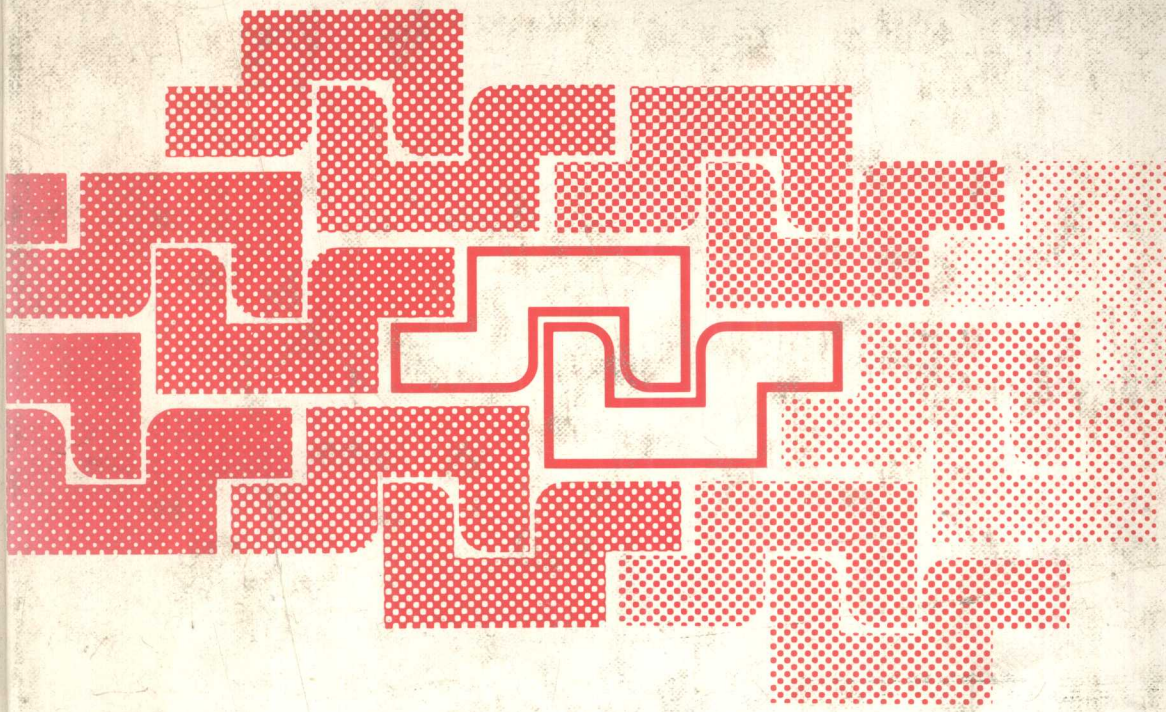

Targeting of Drugs With Synthetic Systems

Edited by
Gregory Gregoriadis
Judith Senior and
George Poste

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Targeting of Drugs With Synthetic Systems

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PREFACE

Targeting of drugs via carrier systems to sites in the body in need of pharmacologic intervention is a rapidly growing area of research in the treatment or prevention of disease. It has evolved from the need to preferentially deliver drugs, enzymes, vitamins, hormones, antigens, etc. to target cells and organs so as to avoid toxicity, waste of drugs through premature secretion or inactivation and at the same time render treatment more convenient and cost-effective. A wide assortment of naturally occurring or semi-synthetic drug carriers (e.g. antibodies, glycoproteins, lectins, peptide hormones, cells and liposomes), their interaction with relevant receptors and mediation of optimal pharmacological action were discussed in the two previous NATO Advanced Studies Institutes (ASI) of this series, "Targeting of Drugs" and "Receptor-Mediated Targeting of Drugs", the proceedings of which were published by Plenum in 1982 and 1984 respectively.

This book contains the proceedings of the 3rd NATO ASI "Targeting of Drugs with Synthetic Systems" held as before at Cape Sounion, Greece during 24 June-5 July 1985. It deals mostly with man-made carriers such as a variety of polymers, matrices, liposomes and other colloidal microparticles. The twenty chapters discuss the interaction of such carriers with the biological milieu, approaches to bypass the reticuloendothelial system (or, when needed, take advantage of its interception of carriers to optimally deliver drugs to phagocytes) and ways to improve delivery to specific cells, often with the help of carrier-linked ligands. Each of these are dealt with by leading authorities in terms of applications in pharmacology, immunology and medicine and related methodologies.

