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Interaction between New Neoglycoproteins and the D-Man/L-Fuc Receptor of Rabbit Alveolar Macrophages

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New types of neoglycoproteins, β-caseins coupled with ovalbumin-derived asparagine oligosaccharides (AO), aspartate aminotransferase-phosphopyridoxylated AO complex (AAT-PG), and streptavidin-biotinylated AO complex (SA-BAO), were tested for their inhibitory effect on binding of bovine serum albumin derivatized with thiomannoside, Man-AI-BSA [Lee YC, Stowell CP, Krantz MJ (1976) Biochemistry 15:3956-63] by rabbit alveolar macrophages. The β-casein derivatives and the AAT-PG complex increased binding affinity as the number of oligosaccharide chains attached was increased. Their inhibitory potencies were closely related to those of the Man-AI-BSA derivatives [Hoppe CA, Lee YC (1983) J Biol Chem 258:14193-99] on the basis of terminal mannose density. The SA-BAO complex containing three AO chains gave stronger inhibitory potency than the β-casein derivative with three AO residues, suggesting that proper orientation of the oligosaccharides on the protein can affect the receptor-ligand interaction.

Neoglycoproteins have proven to be useful in studies of carbohydrate-binding systems [1, 2]. Most of the earlier types of neoglycoproteins were prepared by non-specifically modifying amino groups of proteins with mono- or disaccharide derivatives. Recently, several new types of neoglycoproteins containing complex oligosaccharide chains have been developed. These include, β-casein covalently coupled with ovalbumin-derived

Abbreviations: BSA, bovine serum albumin; Man_{43}-BSA, BSA derivative containing on average 43 residues of Man linked through an amidino-linkage [7]; AO, asparagine oligosaccharide (Man_5-$GlcNAc_2$-Asn) from ovalbumin; AAT, aspartate aminotransferase; PG, phosphopyridoxylated AO; SA, streptavidin; BAO, biotinylated AO.

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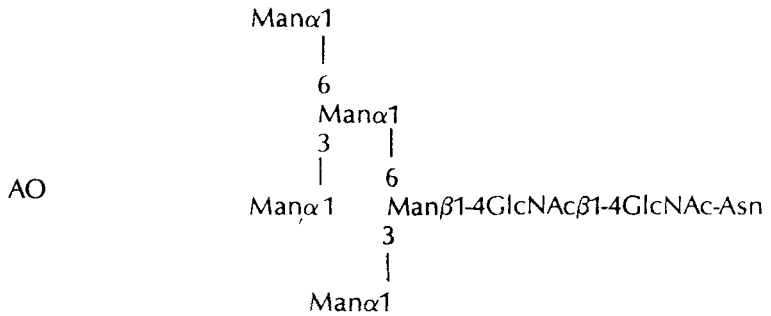

asparagine oligosaccharides (AO), aspartate aminotransferase (AAT) non-covalently complexed with phosphopyridoxylated AO (AAT-PG), and streptavidin (SA) complexed with biotinylated AO (BAO) (SA-BAO). Some characterizations of these neoglycoproteins have been reported [3-6]. These neoglycoproteins have several unique features that were not available in the earlier neoglycoproteins. They provide a larger number of terminal sugars per site of attachment on the protein. Beta-casein derivatives, which were prepared by transglutaminase action, possess the oligosaccharide units on one to seven specific sites dictated by the transglutaminase specificity. Two noncovalent neoglycoproteins, AAT-PG and SA-BAO, have other unique features: 1) the location and the number of oligosaccharides on the protein are known, 2) a part of the oligosaccharide chain is "buried" in the pocket of the active sites. These features of the new neoglycoproteins allow us to examine different aspects of the carbohydrate-receptor interaction. In this study, these new neoglycoproteins were examined in the rabbit alveolar macrophage D-Man/L-Fuc receptor.

