Carbohydrate Research, 61 (1978) 159–168

LECTIN BINDING TO MOSQUITO *Aedes aegyptii* AND HUMAN KB CELLS: STRUCTURAL COMPARISONS OF MEMBRANE OLIGOSACCHARIDES

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(Received August 1st, 1977; accepted for publication September 3rd, 1977)

ABSTRACT

High-capacity adsorbents for lectins, including *Lotus tetragonolobus* L-fucose-binding protein, were readily prepared by conjugation of monosaccharides with commercially available, epoxy-activated Sepharose. Purified, radioiodinated lectins were bound to cells of the mosquito *Aedes aegyptii* and of human KB tumour. Relative to human KB cells, mosquito cells bound less of lectins specific for the sugars (L-fucose and D-galactose) that are terminal residues in many mammalian glycoproteins, whereas the number of binding sites of lectins specific for core-region sugars (D-mannose and 2-acetamido-2-deoxy-D-glucose) were similar. Neuraminidase, which greatly enhanced binding of peanut agglutinin or soybean agglutinin to human KB cells, had negligible effects on binding of these lectins to mosquito cells. The comparative structures of surface oligosaccharides of mosquito and KB cells are discussed in relation to the lectin-binding studies.

INTRODUCTION

The structures of glycoproteins and glycolipids of invertebrate cells have not been studied in any detail. However, the widespread occurrence of complex carbohydrates at cell surfaces, where they are presumed to take part in many important biological processes[1], seems likely to extend to these cells. In view of its comparative simplicity and well understood genetics, the insect embryo, particularly of Diptera species[2], is an attractive tissue for examining the biological properties of cell-surface carbohydrates and prompted the present study.

It has been known for many years that invertebrate cells do not contain gangliosides or glycoproteins having sialic acids as their covalent terminal carbohydrates[3–8]. Little is known, however, of the oligosaccharide sequences in insect glycoproteins or glycolipids for comparison with the sequences contiguous with terminal sialic acid in vertebrate-cell glycoproteins. In this paper, several lectins of well defined specificity have been used as probes to compare the surface-oligosaccha-

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

ride compositions of a cultured mosquito, *Aedes aegyptii* cell-line, and the human KB tumour cell-line.

MATERIALS AND METHODS

Materials. — Acrylamide, *N,N'*-methylenebis(acrylamide), and tetramethylenediamine were obtained from BDH Chemicals Ltd., Poole, U.K. Concanavalin A, ricin (*Ricinus communis* agglutinin of molecular weight 60,000), wheat-germ agglutinin, and the L-fucose-binding protein (from *Lotus tetragonolobus*) were obtained from Miles Laboratories, Stoke Poges, U.K. *Lens culinaris* agglutinin (Lch) was kindly provided by Dr. D. Snary of this Institute. Soybean agglutinin and peanut agglutinin were generous gifts from Dr. N. Sharon, Weizmann Institute, Rehovoth, Israel, who also gave us ample supplies of *N*-(6-aminohexanoyl)-β-D-galactopyranosylamine and *N*-(6-aminohexanoyl)-β-L-fucopyranosylamine. Epoxy-activated Sepharose 6B and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Neuraminidase from *Vibrio cholerae* (EC 3.2.1.18) was obtained from Behringwerke–Hoechst, London, U.K. as a solution (500 units/ml) in 0.05M sodium acetate buffer, pH 5.5, containing 9 mg/ml of sodium chloride and 1 mg/ml of calcium chloride. The enzyme was diluted in this buffer to 50 units/ml before use.

Human tumour KB cells were grown at 36° in monolayer culture as described earlier[9,10]. Mosquito cell-line established[11] from *Aedes aegyptii* larvae was kindly supplied by Dr. M. G. R. Varma, London School of Hygiene and Tropical Medicine, London, U.K. The cells were grown in monolayer culture in Varma–Pudney medium[11] at the optimal temperature of 28°. Cells were detached by scraping, and transferred weekly at 1:100 dilution. KB cells or *Aedes* cells were washed as monolayers in phosphate-buffered saline [sodium chloride (8.0 g), potassium chloride (0.29 g), disodium hydrogenphosphate (1.159 g), potassium dihydrogenphosphate (0.29 g), calcium chloride (0.19 g), and magnesium chloride (0.19 g) diluted to 1 litre with water, pH 7.04] before the binding experiments.