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April 1986

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CONTENTS

MANNOSE BINDING PROTEINS IN THE LIVER AND BLOOD

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INTRODUCTION

The survival of glycoproteins in the circulation is determined by the nature of the terminal non-reducing sugar on their oligosaccharides. Glycoproteins terminating in sialic acid have plasma half-lives measured in days whereas those terminating in other sugars are cleared rapidly from the blood (a few minutes) mainly by the liver. The first mechanism to be defined was the hepatocyte galactose receptor which mediated the clearance of galactose-terminated glycoproteins from the blood (Ashwell & Morell, 1974). Around this time, groups interested in enzyme replacement for lysosomal storage disorders noted that various rat lysosomal enzymes (e.g. β-glucuronidase) administered intravenously were also rapidly cleared by the liver (Achord et al., 1977a; Stahl et al., 1976a,b; Schlesinger et al, 1976). Uptake was carbohydrate mediated since it was abolished by periodate treatment of the lysosomal enzymes. However in the case of lysosomal enzymes hepatic uptake was inhibited, not by galactose, but both by mannan (a mannose-terminated proteoglycan from yeast cell walls) and agalactoorosomucoid (N-acetylglucosamine-terminated) (Stahl et al., 1976c; Achord et al., 1977b). Thus this novel receptor (in this paper referred to as the mannose receptor) recognised glycoproteins terminating in two sugars, either mannose (Man) or N-acetylglucosamine (GlcNAc). Furthermore the receptors were present, not on hepatocytes, but on the sinusoidal lining cells of the liver (Achord et al., 1978; Schlesinger et al., 1978; Steer & Clarenburg, 1979).

Mannose receptors have subsequently been shown to be present on alveolar (Stahl et al., 1978) and peritoneal (Ezekowitz et al., 1980; Imber et al., 1982) macrophages and cultured (but not fresh) circulating mononuclear cells and bone marrow cells (Shepherd et al., 1982; Kataoka & Tavassoli, 1985). However in the clearance of circulating glycoproteins these cells play a minor role, the great majority being cleared by the hepatic mannose receptors (Schlesinger et al., 1980; Parise et al., 1984). Finally, an hepatic mannose binding protein isolated from whole rabbit, rat and human liver, which was originally considered to be the sinusoidal cell mannose receptor (Kawasaki et al., 1978; Townsend & Stahl, 1981; Mizuno et al., 1981) has now been shown to be a distinct intracellular hepatocyte protein (Maynard & Baenziger, 1982; Mori et al., 1983; Wild et al., 1983) located within the cisternae of the rough endoplasmic reticulum (Maynard & Baenziger, 1982; Mori et al., 1984). This paper will review recent data on mammalian mannose binding proteins with particular emphasis on those

areas which are of potential relevance to the problems of drug targeting.

CELL SURFACE MANNOSE RECEPTROS

Cellular Localization of Sinusoidal Mannose Receptors

The cellular localisation of the hepatic mannose receptor has been the subject of controversy. The sinusoidal cells of the liver comprise a mixture of Kupffer cells, endothelial cells, fat-storing cells (Ito cells), and (at least in the rat) pit cells. The endothelial cells of the liver sinusoids are specialised cells which differ both structurally and functionally from the endothelial cells lining the rest of the vasculature. Initially the mannose receptor was believed to be located on Kupffer cells (Achord et al., 1978; Schlesinger et al., 1978, 1980), largely because extrahepatic (e.g. alveolar) macrophages also possessed the receptor. A subsequent study, using isolated hepatic endothelial cells and Kupffer cells separated by centrifugal elutriation indicated that the mannose receptor was located principally on endothelial cells (Summerfield et al., 1982).

