crude human urinary proteins on either human recombinant interleukin-6 (rIL-6) or human recombinant interferon-γ (rIFN-γ) or anti IFN-γ receptor (IFN-γ-R) monoclonal antibodies (McAb) yielded the two respective soluble receptors in significant amounts. A single sequence of 30 amino acid residues was obtained by N-terminal microsequencing of the protein peak purified in tandem by affinity chromatography on an IL-6 column and reveresed-phase high-performance liquid chromatography. This sequence was identical with the predicted N-terminal sequence of IL-6-R as previously reported. The purified IL-6-R retained its biological activity. It was used for the preparation of specific anti IL-6-R monoclonal antibodies. Analysis of the eluted proteins from both IFN-γ and anti IFN-γ-R columns by inhibition of solid-phase radioimmunoassay, enzyme-linked immunosorbent assay, sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blotting proved the existence of soluble IFN-γ-R in normal urine. This finding together with the already known presence of soluble TNF receptors and a soluble IL-2 receptor found both in plasma and in urine indicates that release of soluble cytokine receptors into body fluids is a general phenomenon which occurs under normal physiological conditions.

INTRODUCTION

Soluble extracellular fragments of receptors may serve as natural blockers or enhancers of their respective hormones or cytokines. Only a limited number of such

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soluble receptor fragments have been reported, including receptors for red blood cells[1] and for the Fc portion of immunoglobulins[2]. Among the cytokine receptors, interleukin-2 receptor was the only one reported to be released in a soluble form from activated human lymphoid cells[3]. Moreover, it was detected in body fluids of normal individuals and its level was increased in disease states[4]. To address the question of whether release of soluble cytokine receptors into body fluids is a general phenomenon, we analysed urine from normal donors for the presence of additional cytokine receptors. Affinity chromatography of crude human urinary proteins using either the corresponding ligand [human recombinant interleukin-6 (rIL-6) or recombinant interferon-γ (rIFN-γ)] or the corresponding monoclonal antibodies [anti IFN-γ receptor (IFN-γ-R)] yielded the respective two soluble receptors in significant amounts. It is therefore sugested that shedding of various cytokine receptors is a general phenomenon which occurs under normal physiological conditions.

EXPERIMENTAL

Cytokines

rIL-6 and rIFN-γ (InterPharm Labs., Ness-Ziona, Israel) were purified to homogeneity on an anti IL-6 monoclonal antibody (McAb) column[5] and an IFN-γ McAb column[6], respectively. Iodination of cytokines or receptors was performed by the chloramine-T method to a specific activity of $2.2 \cdot 10^7$ cpm/μg for rIL-6, $2.5 \cdot 10^6$ cpm/μg for rIFN-γ and $5 \cdot 10^7$ cpm/μg for IL-6-R.

Antibodies

McAb No. 34-1[5] was used for affinity purification of IL-6; McAb No. 3-3[7] was used both for affinity purification of rIFN-γ and for coating microtitre plates in a solid-phase radioimmunoassay (sRIA); McAb No. 177-1[8] was used both for immunoaffinity purification of the IFN-γ-R from crude urine and for coating microtitre plates in a double antibody enzyme-linked immunosorbent assay (ELISA). Rabbit anti IFN-γ-R polyclonal antibodies were obtained by immunization with a ligand affinity-purified IFN-γ-R from human placenta.

Immunoaffinity chromatography

Agarose–polyacrylhydrazide beads[9] (BioMakor, Ness-Ziona, Israel) were used in order to couple all the monoclonal antibodies used in immunoaffinity chromatography.

