

SOLUBLE CYTOKINE RECEPTORS ARE PRESENT IN  
NORMAL HUMAN URINE

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Soluble extracellular fragments of receptors may serve as natural blockers of their respective hormones or cytokines. Only a limited number of such soluble receptor fragments were reported, including receptors for RBCs (1) and for the Fc portion of Igs (2). Among the cytokine receptors, IL-2-R 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 analyzed urine of normal donors for the presence of additional cytokine receptors. Affinity chromatography of crude human urinary proteins on either immobilized human rIL-6, immobilized human rIFN- $\gamma$ , or immobilized anti-IFN- $\gamma$ -R mAb yielded the two respective soluble receptors in significant quantities. It is therefore suggested that shedding of various cytokine receptors is a general phenomenon that occurs under normal physiological conditions.

Materials and Methods

*Cytokines.* rIL-6 and rIFN- $\gamma$  (provided by InterPharm laboratories, Ness-Ziona, Israel) were purified to homogeneity on an anti-IL-6 mAb column (5) and an IFN- $\gamma$  mAb column (6), respectively. Iodination of cytokines was performed by the chloramine-T method to a specific activity of  $2.2 \times 10^7$  cpm/ $\mu$ g for rIL-6 and  $2.5 \times 10^6$  cpm/ $\mu$ g for rIFN- $\gamma$ .

*Antibodies.* mAb 34-1 (5) was used for affinity purification of IL-6. mAb 3-3 (7) was used both for affinity purification of rIFN- $\gamma$  and for coating microtiter plates in a solid phase RIA (sRIA). mAb 177-1 (8) was used both for immunoaffinity purification of the IFN- $\gamma$ -R from crude urine and for coating microtiter plates in a double-antibody ELISA. Rabbit anti-IFN- $\gamma$ -R polyclonal antibodies were obtained by immunization with a ligand affinity-purified IFN- $\gamma$ -R from human placenta.

*Ligand Affinity Chromatography of Urine.* Each of the cytokines (2.5 mg) was coupled to Affigel-10 (1 ml; Bio-Rad Laboratories, Richmond, CA). Concentrated crude or partially purified urinary proteins (9) were passed on each of the columns. After washings with PBS, bound proteins were eluted by citric acid (25 mM; pH 2.5) and immediately neutralized.

*Reversed Phase HPLC.* Eluted fractions from the immobilized IL-6 column were further resolved by reversed-phase (rp)HPLC (Aquapore RP-300,  $4.6 \times 30$  mm; Brownlee Labs., Santa Clara, CA) using an acetonitrile gradient in 0.3% aqueous trifluoroacetic acid (TFA). 0.5-ml fractions were collected.

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**SDS-PAGE and Electroblothing.** Proteins resolved by SDS-PAGE (12% acrylamide) under nonreducing conditions (10) were visualized either by silver staining (11) or electroblotted on nitrocellulose sheets as previously described (8). Electroblotted proteins were reacted either with  $^{125}\text{I}$ -rIL-6 ( $10^6$  cpm/ml) or with mAb 177-1 (1:500), followed by  $^{125}\text{I}$ -goat anti-mouse antibodies ( $0.7 \times 10^6$  cpm/ml). The nitrocellulose sheets were then washed, dried, and autoradiographed.

**Inhibition of IFN- $\gamma$  Binding to its mAb in sRIA.** PVC plates coated with anti-IFN- $\gamma$  mAb 3-3 and blocked with BSA were incubated with  $^{125}\text{I}$ -IFN- $\gamma$  ( $10^4$  cpm/well) in the presence or absence of affinity-purified urinary proteins. The wells were then washed, cut, and counted.

**Double Antibody ELISA.** 96-well ELISA plates coated with anti-IFN- $\gamma$ -R mAb 177-1 and blocked with BSA were incubated first with affinity-purified urinary proteins, then with rabbit anti-IFN- $\gamma$ -R serum (1:1,000), and then with goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). The plates were then washed and incubated with a peroxidase substrate (ABTS; Sigma Chemical Co.) and measured by an ELISA reader.

**Protein Determination.** Protein concentrations were determined by the fluorescamine method (12).

**NH<sub>2</sub>-terminal Sequence Analysis.** The HPLC-purified protein was subjected to NH<sub>2</sub>-terminal sequence analysis on a pulsed liquid gas phase protein microsequencer (475A; Applied Biosystems, Inc., Foster City, CA).