This discrepancy has been resolved by three other studies. In an electron microscopic autoradiographic study radiolabelled Man- and GlcNAc-terminated glycoproteins accumulated in Kupffer cells and entothelial cells but not in hepatocytes. However, the internalisation of these ligands by endothelial cells was two to six times greater than that by Kupffer cells (on a cell volume basis) (Hubbard et al., 1979). Similar results were obtained in an in-vitro study, using fractions of isolated Kupffer and endothelial cells prepared by centrifugal elutriation. Both cell types internalised Man- and GlcNAc-terminated glycoproteins, but uptake was greater by endothelial cells (on a cell number basis) (Praaning van Dalen et al., 1982). Finally in a study using a modification of the standard centrifugal elutriation technique for separating hepatic sinusoidal cells a series of fractions were collected and characterized by histochemistry and electron microscopy. These revealed that elutriation yielded cell populations ranging from almost homogeneous Kupffer cell fractions. The uptake of a radiolabelled GlcNAc-terminated glycoprotein (^{125}I-agalactoorosomucoid) was greatest (53% of total uptake) by elutriator fractions containing equal proportions of endothelial and Kupffer cells (Fig. 1) (Parise et al., 1984). Taken together these data indicate that mannose receptors are present on both Kupffer cells and endothelial cells in the liver. It remains to be determined whether these mannose receptors are structurally identical and whether they share a similar spectrum of carbohydrate specificities. Each mannose receptor participates in many internalisation events indicating that it is probably recycled (Stahl et al., 1980).

Effect of Diabetes Mellitus on the Sinusoidal Mannose Receptor

The uptake of Man- and GlcNAc-terminated glycoproteins by isolated hepatic sinusoidal cells is inhibited not only by mannose and N-acetyl-glucosamine but also by glucose, fructose and a glucose-albumin conjugate (Fig. 2). Inhibition by glucose is competitive over a wide range of concentrations and is almost 100% at a glucose concentration of 56mM (Summerfield et al., 1982). Furthermore the rates of plasma clearance, hepatic accumulation and catabolism of GlcNAc-terminated glycoproteins are decreased in diabetic mice or rats (Pizzo et al., 1981; Summerfield et al., 1982). This phenomenon also occures if hyperglycaemia is induced by infusing glucose in the absence of diabetes mellitus (Pizzo et al., 1981). The number of cell surface mannose receptors on rat peritoneal macrophages are reduced when they are cultured in media containing high glucose concentrations (Weiel & Pizzo, 1983). These data indicate that the impaired clearance of glycoproteins by the mannose receptor in diabetes is due to hypergly-

caemia having both a direct inhibitory effect on uptake and down-regulating the surface expression of mannose receptors.

However the effects of diabetes on glycoprotein metabolism by hepatic sinusoidal cells appear to be more complex than simply decreasing uptake.

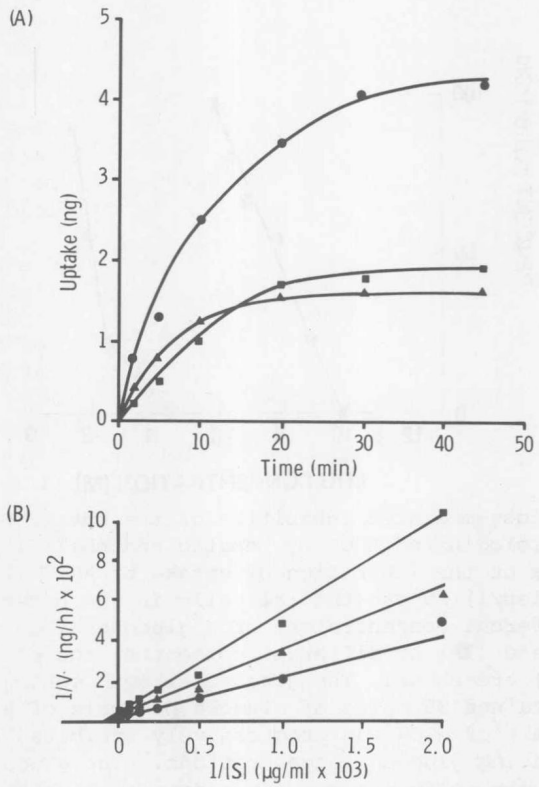

Fig. 1. The specific uptake of radioiodinated agalactorosomucoid (AGOR) by fractions of rat hepatic sinusoidal cells separated by centrifugal elutriation: endothelial cell fraction (▲), mixed cell fraction (●), and Kupffer cell fraction (■). A, the time dependence of specific uptake of AGOR (14 nmol/l). B, the concentration dependence of specific uptake of AGOR. Reproduced with permission from Parise et al., (1984).