Ligand affinity chromatography of urine

Each of the cytokines (2.5 mg) was coupled to Affigel-10 (1 ml) (Bio-Rad Labs. Richmond, CA, U.S.A.). Concentrated crude or partially purified urinary proteins[10] were placed on each of the columns. Following washing with phosphate-buffered saline (PBS), bound proteins were eluted with citric acid (25 m*M*, pH 2.5) and immediately neutralized.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

Eluted fractions from the immobilized IL-6 column were further resolved by RP-HPLC on an Aquapore RP-300, column (30 × 4.6 mm I.D.) (Brownlee Labs.,

Santa Clara, CA, U.S.A.) using an acetonitrile gradient in 0.3% aqueous trifluoroacetic acid (TFA). Fractions of 0.5 ml were collected.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting

Proteins resolved by SDS-PAGE (12%) under non-reducing conditions[11] were rendered visible either by silver staining[12] or electroblotting on nitrocellulose sheets as described previously[8]. Electroblotted proteins were reacted either with [^{125}I]rIL-6 ($1 \cdot 10^6$ cpm/ml) or with McAb No. 177-1 (1:500), followed by [^{125}I]goat anti mouse antibodies ($0.7 \cdot 10^6$ cpm/ml). The nitrocellulose sheets were then washed, dried and autoradiographed.

Hybridoma growth factor (HGF) activity assay[13]

The bioactivity of the IL-6 was measured by stimulation of [^{3}H]thymidine. The assay was performed in the presence or absence of a soluble preparation of IL-6-R. One unit of IL-6 is defined as the amount of protein that gives 50% of the maximum effect in the assay.

Protein determination

Protein concentrations were determined by the fluorescamine method[14].

N-Terminal sequence analysis

The HPLC-purified protein was subjected to N-terminal sequence analysis on a pulsed liquid–gas-phase protein microsequencer (Model 475A; Applied Biosystems; Foster City, CA, U.S.A.).

RESULTS

Affinity chromatography on an immobilized rIL-6 column was used as the main step of IL-6 receptor (IL-6-R) purification. A concentrate of crude urinary proteins (1.5 g of protein in 100 ml obtained from 40 l of urine) was placed on the IL-6 column and bound proteins (50 μg) were eluted by low pH. Further purification was achieved by RP-HPLC which yielded a major protein peak eluting at 39% acetonitrile (14 μg)[15]. On scale-up of these procedures, 110 μg of pure protein were obtained from CM-Sepharose-purified urinary proteins[10], starting with 900 l of urine. Analysis by SDS-PAGE of the protein peak from the RP-HPLC column gave a single broad band with an apparent molecular weight of 50 000 under non-reducing conditions. Following electroblotting of the partially purified protein sample from the affinity column eluate, only the mol.wt. 50 000 band reacted specifically with [^{125}I]IL-6 (Fig. 1). The protein peak from the RP-HPLC was further characterized by N-terminal microsequencing and a single sequence of 30 amino acid residues was obtained (single-letter code): LAPRR(C)PAQEVARGVLTSLPGDSVTLT(C)PG. This sequence was identical with the predicted N-terminal sequence of IL-6-R reported previously[16]. The apparent molecular weight of this fragment may correspond to the extracellular portion of mature IL-6-R.

The ligand affinity-purified IL-6-R retained its biological activity: it enhanced the HGF activity of IL-6 on mouse plasmacytoma cells in a dose-dependent manner

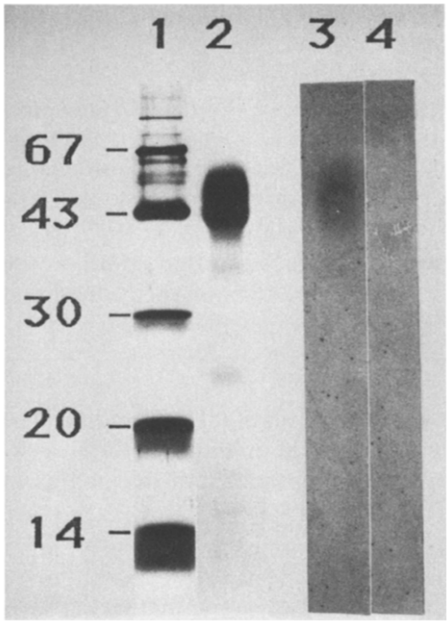

Fig. 1. Analysis of purified IL-6-R by SDS-PAGE and by [^{125}I]rIL-6 binding. Silver-stained SDS-PAGE: lane 1 = molecular weight (kilodalton) markers; lane 2 = aliquot (850 ng) of HPLC peak fraction (39% acetonitrile)[15]. Autoradiograph of [^{125}I]rIL-6 bound to electroblotted proteins: lane 3 = ligand (IL-6)–affinity-purified urinary proteins (LAPUP, 1.8 μg); lane 4 = L(IFN-γ)–APUP (1.5 μg, negative control).

