Involvement of the Kidney in Catabolism of Human Leukocyte Interferon

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SUMMARY

The metabolic fate of human leukocyte interferon (HuIFN-α) was studied after intravenous injection into rats and cynomolgus monkeys. At various intervals the animals were sacrificed and the HuIFN-α content determined in serum and various tissues. HuIFN-α quickly disappeared from the circulation and was found mainly in the kidneys, in which levels were at least 7- to 10-fold higher than in the liver, spleen, lungs, heart, brain and muscles. No interferon was detected in urine. Subcellular fractionation of kidney revealed that the mitochondrial-lysosomal fraction (15,000 g) had a high HuIFN-α content. It was also found that HuIFN-α was rapidly inactivated by two types of proteinases found in the lysosomal fractions of rat, monkey and human kidneys, with an optimal pH of 3 to 4. The inactivation was partially inhibited by either pepstatin or leupeptin. Inactivation was totally prevented by a mixture of both inhibitors. Since it is known that interferon is scantily excreted in urine, our findings suggest that the kidney serves as a main site for its degradation.

INTRODUCTION

Human leukocyte interferon (HuIFN-α) is one of the stable proteins and retains its activity for a long period under various storage conditions (Fantes, 1973; Mogensen & Cantell, 1977). However, its clearance from blood is rapid (Cantell & Pyhälä, 1976), suggesting that the large quantities required for therapeutic purposes may result, at least in part, from its short biological life.

HuIFN-α has little if any biological activity in rats or rabbits; however, both types of animals exhibit similar clearance patterns from the circulatory system as do humans (Cantell & Pyhälä, 1973; Skreko et al., 1973; Emödi et al., 1975 a; Cantell & Pyhälä, 1976). On the other hand, only a small amount of HuIFN-α can be found in body excretions (Emödi et al., 1975 a, b). It seems, therefore, that its disappearance from the circulating blood system may be a result of uptake by body tissues or rapid degradation, or a combination of both, and its clearance or catabolism still remains obscure.

Our present work, performed with rats and with cynomolgus monkeys, which are sensitive to HuIFN-α treatment (Hilfenhaus et al., 1975, 1977), suggests that the kidney is a major site of interferon catabolism.

METHODS

Media for cell cultivation. Eagle’s minimum essential medium (MEM) supplemented with 10% calf serum was used. Pepstatin was kindly donated by Professor H. Umazawa, from the Peptide Institute, Osaka, Japan. Stock solutions were made in 0.1 M-acetate buffer pH 3.5, at concentrations of 1 mg/ml. Leupeptin was purchased from Sigma and was dissolved in a
saline solution at a concentration of 1 mg/ml. All other reagents were obtained from commercial sources and were of analytical grade when available.

**Human leukocyte interferon.** Separation of leukocytes from blood donations was performed by the Israeli Central Blood Bank. After separation, HuIFN-α was prepared and partially purified according to the method of Cantell (Mogensen & Cantell, 1977; Cantell & Hirvonen, 1977). Interferon preparations used in all experiments had a specific activity of $3 \times 10^6$ units/mg protein.

**Interferon assay.** Determination of interferon activity was performed in microassay plates (Tilles & Finland, 1968) using MDBK cells and vesicular stomatitis virus as a challenge virus. The MDBK cells were grown in MEM containing 10% heated calf serum. Wells of microtitre plates were first filled with medium. The tested interferon sample was added to the first well and serials of twofold dilutions were made. Each well was then seeded with the MDBK cells and after 24 h of incubation at 37 °C the virus was added. The titrations were scored microscopically 24 h later. The highest dilution of the titrated sample causing approx. 50% protection of cells was considered the endpoint. All results were corrected for the reference standard interferon (MRC research standard B 69/19).