Materials and Methods

Materials

Man_{43}BSA was prepared as described [7]. Asparagine oligosaccharide (AO: Man_5-$GlcNAc_2$-Asn) from ovalbumin was isolated by cation-exchange chromatography according to the method of Huang *et al.* [8]. Bovine β-casein was purified from casein powder as described by McKenzie [9]. Beta-caseins coupled with 6-aminohexanoyl AO (6ahAO) were prepared by the transglutaminase method reported earlier [3, 6]. All the ε-amino groups of purified β-casein were first modified with ethyl acetimidate to prevent cross-linking of β-casein by transglutaminase. Each β-casein derivative coupled with 6ahAO to a different degree was separated by affinity chromatography on Con A-Sepharose [6] and then tested for purity by SDS-PAGE [10]. Aspartate aminotransferase complexed with two phosphopyridoxylated AO (AAT-PG) and streptavidin complexed with three biotinylated AO (SA-BAO) were prepared as described previously [4, 5]. Man_{43}-BSA was radioiodinated using a modified Chloramine T method [11] and was used within two to three weeks. Transglutaminase was purified from guinea pig liver [12]. Ovalbumin (twice crystallized), casein powder, Sepharose G-100, and mineral oil were obtained from Sigma Chemical Co., St Louis, MO, USA., silicon oil (DC 550 fluid) from Accumetric Inc. (Elizabethtown, KY, USA), BSA from Armour Pharmaceutical Co. (Chicago, IL, USA). All other chemicals used in this work were of the highest purity available commercially and were used without further purification.

Cells

Lung macrophages were isolated from New Zealand white male rabbits as reported earlier [13]. The cells used for these experiments were routinely > 95% viable by trypan blue exclusion and by measurement of intracellular and extracellular lactate dehydrogenase activity [14].

Inhibition and Stimulation Assay

Inhibitory potency of test ligands on Man_{43}-BSA-binding to rabbit alveolar macrophages was assayed as follows. Cells (10^6 cells/ml) and ^{125}I-Man_{43}-BSA (0.12 nM) were incubated in the presence of each ligand in various concentrations for 6 h at 2°C in capped 12 × 75 mm polystyrene tubes (Sarstedt, Princeton, NJ, USA; Cat. no. 2058) which were rotated vertically (end-over-end) at 6 rpm. Assays were initiated by adding cells to incubation mixtures containing ^{125}I-Man_{43}-BSA and test ligand at 2°C in the following incubation medium: 0.12 M NaCl, 5 mM KCl, 2 mM $CaCl_2$, $2H_2O$, 1 mM $MgSO_4$ $7H_2O$, 17 μM (w/v) phenol red, 15 mM sodium piperazine-*N,N'*-bis(2-ethanesulfonate), and 15 mM *N,N*-bis(2-hydroxyethyl)amino-ethanesulfonic acid adjusted to pH 6.7 with NaOH. To determine cell-associated ^{125}I-Man_{43}-BSA in the assays, duplicate samples (200 μl) were taken from each assay tube and the cells were centrifuged for 1 min through a mixture of 4/1 (by vol) silicone oil-mineral oil in a 0.4 ml polypropylene microfuge tube (Sarstedt; Cat. no. 710) by using an Eppendorf Model 5412 microcentrifuge. After the centrifugation, the polypropylene tube was cut at the middle of the oil layer, and the tips containing the cell pellet were measured for radioactivity. Nonspecifically bound ^{125}I-Man_{43}-BSA was determined by adding EDTA to the incubation mixture to a final concentration of 10 mM. The concentration of a test ligand causing 50% inbibition of the binding to the receptor (I_{50}) was obtained by plotting the per cent inhibition against the logarithm of the inhibitor concentration. Some of the results obtained from the assays were analyzed using a non-linear regression program LIGAND [15].

Results and Discussion

In our earlier studies of the Man-receptor on rabbit alveolar macrophages, it was observed that even with a modest increase in the number of Man on BSA, there were dramatic increases in the binding affinity ("cluster effect"). The casein derivatives used in this study also showed a similar "cluster effect" (Fig. 1 and Table 1). In order to relate the cluster effects between the BSA derivatives and the casein derivatives, a plot of Log[Kd] against sugar density was made (Fig. 2). It shows that the dependence of Kd on the Man density between the two series was quite similar. The fact that the Kd decreased (binding affinity increases) as the number of AO chain increased suggests that for the higher substituted casein derivatives, more than one AO chain is participating in the binding of the casein module, as was interpreted for other similar "cluster effects" [2].