Compartmental analysis of the metabolism of intravenously administered ^{125}I-agalactoorosomucoid (^{125}I-AGOR) in rats revealed another effect of diabetes. Not only was ^{125}I-AGOR uptake decreased but the ligand catabolic rate was greatly diminished indicating that diabetes also affected intra-

cellular processing. These effects could be reversed by insulin treatment. Unexpectedly insulin also prolonged the intracellular transport time, the period between ligand internalisation and delivery to the lysosomes (Tanaka et al., 1985).

The functional consequences of impaired mannose receptor function in diabetes are unknown. The inhibition of AGOR uptake by fructose (Summerfield

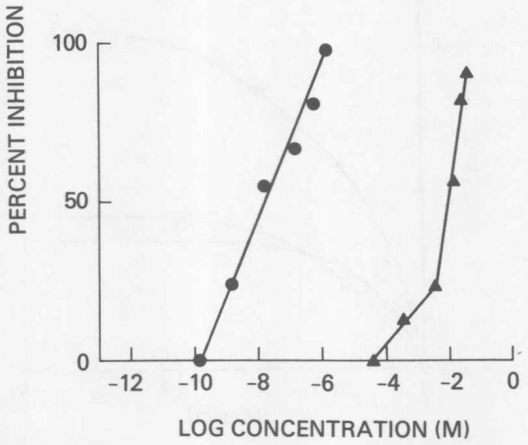

Fig. 2. Glucose-mediated inhibition of the uptake of radioiodinated AGOR by hepatic endothelial cells. Data on the inhibition of uptake of AGOR (14 nmoles/l) by endothelial cells in the presence of different concentraions of a glucose-albumin conjugate (●) or different concentrations of glucose (▲) are shown. The glucose-albumin conjugate contained 35 moles of glucose per mole of albumin. Uptake of AGOR was progressively inhibited by increasing glucose concentrations. The glucose-albumin conjugate was six orders of magnitude more potent an inhibitor (on a molar basis) than glucose. Reproduced with permission from Summerfield et al., (1982).

et al., 1982) is of particular interest because a fructose residue is the terminal sugar of spontaneously glycosylated proteins such as haemoglobin Alc and glycosylated albumin. The terminal fructose residue of such glycoproteins is generated by an Amadori rearrangement of the Schiff base formed between the free amino groups of the protein and glucose (Koenig et al., 1977). However for a glycosylated protein to be internalized by the mannose receptor depends largely on the degree of substitution of the protein with sugar residues (Krantz et al., 1976). At present, there is no

evidence that sufficient glycosylation of proteins occurs in diabetes mellitus to permit their uptake by the mannose receptor. Conversely, the accumulation of glycosylated proteins in plasma in diabetes mellitus (Bunn, 1981) could be due, at least in part, to hyperglycaemia inhibiting their uptake by the mannose recpetor. Yeasts (Warr, 1980) and some bacteria (Perry & Ofek, 1984) are also cleared by the mannose receptor. Thus it is possible that inhibition of the normal function of the hepatic mannose receptor by hyperglycaemia may contribute to abnormal metabolism of glycoproteins and the increased incidence of fungal and bacterial infections in diabetes mellitus.

Effects of Other Agents on the Sinusoidal Mannose Receptor

Several other agents have been shown to influence the hepatic mannose receptor. Some of these studies have been performed on peritoneal or alveolar macrophages and so the evidence that the agents affect the hepatic mannose receptor is indirect. A further problem is that some agents alter the population of local macrophages by causing recruitment of cells from other sites (usually bone marrow). This phenomenon probably accounts for the differing reports of the effect of Bacillus Calmette Guerin (BCG) on the mannose receptor. In studies with isolated peritoneal macrophages, pretreatment with BCG caused a selective diminution of ligand binding to the mannose receptor on these cells (Imber et al., 1982; Ezekowitz et al., 1980). It was concluded that BCG down-regulated the macrophage mannose receptor. However, in another study, BCG was found to cause a great increase in the number of hepatic sinusoidal cells due to the accumulation of mononuclear cells morphologically distinct from the resident Kupffer and endothelial cells. Furthermore although the uptake of glycoprotein per cell was reduced (Fig. 3), in vivo the total hepatic uptake of glycoprotein by BCG treated rats was not different from control animals (Table 1) (Parise et al., 1984). Thus it appeared that, in the liver, BCG caused the recruitment and accumulation of extrahepatic mononuclear cells in the liver but did not alter mannose receptor expression on the resident sinusoidal cells.