**Animals.** Sprague–Dawley rats of approx. 200 g wt. each and cynomolgus monkeys of approx. 3-5 kg wt. each were used. Rats were injected with $1 \times 10^6$ units of interferon in the tail vein. Injected rats were killed by decapitation and their organs were immediately removed. Monkeys were sedated by intramuscular injection of Sernylan (phencyclidine–HCl) 1 mg/kg before the interferon injection. Each monkey was injected in the antecubital vein with $7 \times 10^6$ units of HuIFN-α. Blood samples were collected from the uninjected arm.

**Human kidneys.** Human kidneys were obtained immediately after surgical removal from patients and undamaged portions were used.

**Subcellular fractionation.** After removal, organs were rinsed thoroughly with ice-cold 0·3 M-sucrose, 1 mM-EDTA pH 7, minced rapidly and homogenized 1:8 (w/v) with the same solution in Potter-Elvehjem homogenizer and centrifuged at 140 g for 10 min to remove cell debris. Isolations of kidney or liver mitochondrial-lysosomal and microsomal fractions were done according to Johnson & Lardy (1967). Homogenates were centrifuged at 600 g for 10 min and the supernatant was spun again at 15000 g for 5 min to produce mitochondrial-lysosomal fractions. The supernatant was then centrifuged at 100000 g for 60 min to produce a microsomal pellet. Isolation of separate mitochondrial and lysosomal fractions from kidneys was carried out according to Maunsbach (1974). Before assay of the interferon content, each fraction was brought to 0·1% with Triton X-100 and then diluted as required.

**Urine collection.** Urine was collected by quickly grabbing and holding the rats over a Petri dish with a few drops collected each time. The lower limit of interferon detection in urine is 10 units/ml.

**Inactivation of interferon by tissue homogenates and subcellular fractions.** The reaction mixture (1 ml) contained 100 mM-buffer (glycine buffer pH 2 to 3; sodium acetate buffer pH 3 to 6; sodium phosphate buffer pH 6 to 8; tris–HCl buffer pH 8 to 9), 3000 units of HuIFN-α and 100 µl of homogenate or 100 µg protein of subcellular fraction. The reaction was carried out at 37 °C for 60 min and was terminated by diluting the reaction mixture 20-fold with ice-cold MEM containing 10% calf serum. Aliquots were taken for interferon assay.

**Protein determination.** Protein was determined according to Lowry et al. (1951).

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**RESULTS**

**Distribution of HuIFN-α in body tissues of intravenously injected rats and monkeys**

$1 \times 10^6$ units of HuIFN-α were injected intravenously into rats. The animals were sacrificed at 5, 30, 90, 240, 360, 480 min after injection and the interferon content in serum,
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Fig. 1. Interferon concentration in body tissues of rats and monkeys after intravenous injection. (a) HuIFN-α concentration in rat tissues at different times (min) after intravenous injection; each value is a mean of three injected rats. (b) HuIFN-α concentration in monkey tissues 20 min after intravenous injection. The values are the mean from two monkeys.

heart, lung, spleen, brain, calf muscle, liver and kidney was determined. The highest level of HuIFN-α was found in the kidney (Fig. 1a). This concentration was at least 10 times higher than in the other organs and remained high for at least 30 min, even when the serum had begun to fall. However, after 90 min, the concentration in the kidney decreased to about 10% of its maximal level at 5 and 30 min after injection. The maximal amount of HuIFN-α that was found in the examined tissues (excluding serum) at 5 and 30 min after injection did not exceed 10 to 15% of the total injected interferon. The HuIFN-α content of urine was assayed 5, 15, 30, 60 and 120 min after interferon injection of 1 x 10⁶ units. No interferon could be detected in urine. A control experiment showed that incubation of HuIFN-α with urine for 20 h at room temperature or 37 °C did not result in any loss of interferon activity.

Similar interferon distribution was also observed in the monkey. The highest interferon level at 20 min after intravenous injection of 7 x 10⁶ units of HuIFN-α was found in the kidneys, where its concentration was about 7 times higher than in other organs (Fig. 1b). The total amount of HuIFN-α found in the tissues at that time was also about 10% of the total interferon injected.