The number of bound casein derivatives changed little (approximately 70 000 per cell) as the number of AO was increased from one to three. This value is somewhat smaller than the comparable values for the Man-BSA containing equivalent number of Man (75 000-100 000), but is not unreasonable. Although the sites of AO modification on the casein molecules are dictated by the sites of glutamine residues [3, 18] and the seven

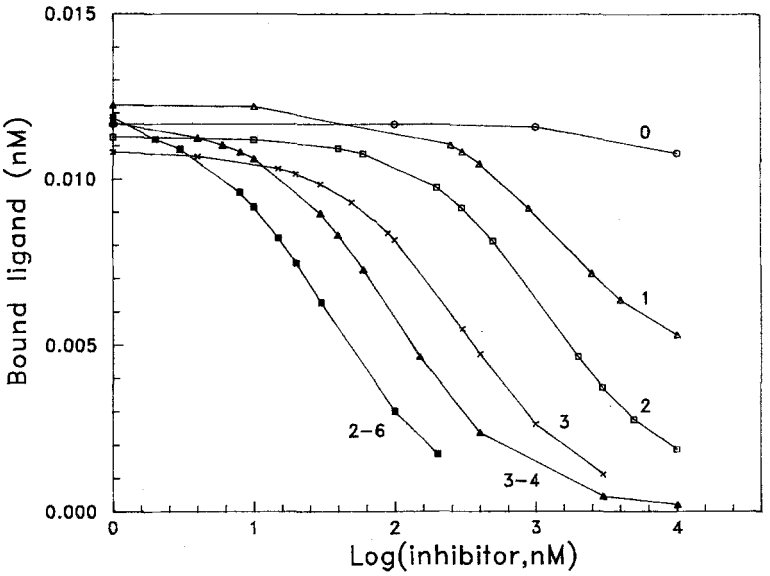

Figure 1. Inhibition of Man_{43}-BSA binding by β-casein derivatives.
Macrophages (10^6 cells/ml) and ^{125}I-Man_{43}-BSA (0.12 nM) were incubated in the presence of β-casein derivatives coupled with *n* AO residues: (○), *n* = O; (△), *n* 1; (□), *n* =2; (×), *n* = 3; (▲), *n* = 3-4; (■), *n* = 2-6, at the indicated concentrations for 6 h at 2°C, and specifically bound Man_{43}-BSA was measured by centrifugation through oil as described in the Materials and Methods section. The best-fit parameters (Table 1) were determined using the program LIGAND. The number designated for each curve is the number of AO chains on the casein molecule.

sites of the AO attachment are supposed to be in the random coil or beta-sheet region of the molecule [19] the exact geometry of the terminal Man in these derivatives is not known.

Binding of different ligands to rabbit hepatocytes was analyzed by regarding the cell surface as a lattice of receptor molecules [20]. A synthetic hexavalent ligand, di-*tris*-lac, was bound to the hepatocytes in larger amounts than asialoorosomucoid (ASOR), although their apparent Kd's were quite similar. Using this line of argument, it is predicted that a larger number of AO should be bound to the macrophage surface than the casein derivative with one (or more) AO chain. Indeed, at saturation, approximately 109 000 AO molecules were bound to each cell, as compared to approximately 70 000 for the casein derivatives. However, the difference is much smaller than that between di-*tris*-lac and ASOR. This may be because of the relative inflexibility of the AO mannosyl chains in comparison to the di-*tris*-lac molecule, which contains two hexyl chains to allow flexibility.

However, a more interesting observation was that the Kd from Tyr-6ahAO (approximately 300 nM) was much lower than that of casein with one AO chain (approximately 1200), determined from the data of the inhibition assay using the LIGAND program. This is also a deviation from the general relationship of Kd/Man density shown in Fig. 2. This may be interepreted as a case of "binding enhancement" such as found by Hoppe and

Table 1. Summary of binding parameters of various ligands. The ligands were tested for the inhibitory effects on Man_{43}-BSA-binding by rabbit alveolar macrophages. The data were analyzed with LIGAND, and the best-fit parameters are shown.