In contrast, iron loading of hepatic sinusoidal cells by injections of iron sorbitol profoundly reduced hepatic glycoprotein uptake and caused a twofold increase in the proportion of ligand remaining in the circulation (Table 1). Iron loading caused a selective suppression of glycoprotein uptake by the fraction of cells containing equal numbers of Kupffer and endothelial cells (Fig. 3), that fraction containing the greatest density of mannose receptors (Parise et al., 1984). The mechanism by which iron appears to down-regulate the expression of the mannose receptor is unclear, however it is a specific effect since other agents, such as latex particles (Fig. 3), which are also phagocytosed by Kupffer cells have no influence (Parise et al., 1984).

Two manoeuvres have been described which up-regulate the cell surface expression of the mannose receptor. A 24h fast (Summerfield et al., 1982) or pretreatment with dexamethasone (Shepherd et al., 1985) increase the amount of ligand bound by isolated sinusoidal cells or cultured bone marrow cells about threefold without altering the apparent uptake (the affinity of the receptor for the ligand).

The consequences of the alterations of glycoprotein metabolism that can be induced by external factors acting on the mannose receptor remain to be defined. Dexamethasone treatment, as well as up-regulating the expression of the mannose receptor, also reduced the extracellular levels (in the culture medium) of hexosaminidase (a lysosomal enzyme) suggesting that the mannose receptor may play a role in regulating the extracellular levels of lysosomal enzymes (Shepherd et al., 1985). The demonstration that iron overload has a potent depressant effect on the hepatic sinusoidal

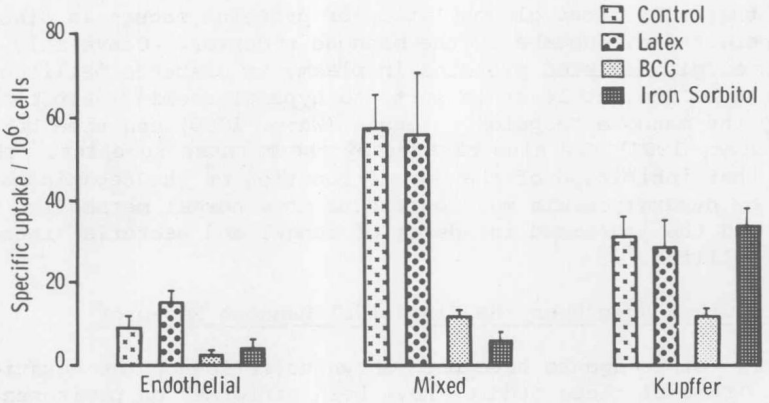

Fig. 3. The specific uptake of radioiodinated AGOR by rat hepatic sinusoidal cells separated by centrifugal elutriation into endothelial cell, mixed cell and Kupffer cell fractions. The uptake by sinusoidal cells from control rats and rats after latex, bacillus Calmette-Guerin and iron sorbitol are shown. Reproduced with permission from Parise et al. (1984).

mannose receptor raises the possibility that marked accumulation of lysosomal enzymes may occur in clinical states of iron overload such as haemochromatosis and haemosiderosis in which the cellular iron burdens are much greater.

Effects of Glycoprotein Structure on Uptake and Processing by the Hepatic Mannose Receptor.

There are several stages in the metabolism of a glycoprotein that is targeted for the hepatic mannose receptor. The glycoprotein must first bind to the mannose receptor. The ligand-receptor complex is internalized and transported into the cell in endosomes. In the endosome compartment the ligand-receptor complex is disrupted by acidification of the endosome (Forgac et al., 1983). The endosomes then form another specialized organelle, the compartment for uncoupling the ligand-receptor complex (CURL) where the ligand is segregated from the receptor (Geuze et al., 1983). The receptor is recycled to the cell surface for reutilization and the glycoprotein is transported to the lysosomes to be catabolized.

