Metabolism of Interferon: Hepatic Clearance of Native and Desialylated Interferon

By V. BOCCI, A. PACINI, G. P. PESSINA, V. BARGIGLI AND M. RUSSI

Istituto di Fisiologia Generale and Istituto di Microbiologia, University of Siena, 53100 Siena, Italy

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SUMMARY

The effect of removal of sialic acid on the survival of rabbit serum and urinary interferon (IF) has been investigated in isolated, perfused rabbit liver preparations. In contrast with native IF, which may be already partially desialylated, IF freed of 80 to 90% of its sialic acid was rapidly cleared from the perfusate of normal livers, or livers pre-treated with actinomycin D. The results suggest that the mechanism of IF catabolism by the liver is similar to that reported for several other circulating glycoproteins.

INTRODUCTION

It has been shown that interferon, whether actively induced or passively administered (Oh, 1966; Bocci, Russi & Rita, 1967; Pyhälä & Cantell, 1974; Emödi et al. 1975), disappears very rapidly from the circulation. The results obtained in studies designed to see whether IF is lost through the kidney or the intestinal epithelium (Bocci et al. 1968a), is bound to cell membranes (Bourgeade, 1974; Dianzani & Baron, 1975), or is catabolized by the liver (Bocci et al. 1968b), have invariably shown that these sites are of little importance. Thus the main site of IF catabolism remains elusive. IF loses some activity when simply incubated with body fluids (Cesario, Mandell & Tilles, 1973), but this is hardly surprising in view of its instability. If the main catabolic pathways were known, it might perhaps be possible to delay IF catabolism, and so maintain therapeutic levels of IF in the plasma for a longer time. To this same end, a study of the most efficient route of IF administration has also been reported (Emödi et al. 1975).

When we studied the catabolic role of the liver (Bocci et al. 1968b), we noticed at first a modest loss of activity with a tendency to a slight increase after about 90 min of perfusion. The results did not indicate a decisive role for the liver, but at that time we knew very little about the chemical nature of IF and the possible implications of this.

That rabbit IF is a heterogeneous mixture of glycoproteins containing sialic acid (N-acetylneuraminic acid, NANA) was shown later and independently by Schonne, Billiau & de Somer (1970) and ourselves (Viti et al. 1970; Bocci et al. 1971). This result has been amply confirmed and extended (Dorner, Scriba & Weil, 1973; Besancon & Ankel, 1974; Davey et al. 1974; Knight, 1975; Bose et al. 1976). Meanwhile Ashwell & Morell (1974) have developed a new concept for the role of NANA in regulating the time for which glycoproteins remain in the circulation. When NANA is cleaved by the action of neuraminidase (NANase), galactose becomes exposed as the terminal sugar of the protein-linked carbohydrate chain and acts as a specific determinant recognized by hepatic receptors (Pricer & Ashwell, 1971).
On this basis, and because the liver probably has a secondary role as an organ desialylating IF, it seemed to us important to re-evaluate its role in IF catabolism by testing native and desialylated IF.

**METHODS**

*Preparation, purification and assay of IF.* In most of the experiments reported here, one pool of rabbit serum IF and one of urinary IF were used. These were obtained in June 1974 from rabbits given an intravenous inoculation of about $10^9$ p.f.u. of Newcastle disease virus. Serum and urine were collected from the rabbits, acidified, exhaustively dialysed and freeze-dried as previously described (Bocci *et al.* 1968a). The serum pool contained 48 reference rabbit interferon units/mg protein, and the pooled urine 1150 units/mg protein. In order to remove small yet undialysable glycoproteins and glycopeptides (Bourrillon, 1972), the crude IF was subjected to extensive ultrafiltration with an Amicon system model 401, using 76 mm PM-30 Amicon Diaflo membranes. There was almost quantitative recovery of urinary IF, and its specific activity increased to 14000 reference IF units/mg protein with a mean potency of $3.8 \times 10^6 \pm 0.3 \times 10^5$ reference IF units/ml (mean of 23 titrations). The serum IF pool contained $0.3 \times 10^4 \pm 0.4 \times 10^3$ reference IF units/ml (mean of 8 titrations). In some recent confirmatory studies, further batches of serum and urinary IF, prepared in 1976, were used. These contained, respectively, 210 and 3800 reference IF units/mg protein, but after purification by ultrafiltration, the urinary preparation contained 52000 reference units/mg protein.

