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Binding of Plant Lectins to Mycoplasma Cells and Membranes

ITZHAK KAHANE* AND JOSEPH G. TULLY

Biomembrane Research Laboratory, Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel, and Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014*

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The binding of iodinated wheat germ agglutinin, *Ricinus communis* agglutinin, and concanavalin A to mycoplasma cells and membranes was examined. All mycoplasmas studied specifically bound concanavalin A or *R. communis* agglutinin and, to a lesser degree, wheat germ agglutinin. The binding of lectins to whole cells was similar to that recorded for membranes, suggesting that significant binding only occurred on the outer surface of the mycoplasma membrane. Proteolysis of the membrane almost always increased the capacity to bind lectins, which indicates that additional carbohydrate groups on the mycoplasma membrane are masked by a protein layer or protein complexes on the membrane. The observation that carbohydrates are apparently exposed on the surface of mycoplasma membranes should stimulate more concentrated study on the isolation and chemical characterization of these substances since it is quite likely that they are responsible for a variety of reactions between mycoplasmas and host cells.

A variety of carbohydrate complexes (e.g., glycolipids, glycoproteins, and polysaccharides) are found in the mycoplasmas, but they comprise only a minor fraction of the total membrane components (20, 26). This fact has made the study of these complexes difficult and, in fact, only a few of them have been characterized (8, 9, 19, 27). Nevertheless, even the scanty information available points to the importance of the carbohydrate residues of these complexes. Thus, glycolipids were reported to be involved in the immunogeneity of *Mycoplasma pneumoniae* cells (3, 5, 19, 21), and Goel and Lemcke (9) purified from *M. gallisepticum* a glycoprotein that is involved in hemagglutination caused by this organism. Only further investigation can reveal how many of the other mycoplasmas that absorb to erythrocytes and other cells (10, 20) do involve a similar glycoprotein. Both these glycolipids and the glycoprotein had carbohydrate residues exposed on the outer surface of the membrane. In view of the possible importance of the exposed carbohydrate residues, it seemed of great interest to test how common this phenomenon is in mycoplasmas. Because of the small amounts of carbohydrates in mycoplasma membranes, we decided to use indirect means of determining their presence and location. Plant lectins with known sugar specificity to detect exposed carbohydrate residues seemed suitable for this purpose, especially since they have been used in similar studies on membranes of other organisms (11, 15, 16, 18). Recently, Schiefer et al. (24) reported that agglutination was induced by lectins in several mycoplasmas. However, it is well established that the binding of lectins to cells via their carbohydrate residues does not necessarily result in agglutination (18). Therefore, binding of concanavalin A (ConA), *Ricinus communis* agglutinin (RCA), wheat germ agglutinin (WGA), and lima bean agglutinin (LBA), which are specific for carbohydrates commonly found in mycoplasmas, was studied. The experiments were aimed at answering two main questions. (i) Do mycoplasmas have carbohydrates exposed on the surface of the membrane? (ii) Are these carbohydrates exposed on both surfaces of the membranes, or is there asymmetry in their distribution?

(A preliminary report of this study was presented at the International Congress on Mycoplasmas of Man, Animals, Plants and Insects, Bordeaux, France, 1974, and at the 1974 Meeting of the Israeli Microbiological Association [Israel J. Med. Sci. 11:499, 1975].)

MATERIALS AND METHODS

Organisms and growth conditions. The organisms (see Table 1) were grown in standard mycoplasma media containing either 20% horse serum or 1% bovine serum fraction, and appropriate supple-

ments of L-arginine or glucose (20, 22). The cultures were incubated aerobically at 37°C for 18 to 48 h, and the cells were harvested at optimal turbidity but before extreme changes in pH had occurred.

Cells and membrane preparations. The cells were harvested by centrifugation (10,000 × *g* for 20 min at 4°C) and under the same conditions were washed three times in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.2) containing 0.2 M NaCl. Membranes were derived from the organisms by osmotic shock and were washed as described previously (14). *M. pneumoniae* and *M. gallisepticum* cells were loaded with 2 M glycerol prior to osmotic shock (14).