Preparation of sugar–Sepharose conjugates. — The ligand *N*-(6-aminohexanoyl)-β-D-galactopyranosylamine (100 mg) or *N*-6-aminohexanoyl-β-L-fucopyranosylamine[12] in 0.1M sodium hydrogencarbonate buffer (pH 8.3) containing 0.5M sodium chloride (10 ml) was coupled with cyanogen bromide-activated Sepharose 4B (2 g) by recommended procedures (Affinity chromatography: Principles and Methods, Pharmacia). The conjugate was finally washed extensively with phosphate-buffered saline, and packed into a column (5 ml total volume).

Coupling to Sepharose of 2-acetamido-2-deoxy-D-galactose (100 mg) or L-fucose (100 mg) was also performed[13] by adding the monosaccharide to epoxy-Sepharose 6B (2 g) suspended in 0.1M sodium hydroxide (10 ml) with vigorous shaking. The gel was then washed with 0.1M sodium hydroxide (250 ml), water (250 ml), and then alternately with 50mM Tris–HCl buffer (pH 8) containing 0.5M sodium chloride (250 ml) and 0.05M sodium formate buffer (pH 4)–0.5M sodium chloride (250 ml).

Finally, after washing in phosphate-buffered saline, the gel was packed into a column (5 ml total volume).

Affinity chromatography. — Iodinated lectin (0.1 mg) was applied in phosphate-buffered saline to the column at 4°. The column was eluted with the same buffer and fractions (1 ml) were collected at 10–12 ml/h. The bound lectin was then desorbed from the gel by a hapten (0.1M). Each fraction was counted for ^{125}I in a gamma spectrometer, and lectin-containing fractions were combined, diluted with non-radioactive lectin to a known specific activity (100 μg/ml, 1–4 × 10^6 c.p.m./mg protein), and dialysed against phosphate-buffered saline. Finally, bovine serum albumin was added to a final concentration of 50 μg/ml, and the solutions were stored at −20°.

Iodination of lectins. — Lectins were labelled with ^{125}I by lactoperoxidase-catalysed iodination in the presence of an appropriate sugar hapten of the lectin and purified by affinity chromatography. The iodination and preparation of ^{125}I-labelled ricin, concanavalin A, and *Lens culinaris* lectin by affinity chromatography on Sepharose or Sephadex and elution with 0.1M D-galactose or 0.1M methyl α-D-mannoside, respectively, have been described fully elsewhere[14,15]. Other lectins were labelled similarly and purified on conjugated sugar–Sepharose columns. Soybean agglutinin (0.1 mg) was dissolved in phosphate-buffered saline (1.0 ml) containing 5mM D-glucose, 272mM 2-acetamido-2-deoxy-D-galactose, lactoperoxidase (40 μg, 40–50 units/mg, Sigma Chemical Corp., London), D-glucose oxidase (0.4 units/ml, Type V from *Aspergillus niger*, 200 units/mg, Sigma) and 500 μCi of carrier-free [^{125}I]iodide (Radiochemical Centre, Amersham, U.K.). After incubation for 30 min at room temperature, the mixture was dialysed against saline at 4° prior to purification by affinity chromatography on Sepharose 4B–N-(6-aminohexanoyl)-β-D-galactopyranosylamine and elution with 0.1M D-galactose[12,16]. Other preparations were purified by chromatography on a conjugate of 2-acetamido-2-deoxy-D-galactose and epoxy-activated Sepharose 6B, with identical results. Peanut agglutinin[17,18] and the *Lotus* fucose-binding protein(s)[12,16] were labelled with lactoperoxidase in the same way, and purified by adsorption either onto Sepharose 4B–N-(6-aminohexanoyl)-β-D-galactopyranosylamine or onto Sepharose 4B–N-(6-aminohexanoyl)-β-L-fucopyranosylamine, respectively, and elution with 0.1M hapten sugar. L-Fucose, conjugated with epoxy-activated Sepharose, was also used successfully to purify iodinated *Lotus* lectin.