For convenience in reference, the mixture of serum proteins and of urinary proteins containing IF activity are here termed serum IF and urinary IF.

Proteins were measured either by dry weight, by the method of Gornall, Bardawill & David (1949), or Lowry *et al.* (1951), using a crystallized sample of bovine serum albumin as a standard.

Interferon was titrated in serially propagated cultures of RK13 cells by measuring inhibition of vesicular stomatitis virus by a plaque reduction method, as described earlier (Bocci *et al.* 1968a). All titres are given in terms of the unit assigned to the research standard for rabbit IF (preparation Go 19-901-028, obtained through the courtesy of the Research Resources Branch, National Institute of Allergy and Infectious Diseases, Rockville, Maryland 20852).

It has been shown that rabbit urinary IF has the same physicochemical and biological properties as serum IF (Bocci *et al.* 1968a) and is probably derived from the plasma pool. Because urinary IF has high specific activity and is easily obtained in large amounts, it is very convenient for use in rabbit experiments and we assume that its metabolic behaviour is similar to serum IF.

*Desialylation of IF.* Desialylation of serum IF and of crude and purified urinary IF was carried out using highly purified NANase from *Vibrio cholerae* (Behringwerke), as described by Viti *et al.* (1970). The NANA content was measured according to Aminoff (1961). Total NANA content was measured from the same sample hydrolysed in 0.075 N-H$_2$SO$_4$ at 80 °C for 1 h. The residual NANA content is expressed as a percentage of that at the start of incubation. NANase was inactivated by shifting the pH from 5.5 to 2.0. The sample was left at the latter pH for 30 min at 37 °C and then equilibrated by dialysis against phosphate-buffered saline at pH 7.4. No significant NANase activity could be detected after this treatment.

*Binding of IF by liver membranes.* Plasma membranes from rabbit liver were isolated according to Ray (1970). Some were heat-denatured at 56 °C for 10 min, and some were treated with NANase for 3 h and then centrifuged and washed twice to remove the enzyme.
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Not more than 63% of the total membrane-bound NANA could be removed. Known amounts of purified native and desialylated urinary IF were incubated with the same amounts of normal, desialylated and heat-denatured membranes. After incubation, the residual unbound IF was titrated in the supernatants and the missing activity was termed membrane-bound IF and expressed as a percentage.

Liver perfusion. Livers were excised from normal rabbits (2.8 to 3 kg) fasted for about 20 h. When desired, actinomycin D (0.7 mg/kg body weight) was injected through a mesenteric vein 80 to 100 min before starting the perfusion. This amount of actinomycin D should completely inhibit IF synthesis for the next 12 h (Ho & Kono, 1965).

Perfusions were carried out as previously described (Bocci et al. 1968b) according to the method of Miller et al. (1951) with some modifications to improve the viability of the preparation. We have used a very efficient rotating disc oxygenator, and the perfusion medium (400 ml) consisted of fresh heparinized rabbit blood (88%) and homologous serum (12%), with additional glucose (0.9 g/l), penicillin (400,000 units/l) and streptomycin (0.2 g/l). Livers treated with actinomycin D were perfused with blood from which nearly all leucocytes had been removed. After a 15 min period of equilibration, serum or urinary (crude and purified) IF was added to the perfusate in amounts of about 1 x 10⁶ reference units.