Proteolytic digestion of the membranes. Samples (0.2 ml) of membrane suspension (2 mg of protein per ml) in 0.05 M Tris-hydrochloride (pH 8.0) were incubated at 37°C with 10 μg of L-1-tosylamido-2-phenylethylchloromethyl-1-ketone-treated trypsin (Worthington Biochemicals Corp.) or Pronase grade A (Calbiochem). For the Pronase digestion, 0.005 M $MgCl_2$ was included in the buffer. After 15 min, the trypsin was inactivated with trypsin inhibitor (16 μg), and the Pronase was inactivated by the addition of 0.005 M sodium ethylenediaminetetraacetate (pH 7.2). The membrane residues were washed three times in 0.05 M Tris-hydrochloride (pH 7.2).

Plant lectins and their iodination. The plant lectins were purified by affinity chromatography, utilizing techniques described earlier for the isolation of WGA (13), LBA (7), RCA (30), and ConA (1). All lectins were analyzed for purity and sugar specificity. They were labeled with ^{125}I by the chloramine T reaction (29) and repurified by the affinity chromatography systems mentioned above.

Binding of lectins to membranes or cells. Mycoplasma membrane suspensions (400 μg of protein in 0.2 ml of 0.05 M Tris-hydrochloride, pH 7.2) were incubated at 37°C for 5 min. ^{125}I-labeled lectin (20 μg of protein in 0.2 ml of the same buffer) was added, and the reaction continued for an additional 15 min, at which time it was terminated with cold buffer. The membranes were washed three times by centrifugation (15,000 × *g* for 15 min at 4°C), and the amount of bound lectin was determined. The specificity of the binding was estimated by displacing the membrane-bound lectin by the specific sugar. Thus, the membranes were immersed for 15 min at 37°C in 0.05 M Tris-hydrochloride buffer (pH 7.2; 2 ml) containing 0.1 M of the specific sugar (*N*-acetyl-D-glucosamine, D-galactose, or α-methyl-D-mannoside for WGA, RCA, and ConA, respectively). Nonspecific displacement by the sugars was estimated by the same procedure but with D-galactose, α-methyl-D-mannoside, and *N*-acetyl-D-glucosamine for WGA, RCA, and ConA, respectively. The binding of the lectins to cell suspensions followed the same protocol as that for membrane suspensions, except that 600 μg of cell protein was used and the buffer contained 0.15 M NaCl.

Analytical procedures. Protein was determined by the method of Lowry et al. (17). Membrane lipids were extracted with chloroform-methanol (2:1, vol/vol) as described previously (14). Carbohydrates were estimated by gas-liquid chromatography, using a 3% SE 30 column and following the procedure of Clamp (6).

RESULTS

Binding of lectins to membranes. The varying relationship between the binding of lectins and increasing amounts of membranes of *Acholeplasma laidlawii* and *M. pneumoniae* is presented in Fig. 1. There was a leveling off in lectin binding when membrane quantities exceeded 1 mg of protein per ml. The binding of WGA to small amounts of *M. pneumoniae* membranes was an exception to these observations. LBA and WGA, lectins specific for *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine, respectively, bound to *A. laidlawii* membranes at a molar ratio of ~2:1. These sugars have been identified at a molar ratio of 3:1 in the polymer comprising most of the hexosamine in these membranes (8). Thus, it is suggested that in *A. laidlawii* LBA and WGA are primarily bound to this polymer.

The binding of lectins to other mycoplasma membranes is presented in Table 1. All membranes bound some lectins, and the amount of bound ConA and RCA always exceeded that of WGA. The binding seemed specific, since significant amounts of the bound lectin were displaced by the specific sugar (Table 1), whereas only 8 to 10% of the bound lectins were displaced by nonspecific sugars.

The binding of lectins varied in different batches of membranes (cf. Tables 1 and 3), possibly indicating differences in exposed sites or variations in total carbohydrate content. It is therefore difficult to determine whether variations in the amount of lectin bound to two strains of one *Mycoplasma* species (Table 1) are significant.