Ligands	No. of carbohydrate chains/protein	I_{50} (nM)	K_d (nM)	Sites/cell[a]
AO	1	—	296[b]	108 600[b]
β-casein	1	3020	1163	74 000
	2	1202	980	72 000
	3	295	253	65 900
	3-4	83	81	68 400
	2-6	17	28	75 700
AAT-PG	2	1995	1053	76 300
ST-BAO	3	40	33	80 000
Man_5-BSA	5[d]	—	820[c]	130 000[c]
Man_{13}-BSA	13[d]	—	113[d]	115 000[c]
Man_{24}-BSA	24[d]	—	13[c]	37 900[c]
Man_{43}-BSA	43[d]	—	2[c]	42 700[c]

[a] The number of sites was regarded as equal to the number of ligands bound.
[b] The best-fit parameters were determined from the direct binding data of AO [16].
[c] Taken from Hoppe and Lee [17].
[d] As monosaccharide.

Lee [13] for the same cells. Binding of iodinated Man_{43}-BSA was greatly enhanced in the presence of 0.1 M Man or L-Fuc. This was rationalized as Man monosaccharides, being small and mobile, and with relatively weak binding affinity to the receptor molecule, can cause rearrangement of the kinetically formed Man_{43}-BSA-receptor complex into a thermodynamically more favorable complex. We have established that not only Man and L-Fuc, but also small Man-containing compounds can cause enhancement of binding (Ohsumi *et al.*, unpublished results) under similar conditions. It is possible that AO, fulfilling the requirement for enhancement of binding as assumed above, enhances binding of itself.

Binding of AAT-PG fits into the relationship presented in Fig. 2 reasonably well. AAT is composed of two identical subunits of 46 kDa each, and possesses a pseudo-two fold symmetry axis, and the binding sites for the coenzyme are orientated in diametrically opposite directions (180°) [21, 22]. Therefore, the phosphopyridoxylated AO, at least near the coenzyme binding sites, should be pointing in opposite directions. When this complex (AAT-PG) was treated with α-mannosidase, only one Man could be released. Despite these apparent unfavorable expectations of the oligosaccharide chain orientation, AAT-PG behaves quite in line with most of the ligands shown in Fig. 2. It is interesting to note that AAT-PG binds to Concanavalin A quite well [4], somewhat contrasting with the results of the mannosidase reaction. It should be noted that although the dissociation constant for the AAT-PG complex has not been determined, it has been empirically determined that at a concentration of 10^{-7} M, less than 5% of the complex dissociates after 6 h incubation in the absence of pyridoxine derivatives or phosphate [4]. Thus, under the experimental conditions used here, the complex should remain intact over the major part of the concentration range studied.

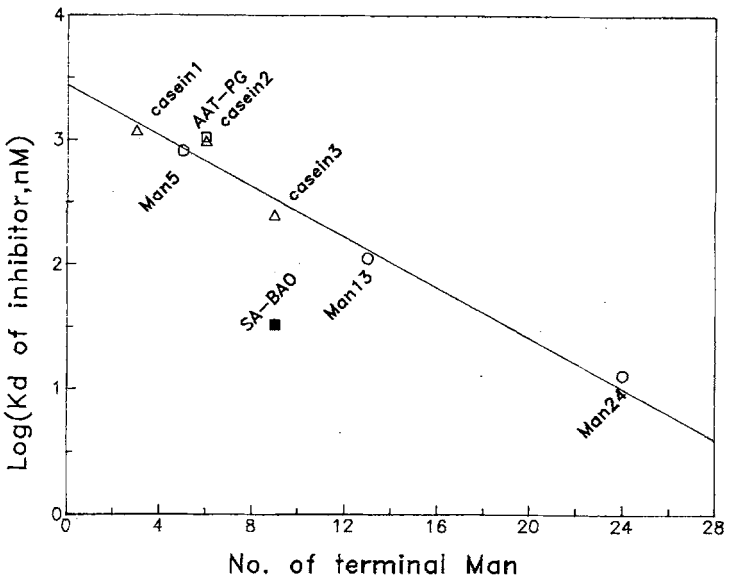

Figure 2. The plot of Log[Kd] *vs.* the number of terminal D-mannose residue of various ligands.
The results of inhibition of Man_{43}-BSA binding by β-casein derivatives (△), Man_n-BSA (○), AAT-PG (□), and SA-BAO (■) (Figs. 1 and 3) were analysed with LIGAND (Table 1). The logarithm of the best-fit Kd values was plotted against the number of terminal D-mannose residue of the inhibitors.

