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Wheat Germ Agglutinin Blockage of Chlamydial Attachment Sites: Antagonism by *N*-Acetyl-D-Glucosamine

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Addition of 2 to 10 μg of wheat germ agglutinin (WGA), a lectin from *Triticum vulgaris* specific for *N*-acetyl-D-glucosamine, per ml to suspensions of mouse fibroblasts (L cells) blocked the attachment of ^{14}C-labeled *Chlamydia psittaci* 6BC to the L-cell surface. WGA and strain 6BC competed for similar sites on L cells, but once bound, one was not replaced by the other. *N*-Acetyl-D-glucosamine, but not other monosaccharides of related structure, antagonized the blocking action of WGA. Lectins with specificities other than that of WGA prevented chlamydial attachment only at much higher concentrations or not at all. Exposure of L cells to trypsin and to high multiplicities of strain 6BC decreased the amount of subsequently added ^{3}H-labeled WGA that was bound by these cells. WGA also blocked the attachment of strain 6BC to other established cell lines of murine, simian, and human origin. A lymphogranuloma venereum strain (440L) of *C. trachomatis* was just as sensitive to the blocking action of WGA as was strain 6BC. It appears that the attachment of both *C. psittaci* and *C. trachomatis* to host cells of diverse origin involves an *N*-acetyl-D-glucosamine-containing entity that binds WGA with high affinity.

The 6BC strain of *Chlamydia psittaci* and the lymphogranuloma venereum 440L strain of *C. trachomatis* are phagocytized by L and HeLa 229 cells much more efficiently than are polystyrene latex spheres and *Escherichia coli* K-12 (4, 6). This disproportionately efficient uptake of chlamydiae is abolished by incubation of host cells with trypsin and other proteases, and regeneration of the protease-sensitive component of the cell surface receptor for chlamydiae is inhibited by cycloheximide (4, 6). These observations led Byrne (4) to suggest that this component might be protein or glycoprotein in nature.

Carbohydrates are being increasingly identified as part of the cell surface receptors for a variety of microorganisms (15), and lectins, which are proteins that bind specifically to sugar moieties (14), have been useful tools in these investigations. Therefore, the role of carbohydrates on host cell surfaces in the interaction between chlamydiae and host cells that leads to attachment and ingestion of these parasites was studied by measuring the ability of lectins to block this interaction.

MATERIALS AND METHODS

Growth of L cells. The 5b clone (19) of mouse fibroblasts (L cells) was grown by the method of Hatch (10) in medium 199 containing 0.1% sodium bicarbonate (5% CO_2-95% air), 200 μg of streptomycin sulfate per ml, and heat-inactivated fetal calf serum (5% for suspensions; 10% for monolayers). L cells were counted in a Coulter Counter (model Zb; Coulter Electronics, Inc., Hialeah, Fla.). Cell viability was routinely estimated by the trypan blue exclusion test (12). On some occasions, the ability of single L cells to form colonies on a plastic substrate was also determined (12). Periodic cultures for mycoplasma were negative (Flow Laboratories, Inc., Rockville, Md.).

Growth, purification, and titration of chlamydiae. The 6BC strain of *C. psittaci* and the lymphogranuloma venereum 440L strain of *C. trachomatis* were grown in and harvested from L cells by the procedure of Hatch (10). Chlamydial infectivity was assayed in terms of the 50% infectious dose (ID_{50}) for L cells (10).

The two chlamydial strains were isotopically labeled by the method of Byrne (4). L cells were infected in suspension with 10 ID_{50} of chlamydiae per host cell in a medium containing 1 to 2 μCi of a ^{14}C-labeled amino acid mixture per ml (New England Nuclear Corp., Boston, Mass.; average specific activity, 259 mCi/mmol) and 2 μg of cycloheximide per ml to inhibit incorporation of the label into host protein (1). The labeled chlamydiae were purified as described by Hatch (10). When centrifuged on continuous Renografin gradients (8), purified ^{14}C-labeled chlamydiae yielded a single peak of radioactivity and infectivity. Most experiments were done with a single preparation of ^{14}C-labeled *C. psittaci*.

Measurement of chlamydial interaction with host cells. The association of ^{14}C-labeled *C. psittaci* or *C. trachomatis* with L cells in suspension was

followed by a modification of the Byrne procedure (4) which measures total host cell-associated chlamydiae, that is, both ingested chlamydiae and chlamydiae that have become attached but are not ingested. The temperature of incubation was 37°C, and the L-cell density was 10^6/ml. Other conditions of interaction between host cells and chlamydiae are specified in the tables and figures. L cells that had been exposed to labeled chlamydiae were centrifuged for 5 min at 300 × *g*, washed twice with phosphate-buffered saline, dissolved in 2 ml of 1% sodium dodecyl sulfate, and transferred to a scintillation vial. The centrifuge tube was rinsed with 2 ml of distilled water, which was then added to the scintillation vial. After 7 ml of a scintillation cocktail (Insta-Gel, Packard Instrument Co., LaGrange, Ill.) was added and the mixture was shaken thoroughly, the samples were counted in a liquid scintillation counter (Packard Instrument Co.). When ^{14}C-labeled *C. psittaci* interacted with host cells in monolayer, the assay procedure was that of Byrne (5), except that, after the cells had been dispersed with trypsin, the total cell-associated counts were measured as just described for suspension cultures. The monolayers were washed with growth medium instead of phosphate-buffered saline.