(Fig. 2) while the IL-6 alone at the dose used (0.5 unit/ml) did not stimulate HGF activity at all. Taga *et al.* [17] recently reported that the soluble part of the rIL-6-R increased the growth-inhibitory effect of human IL-6 on mouse M1 cells. Moreover, our soluble IL-6-R strongly increased the anti-growth effect of IL-6 on human breast ductal carcinoma cells T47[18,19].

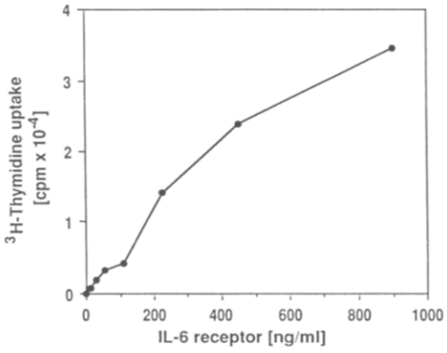

Fig. 2. Enhancement of IL-6 HGF activity by L(IL-6)–APUP on T1165 mouse plasmacytoma cells. Background [^{3}H]thymidine uptake (5000 cpm) of the cells was subtracted from all the readings.

TABLE I

SCREENING OF HYBRIDOMAS BY AN INVERTED sRIA

Hybridoma No.	*cpm*	*Ig class*	*Hybridoma No.*	*cpm*	*Ig class*
4.4	20455	IgG1	36	1455	IgM
5	1085	IgM	37	9640	IgG1
17.1	36565	IgG2a	38.4	35975	IgG1
20.2	31450	IgG1	39.1	5195	IgG2
22	11465	IgG2	40	1415	IgG1
24.2	8850	IgG1	41	1870	IgG1
25	2000	IgG2a	42.5	33565	IgG1
28.7	1645	IgG1	43	1255	IgG1
29	4165	Not done	46	6090	Not done
30.8	1755	IgM	48	18000	IgG1
31	3060	Not done	49	8000	IgM
32.5	31465	IgG1	50.3	28440	IgG1
33.2	14875	IgG1	51	1075	IgG1
34.1	33480	IgG1	52	3945	IgM
35.2	35495	IgG3	53.4	3440	IgG1

The pure soluble IL-6-R preparation was used in order to prepare monoclonal antibodies. Fusion of NSO cells with spleen cells from a Balb/c mouse that received five injections (2.5 μg per injection) was performed. An inverted solid-phase RIA was used for screening. Briefly, microtitre plates were coated with pure goat anti-mouse antibodies and washed and incubated with the hybridoma supernatants. The specific antibodies were detected by a pure preparation of an iodinated soluble IL-6-R. Thirty anti IL-6-R antibodies were obtained out of 800 hybridomas screened (Table I). Some of the antibodies were found to be suitable for detection of IL-6-R in Western blots. They are now being characterized for neutralization of the biological activity of IL-6 and for immunoaffinity purification of IL-6-R from different sources.

In parallel we searched for the possible existence of soluble IFN-γ-R or other IFN-γ binding proteins. Two approaches were attempted. In one ligand affinity chromatography and in the other immunoaffinity chromatography was used. Application of crude proteins from 125 l of urine on the rIFN-γ column[20] and elution at low pH yielded 37 μg of IFN-γ binding proteins. Application of crude proteins from 100 l of urine on an immobilized anti IFN-γ-R McAb No. 177-1[8] and elution at low pH yielded 70 μg of proteins. Analysis of the eluted proteins from both columns by SDS-PAGE, under non-reducing conditions followed by silver staining revealed a similar pattern of protein bands (Fig. 3). Following electroblotting onto nitrocellulose membrane, only the mol. wt. 40000 protein in both preparations reacted specifically with the anti IFN-γ-R McAb (Fig. 3). The same band also reacted specifically with [^{125}I]IFN-γ but the signal was faint (data not shown). From the results obtained it was concluded that the protein showing a molecular weight of 40000 is the ligand-binding domain of the IFN-γ receptor. Further confirmation of the identity of the IFN-γ binding protein as IFN-γ receptor was obtained by two additonal tests. The eluate from the rIFN-γ column inhibited in a dose-dependent manner the binding of [^{125}I]IFN-γ to anti IFN-γ McAb in an sRIA[15], and the same eluate gave a specific signal in a double antibody ELISA based on monoclonal and polyclonal anti IFN-γ-R antibodies[15].