Subcellular distribution of injected interferon in rat and monkey kidneys

Rat kidneys were removed 20 min after intravenous HuIFN-α injection, fractionated and each subcellular fraction was assayed for HuIFN-α content. The results are presented in Table 1 and show clearly that interferon penetrated into kidney cells. About 30% of the interferon which accumulated in the kidneys was found in the mitochondrial-lysosomal fraction and about 65% in the 100000 g supernatant. Similar results were obtained with monkey kidneys, where 25% of the interferon was associated with mitochondrial-lysosomal fraction and about 70% was found in the supernatant. In control experiments in which HuIFN-α was homogenized together with kidneys from uninjected rats, almost no interferon was found in the mitochondrial-lysosomal fraction (Table 1). No appreciable HuIFN-α was
Fig. 2. Effect of pH on HuIFN-α degradation by kidney homogenates. A 100 μl amount of rat (●), monkey (○) and human (△) kidney homogenate was incubated for 60 min at 37 °C with 3000 units of HuIFN-α at different pH values.

Table 1. Distribution of HuIFN-α in subcellular fractions of rat and monkey kidney homogenates

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Injected into rats*</th>
<th>Injected into monkeys*</th>
<th>Added to rat kidney homogenates†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units/kidney fraction</td>
<td>Units/mg protein</td>
<td>Units/kidney fraction</td>
</tr>
<tr>
<td>Cell debris</td>
<td>800</td>
<td>20</td>
<td>2500</td>
</tr>
<tr>
<td>Mitochondria-lysosomes</td>
<td>15 000</td>
<td>400</td>
<td>40 000</td>
</tr>
<tr>
<td>Microsomes</td>
<td>3000</td>
<td>100</td>
<td>5000</td>
</tr>
<tr>
<td>Supernatant</td>
<td>35 000</td>
<td>500</td>
<td>120 000</td>
</tr>
</tbody>
</table>

* Rats were given 1 × 10⁶ units HuIFN-α intravenously and monkeys were given 7 × 10⁶ units HuIFN-α intravenously and sacrificed 20 min later. Kidneys were removed, homogenized and fractions assayed for HuIFN-α activity.

† Kidneys of non-injected rats were homogenized with 65 000 units HuIFN-α/g wet tissue and the total HuIFN-α content of each subcellular fraction measured.

detected in subcellular fractions from liver of injected rats 20 min after interferon injection, although small amounts were found in the supernatant.

Inactivation of HuIFN-α by rat, monkey and human kidney subcellular fractions

Since interferon was found to accumulate in rat and monkey kidneys, this experiment was designed to determine whether interferon could be inactivated by tissue homogenates and by subcellular fractions. Homogenates and subcellular fractions of rat, monkey and human kidneys were prepared and incubated with HuIFN-α at pH values ranging from 2 to 9. As shown in Fig. 2, all three homogenates caused inactivation of interferon activity, the effect being maximal between pH 3-0 to 4-0. Incubation of the different subcellular fractions with interferon showed that in all three species the inactivation was caused by the lysosomal fraction only. Complete inactivation of 3000 units of interferon at pH 3-5 was achieved in 60 min with 100 μg protein of the lysosomal fraction while 300 μg protein of the mitochondrial, microsomal and supernatant fractions under the same conditions caused no inactivation at all. These fractions at a higher protein concentration caused some inactivation of interferon and this was attributed to small amounts of lysosomal contamination. Purified lysosomal
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Fig. 3. Inhibition of interferon degradation in lysosomal fractions by pepstatin and leupeptin. (a) Rat, (b) monkey and (c) human lysosomal fractions were incubated at 37 °C at pH 3.5 for 60 min with 3000 units of HuIFN-α. ●, Control, no inhibitor; ▲, 5 μg pepstatin; ○, 5 μg leupeptin; △, 5 μg pepstatin + 5 μg leupeptin.

fractions of rat, monkey and human kidneys showed the same pH dependence of interferon inactivation as did the homogenates.