## Results

Affinity chromatography on an immobilized rIL-6 column was used as the main step of IL-6-R purification. A concentrate of crude urinary proteins (1.5 g protein in 100 ml obtained from 40 liters of urine) was passed on the IL-6 column, and bound proteins (50  $\mu\text{g}$ ) were eluted by low pH. Further purification was achieved by RP-HPLC, which yielded a major protein peak (14  $\mu\text{g}$ ) (Fig. 1, fr. 42). On scale up of these procedures, 110  $\mu\text{g}$  of pure protein was obtained from CM-Sepharose-purified urinary proteins (9), starting with 900 liters of urine. Analysis by SDS-PAGE of the protein peak from the RP-HPLC column gave a single broad band of  $M_r$   $5 \times 10^4 \pm 10^4$  under nonreducing conditions. After electroblotting of the partially

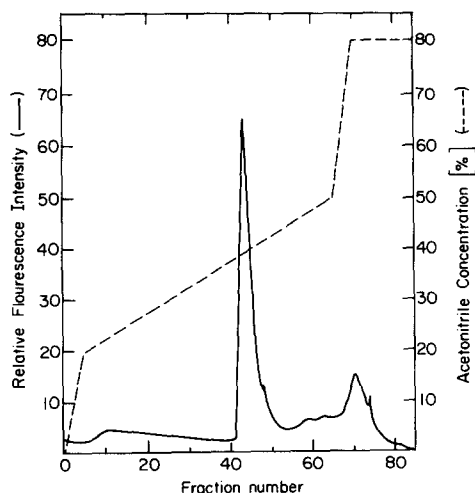


FIGURE 1. Purification of the urinary IL-6-R by RP-HPLC. Ligand (IL-6) affinity-purified urinary proteins were applied to the HPLC column, eluted at 0.5 ml/min by an acetonitrile gradient (dotted line), and the protein was monitored by a preparative fluorescamine system (12) (solid line).

purified protein sample from the affinity column eluate, only the  $5 \times 10^4 \pm 10^4$  band reacted specifically with  $^{125}\text{I}$ -IL-6 (Fig. 2). The protein peak from the RP-HPLC was further characterized by  $\text{NH}_2$ -terminal microsequencing and a single sequence of 30 amino acid residues was obtained: L A P R R (C) P A Q E V A R G V L T S L P G D S V T L T(C) P G. This sequence was identical to the predicted  $\text{NH}_2$ -terminal sequence of IL-6-R as previously reported (13); cysteine (C) is not detectable in the protein microsequencer. The  $\text{NH}_2$  terminus of this natural receptor fragment confirms the presumed cleavage point of the signal peptide of the primary translation product (13). The apparent molecular weight of this fragment may correspond to the extracellular portion of mature IL-6-R.

We then searched for the possible existence of soluble IFN- $\gamma$ -R or other IFN- $\gamma$  binding proteins. Two approaches were attempted. In the first, ligand affinity chromatography was used, while in the second, immunoaffinity chromatography was used. Application of crude proteins from 125 liters of urine on the rIFN- $\gamma$  column (14) and elution at low pH yielded 37  $\mu\text{g}$  of IFN- $\gamma$  binding proteins. Application of crude proteins from 100 liters of urine on an immobilized anti-IFN- $\gamma$ -R mAb 177-1 (8) and elution at low pH yielded 70  $\mu\text{g}$  of proteins. Analysis of the eluted proteins from both columns by SDS-PAGE, under nonreducing conditions, followed by silver staining, revealed a similar pattern of protein bands, corresponding to proteins of  $M_r$   $4 \times 10^4$ ,  $5.3 \times 10^4$ , and two protein bands  $>9.3 \times 10^4$  (Fig. 3, lanes 2 and 3). After electroblotting onto nitrocellulose membrane, only the 40-kD protein in both preparations reacted specifically with the anti-IFN- $\gamma$ -R mAb (Fig. 3, lanes 4 and 5). The same band also reacted specifically with  $^{125}\text{I}$ -IFN- $\gamma$ , but the signal was faint (data not shown). From the results obtained it was concluded that 40-kD protein is the ligand binding domain of the IFN- $\gamma$ -R. Further confirmation of the identity of the IFN- $\gamma$  binding protein as IFN- $\gamma$ -R was obtained by two additional tests. The eluate of the rIFN- $\gamma$  column inhibited, in a dose-dependent manner, the binding of  $^{125}\text{I}$ -IFN- $\gamma$  to anti-IFN- $\gamma$  mAb 3-3 in an sRIA (Fig. 4 a). The same eluate gave a specific signal in a double-antibody ELISA based on monoclonal and polyclonal anti-IFN- $\gamma$ -R antibodies (Fig. 4 b).

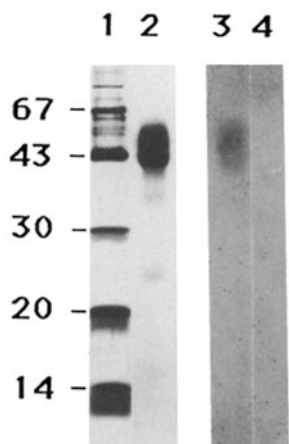


FIGURE 2. Analysis of the purified IL-6-R by SDS-PAGE and by  $^{125}\text{I}$ -rIL-6 binding. Silver-stained SDS-PAGE: lane 1, molecular mass (kD) markers; lane 2, aliquot (850 ng) of HPLC fraction 42 (Fig. 1). Autoradiograph of  $^{125}\text{I}$ -rIL-6 bound to electroblotted proteins: lane 3, ligand (IL-6) affinity-purified urinary proteins (1.8  $\mu\text{g}$ ); lane 4, ligand (IFN- $\gamma$ ) affinity-purified urinary proteins (1.5  $\mu\text{g}$ , negative control).