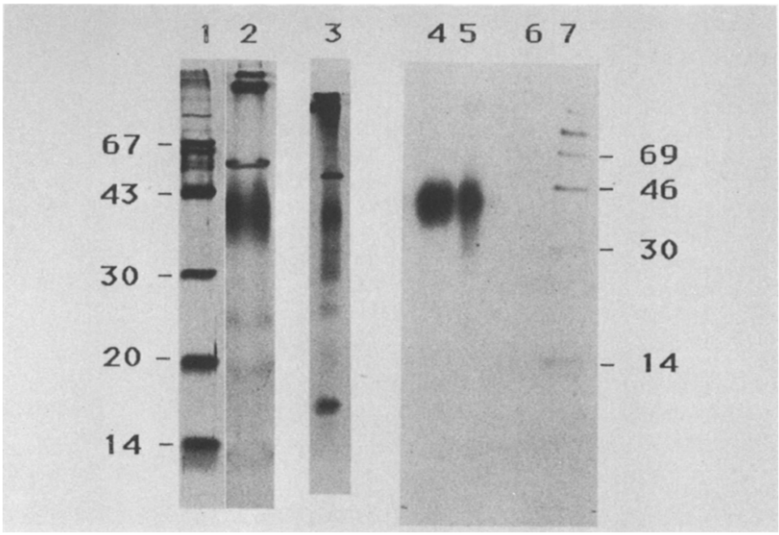

Fig. 3. Analysis of IFN-γ binding proteins by SDS-PAGE and by Western blotting. Silver-stained SDS-PAGE: lane 1 = molecular weight (kilodalton) markers; lane 2 = L(IFN-γ)–APUP (300 ng); lane 3 = immunoaffinity (anti IFN-γ-R McAb)-purified urinary proteins (IAPUP, 250 ng). Autoradiograph of a Western blot performed with anti IFN-γ-R McAb: lane 4 = L(IFN-γ)–APUP (600 ng); lane 5 = IAPUP (900 ng); lane 6 = L(IL-6)–APUP (1200 ng, negative control); lane 7 = ^{14}C molecular weight (kilodalton) markers.

DISCUSSION

This study has demonstrated the convenience and success of ligand and immunoaffinity chromatography in the purification of proteins that are present in trace amounts in very crude protein mixtures. It also proves the existence of specific receptors for the IL-6 and IFN-γ cytokines in normal human urine. Moreover, the purified IL-6-R retained its biological activity; it enhanced the growth-stimulatory action of subminimal doses of IL-6 on mouse cells and also increased the growth-inhibitory effect of IL-6 on human breast cancer cells. This finding, together with the already known presence of soluble IL-2-R in both plasma and urine, indicates that release of soluble cytokine receptors into body fluids is a general phenomenon that occurs under normal physiological conditions, and might have significant consequences *in vivo*. We recently found that a urinary protein that inhibits TNF activity[21], shown by us and others to function by binding TNF specifically[10,22], also represents a soluble version of a cell surface TNF receptor[23]. Soluble receptors might be derived either by shedding of cell surface receptors or by a separate biosynthetic pathway starting from alternatively spliced mRNA or even from a distinct gene. However, the protein sequence identity of the soluble part of the IL-6-R described by us and the reported IL-6-R cDNA indicates that both proteins are derived from the same gene. The observed prevalence of soluble cytokine receptors suggests that they may have an immunoregulatory role, either by participation in the process of

eliminating cytokines via the kidney or, if present in the plasma as shown for IL-2-R, by modulating the availability of their corresponding cytokines.

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