THE INFLUENCE OF GLYCOSIDASES AND LECTINS ON INSULIN BINDING TO ZAJDELA HEPATOMA CELLS

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1. Introduction

Numerous studies insist upon the importance of cell surface carbohydrates for a variety of cell recognition phenomena. In particular, glycoconjugates have been shown to be involved in the binding by cell membrane receptors of certain bacterial toxins, lectins and hormones [1–4].

For insulin, carbohydrates were implicated in specific hormone binding [5–11]. For normal hepatic cells, terminal galactose residues and accessible mannose groups were involved in high affinity insulin binding [6]. Our studies have analysed the insulin binding characteristics of transformed hepatic cells (Zajdela rat ascites hepatoma cells). These results showed that the number of specific insulin receptors was decreased as compared to normal hepatocytes but that their characteristics were not modified (pH and temperature dependance, curvilinear plots obtained by Scatchard analysis, dissociation of bound insulin increased by insulin). However, terminal β-galactose groups were not implicated in the binding process [7].

To explain this difference between normal and transformed hepatic cells and to study the nature of carbohydrate residues involved in insulin binding to hepatoma cells, the effect of different glycosidases and lectins was investigated.

Our results indicate the importance of sialogalactoglycopeptides for insulin binding. They lead to the hypothesis of a model concerning the role of glycoconjugates in the insulin binding process, which is discussed here.

Preliminary reports of these findings have been published [9].

Abbreviations: BSA, bovine serum albumin; con A, concanavalin A; Fuc, α-fucose; Gal, β-galactose; Glc NAc, *N*-acetylglucosamine; KRP, Krebs-Ringer phosphate; Man, α-mannose; NeuNAc, *N*-acetyl-neuraminic acid; PNA, peanut agglutinin; RCA, *Ricinus communis* agglutinin I; SBA, soyabean agglutinin; UeAF, *Ulex europeus* agglutinin; WGA, wheat germ agglutinin

2. Materials and methods

2.1. *Preparation of cells and insulin binding studies*

Zajdela ascites hepatoma cells (strain D) were transplanted and prepared, as in [7], in Krebs-Ringer phosphate buffer (pH 7.6) 1.5% BSA. Insulin binding experiments using [^{125}I]insulin (50–100 μCi/μg Radiochemical Centre, Amersham) and a native insulin (Novo Labs, Pork Insulin MC) were performed at 4°C as in [7] except that at the end of the incubation period the cells were washed once. All experiments were repeated at least 3 times.

2.2. *Enzymic digestions*

Two neuraminidases from *Clostridium perfringens* (1–5 mU/10^6 cells, Worthington NEUA 18 U/mg) and from *Vibrio cholerae* (0.5–5 U/10^6 cells, Institut Behring ORKD 500 U/ml), β-galactosidase (0–500 mU/10^6 cells, Worthington BGC 500 U/mg) and α-L-fucosidase from beef kidney (1–5 mU/10^6 cells Boehringer Mannheim 1 U/mg) were employed. Digestions were for 30 min at 37°C as in [7].

2.3. *Effect of lectins*

Lectins used were: *Ricinus communis* agglutinin I (type 120 from castor beans), peanut agglutinin (from *Arachis hypogea*), soyabean agglutinin (from soyabean *Glycine max*), *Ulex europeus* agglutinin (from gorse seeds) and wheat germ agglutinin (from wheat germ *Triticum vulgare*), from Reactifs IBF

(Pharmindustrie), concanavalin A (from jack bean *Canavalia ensiformis*) from Worthington Biochem. The saccharide binding specificities of the different lectins were [12–14]: for RCA_I and PNA, terminal β-galactose (Gal); for SBA, terminal galactose and *N*-acetyl-galactosamine; for UeAf, terminal α-Fuc; for WGA, terminal or internal *N*-acetyl-glucosamine and *N*-acetyl-neuraminic acid; for con A, terminal or internal α-mannose and α-glucose.