An exception to the relationship shown in Fig. 2 is SA-BAO. The inhibitory potency of the SA-BAO (I_{50} = 40 nM) was significantly higher than that of the β-casein derivative possessing three AO chains (I_{50} = 295 nM), as shown in Fig. 3 and Table 1. The effectiveness of the SA-BAO may lie in the tight organization of the streptavidin subunits. In the avidin-biotin complex the four identical subunits form a spheroidal structure (6 × 5.5 × 4 nm) with 222 symmetry, where the biotin binding sites are arranged in pairs on the 5.5 nm faces [23]. There is no major conformational change accompanying the binding of biotin [24]. The binding site has been estimated to be buried 0.9 nm deep below the van der Waals surface of avidin [25]. Streptavidin is remarkably similar to avidin, especially with respect to stoichiometry of biotin binding and subunit molecular weight [26, 27]. In fact, it has been shown that both the avidin-BAO and the SA-BAO complex behaved in a similar way on α-mannosidase and endo-glycosidase H treatments [5]. It is possible that the SA-BAO complex forms a spheroidal structure similar to that of the avidin-BAO complex, where two of the three oligosaccharides are arranged almost in parallel on the 55 nm face. That the SA-BAO is a more potent ligand than the casein with equivalent number of AO chains or the Man-BSA with equivalent number of mannosyl residues may be due to the juxtaposition of two of the three AO chains in SA-BAO, providing a more suitable topographical arrangement of the mannosyl residues for optimal binding.

In summary, the new types of neoglycoproteins are more efficient ligands on the basis of points of attachment on the proteins. In one of them (SA-BAO), the orientation of the

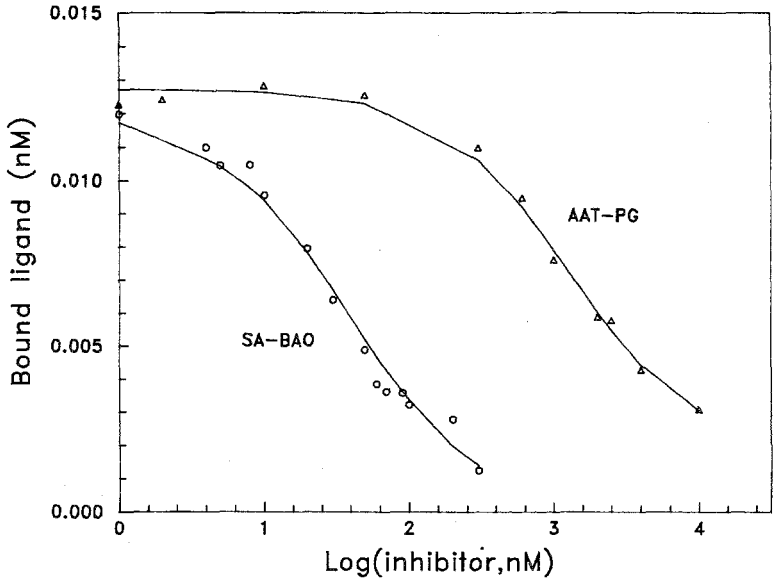

Figure 3. Inhibition of noncovalent neoglycoproteins: AAT-PG and SA-BAO.
Macrophages (10^6 cells/ml) and ^{125}I-Man_{43}-BSA (0.12 nM) were incubated in the presence of AAT-PG (△) or SA-BAO (○) at the indicated concentrations for 6 h at 2°C, and specifically bound Man_{43}-BSA was determined as described for Fig. 1. The best-fit parameters (Table 1) were determined with LIGAND.

oligosaccharide extended from the protein matrix seems to affect the receptor-ligand interaction. These new neoglycoproteins offer attractive alternatives to the previous types of neoglycoproteins, especially when naturally derived oligosaccharides are the subject of study.

Acknowledgements

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