Binding assays. — KB or mosquito cells were grown to high cell-density (1–2 × 10^6 cells/dish) on Falcon, 35-mm diameter, culture dishes (Flow Laboratories, Irvine, Scotland), washed three times with phosphate-buffered saline (each 5 ml), and incubated either at 2 or 28° with increasing amounts of [^{125}I]lectins (0–100 μg) in 1 ml of phosphate-buffered saline. In control experiments, the lectin solutions contained a hapten monosaccharide at 0.06M final concentration. After incubation for 1 h (sufficient for maximal binding as shown in preliminary experiments), the unbound lectins were removed by washing the monolayers several times with phosphate-buffered saline at either 2 or 28°, as appropriate. Then cells were fixed by

washing at 2° successively with 2% phosphotungstic acid–10% perchloric acid followed by ethanol, dried at air temperature, and dissolved in 0.5M sodium hydroxide (1 ml). Portions (0.1 ml) were then assayed for protein by the Lowry procedure[33] and counted for ^{125}I in a gamma spectrometer. Duplicate cultures grown exactly as just described were trypsinized[15] and the numbers of cells recovered were counted.

Treatment with neuraminidase. — Cells growing on Falcon, 35-mm diameter dishes in monolayer cultures were incubated with neuraminidase (50 units/ml) for 45 min at 28°. The cells were then washed 3 times with phosphate-buffered saline to remove neuraminidase, and treated with [^{125}I]lectins as already described.

SDS-poly(acrylamide) gel electrophoresis. — Samples of purified ^{125}I-labelled lectins were heated for 5 min at 90° in 1% dodecyl sodium sulfate–10mM phosphate buffer (pH 7.0) with 1% 2-mercaptoethanol to ensure complete dissociation. The samples (0.1 ml) were then examined in the system described by Weber and Osborn[19]. Gels were stained with 0.1% (w/v) Coomassie Blue in methanol–acetic acid–water (40:7:53 v/v) for 4 h and destained in methanol–acetic acid–water (5:7:88 v/v). Other gels were cut into 2-mm slices before staining, and each slice was counted for ^{125}I radioactivity.

RESULTS

Purity of iodinated lectins. — The iodinated lectins used in this study were purified from reaction products by affinity chromatography on suitable support-columns and by elution with a specific sugar hapten. The recently available (Pharmacia), epoxy-activated Sepharose resin was used to prepare 2-acetamido-2-deoxy-D-galactose and L-fucose conjugates, and these proved to be very efficient adsorbents for soybean agglutinin (confirming Vretblad[13]) and for *Lotus* L-fucose-binding protein, respectively. Purification of iodinated soybean agglutinin and *Lotus* lectin on Sepharose–*N*-(6-aminohexanoyl)-β-D-galactosylamine or -β-L-fucosylamine, respectively, was also performed by established procedures[12], with similar results. The purity of each lectin was confirmed by polyacrylamide-gel electrophoresis. In each case, a single polypeptide band was detected that corresponded exactly with the peak of radioactivity. The subunit molecular weights were calculated from the migration relative to standard proteins; for soybean agglutinin (30,000), peanut agglutinin (25,000), L-fucose-binding protein (25,000), concanavalin A (27,000), and *Lens culinaris* agglutinin (20,000) the values were in excellent agreement with literature values[12,16]. The molecular weights calculated for unreduced lectins and the numbers of sugar-binding sites are shown in Table I. Ricin[20] gave two subunits (molecular weights 27,000 and 32,000), the larger of which was iodinated slightly more efficiently than the smaller; the unreduced protein had a calculated molecular weight of 60,000 (Table I).

