The Role of Sialic Acid in Determining the Survival of Glycoproteins in the Circulation

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SUMMARY

Evidence is presented to indicate a generalized role for the terminal sialic acid residues of circulating glycoproteins. Upon injection into rats, all of the desialylated plasma proteins tested, with the exception of transferrin, were promptly removed from the circulation and were recovered from the liver. The materials examined included orosomucoid, fetuin, ceruloplasmin, haptoglobin, α2-macroglobulin, thyroglobulin, lactoferrin, and the two gonadotropic hormones, human chorionic gonadotropin and follicle-stimulating hormone.

Competitive inhibition of hepatic uptake was demonstrated by the injection of tracer amounts of 64Cu-ceruloplasmin together with substantive amounts of the above-mentioned desialylated proteins or the glycopeptides derived from them. Uptake was not inhibited by the fully sialylated protein or their glycopeptides.

A previous report in this series (1) documented the observation that enzymatic removal of sialic acid from ceruloplasmin resulted in a preparation which, upon intravenous injection into rabbits, was rapidly transferred from the circulation into the parenchymal cells of the liver. Subsequent quantitative studies in rats (2) established that the cleavage of only a small fraction of the sialic acid residues of ceruloplasmin was sufficient to effect the removal of these molecules from the circulation.

At the time of the original observation, the generality of this phenomenon was tested with transferrin, the iron-binding protein of normal serum. Injection of a preparation of transferrin from which all of the sialic acid had been removed enzymatically resulted in a serum survival time indistinguishable from that of the fully sialylated protein (1). As a consequence, we concluded that the striking effect of neuraminidase upon ceruloplasmin was, if not unique, at least not characteristic of all glycoproteins.

Reinvestigation of this phenomenon, the results of which are presented in this paper, suggests that it is transferrin which is the exception with respect to the function of sialic acid, and that sialic acid plays a previously unrecognized role in regulating survival in the circulation of the large majority of glycoproteins tested.

MATERIALS AND METHODS

Clostridium perfringens neuraminidase (1.25 units per mg), Pronase (Grade B), and galactose oxidase (20 units per mg) were obtained from Worthington, Calbiochem, and General Biochemicals, respectively. The crude galactose oxidase was partially purified before use as follows. The contents of one vial, 125 units in 6.25 mg, were dissolved in 5 ml of 0.025 M sodium phosphate buffer, pH 7.0. This solution was put on a column of DEAE-cellulose (1 x 5 cm) which had been equilibrated with the same buffer. The filtrate (10 ml) was made 1% in NaCl and precipitated by the addition of 3 volumes of ethanol-chloroform (9:1, v/v). After brief centrifugation, the pellet was dissolved in a small amount of the above buffer and any insoluble material was removed by further centrifugation. The specific activity of the purified preparation increased 9-fold and the original hexose content of 38% decreased 20-fold. 125Iodine (15 Ci per mg) and potassium borotritide (3.53 Ci per mmole) were obtained from Amersham/Searle Corporation, Chicago, Illinois. The latter was prepared as a 0.01 M solution in cold 0.01 N KOH shortly before use. Heat-denatured 125I-albumin was a commercial product of Squibb. Copper-64 (20 mCi per mg) was supplied by New England Nuclear and was used for the preparation of 64Cu-ceruloplasmin, as described previously (1).

We are indebted to Dr. John B. Robbins for the preparation of α2-macroglobulin and lactoferrin, to Dr. Philip Aisen for crystalline human transferrin, and to Dr. Harold Edelhoch for the bovine thyroglobulin. Crystalline ceruloplasmin was prepared from Cohn's fraction IV of human plasma (3) and orosomucoid (α1-acid glycoprotein) from human serum by the procedure of Whitehead and Sammons (4). Haptoglobin was isolated from human serum by a modification (5) of the method of Connell and Shaw (6). Bovine fetuin was obtained from Grand Island...
Table I

Effect of asialoorosomucoid on hepatic uptake of 
125I-asialoceruloplasmin

A tracer amount (0.3 mg) of 125I-asialoceruloplasmin was injected into the tail vein of male albino rats. In Experiments 2 to 4, the indicated amounts of various preparations were added to the tracer. The animals were killed after 14 min and the liver radioactivity was determined as described in the legend to Fig. 1.