Cells ($1-2 \times 10^6$) were incubated with various concentrations of lectins, either at 22°C (UeAF, con A) or at 4°C (PNA, SBA, RCA, WGA), in order to ensure good conditions for binding [15], for 1 h in 0.5 ml KRP buffer 1.5% BSA, (pH 7.6). Insulin binding experiments were then done at 4°C as above. Control tubes for the specificity of lectin binding were assayed with lectin specific sugars, i.e., lactose (Reactifs IBF, Pharmindustrie) for RCA, PNA and SBA, fucose (Sigma Chem.) for UeAF and Glc NAc (Sigma) for WGA, lectins having been preincubated for 20 min at 22°C in the presence of 0.05 M of the respective sugar prior to addition of the cells. The effect of lectins was also tested in some cases after neuraminidase treatment of hepatoma cells.

In some cases, cell agglutination was observed when high lectin concentrations were added to the cells. However this agglutination did not impede insulin binding since maximal agglutination could occur without a decrease in insulin binding. In particular for certain lectin concentrations, cell agglutination was observed when RCA, PNA, SBA or WGA were incubated with intact or desialylated cells even when the insulin binding was not decreased: the effect of RCA, PNA or SBA on intact cells, the effect of WGA plus Glc NAc on intact cells.

We also verified that the effect of lectins was specific and not toxic by showing that addition of specific sugars impeded the inhibitory effect of lectins upon insulin binding. If a non-specific sugar at the same molarity was added to the preparation, the lectin effect was the same as when in its absence even though the osmolarity was increased.

3. Results and discussion

3.1. *Effect of sialidase, β-galactosidase and galactose specific lectins*

Two different neuraminidases (from *Clostridium perfringens* and *Vibrio cholerae*) were tested. In each case insulin binding was not modified after enzymic treatment of Zajdela hepatoma cells even with enzyme concentrations hydrolyzing 1.5 μg sialic acid/10^6 cells.

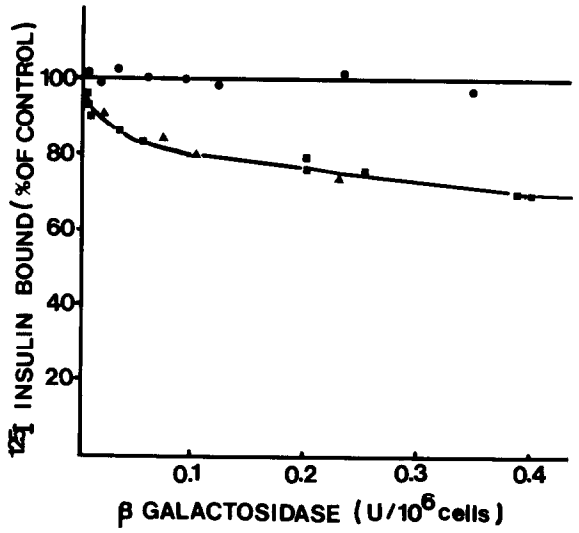


Fig.1. Effect of β-galactosidase digestion upon the specific insulin binding by Zajdela hepatoma cells. Cells were digested with various concentrations of: (•) β-galactosidase alone; (▪) β-galactosidase plus neuraminidase from *Clostridium perfringens* (5 mU/10^6 cells); (▲) β-galactosidase plus neuraminidase from *Vibrio cholerae* (0.5 U/10^6 cells); under the conditions indicated in the text.

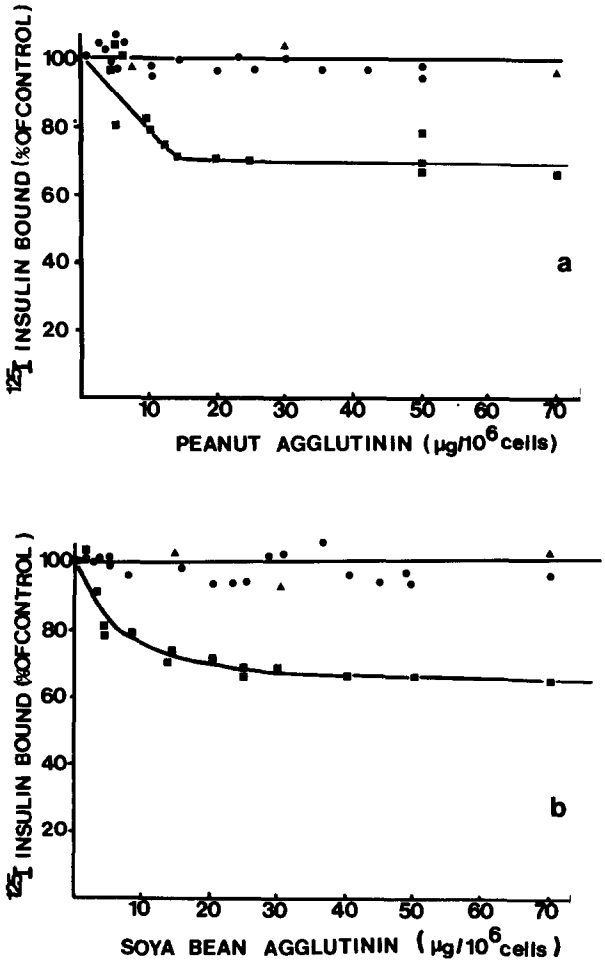
Similarly, when β-galactosidase was employed, insulin binding was not modified. However, when hepatoma cells were digested with sialidase plus β-galactosidase, an inhibition of specific insulin binding to cells was observed (fig.1). This inhibition increased with increasing concentrations of β-galactosidase and was 30% of the total binding when employing 0.4 U enzyme/10^6 cells. Scatchard analysis showed that the inhibition only affected the number of high affinity sites (table 1), the dissociation constants remaining unmodified.