Effect of pepstatin and leupeptin on interferon inactivation of lysosomal fractions

The pH dependency of interferon inactivation by lysosomal fractions from human, monkey and rat kidneys suggested that the inactivation may result from proteolytic degradation by cathepsins (Barrett & Heath, 1977). An attempt to prevent the inactivation was, therefore, made by pepstatin and/or leupeptin, by adding the respective specific inhibitors of carboxyl and thiol lysosomal proteinases. As shown in Fig. 3, each of the inhibitors separately caused partial inhibition of the degradation process. Practically full inhibition was achieved by the addition of both inhibitors, clearly indicating that inactivation of interferon results from proteolytic degradation.

DISCUSSION

Disappearance of exogenous intravenously injected interferon from the serum of rats, monkeys and humans is very rapid (Skreko et al., 1973; Emödi et al., 1975a; Cantell & Pyhälä, 1976). One of the possibilities is that interferon is rapidly taken up by several organs or tissues in which it is extensively inactivated. Examination of several organs of rats and monkeys after injection revealed that the highest interferon concentrations were found in the kidney. These concentrations were 7 to 10 times higher than in other organs and remained high as the concentration in the blood started to decrease. Moreover, the data (Table 1) present clear evidence that the interferon penetrated into the kidney cells and was concentrated in the mitochondrial-lysosomal fraction.

The question, however, arises as to the source of interferon found in the supernatant fraction of the kidney (Table 1). Three possibilities may be considered: renal plasma, tubular fluid and cell sap. After 30 min, the rat kidney and serum contents of HuIFN-α were correspondingly 50000 and 10000 units/g. In the monkey (after 20 min) the respective values were 20000 and 3000 units/g. On the other hand, no HuIFN-α was found in urine and in separate experiments, we have shown that exogenous HuIFN-α added to urine retains its activity. These findings rule out the possibility that significant amounts of HuIFN-α found in the supernatant result from renal plasma and make it most unlikely that it comes from the tubular fluid. If one assumes that HuIFN-α follows the classical path of proteins in the kidney, namely, tubular reabsorption, followed by intralysosomal catabolism (Maunsbach, 1969; Pacini et al., 1980), the 65 to 70% of HuIFN-α found in the supernatant fraction may represent interferon that was endocytosed prior to the uptake by the lysosomes. One has to remember that $1 \times 10^6$ units of interferon consist only of about 1 μg of that protein.
Therefore, the absolute amount is very small. In addition, in vitro experiments indicated rapid degradation of the interferon by rat, monkey and human lysosomal fractions. This degradation process was found to be due to the proteolytic activity of carboxyl and thiol proteinases which could be completely inhibited by both pepstatin and leupeptin (Fig. 3).

The organ distribution of HuIFN-α was similar in rats and monkeys and it may be expected that degradation of HuIFN-α in humans follows a similar pathway. The fact that cynomolgus monkeys respond clinically to HuIFN-α substantiates this suggestion. Although it cannot be ruled out that liver and other organs are capable of degrading interferon, the perfusion experiments of Bocci et al. (1968) suggest that rabbit interferon in its native form was almost not catabolized by the liver and inactivation could be achieved only after desialylation (Bocci et al., 1977). On the other hand, Mogensen et al. (1974) did not find any difference in HuIFN-α clearance from the rabbit’s blood system after desialylation. Their results are supported by the data of Dr M. Rubenstein, Weizmann Institute (personal communication) that HuIFN-α does not seem to contain any carbohydrate moieties. It seems, therefore, that although inactivation of interferon by liver and other organs cannot be excluded, the kidney serves as a main site of interferon degradation. This conclusion is further supported by our recent experiments in which ligation of the kidneys followed by interferon infusion resulted in a progressive (up to 10-fold) increase of serum interferon as compared to the non-ligated animals (T. Bino et al., unpublished results).

Our present data do not indicate, however, whether HuIFN-α is taken up directly from circulation or is reabsorbed by the tubules. Further experiments aimed at answering this question are now in progress.

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REFERENCES


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