Carbohydrates in *M. hominis* membranes. The fact that the binding of lectins to *M. hominis* membranes resembled their binding to most other mycoplasma membranes tested (Table 1) was somewhat unexpected. Although glycolipids are the predominant carbohydrate-containing substances in most other mycoplasmas, previous studies (23) on this species indicated the absence of glycolipids. This suggested two possibilities: the occurrence of nonspecific binding or the presence of other carbohydrate-bearing substances in the membrane. The latter possibility is supported by the data obtained here indicating that *M. hominis* has an abundance of carbohydrates with a specificity for the binding of WGA, RCA, and ConA lectins (Table 2). The major part of the carbohydrates was in the lipid-extracted membrane residue (Table

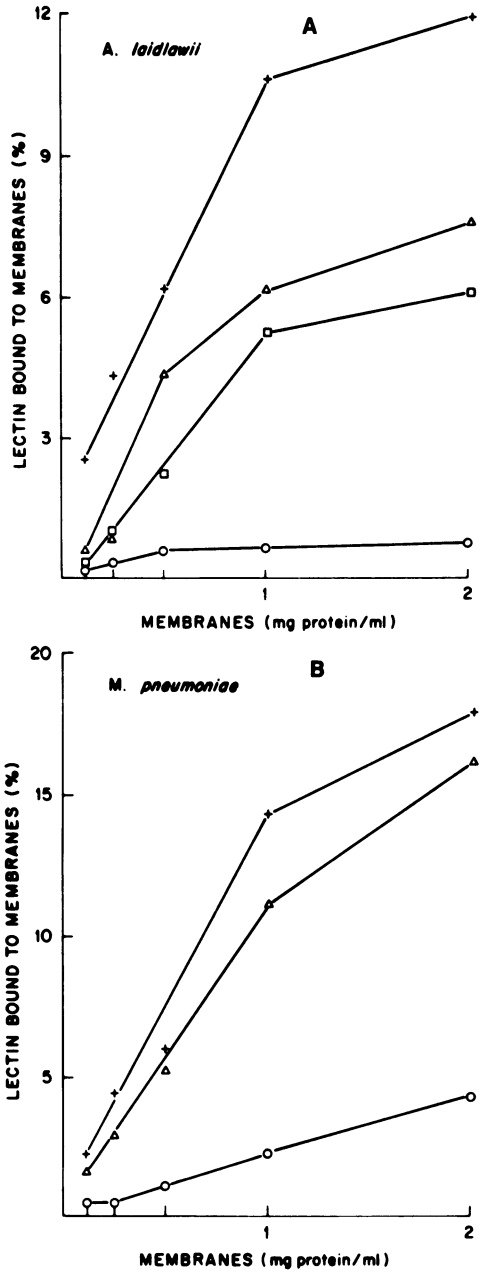

FIG. 1. *Binding of lectins to mycoplasma membranes. (A) A. laidlawii; (B) M. pneumoniae. Membrane suspensions were incubated with ^{125}I-labeled lectins for 15 min at 37°C. The membranes were washed, and the amount of bound lectin was determined. Symbols: ○, WGA; □, LBA; △, ConA; +, RCA.*

2). The nature of the substances containing the carbohydrates is still to be elucidated.

Binding of lectins to cells and their derived membranes. A comparison of the binding of lectins to whole mycoplasma cells and to membranes was carried out to provide some information on the possible location of exposed binding sites. These experiments were based on the assumption that when incubated with intact cells the lectins have access only to carbohydrates on the outer surface of the membrane, whereas when incubated with isolated membranes, which are nonsealed vesicles, the lectins can approach both the outer and cytoplasmic membrane surfaces.

When binding was calculated per milligram of membrane protein, it was found that in a given species the amounts of lectin bound to cells and to membrane preparations were similar (Table 3). These results indicate that only the exterior membrane surface has sites that are capable of binding lectins and, therefore, probably contains the carbohydrate residues. The question as to whether the inside surface of membrane is able to bind lectins remains unanswered since we have no evidence that the lectins reached this surface. However, a study of the location of mycoplasma membrane proteins, by similar techniques, indicated that labeled compounds were able to reach inner membrane sites (2, 13).

Binding of lectins to protease-digested membranes. Some insight into the chemical properties of the binding sites was provided by protease digestion of the membranes (Table 4). Trypsin, as expected, was a milder protease than Pronase. Both trypsin and Pronase treatment resulted in a marked increase in lectin binding in most of the tested membranes, indicating that many binding sites on the native membranes were masked by peptides. In only a few instances did proteolytic treatment lower the binding capacity, either by digestion of glycopeptides, release of polysaccharides or lipids, or changes in the orientation of nondigested components.