Concordant results were observed when the effect of 3 galactose-specific lectins (RCA, PNA, SBA) was studied. When intact Zajdela hepatoma cells were used no modification in insulin binding was observed. On the contrary, when the cells were pretreated with sialidase, the addition of the lectins SBA, PNA or RCA resulted in an inhibition of 30–35% of insulin binding (fig.2). This effect could be accorded to specific lectin binding to galactose and toxic alterations could be excluded as it was reversed when lectins

Table 1
Effect of various glycosidases and lectins on the number of high and low affinity insulin binding sites

Agent		Variation in the number of:	
		High affinity sites (%)	Low affinity sites (%)
Control		0	0
β-Galactosidase	(0.2 U/10^6 cells)	0	0
Neuraminidase	(5 mU/10^6 cells)	0	0
β-Galactosidase + neuraminidase	(0.2 U/10^6 cells) (5 mU/10^6 cells)	−20	0
α-Fucosidase	(5 mU/10^6 cells)	0	0
Ulex europeus	(20 μg/10^6 cells)	−20	−20
Concanavalin A	(20 μg/10^6 cells)	−30	−5

Insulin binding characteristics were determined from Scatchard analysis as in [7], experiments being repeated ⩾3 times. For control cells the number of high affinity binding sites was 460/cell and for low affinity sites 11 000/cell



were preincubated with lactose (fig.2).

Thus our results using enzymes and lectins indicate the presence of β-galactose groups implicated in high affinity insulin binding to hepatoma cells. However these galactose residues are not directly accessible to binding being masked by sialic acid moieties probably linked to galactoses and the presence of which does not modify insulin binding characteristics.

The non-involvement of sialic acid in insulin binding was proposed for normal cells [6,16] and galacto-

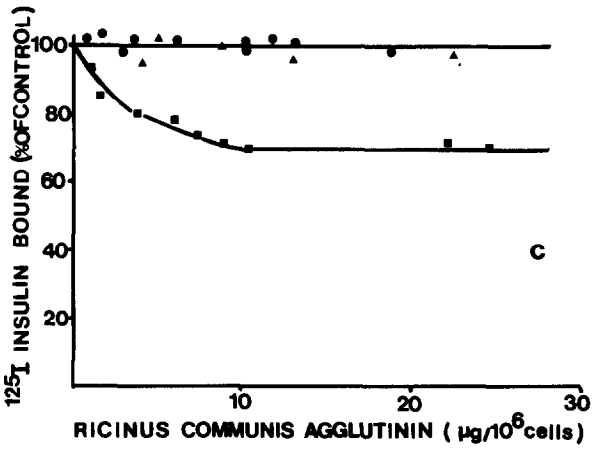


Fig.2. Effect of galactose specific lectins PNS, SBA and RCA on insulin binding to Zajdela hepatoma cells. (a) Effect of PNA, (b) effect of SBA, (c) effect of RCA, on: (●) intact cells; (■) desialylated cells; (▲) desialylated cells + lactose. For experimental details see text.

glycoconjugates playing a role in high affinity insulin binding were directly revealed for normal hepatic plasma membranes [6].