Numbers of binding sites. — The results of several experiments using mosquito *(Aedes aegyptii)* cells and human tumour KB cells are collected in Figs. 1–4. The binding to cells was drastically decreased, to less than 10%, by an appropriate hapten

TABLE I

BINDING OF LECTINS TO CELLS[a]

Lectin[b]	*Mol. wt.*	*Saccharide*			*Lectin-binding sites* $\times 10^{-6}$*/cell*	
		Binding sites/mol	*Specificity*	*Temp.* (°)	*Mosquito cells*	*KB cells*
Concanavalin A	102,000	4	D-Mannose	2	22	48
				28	23	79
Lens culinaris	60,000	2	D-Mannose	2	28	65
				28	38	114
Ricin	60,000	1	D-Galactose, 2-acetamido-2-deoxy-D-galactose	2	13	162
				28	16	224
Peanut	110,000	ND	D-Galactose	2	< 1	2
				28	< 1	2
				28[c]	6	50
Soybean	120,000	2	D-Galactose, 2-acetamido-2-deoxy-D-galactose	28	20	<1
				28[c]	20	40
Lotus	120,000	4	L-Fucose	2	3	37
				28	3	44

[a]The binding curves shown in Fig. 1–4 were analysed by Scatchard's method (insets, Figs. 1–4) to compute the numbers of binding sites/cell. [b]See ref. 12, 16. [c]After neuraminidase.

sugar, for example, D-galactose in the case of ricin, soybean agglutinin, or peanut agglutinin, L-fucose in the case of the *Lotus* lectin, and methyl α-D-mannoside for concanavalin A or *Lens culinaris* lectin. We have not systematically examined the effects on lectin binding to the cells of such variables as pH or ionic conditions. However, under strictly comparable conditions of pH, ionic conditions and temperature, several lectins show simple saturation-binding to both cell types. The binding data, when corrected for non-specific binding in the presence of hapten sugar and plotted in the Scatchard relation[21,22], yield the total number of binding sites per cell (Table I). In most instances, the plots are linear, indicating that binding takes place bimolecularly between each lectin molecule and a single receptor. However, deviation from a simple, linear Scatchard-plot was found in several cases (Figs. 1–4 insets).

Binding of concanavalin A to mosquito cells showed positive cooperativity. This conclusion could be deduced most clearly (Fig. 3c) from the shape of the Scatchard plot of the binding data and is commented upon further in the Discussion. In contrast, binding of concanavalin A to KB cells converted to a linear Scatchard plot (Fig. 3d). In several instances curvilinear plots, concave upwards, were obtained. Discontinuities of this kind in the Scatchard plots were obtained from the binding data of *Lotus* lectin to KB cells (Fig. 4b), ricin to KB cells (Fig. 4d), and *Lens culinaris* lectin to both cell types (Fig. 3a and b). This deviation may be due to a variety of

causes, the most probable being the presence of multiple classes of binding sites that have different affinities for the lectins, although alternative interpretations based on negative cooperativity of binding have been proposed recently[31,32]. Table I gives the total numbers of binding sites, assuming the former interpretation.