DISCUSSION

Carbohydrates are only minor components of mycoplasma membranes (20) and may be distributed among four groups of substances: glycolipids (26), polysaccharides (8), lipopolysaccharides (27, 28), and glycoproteins (9, 13). For studying the topology of the carbohydrate-including substances of the membrane, lectins were used and found to be sensitive tools (4, 15, 16, 18). Recently, Schiefer et al. (24) extended such studies to mycoplasmas. His group tested crude lectin preparations for their ability to agglutinate several mycoplasmas. The preparation from *Canavalia ensiformis* (crude ConA), which is specific for D-mannose, D-glucose, and

TABLE 1. *Binding of ^{125}I-labeled lectins to mycoplasma membranes*

Organism	Strain	Lectin bound to membrane (% of total)[a]		
		WGA	RCA	ConA
M. neurolyticum	Type A	1.1 (ND)[b,c]	3.3 (86)	5.9 (85)
M. bovigenitalium	PG11	1.3 (ND)	19.6 (88)	15.3 (80)
M. spumans	PG13	2.6 (ND)	6.4 (88)	15.3 (82)
M. spumans	MH2939	3.4 (85)	8.9 (75)	25.5 (74)
M. canis	PG14	1.2 (ND)	7.4 (84)	6.0 (85)
M. gallinarum	PG16	0.9 (ND)	5.3 (70)	9.4 (34)
M. hominis	PG21	0.5 (ND)	7.2 (49)	9.8 (69)
M. gallisepticum	PG31	1.9 (ND)	5.2 (42)	6.7 (53)
M. gallisepticum	S6	1.3 (99)	5.1 (55)	4.4 (64)
Mycoplasma sp.	Iowa 695	0.9 (92)	6.6 (36)	10.1 (64)
M. meleagridis	17529	1.9 (75)	12.3 (35)	9.9 (25)
M. pneumoniae	FH	4.4 (74)	18.1 (71)	16.2 (67)
M. pulmonis	PG34	1.7 (100)	9.7 (67)	8.6 (37)
M. mycoides subsp. *mycoides*	UM30847	1.6 (90)	7.7 (67)	16.3 (51)
A. laidlawii	PG8	0.9 (61)	12.0 (61)	7.8 (33)
A. axanthum	S743	1.2 (74)	17.1 (71)	8.1 (67)
A. granularum	BTS39	3.8 (90)	22.1 (73)	16.1 (34)

[a] The binding of 20 μg of lectins to 400 μg of membrane protein was performed as described in the text.

[b] The specificity of the binding was estimated by displacing the membrane-bound lectin by the specific sugar (*N*-acetyl-D-glucosamine, D-galactose, or α-methyl-D-mannoside for WGA, RCA, and ConA, respectively). The numbers in parentheses are the percentages of displacement.

[c] ND, Not done.

TABLE 2. *Carbohydrate composition of M. hominis membranes*

Determination	Carbohydrate (nmol/mg of membrane protein)					
	Fucose (?)	Mannose	Galactose	Glucose	Glucosamine	Galactosamine
Native membranes	8.7	9.8	13.0	70.8	11.8	0
Lipid-extracted membranes	8.0	10.5	12.2	61.1	13.1	0
Lipid extract	0	TR[a]	TR	5.0	0	0

[a] TR, Trace.

TABLE 3. *Binding of lectins to mycoplasma cells and their derived membranes*

Organism	Strain	Prepn	% Lectin bound		
			WGA	RCA	ConA
M. hominis	PG21	Cells[a]	2.9	9.4	18.5
		Membranes[b]	2.6	9.3	19.1
M. gallisepticum	S6	Cells	3.3	12.1	7.9
		Membranes	4.5	9.6	7.8
M. mycoides subsp. *mycoides*	UM30847	Cells	1.7	6.0	9.5
		Membranes	1.8	6.5	10.0
A. laidlawii	PG8	Cells	0.6	13.9	10.7
		Membranes	0.6	13.6	11.7

[a] For mycoplasma cells, 600 μg of protein was used to bind the lectins; for *A. laidlawii* cells, 1 mg of protein was used.

[b] For mycoplasma membranes, 400 μg of protein was used to bind the lectins.