For hepatoma cells, the intervention of sialic acid in this process could be related to an increased sialic acid content found on the surface of different hepatoma strains [17–19], galactose residues being accessible only after neuraminidase treatment. On the contrary, for normal hepatic cells, receptors for RCA [20] and for insulin [6], possessing terminal galactose moieties, were directly revealed.

3.2. *Effect of α-fucosidase and* Ulex europeus *lectin*

When hepatoma cells were digested with α-fucosidase, no modification in insulin binding was noted. However when the effect of the lectin UeAF, specific for fucose, was investigated a 20% inhibition of insulin binding was observed with 10 μg lectin/10^6 cells (fig.3a) and this effect was specific for fucose. When desialylated cells were used, the same results were observed as for intact cells. Scatchard analysis of this effect showed a 20% reduction in the number of high and low affinity insulin binding sites (table 1) without modification in their affinity.

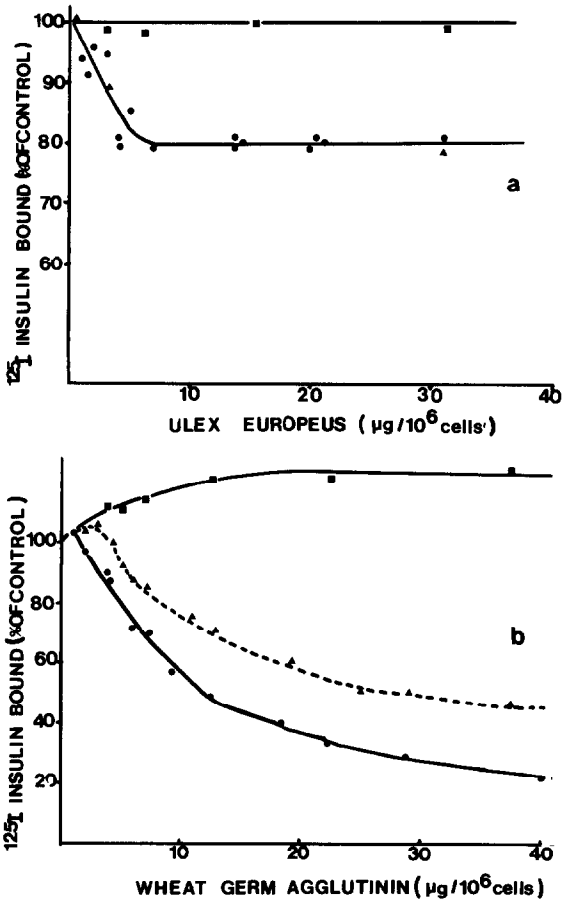


Fig.3. Effect of UeAF and WGA on specific insulin binding to Zajdela hepatoma cells. (a) Effect of UeAF upon: (•) intact cells; (▪) intact cells + fucose; (▲) desialylated cells. (b) Effect of WGA upon: (•) intact cells; (▪) intact cells + Glc NAc; (▲) desialylated cells.

Thus, for Zajdela hepatoma cells, although the hydrolysis of fucose residues by fucosidase did not modify insulin binding, the binding of UeAF to fucose resulted in an inhibition. Studies with normal hepatic cells concerning the effect of fucosidase and UeAF did not reveal any modification in either case ([6] and in press). Thus it seems likely that, for hepatoma cells, fucose residues are linked or close to sialogalactoglycoconjugates involved in insulin binding. These fucose groups are not implicated in the insulin recognition process. However, when *Ulex* lectin is attached to fucose moieties, steric occupation of insulin receptors may partially impede hormone binding.

The presence of excess fucose residues in glycoconjugates of Zajdela hepatoma cells involved in insulin binding could be related to an increased cell surface fucose content as found in surface glycoproteins for hepatoma cells [17,18,21].

3.3. *WGA*

The effect of WGA on insulin binding to intact hepatoma cells was investigated. A biphasic effect was noted: a slight enhancement with low concentrations of the lectin and a marked inhibition with higher concentrations (fig.3b), insulin binding being decreased by 75% with WGA at 40 μg/10^6 cells. If WGA was preincubated with its specific sugar, Glc NAc, no inhibition could be noted. To the contrary, insulin binding was slightly enhanced (fig.3b). These results suggest that the increased insulin binding, observed when using low lectin concentrations and which is not reversed by Glc NAc, could be due to non-specific lectin effects.