<table>
<thead>
<tr>
<th>Experiment no. and addition</th>
<th>Amount</th>
<th>Radioactivity recovered in the liver %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Asialotransferrin</td>
<td>3.9</td>
<td>99</td>
</tr>
<tr>
<td>3. Orosomucoid</td>
<td>8.1</td>
<td>99</td>
</tr>
<tr>
<td>4. Asialoorosomucoid</td>
<td>8.7</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Human pituitary follicle-stimulating hormone (LER 8690) was the generous gift of Dr. Griff Ross. This material was iodinated with 125I by the method of Greenwood and Hunter (13) to yield a specific activity of 35 to 75 mCi per mg. The iodinated protein was separated from the unreacted isotope by passage through a Sephadex G-25 column and the extent of radiation damage was ascertained by the double antibody technique (14). Approximately 65 to 70% of the radioactivity was precipitated in the presence of excess antibody. For the preparation of the asialo derivative, 1 mg of carrier hormone (Albert D-RA, 50 i.u. per mg) was added to 1 ml of 0.05 M sodium acetate buffer, pH 5.6, in 0.15 M NaCl containing 10 μCi of 125I-FSH.1 To this were added 20 μg of C. perfringens neuraminidase and the sample was incubated at 37° for 20 min. The release of sialic acid was determined by the method of Warren (15). The neuraminidase was readily removed by affinity chromatography on a column of Sepharose-4B which had been coupled with rabbit antineuraminidase antibody (16). The iodination and desialylation of HCG were carried out in a similar fashion with essentially identical results. The procedures for the enzymatic removal of sialic acid from ceruloplasmin and for tritiation of the resulting asialoprotein previously reported (3) were followed for each of the other glycoproteins described in this report.

The glycopeptides of ceruloplasmin, transferrin, and orosomucoid were prepared by the method of Jamieson (17, 18). Following the separation on Sephadex G-25, the glycopeptide solution was evaporated to dryness over NaOH and redissolved in 0.1 M sodium acetate, pH 5.6, containing 0.17 M NaCl. The sialic acid concentration was adjusted to that of a 1% solution of the protein from which the glycopeptide fraction was derived. Removal of sialic acid was accomplished by hydrolysis in 0.1 N HCl at 85° for 30 min, followed by neutralization to pH 5.6 with NaOH.

RESULTS

Injection of a number of tritiated asialoproteins into rats gave rise to the pattern of survival curves illustrated in Fig. 1. In contradistinction to the survival curves of native ceruloplasmin and asialotransferrin, the asialo derivatives of all the other glycoproteins disappeared promptly from the plasma. Not shown on the figure are the control curves for 125I-labeled prep-

1 The abbreviations used are: FSH, follicle-stimulating hormone; HCG, human chorionic gonadotropin.
A 4O

20 40 60 80 100 120 140
MINUTES

FIG. 2. Effect of increasing amounts of asialoorosomucoid upon the circulation time of ⁴⁰Cu-asialoceruloplasmin. Conditions were as described in the legend to Fig. 1. All rats were injected with ⁴⁰Cu-asialoceruloplasmin (0.3 mg) with or without addition of varying amounts of unlabeled asialoorosomucoid. The dashed line shows the decrease in plasma concentration of fully sialylated ⁴⁰Cu-ceruloplasmin resulting from both diffusion into extravascular space and normal catabolism. Additions of asialoorosomucoid: △, none; ○, 0.75 mg; ●, 2.0 mg; ♦, 8.5 mg.

arations of orosomucoid, fetuin, and α₂-macroglobulin, all of which were indistinguishable from intact ⁴⁰Cu-ceruloplasmin during the initial 60 min. In the case of ¹²⁵I-thyroglobulin, the survival time was significantly greater than that of the asialo derivative, although the difference was less marked than with the other preparations shown in Fig. 1.