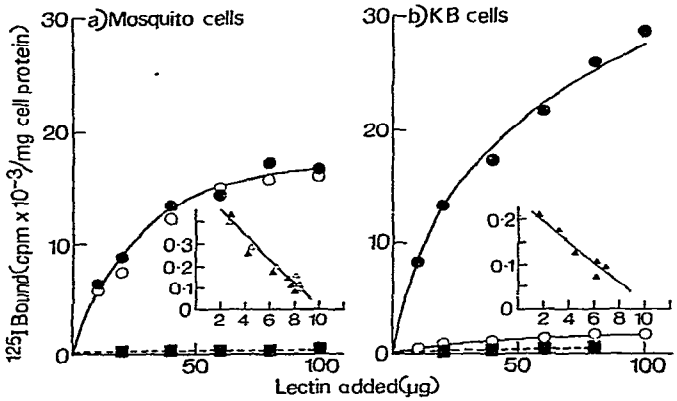

Fig. 1. Binding of ^{125}I-soybean agglutinin to mosquito cells (*a*), and KB cells (*b*), and the effect of neuraminidase. Increasing amounts of radio-iodinated and affinity chromatography-purified lectin were added to cells, or cells treated previously with neuraminidase as described in the Methods section. Controls contained neuraminidase-treated cells incubated under identical conditions with lectin in the presence of 20mM 2-acetamido-2-deoxy-D-galactose. After incubation for 60 min at 28°, the proportion of lectin bound to the washed cells was determined as described in the Methods section. *Inset*: Scatchard plots of binding data; abscissa, μg of lectin bound; ordinate, bound/free. Before neuraminidase, ○ △; after neuraminidase, ● ▲; after neuraminidase plus 2-acetamido-2-deoxy-D-galactose, ■.

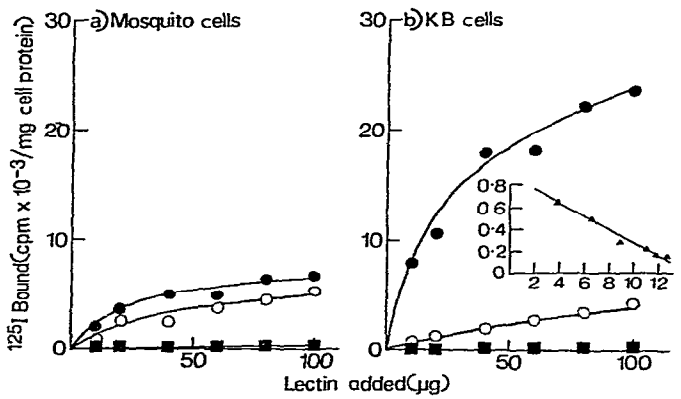

Fig. 2. Binding of ^{125}I-peanut agglutinin (*a*) to mosquito cells and (*b*) to KB cells before and after treatment with neuraminidase. See legend to Fig. 1 for details. Before neuraminidase, ○; after neuraminidase, ● ▲; neuraminidase-treated cells plus D-galactose, ■.

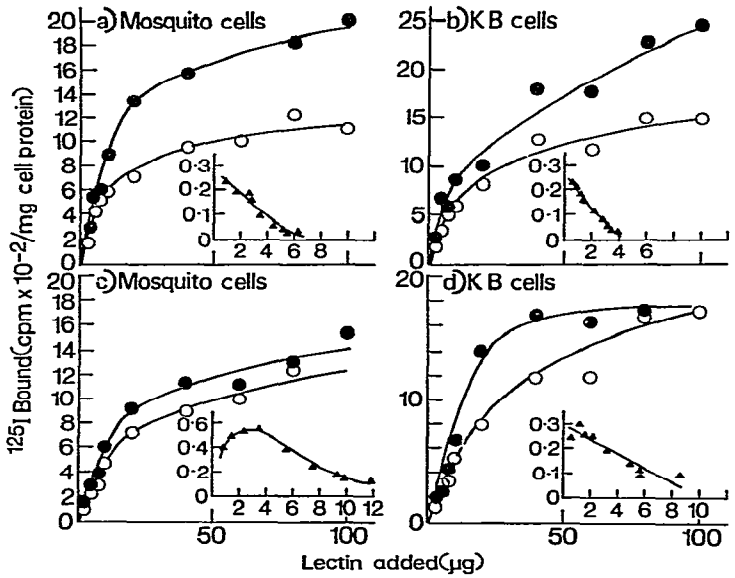

Fig. 3. Binding of ^{125}I-*Lens culinaris* agglutinin (*a* and *b*) or concanavalin A (*c* and *d*) to mosquito cells and KB cells at 2° (open circles) or 28° (closed circles). Inset: ▲, Scatchard plots of data obtained at 2°. See Fig. 1 for details.