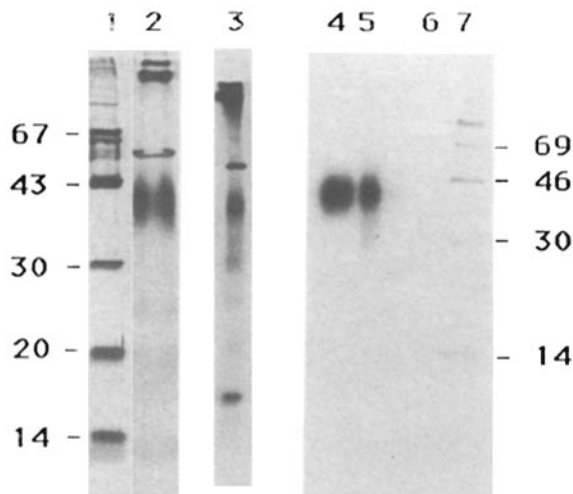


FIGURE 3. Analysis of IFN- $\gamma$  binding proteins by SDS-PAGE and by Western blotting. Silver-stained SDS-PAGE: lane 1, molecular mass (kD) markers; lane 2, ligand (IFN- $\gamma$ ) affinity-purified urinary proteins (300 ng); lane 3, immunoaffinity (anti-IFN- $\gamma$ -R mAb)-purified urinary proteins (250 ng). Autoradiograph of a Western blot performed with anti-IFN- $\gamma$ -R mAb: lane 4, ligand (IFN- $\gamma$ ) affinity-purified urinary proteins (600 ng); lane 5, immunoaffinity (anti-IFN- $\gamma$ -R mAb)-purified urinary proteins (900 ng); lane 6, ligand (IL-6) affinity-purified urinary proteins (1,200 ng, negative control); lane 7, <sup>14</sup>C molecular mass (kD) markers.

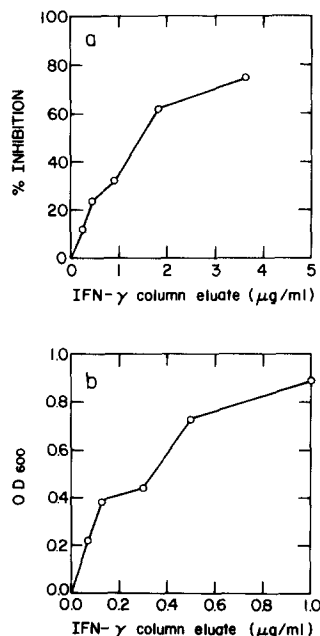


FIGURE 4. (a) Inhibition of <sup>125</sup>I-rIFN- $\gamma$  binding in sRIA by ligand (IFN- $\gamma$ ) affinity-purified urinary proteins. Maximal binding with <sup>125</sup>I-rIFN- $\gamma$  alone was 4,300 cpm. Background binding (200 cpm) was determined in the presence of excess unlabeled rIFN- $\gamma$  and was subtracted from all readings. Immunoaffinity-purified IFN- $\gamma$ -R from human placenta (8) (8  $\mu$ g/ml) was used as a positive control and gave 80% inhibition. Ligand (IL-6) affinity-purified urinary protein (10  $\mu$ g/ml) was used as a negative control and gave only 10% inhibition. (b) Characterization of IFN- $\gamma$  binding proteins as receptors by a double-antibody ELISA. Immunoaffinity-purified IFN- $\gamma$ -R from human placenta (8) (0.5  $\mu$ g/ml) was used as a positive control and gave 0.45 OD<sub>600</sub>. Ligand (IL-6) affinity-purified urinary proteins (0.5  $\mu$ g/ml) were used as a negative control and gave 0.06 OD<sub>600</sub>.

### Discussion

The present study proves the existence of specific receptors for the IL-6 and IFN- $\gamma$  cytokines in normal human urine. 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. We recently found that a urinary protein that inhibits TNF activity (15), shown by us and others to function by binding TNF specifically (9, 16), also represents a soluble version of a cell surface TNF receptor (Engelmann,

H., D. Novick, and D. Wallach, manuscript submitted for publication). Soluble receptors can 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.

### Summary

Affinity chromatography of crude human urinary proteins on either human rIL-6, human rIFN- $\gamma$ , or anti-IFN- $\gamma$ -R mAb yielded the two respective soluble receptors in significant quantities. A single sequence of 30 amino acid residues was obtained by NH<sub>2</sub>-terminal microsequencing of the protein peak purified in tandem by affinity chromatography on an IL-6 column and reversed-phase HPLC. This sequence was identical to the predicted NH<sub>2</sub>-terminal sequence of IL-6-R as previously reported. Analysis of the eluted proteins from both IFN- $\gamma$  and anti-IFN- $\gamma$ -R columns by inhibition of solid phase RIA, ELISA, SDS-PAGE, and Western blotting proved the existence of soluble IFN- $\gamma$ -R in normal urine. Our finding, together with the already known presence of urinary TNF binding proteins and a soluble IL-2-R both in plasma and in urine, indicates that release of soluble cytokine receptors into body fluids is a general phenomenon that occurs under normal physiological conditions.

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