In every instance, the major portion of the radioactivity lost from the plasma was recovered in the liver. Consequently, the competitive inhibition of these various desialylated proteins, with respect to hepatic uptake, was investigated. The initial experiments revealed that when tracer amounts of ⁴⁰Cu-asialoceruloplasmin (0.3 mg) were injected into a rat and the animal was killed 14 min later, approximately 98% of the total radioactivity was recovered from the liver. However, when unlabeled asialoorosomucoid (8.7 mg) was injected simultaneously with ⁴⁰Cu-asialoceruloplasmin, the amount of radioactivity recoverable from the liver was less than 4% (Table I). Neither intact orosomucoid nor asialotransferrin reduced the hepatic uptake of asialoceruloplasmin.

The effects of varying both the amount of inhibitor used and the period of observation were next investigated and the results are illustrated in Fig. 2. With increasing doses of unlabeled asialoorosomucoid as the inhibitor, the survival time of the labeled tracer rose sharply from less than 5 min to more than 2 hours. Analogous experiments with other desialylated proteins yielded similar although less striking results. When asialohaptoglobin (10 mg) was tested as an inhibitor, the half-life of the tracer increased 8-fold to 40 min, whereas with desialylated preparations of α₂-macroglobulin (10 mg), thyroglobulin (20 mg), and lactoferrin (5 mg), the half-life was approximately doubled. Comparable amounts of the fully sialylated proteins were uniformly ineffective in lengthening the survival time of the asialoceruloplasmin.

It seemed clear from the foregoing that the amount of inhibitor in the circulation changed constantly with time and that the hepatic uptake of the tracer occurred only after a critical serum concentration of the inhibitor was reached. In order to define this relationship more precisely, a series of experiments was undertaken wherein both inhibitor and asialoceruloplasmin tracer were labeled with tritium and copper-⁶⁴, respectively.

The survival curves resulting from the simultaneous injection of ¹²⁵I-asialoorosomucoid (10 mg) and ⁴⁰Cu-asialoceruloplasmin (0.3 mg) are shown in Fig. 3. For approximately 2 hours, the latter remained viable in the circulation, during which time the concentration of the former decreased steadily. As the plasma
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0 20 40 60 80 100 120 140 MINUTES

FIG. 5. Relationship of plasma concentration of asialohippotoglobin to its inhibitory effect. Conditions were as described in the legend to Fig. 1. The combined injection contained \( ^{64}\text{Cu}-\text{asialoceruloplasmin} \) (0.3 mg) and \( ^{3}H\)-asialohippotoglobin (10 mg, 0.2 \( \times \) \( 10^{8} \) dpm). ○, copper-64; ●, tritium.

concentration of the tritiated inhibitor approached 1% of the injected dose per ml, the slope of both survival curves increased sharply and both proteins disappeared rapidly from the circulation. A similar pattern of behavior was seen when tritiated asialofetuin was used as the inhibitor (Fig. 4). In the case of haptoglobin, the tracer preparation disappeared at a constant, although markedly diminished, rate throughout the entire 2-hour period (Fig. 5).

A more detailed study of survival time as a function of dosage is shown in Fig. 6. The half-life of injected \( ^{64}\text{Cu}-\text{asialoceruloplasmin} \) was neither constant nor proportional to the dosage. Instead, it decreased intermittently throughout the experiment as a function of the changing plasma concentration rather than of the amount injected.

As a consequence of the demonstration that several desialylated glycoproteins acted effectively as inhibitors of hepatic uptake, it appeared unlikely that specific conformational modification was responsible for the inhibitory activity. In order to exclude this possibility and to define more precisely the nature of the inhibitor, the intact glycoprotein was destroyed by treatment with Pronase and the resulting glycopeptide moiety was recovered. Upon injection into rats, the glycopeptides isolated from ceruloplasmin and from orosomucoid had no effect on the survival of the labeled tracer in the circulation. However, upon desialylation, the glycopeptides from both proteins were effective in inhibiting uptake of asialoceruloplasmin by the liver (Fig. 7).