Surprisingly little data exists on the influence of glycoprotein structure on their uptake and processing by the hepatic mannose receptor despite its potential importance for drug and enzyme targeting. However there are observations that indicate that glycoprotein structure exerts an important effect. In contrast to glycoproteins such as AGOR, which has an hepatic half-life of approximately 30 min (Taylor et al., 1985), several mannose-terminated lysosomal enzymes are not rapidly catabolized after their uptake by hepatic sinusoidal cells (Schlesinger et al., 1978; Steer et al., 1979) even though they have been transported to lysosomes (Schlesinger et al.,

Table 1. In-vivo metabolism of ^{125}I-AGOR

	n	Distribution*				
		Liver	Blood	Lung	Spleen	Recovery**
Control	4	60±1.4	12.3±1.4	0.5±0.1	0.9±0.1	82±5.0
Latex	3	58±0.7	8.0±0.8	0.3±0.0	1.0±0.2	74±0.5
BCG	3	61.5±3.8	11.2±2.3	0.6±0.1	1.4±0.1	86±5.0
Iron sorbitol	3	39.3±0.4‡	24.7±0.6	0.9±0.1	1.9±0.4	82±2.0
Sorbitol	5	81.2±7.2	6.9±1.3	0.2±0.004	0.4±0.04	93±6.0

AGOR = Agalacto-orosomucoid; BCG = Bacillus Calmette-Guerin
Values represent mean ± SEM for each group
*Distribution is expressed as percent injected radioactivity accumulating in an organ by 10 min.
**Recovery is expressed as percent of injected radioactivity that was recovered in the organs examined (including the kidneys).
‡Significantly lower than control ($p < 0.05$)
Reproduced with permission from Parise et al., (1984).

1976; Brown et al., 1978). Undoubtedly structural features that protect from lysosomal degradation are critical properties of lysosomal enzymes.

The binding rate of neoglycoproteins to the mannose receptor is dependent on the density of substituted sugars (Schlesinger et al., 1980; Hoppe & Lee, 1983), the type of substituted sugar (the receptor has a greater affinity for mannose than glucose) and the geometry of the oligosaccharide (the receptor has greater affinity for complex oligosaccharides than a similar number of monosaccharides) (Taylor et al., 1985). In contrast the rate of ligand internalisation is independent of the receptor binding rate constants (Hoppe & Lee, 1983; Taylor et al., 1985). Intracellular transport time, which is a composite of the events from ligand internalization to its delivery to the lysosomes and catabolism have been studied by compartmental analysis of in-vivo glycoprotein metabolism in the rat (Taylor et al., 1985). Transport was significantly slower for the Man-BSA (a mannose-terminated neoglycoprotein) than for the other ligands (e.g. AGOR) suggesting that receptor-ligand uncoupling was slower for Man-BSA (for which the receptor had the highest affinity in these studies), or that extra-lysosomal catabolism of the other ligands occurred.

Many studies have shown that deglycosylated forms of glycoproteins are catabolized more rapidly than the original glycoprotein (Wang & Hirs, 1977; Olden et al., 1977; Loh & Gainer, 1978; Brown et al., 1979; Chu & Maley, 1980; Olden et al., 1982; Bernard et al., 1982). Furthermore, swainsonine, an inhibitor of lysosomal α-mannosidase, has been shown to cause total inhibition of proteolytic degradation of endocytosed asialoglycoproteins (Winkler & Segal, 1984). These data indicate that deglycosylation of glycoproteins is the rate-limiting step in their catabolism. But catabolism also seems to be determined by the nature of the carbohydrate on the glycoprotein. It was greater for glucose-terminated than

mannose-terminated BSA and slowest for AGOR which bears a complex oligosaccharide (Taylor et al., 1985).

Thus it is evident that the structure of a glycoprotein has a critical effect on its processing by the mannose receptor. An understanding of the structural features that influcence hepatic uptake, transport and catabolism will be of value in drug targeting and for enzyme replacement in lysosomal storage disorders.