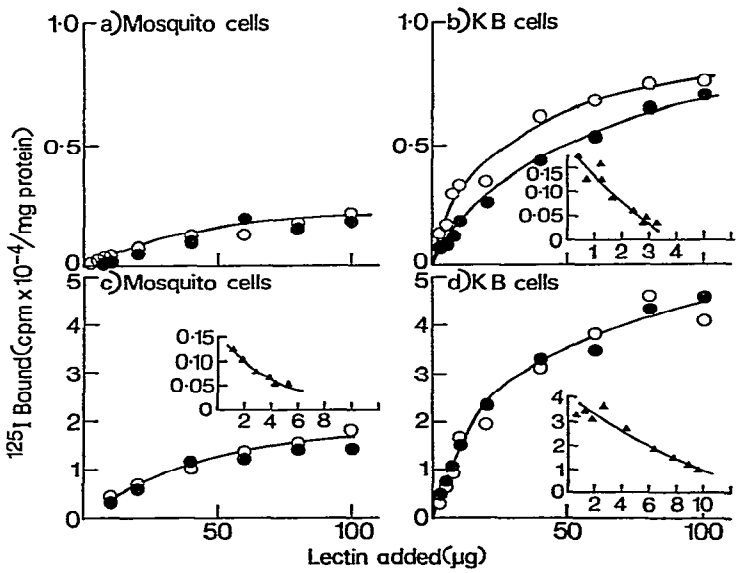

Fig. 4. Binding of L-fucose-binding protein from *Lotus tetragonolobus* (*a* and *b*) or ricin (*c* and *d*) to mosquito cells or KB cells at 2° (open circles) or 28° (closed circles). Inset: ▲, Scatchard plots of data obtained at 2°. See Fig. 1 for details.

DISCUSSION

Our results (Table I) show that *A. aegyptii* cells resemble human KB cells in the variety and number of lectin-binding sites, indicating similarities in their surface-carbohydrate constitutions. Several interesting differences are apparent, however. In the first place, the number of surface-carbohydrate receptors for ricin is greatly decreased in mosquito cells relative to KB cells. The decrease (90%) is very similar to that obtained in certain lines of kidney cells of baby hamster, selected for resistance to ricin toxicity[14,15]. Unlike these latter BHK cell-lines, the mosquito cells, however, are sensitive to the toxin (T. D. Butters and R. C. Hughes, unpublished results) and presumably retain a sufficient number of ricin receptors to mediate the toxicity of ricin. The decreased number of ricin-binding sites on mosquito cells indicates, however, a lower content of terminal D-galactose and/or 2-acetamido-2-deoxy-D-galactose residues in surface oligosaccharides. Ricin binds well to such desialylated glycoproteins as α_1-acid glycoprotein[23,25], which has 2-acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl end-groups, or to desialoylated fetuin, which contains the same terminal sequence and in addition 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-β-D-galactopyranosyl end-groups.

The latter sequence is the most specific binding site for peanut agglutinin, which is often considered a diagnostic reagent for this sequence[12,16–18]. However, the lectin is also inhibited by other β-D-galactosides and weakly by α-D-galactosides. The high binding of peanut agglutinin to human KB cells after treatment with neuraminidase suggests that glycoproteins containing one or more of these terminal sequences is present and substituted by sialic acid. Glycophorin, the major glycoprotein of human erythrocytes, also binds peanut agglutinin avidly, but only after treatment with neuraminidase[18]. Binding is to the 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-galactose sequence linked in *O*-glycosidic linkage to serine or threonine residues of the polypeptide moiety. Although treatment with neuraminidase enhanced peanut agglutinin binding to KB cells by approximately 25 fold (indicating that oligosaccharide chains similar to these are present), it is notable that a considerable number binding sites is available on KB cells before removal of terminal sialic acid. The nature of these sites is unknown and, of course, may be unrelated to the sites revealed by treatment with neuraminidase.

The small but definite level of binding of peanut agglutinin to mosquito cells (Table I) is virtually unchanged by treatment with neuraminidase. Therefore, the enhancement of binding to KB (Fig. 2) and other cells[12,16–18] by treatment with this enzyme is probably due to removal of sialic acid residues and exposure of receptors, rather than to such trivial reasons as exposure of non-specific binding sites or binding of lectin to surface-adsorbed neuraminidase.