The inhibition of insulin binding by desialylated cells, after WGA action, could be explained by the binding of the lectin to Glc NAc residues, even when in the non-terminal non-reducing position [12]. This effect indicated the importance of Glc NAc moieties in the insulin receptor. The differences noted between desialylated and intact hepatoma cells could be accounted to the binding of WGA to sialic acid residues [12,13,22]. Since WGA posseses 4 carbohydrate

binding regions, multivalent interactions with surface sialoglycoconjugates [16] could result in partial steric occupation of insulin binding sites.

The effect of con A was also studied as in [7]. A 30% inhibition of insulin binding was noted when using moderate con A concentrations: this inhibition affected the number of high affinity sites (table 1).

The effect of con A and WGA on insulin binding was described for normal cells [5,6,11,23] and led to the conclusion that common insulin and con A binding sites exist and that the insulin receptor has a glycosidic structure.

Thus our studies with glycosidases and lectins indicate that galactose residues are implicated in insulin binding to high affinity sites: mannose and *N*-acetyl-glucosamine molecules probably form the core of this glycoconjugate. Furthermore, for Zajdela hepatoma cells, sialic acid and fucose groups are either linked or close to this (or these) glycopeptide(s).

This carbohydrate composition has been described for certain types of N-linked oligosaccharide side-chains usually possessing a core sequence containing Man and Glc NAc, branched chains linked via the sequence Gal–Glc NAc to internal Man residues, NeuNAc groups in non-reducing terminal positions and sometimes Fuc linked to Gal or Glc NAc [24], and in particular for con A receptor fractions isolated from rat hepatomas [25]. These structures were found on the surface of normal and transformed cells [26,27], the latter possessing an increased number of terminal sialic acid and fucose residues [28].

It is difficult to relate this low specificity of the glycosidic structure to highly specific insulin binding. Thus it is tempting to speculate that this (or these) glycopeptide(s) is (are) not directly involved in initial insulin binding to an acceptor site, but that the interaction of the carbohydrate moiety with the insulin–acceptor complex transforms the low affinity linkage to a high affinity one. This glycosidic struture may be a part of, or functionally linked to, the receptor molecule and thus could modulate insulin specific action.

This hypothesis for the functional heterogeneity of insulin receptors was studied [29]. It was postulated that initially insulin binds to a low affinity cell surface receptor and that secondly some parts of this complex are converted to a high affinity form.

Other works demonstrate the importance of a cell membrane glycoprotein in the insulin binding process [10,30]. Thus the interaction of the homogeneous low affinity insulin binding species with a membrane glycoprotein resulted in the appearance of a high affinity binding plateau, suggesting that this glycoprotein may represent an effector, or a non-recognition moiety, of the receptor oligomer, that confers special binding properties to the receptor complex [10].

Studies using normal liver plasma membranes indicate that two membrane galactoglycoproteins are involved in the specific insulin recognition process [8,9].

In [11] con A-like binding sites were speculated to exist on the cell membrane and are a part of, or functionally linked to, the insulin receptor. This carbohydrate moiety present on the membrane might affect the binding of insulin and control its action.

Thus the following attractive hypothesis could be put forward: in normal cells a *N*-glycopeptidic structure containing Man, Glc NAc and Gal is a constituant of a glycoprotein linked to or close to the acceptor site for insulin. The hormone binds initially to the acceptor molecule with a low affinity and secondly this association with the glycopeptidic moiety gives rise to a high affinity complex which can modulate the hormone action. This glycoproteic fraction may form a non-recognition part of the receptor oligomer or may contain effector molecule(s) with which the insulin receptor may interact within the plane of the membrane, as postulated by the mobile receptor paradigm [31].

An alternative explanation, although less probable, could be considered in regard to the theory of negative cooperativity [31,32]: the glycosidic fraction could be involved in site–site interactions, its position of fixation to the insulin receptor being partially masked in the high affinity form of the molecule.

For hepatoma cells, this glycopeptidic moiety possesses added sialic acid and fucose residues. This increased glycosylation for transformed hepatocytes does not modify their insulin binding characteristics as demonstrated both previously and here. The differences observed between normal and transformed cells in the effect of lectins and enzymes could be explained by modification in the surface receptor environment with an increased sialic acid and fucose content.

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