In clear contrast, neither the intact nor the asialoglycopeptides isolated from transferrin inhibited the rapid accumulation of tracer in the liver (Table II). Similarly, free galactose, \( \alpha \)-methyl-\( \beta \)-galactoside (30 mg), and lactose (30 mg) were ineffective as inhibiting agents.

With the realization that the viability of many (if not most) of the serum glycoproteins depends upon the presence of their normal complement of sialic acid residues, consideration was given to another category of biologically potent glycoproteins, i.e., the gonadotropic hormones. The biological activity of two members of this group, human chorionic gonadotropin and...
TABLE II
Comparison of inhibitory effectiveness of glycopeptides isolated from transferrin and orosomucoid

A tracer amount (0.2 mg) of $^{64}$Cu-asialoceruloplasmin was injected into the tail vein of male albino rats. In Experiments 2 to 5, the indicated amounts of various preparations were added to the tracer. All plasma samples were collected 15 min after injection and the radioactivity determined as described in the legend to Fig. 1.

<table>
<thead>
<tr>
<th>Experiment no. and source of glycopeptides</th>
<th>Amount* (mg)</th>
<th>Radioactivity remaining in circulation (% dose/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None ..................................</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>2. Native transferrin .....................</td>
<td>30</td>
<td>0.1</td>
</tr>
<tr>
<td>3. Desialylated transferrin .............</td>
<td>30</td>
<td>0.2</td>
</tr>
<tr>
<td>4. Native orosomucoid ...................</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>5. Desialylated orosomucoid ............</td>
<td>10</td>
<td>9.8</td>
</tr>
</tbody>
</table>

* The weight of glycoprotein from which the added glycopeptides were derived.

Fig. 8. Plasma survival time of native and desialylated human chorionic gonadotropin in the rat. The preparation of HCG was purified, labeled with $^{125}$I, and desialylated as described under "Materials and Methods." Each injection (1.0 ml) contained 2.0 to 2.6 × 10⁶ cpm, of which 90% was precipitable with specific antisera. Injections were made in the tail vein of Sprague-Dawley female rats weighing about 200 g. At the indicated time intervals, blood was collected in heparinized tubes and centrifuged, and the residual radioactivity was determined on an aliquot of the plasma. The same aliquot was then assayed by the double antibody technique and all values were corrected for immune precipitable counts. ●, $^{125}$I-HCG; ▲, $^{125}$I-asialo-HCG plus 5 mg of asialoorosomucoid; ■, $^{125}$I-asialo-FSH. The slope of this curve was unaffected by the addition of 5 mg of fully sialylated orosomucoid. In either case, the liver contained 77% of the recoverable radioactivity at the end of the experiment (30 min), in the lowermost curve.

Fig. 9. Plasma survival time of native and desialylated follicle-stimulating hormone in the rat. The conditions of this experiment were similar to those described in the legend to Fig. 8 with the exception that 3.0 to 3.6 × 10⁶ cpm of $^{125}$I-FSH were injected, of which approximately 70% was precipitable by specific antisera. ●, $^{125}$I-FSH; ▲, $^{125}$I-asialo-FSH plus 5 mg of asialoorosomucoid; ■, $^{125}$I-asialo-FSH. The slope of this curve was unaffected by the addition of 5 mg of fully sialylated orosomucoid. In either case, the liver contained 72% of the recoverable radioactivity at the end of the experiment (30 min), in the lowermost curve.

Differentiation from Phagocytosis—The rapid clearance of neuraminidase-treated glycoproteins and their resultant localization in the liver is reminiscent of the activity of the systemic reticuloendothelial cells in clearing the circulation of foreign particles. The ability of these cells to phagocytize a variety of such materials as bacteria, carbon suspensions, and denatured proteins has been correlated with their role in cellular defense. No such mechanism is apparent in the highly selective removal of
Inhibitor specificity for hepatic uptake by Kupffer cells and hepatocytes

In Experiments 1 and 2, 131I-heat-denatured albumin (0.5 mg) was injected and in Experiments 3, 4, and 5 64Cu-asialoceruloplasmin (0.3 mg) was injected. The animals were killed 10 min after injection and the liver radioactivity determined as described in the legend to Fig. 1.