TABLE 4. *Changes in binding sites for lectins after partial proteolysis of mycoplasma membranes*

Organism	Strain	Protease treatment		Lectin bound to membrane residue (% of control)[a]		
		Enzyme	% Protein digested	WGA	RCA	ConA
M. neurolyticum	Type A	Trypsin	41	86	208	158
		Pronase	50	68	307	186
M. pneumoniae	FH	Trypsin	13	160	135	137
		Pronase	48	145	112	114
M. spumans	PG13	Trypsin	34	ND[b]	244	235
		Pronase	51	ND	122	139
M. gallisepticum	PG31	Trypsin	23	160	138	164
		Pronase	53	202	154	183
M. gallisepticum	S6	Trypsin	19	120	152	127
		Pronase	56	51	156	151
A. laidlawii	PG8	Trypsin	9	ND	143	168
		Pronase	55	ND	142	172

[a] The results are expressed as percentages of lectin bound to digested membranes when compared with control membranes, which were not digested but washed in the same manner.
[b] ND, Not done.

related disaccharides, did agglutinate 7 of 10 species. Second best was the preparation of *R. communis*, specific for D-galactose, lactose, and L-arabinose. Schiefer et al. (24), using also proteolytic treatment, concluded that the glycolipids are the major lectin-binding substance. Interestingly, *A. laidlawii* cells, whose membranes include about 40% glycolipids as glucose and galactose derivatives, did not agglutinate even after proteolysis. More recently, Schiefer's group demonstrated, by electron microscopy, the binding of ConA to *M. mycoides* subsp. *capri* (25).

On the whole, the agglutination studies indicated that the majority of mycoplasmas tested had carbohydrates exposed on the cell surface. However, this technique has one major drawback, namely, that there is no direct correlation between binding of the lectin and agglutination, since, although binding always precedes agglutination, the latter does not invariably occur even if binding takes place (18). Thus, it seemed more valuable to estimate the binding of lectins to exposed carbohydrates. Using this approach, we showed that in all mycoplasmas studied to date a significant amount of binding sites for the tested lectins occur on the membranes (Table 1). These sites appear to be located primarily on the exterior surface of the membrane (Table 3).

Throughout our experiments, the binding of lectins proved to be a sensitive indicator for carbohydrates exposed on the surface of membranes. However, it must be borne in mind that there are several limitations to this approach. The problem of specific binding is illustrated by the fact that the specific carbohydrates, even at high concentrations (0.1 M), do not always detach all of the bound lectin (Table 1). There is also the question of nonspecific binding, as evidenced by detachment of lectin by nonspecific carbohydrates (in our experiments, this accounted for about 8% of the bound lectins). A second factor to be considered is that the bound lectins might perturb the membranes and thus cause changes in membrane architecture and properties (11). Avoidance of this problem necessitates the use of moderate amounts of lectins, and this dictated the amount of lectin used throughout our experiments (Table 1). A third factor is the tendency of lectins to aggregate (15), thus complicating the calculation of the number of lectin molecules in the system. For this reason, our results are expressed as percentages of the lectin that did bind rather than as the number of lectin molecules per membrane.

Despite these limitations, lectin binding was more sensitive than the agglutination test (24). Thus, no agglutination of *M. hominis* and *A. laidlawii* cells was reported to occur with crude ConA and RCA (24), although the purified lectins did bind to these cell membranes (Table 1). Moreover, agglutination tests are complicated further by the tendency of many mycoplasmas to grow in clumps. Even the membranes of

many mycoplasmas tend to agglutinate upon centrifugation. This hampered our attempts to evaluate the increase in agglutinability of the membranes, especially after proteolytic treatment. On the other hand, the lectins we employed were tested by Schiefer and found to agglutinate mycoplasma membranes and cells (H.-G. Schiefer and I. Kahane, unpublished data).

Some information on the chemical nature of the different binding sites was revealed by the binding of lectins to membranes after proteolytic digestion (Table 4). The outcome of these experiments depends on the resistance of the lectin-binding sites to proteolysis. Thus, if the sites are part of a digestable glycoprotein, there will be a decrease in binding. On the other hand, should a protein mask glycolipids or polysaccharides, its removal will expose new sites, resulting in an increase in binding. However, if both processes do occur at the same time, the results will be more complex, since the binding will be a net result that will not allow an evaluation of the separate processes. An even more complex situation might result if a polysaccharide loosely bound to protein is released from the membrane after proteolysis. The observed decrease could be interpreted as loss of glycoprotein. Considering this and the results in Table 4, it can be suggested that glycolipids or other protease-resistant sites were unmasked in the majority of membranes. Only in a few mycoplasmas did a decrease in binding occur, which suggests digestion of glycoproteins, removal of binding sites attached to glycoproteins, or even rearrangement of the membrane constituents after digestion. The exact answer will be revealed only when the nature of the different carbohydrate-containing substances is more completely studied.

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