In view of the very rapid clearance of desialylated IF, it was essential to calculate the initial concentration of IF in the perfusate by taking into account the volume and total units of IF added to the volume of perfusate less the blood cell volume – mean haematocrit 30%, plus an average of 20 ml, i.e. the liver extracellular volume at the beginning of perfusion. This total volume agreed well with that calculated by the dilution of Evans Blue (0.6 mg) in the perfusate. Samples of blood perfusate were taken at intervals up to 2 to 4 h, and IF titres in the plasma were expressed as a percentage of the initial value.

In a few experiments the same liver (normal or pre-treated with actinomycin D) was tested as follows: after the preliminary stabilization, a dose of native or desialylated IF was added to the perfusate and three samples were withdrawn during the following 50 min. Immediately thereafter, the whole perfusate was discarded, and the perfusion circuit was washed with saline and refilled with the same volume of new perfusate. Meanwhile the liver continued to be perfused and washed within an ancillary circuit without recirculation. The change of the perfusate took 5 to 7 min and afterwards the liver was returned to the original system and restabilized for a further 10 min. A sample was then withdrawn to check the amount of residual IF. A new dose of IF was then added to the perfusate and three more samples were taken in the next 50 min.

The viability of the isolated liver as assessed by evaluating bile secretion and levels of glucose, urea, plasma proteins, glutamic-oxaloacetic and glutamic-pyruvic transaminases and alkaline phosphatase in the perfusate, was found to be very satisfactory for the period of perfusion used (Pessina et al. 1976). Recently, we have directly tested liver function in our studies by injecting 15 mg bromosulphophthalein (BSP) in 1 ml of saline at the start of perfusion. The initial BSP concentration was estimated by calculating the dilution of the dose in the plasma volume. The amounts of BSP present in samples of perfusate removed at different times thereafter were measured colorimetrically at 580 nm, and were expressed as the percentage BSP remaining in the perfusate. Since the BSP test can be repeated, it was possible to evaluate the function of the same liver during two successive perfusions, for example when native and then desialylated IF, or vice versa, were tested in the same liver preparation. Such tests showed that the liver maintained its capacity to clear dye from the plasma without impairment during the two perfusions.
RESULTS

Desialylation of serum and urinary IF

Fig. 1 shows the change in NANA content during NANase treatment of urinary and serum IF as a function of time. The release of up to about 80% of NANA from glycoproteins was accompanied only by a small loss of antiviral activity. While a second addition of NANase at 2 h had little further effect, incubation for a longer time (14 h) increased desialylation up to 92%, but at the expense of greater inactivation of IF (about 30%). This confirms previous results obtained by Viti et al. (1970). From the data it remains uncertain if IF, like other glycoproteins, contains NANA inaccessible to the neuraminidase used (Gottschalk & Drzeniek, 1972), and it is impossible to assess the actual desialylation of IF as opposed to the bulk of contaminating glycoproteins. This is probably not a critical drawback, as Van den Hamer et al. (1970) showed that removal of only 20% of NANA from ceruloplasmin was sufficient for the liver to recognize and bind the partially desialylated protein.

Clearance of native and desialylated IF by normal rabbit livers

Experiments illustrating the clearance of native and desialylated urinary and serum IF are presented in Fig. 2 and 3. As shown, only 25 to 40% of native IF disappeared in about 30 min from the recirculating perfusate (the subsequent rise in IF levels is discussed below). This result contrasts sharply with the behaviour of desialylated IF, where the loss was, respectively, 50 and 80%, for the crude and purified urinary preparations. The disappearance of desialylated serum IF was even more marked because only 10% remained in the perfusate after 30 min. Very similar results were obtained with the 1976 batches of IF. Since we excluded in control experiments the possible adsorption and inactivation of IF on the perfusion apparatus, the data suggest that the faster disappearance of desialylated IF is attributable to the binding of most of the desialylated IF to hepatic receptors.

Binding of urinary IF by rabbit liver plasma membranes

Direct demonstration of binding of IF to hepatic plasma membranes would have been desirable, but unfortunately IF could not be isotopically labelled. An indirect demonstration is, however, given in Table 1. Up to 42 and 67% of the native and desialylated IF, respectively, appeared to bind to the membranes. Smaller amounts of IF were bound to
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Fig. 2. Concentration of native (○—○) and desialylated (●—●) urinary interferon (expressed as a percentage of the initial value) in the perfusates of six different livers. (a) Crude IF. (b) Partially purified IF.