<table>
<thead>
<tr>
<th>Experiment no. and radioactive tracer used</th>
<th>Inhibitor added</th>
<th>Amount</th>
<th>Radioactivity recovered in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 131I-Heat-denatured albumin</td>
<td>None</td>
<td>10%</td>
<td>63%</td>
</tr>
<tr>
<td>2. 131I-Heat-denatured albumin</td>
<td>Asialoorosomucoid</td>
<td>20%</td>
<td>98%</td>
</tr>
<tr>
<td>3. 64Cu-Asialoceruloplasmin</td>
<td>None</td>
<td>20%</td>
<td>94%</td>
</tr>
<tr>
<td>4. 64Cu-Asialoceruloplasmin</td>
<td>Noniodinated heat-denatured albumin</td>
<td>10%</td>
<td>4%</td>
</tr>
<tr>
<td>5. 64Cu-Asialoceruloplasmin</td>
<td>Asialoorosomucoid</td>
<td>10%</td>
<td>4%</td>
</tr>
</tbody>
</table>

Upon the simultaneous injection of two asialoproteins of varying susceptibility for clearance, a preferential removal of one is seen. A good example of this variable binding behavior is illustrated in Fig. 3. In the presence of excess asialoorosomucoid, there is a complete exclusion of asialoceruloplasmin from the liver. At the point at which the plasma concentration of the former falls to a critical low level, both proteins disappear rapidly and at approximately the same rate. Asialoorosomucoid, on the other hand, exhibits a binding efficiency for the liver similar to that of asialo-ceruloplasmin, since the latter protein disappears from the plasma continuously and at a constant rate in the presence of excess inhibitor (Fig. 5).

With the development of the inhibition technique, it became possible to re-examine the role of the Kupffer cells in the selective hepatic uptake of asialoglycoproteins and to confirm the earlier historadioautographic evidence for the participation of parenchymal cells in this phenomenon (1). Further confirmation of the nonparticipation of the Kupffer cells in this system has been obtained by comparison of the lack of cross-inhibition of hepatic uptake between the asialo-plasma proteins and a readily phagocytized colloid, heat-denatured 131I-albumin. Upon injection of tracer amounts (0.5 mg) of the latter into a rat, 63% of the total dose was recovered from the liver 10 min later (Table III). These results are inconsistent with the deposition of the asialoglycoproteins in the Kupffer cells and provide additional supportive evidence that the parenchymal cells are the locus of accumulation in the liver.

Further differentiation between the two systems was obtained by comparison of the quantitative aspects of clearance. Detailed studies on the kinetic parameters of phagocytosis indicated the survival time of phagocytized colloids was shown to be proportional to the dose administered and to be constant throughout the duration of the experiment (20). In contradistinction, the survival time of asialoceruloplasmin was not proportional to the dose injected nor was it constant throughout the experiment (Fig. 6). In short, the available evidence points to the identification of the hepatocyte, and not the Kupffer cell, as the site of active accumulation of asialoglycoproteins.

At the present time, little information is available as to the mechanism of binding and transport of desialylated proteins into hepatocytes. The number of binding sites appears, however, to be limited since they are readily saturated by the injection of milligram quantities of an appropriate asialoprotein. The subsequent intracellular protein transport and lysosomal catabolism (21) would then serve to make such sites available for further uptake.

The curiously selective nature of these receptor sites was revealed by the Pommier digestion experiments, which showed that the requirements for binding do not include a conformationally intact protein but are satisfied by the asialoglycopeptides from many proteins (Fig. 6) with the exception of transferrin (Table II). Nevertheless, the composition of the two carbohydrate chains of transferrin does not appear to be strikingly dissimilar to that of the other glycoproteins examined (18).

These results, and the lack of inhibition by simple carbohydrate analogues, suggest a specificity and complexity of the glycopeptide structure which remain to be elucidated.

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logical activity and immunocompetence of the gonadotropic hormones and to Julie Windsor for skillful technical assistance.

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