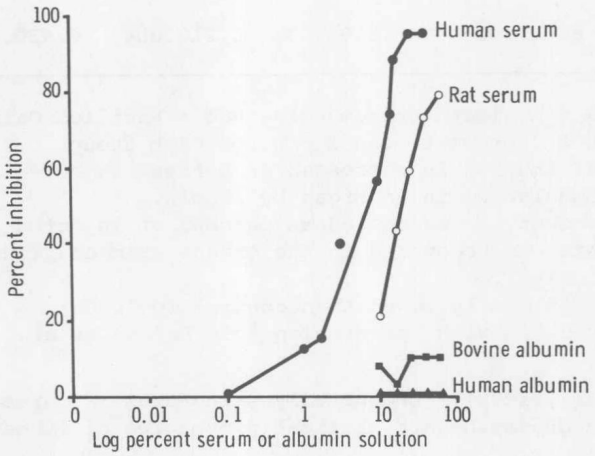

Fig. 4. Inhibition by dialyzed serum of the specific uptake of radioiodinated AGOR by rat hepatic sinusoidal cells. Sinusoidal cells were incubated with AGOR (14nmoles/l) with increasing amounts of dialyzed serum or albumin (5% w/v in glucose-free buffer). Serum, but not albumin, inhibits glycoprotein uptake by hepatic sinusoidal cells. Reproduced with permission from Taylor and Summerfield (1984).

MANNOSE BINDING PROTEINS IN THE BLOOD

It is now recognized that the metabolism of circulating Man- or GlcNAc-terminated glycoproteins is further complicated by the presence of mannose binding proteins in the blood. Human or rat serum inhibit the specific uptake of AGOR by isolated rat hepatic sinusoidal cells (Fig. 4). The serum inhibitors are not glycoproteins that bind to the hepatic mannose receptor but have the properties of lectins that bind Man- or GlcNAc-terminated glycoproteins. They can be isolated from serum by affinity

chromatography and the isolated proteins will inhibit the sinusoidal uptake of glycoproteins in vitro (Taylor & Summerfield, 1984).

The nature of these serum mannose binding proteins is becoming clearer. Human serum contains calcium independent mannose binding proteins, the principal one is mannose specific immunoglobulin (IgG) (Summerfield & Taylor, 1985). A calcium dependent mannose binding protein has been isolated from rabbit and human serum (Kozutsumi et al., 1980; Kawasaki et al., 1983). This calcium dependent serum mannose binding protein, like the hepatocyte mannose binding protein (Townsend & Stahl, 1981), has specificities for GlcNAc and fucose as well as mannose (Summerfield & Taylor, 1985) Furthermore in Western blots, antibodies to the 30k subunit of this serum protein bind the 30k subunits of the hepatocyte mannose binding protein isolated by Wild et al. (1983) (Summerfield & Taylor, 1985). These data indicate that the calcium dependent serum mannose binding protein may originate by secretion from hepatocytes.

Thus serum contains at least two classes of mannose binding proteins; mannose specific, calcium independent immunoglobulins and a calcium dependent protein of broader carbohydrate specificity which is probably secreted by hepatocytes. The central questions that remain are the modes of action and functions of these serum mannose binding proteins. The factors that determine which serum mannose binding protein binds a glycoprotein that enters the circulation and the ways in which the glycoprotein-binding protein complex interacts with the sinusoidal mannose receptor are unknown. Since the binding of glycoprotein to both serum and sinusoidal mannose binding proteins is carbohydrate mediated it seems likely that the glycoprotein dissociates from the serum mannose binding protein prior to binding to the sinusoidal mannose receptor. As to the function of serum mannose binding proteins, they might bind and inactivate noxious glycoproteins (such as lysosomal enzymes, yeasts and bacteria) that enter the circulation prior to their removal by the sinusoidal mannose receptor. Serum mannose binding proteins may function in an analogous way to α 1-antitrypsin and the other components of the serum antiprotease system. The consequences of this system of serum mannose binding proteins for targeting drugs to the sinusoidal mannose receptor remains to be defined.

CONCLUSIONS

Mannose- and N-acetylglucosamine-terminated glycoproteins that enter the circulation are first bound by carbohydrate specific serum binding proteins and then cleared by carbohydrate specific receptors on hepatic sinusoidal cells. Both the structure of the saccharide moiety of the glycoprotein and external factors such as diabetes mellitus or treatment with glucocorticoids influence uptake and processing of the glycoprotein by the sinusoidal mannose receptor. The mannose receptor should be a potent means of targeting drugs to hepatic sinusoidal cells.

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