Fig. 3. Concentrations of native (○—○) and desialylated (●—●) serum interferon (expressed as a percentage of the initial value) in the perfusates of two different livers.
Table 1. Binding of purified urinary interferon by rabbit liver membranes

<table>
<thead>
<tr>
<th>Liver membranes*</th>
<th>Interferon (1500 units)</th>
<th>IF as percentage† of control</th>
<th>Recovered in the supernatant</th>
<th>Membrane-bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>58</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Heat-denatured</td>
<td>Normal</td>
<td>75</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Desialylated</td>
<td>Normal</td>
<td>87</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>Desialylated</td>
<td>33</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Heat-denatured</td>
<td>Desialylated</td>
<td>91</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Desialylated</td>
<td>Desialylated</td>
<td>66</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>Normal</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Incubation carried out at 25 °C for 1 h with gentle mixing in 6 ml of medium composed of 100 mM-NaCl, 50 mM-tris-HCl buffer, pH 7.5, 10 mM-CaCl₂, plus 65 mg of rabbit serum proteins and 31 mg of membranes.
† Average of two experiments.

Fig. 4. Concentrations of native (○—○) and desialylated (●—●) urinary interferon (expressed as a percentage of the initial value) in the perfusates of two different livers pre-treated with actinomycin D.

heat-denatured or desialylated membranes, in agreement with the finding (Pricer & Ashwell, 1971) that effective binding of glycoproteins requires the presence of NANA on the plasma membrane.

Clearance of native and desialylated urinary IF by livers from actinomycin D-treated rabbits

Unfortunately, there were considerable increases in the concentrations of IF in later samples of perfusate, particularly after tests with the crude preparations (Fig. 2 and 3). This made it impossible to determine quantitatively how much IF was taken up by the liver preparations. We carried out control experiments with urinary and serum proteins obtained from normal rabbits, which were treated in the same way as the proteins from rabbits injected with Newcastle disease virus, and with purified urinary IF which had been denatured at 100 °C for 1 h. From these, we ascertained that the newly-synthesized or released IF is not detectable in the perfusate until 60 min after the beginning of perfusion. Thus the phase during which there is major uptake of added IF should not be seriously influenced by this
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IF which appears in the liver preparations. Nevertheless, in order to support our conclusion that desialylated IF is more rapidly cleared from the circulation by the liver, we thought it worthwhile to test livers pre-treated with actinomycin D and perfused with leucocyte-depleted blood. The results reported in Fig. 4 were obtained by perfusing two livers, one with purified urinary native IF and the other with the desialylated IF. As in Fig. 2, the clearance of the desialylated IF was faster than that of the intact IF, and moreover there was no IF increase after 1 h of perfusion.

More conclusive evidence was obtained by the results reported in Fig. 5, where uptake was measured in the same liver by successively testing native and desialylated IF (and vice versa). Hepatic function was checked by BSP clearance, and was virtually unchanged during the experiment, only 17% of the BSP remaining in the perfusate after the first test and 14% after the second test.