Neuraminidase also affects greatly the number of binding sites for soybean agglutinin on KB cells, but has no effect on the binding of this lectin to mosquito cells. It is established[26,27] that soybean agglutinin is specific for terminal α- or β-linked 2-acetamido-2-deoxy-D-galactose or D-galactose residues; substances that have

subterminal D-galactose or 2-acetamido-2-deoxy-D-galactose residues are very weak hapten-inhibitors. The effect of neuraminidase on binding of soybean agglutinin to KB cells is presumably to expose binding sites by removal of sialic acid residues, as in the enhancement of peanut-agglutinin binding by this enzyme. Interestingly, the numbers of binding sites for peanut and soybean agglutinins revealed by treatment of KB cells with neuraminidase are quantitatively similar (Table I), suggesting that the lectins are binding to similar oligosaccharide sequences. Presumably, ricin also binds to these sequences, but there are additional sequences present in these cells that are suitable for binding of ricin, a lectin of less-restricted specificity. It may similarly be suggested that the soybean agglutinin and ricin share binding sites on mosquito cells, as these are quantitatively very similar.

The lack of an effect of neuraminidase on binding of peanut agglutinin or soybean agglutinin to *Aedes* is understandable in view of the absence of sialic acid from these cells[3-6]. L-Fucose, another sugar commonly occupying terminal sites of mammalian glycoproteins, also appears to be a very minor component of these cells, as shown by the binding of the *Lotus* lectin, which amounts to less than 10% of the binding of this lectin to KB cells (Table I). However, some caution is required, as other glycoproteins rich in α-L-fucopyranosyl end-groups also bind poorly the *Lotus* lectin[34].

By contrast to the results discussed thus far, mosquito and KB cells exhibit similar binding of two lectins (concanavalin A and *Lens culinaris* agglutinin) that interact readily with internal sugar residues[16], particularly α-D-mannosyl residues, of mammalian glycoproteins (Table I). Of course, we do not infer from these data that identical, D-mannose-containing oligosaccharide-sequences are binding either of these lectins to the mosquito and KB cells, respectively. Indeed, our chemical evidence suggests that the distribution of D-mannose (and therefore concanavalin A and *Lens culinaris* lectin)-binding sites in mosquito cells is very different from that in KB cells, the mosquito cells being very rich in D-mannose-containing glycolipids (T. D. Butters and R. C. Hughes, unpublished). Nevertheless, the lectin probes do suggest the presence in mosquito cells of α-D-mannose sequences that may be terminal or, alternatively, be situated internally and substituted with terminal D-galactose or 2-acetamido-2-deoxy-D-galactose residues. Unlike KB cells, however, these oligosaccharide sequences of mosquito cells are not substituted further with sialic acid residues.

In addition to these simple correlations of lectin binding and surface-oligosaccharide structure, the results summarized in Table I and Figs. 1–4 point to other differences in membrane structure between the mosquito and human cells. The positive cooperativity exhibited by low concentrations of concanavalin A with mosquito cells but not with KB cells has also been found in thymocyte membranes[28] and whole thymocytes[29,30]. Cooperativity may be the result of interactions between receptor molecules, where the binding of receptors and lectins modulates the binding affinity of the receptors for the binding of additional lectin molecules. Using such multivalent lectins as concanavalin A, polymerization or clustering of receptors in a

fluid membrane has been suggested as one possible explanation of these cooperative effects[30]. Certainly, the valency of concanavalin A plays a role, as divalent succinyl-concanavalin A does not show positive cooperativity[30]. However, it is interesting to speculate that the positive cooperativity of binding by mosquito cells of tetravalent concanavalin A reveals a higher degree of cross-linking of receptors, and therefore a higher mobility, of these cell-membrane receptors than the corresponding receptors of the human tumour KB cell.

In conclusion, we point out the value of highly purified lectins as probes for distinguishing between the cell-surface carbohydrate structure of two cell types as a preliminary to purification and further characterization of these components.

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