DISCUSSION

Our studies have shown that when preparations of urinary and serum rabbit interferon, treated to remove the sialic acid, are added to the perfusate of an isolated liver preparation, the biological activity in the perfusate falls rapidly and to a much greater extent than if native IF is added. To our knowledge this is the first report showing that the clearance of homologous IF is affected by previous treatment with neuraminidase. Our conclusion is at
variance with that of Mogensen et al. (1974) which states that desialylation of human leucocyte IF does not affect its pharmaco-kinetics in rabbit, but it agrees well with a current line of thought (Ashwell & Morell, 1974). One explanation could be a different reactivity of the rabbit liver to rabbit and human IF, although this hypothesis does not apply for human asialotransferrin when injected into rabbits (Regoezzi, Hatton & Wong, 1974). Unfortunately, our results are confused by the unexpected finding of a considerable increase of IF activity in the perfusate after some 2 to 3 h. This contrasts with our previous results (Bocci et al. 1968b). As in the earlier experiments, there were traces of inactivated NDV and endotoxin in the urinary IF preparation, and the added IF would have ‘primed’ production of yet more IF (Stewart, Gosser & Lockart, 1971). However, the system used for liver perfusion in the present experiment was a considerable improvement over that used formerly, with a significant increase in the viability of the organ. Moreover, the perfusate in the former experiments contained about $2.7 \times 10^9$ leukocytes which could have contributed to IF formation (Cantell et al. 1968); the perfusate in the present experiments contained far fewer.

If output of IF by liver cells began shortly after adding interferon to the perfusate, this would have obscured the disappearance of desialylated IF. We have not attempted to resolve our experimental curves, which represent the summation of loss and gain of IF from the perfusate. However, it seems reasonable to conclude that the actual rate of loss must have been faster than that shown by the experimental data. This conclusion is strengthened by the results obtained with livers treated with actinomycin D; this treatment inhibits IF synthesis and no increases in IF titres in the perfusate were observed at 2 to 3 h. Thus, critical evidence that desialylated IF is cleared more rapidly from the perfusate than native IF was obtained by successively testing the two types of IF in liver preparations treated with actinomycin D (Fig. 5).

Some native IF also disappeared from liver perfusates. This may reflect the presence of some partially desialylated IF in the preparation because the well-known charge heterogeneity of IF (Schonne, et al. 1970; Weil & Dorner, 1973) can be related to partially desialylated IF. It remains, however, unclear whether the heterogeneity of rabbit IF is due to loss of neuraminic acid in vivo or to the addition of different amounts of NANA as the terminal sugar during synthesis.

Partially purified urinary IF, when desialylated and added to perfusates, disappeared at about twice the rate of that of crude IF, similarly desialylated. We suggest that the relatively larger amounts of desialylated glycoproteins and glycopeptides in the crude preparation will have had a correspondingly bigger effect in competitively inhibiting the uptake of the desialylated interferon by the liver.

It is possible that desialylated IF is bound to receptors in the liver and later released into the perfusate. This seems unlikely in view of the results in actinomycin-treated livers. Also, there were no significant changes in the perfusate, for example, in calcium content or pH, which would have encouraged elution. Furthermore, if any small amounts of NANase were present in the perfusate, they could have destroyed the capacity of the liver to bind the glycoproteins, particularly in the absence of any competitive substrates, such as plasma glycoproteins and blood cells. However, such amounts do not dissociate asialoglycoproteins from pre-formed complexes with receptors (Hudgin et al. 1974).

We found that plasma membranes from normal liver cells bound desialylated IF effectively but, somewhat surprisingly, there was essentially the same binding of normal IF. This apparent contrast with a difference in clearance rate from perfused livers is not easily explained, but may reflect a difference in the ratio of IF to membrane-binding proteins.
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We assume that the final catabolic step is transfer of IF into hepatic lysosomes, where, like other asialoglycoproteins (Gregoriadis et al. 1970), it is broken down.

If our hypothesis is correct, namely that IF is catabolized by desialylation, followed by breakdown in the liver, it is relevant to ask where the IF (and other circulating glycoproteins) become desialylated. This topic has been reviewed recently (Bocci, 1976): in brief, desialylation is likely to happen in micro environments where the circulation is sluggish, and NANase concentrations may become effective. In particular, we postulate that IF may also be desialylated by membrane-bound NANase when it is transiently attached to the cell membrane. If it returns to the circulation after desialylation, it may then be rapidly and quantitatively taken up by liver cells.

We have recently found that desialylated IF injected into intact rabbits disappears from the circulation significantly faster than untreated interferon. These results will be presented elsewhere (Bocci et al. 1977).

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