The ingestion of unlabeled *C. psittaci* was measured by the method of Byrne (4), which consists of exposing suspended L cells to a single ID_{50} of chlamydiae (10) under appropriate experimental conditions, plating the cells in Leighton tubes, incubating at 37°C for 24 h, staining with Giemsa, and counting the percentage of L cells bearing one or more chlamydial inclusions.

Reagents. Sugars and lectins were purchased from Sigma Chemical Co., St. Louis, Mo. A single batch of wheat germ agglutinin (WGA), a lectin from *Triticum vulgaris*, was used in most experiments. The lyophilized powder was dissolved in phosphate-buffered saline to a concentration of 1 mg/ml and frozen at −90°C in 1-ml portions, one of which was thawed on the day of each experiment. According to the supplier, WGA agglutinates a fresh 2% suspension of type A human erythrocytes at a concentration of 16 μg/ml, and the agglutination is prevented by a 100-fold excess of *N*-acetyl-D-glucosamine (NAG). ^{3}H-labeled WGA (New England Nuclear Corp.) was diluted in unlabeled WGA to a concentration of 10 μg and 4,000 cpm per ml. The amount of ^{3}H-labeled WGA associated with L cells was measured as already described for chlamydiae-associated ^{14}C counts. Concanavalin A (type I) and *Ricinus communis* lectin (type III) were obtained as lyophilized powders, and stock solutions were prepared as already described for WGA. NAG and *N*-acetylneuraminic acid were crystalline preparations, whereas *N*-acetyl-D-galactosamine and *N*-acetyl-D-mannosamine were grade II products. Crystalline trypsin was purchased from Worthington Biochemicals Corp., Freehold, N.J.

RESULTS

WGA inhibits attachment of *C. psittaci* to L cells. When 10 μg of WGA per ml was added to suspended L cells 1 h before inoculation with *C. psittaci*, the number of chlamydial inclusions observed 24 h later was reduced by more than 90% (Fig. 1). When the WGA inhibition was measured in terms of reduction in association of ^{14}C-labeled *C. psittaci* with L cells, an assay that measures the sum of attached-plus-ingested chlamydiae (4–6), an almost identical dosage-response curve was obtained (Fig. 1). This indicates that WGA inhibited inclusion formation by preventing attachment of *C. psittaci* to the host cell surface, because if some later stage in chlamydial infection had been the one primarily affected, association of chlamydial ^{14}C counts with L cells would have been only slightly inhibited. The possibility that the inhibition was due to a reaction between the lectin and *C. psittaci* was eliminated by incubating chlamy-

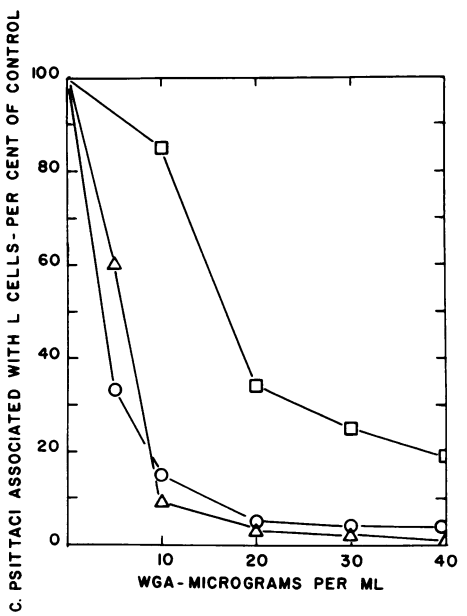

FIG. 1. *Inhibition by WGA of the interactions between L cells and C. psittaci. Indicated concentrations of WGA were added to L cells at 37°C. One hour later, C. psittaci was added, and incubation of L cells, chlamydiae, and WGA was continued for another hour before the extent of interaction between host cells and chlamydiae was measured. Interactions were expressed as percentages of controls, with the interaction observed in the absence of WGA taken as 100%. The multiplicity of infection was 1 ID_{50} per L cell, and 5 × 10^6 cells without added WGA attached 2,000 cpm of labeled chlamydiae.* (○), *Inhibition of inclusion formation in suspensions of L cells. One hour after inoculation of C. psittaci, unattached chlamydiae and WGA were removed by washing, and inclusions were counted after an additional 24 h at 37°C.* (△) *Inhibition of attachment of ^{14}C-labeled C. psittaci to L cells in suspension.* (□) *Inhibition of attachment of ^{14}C-labeled C. psittaci to L cells in monolayer. See the text for details of experimental procedures.*

diae with 50 μg of WGA per ml. When the lectin was removed, there was no inhibition of the association of *C. psittaci* with L cells.

Figure 2 shows the relation between the dosage of WGA and inhibition of chlamydial attachment at low concentrations of the lectin. The 50% inhibitory concentration was approximately 5 μg of WGA per ml.

WGA inhibition of chlamydial attachment to L cells is not due to agglutination or generalized host cell damage. If WGA had agglutinated or injured L cells (17), this could have offered a trivial explanation for the observed inhibition of chlamydial attachment. However, under the conditions of these experiments, as much as 200 μg of WGA per ml did not agglutinate L cells, as observed by phase-contrast microscopy. The viability of L cells was also not impaired. When they were incubated for 2 h at 37°C in WGA concentrations sufficient to give nearly complete inhibition of chlamydial attachment and the unbound lectin was removed by washing, there was little or no decrease in the number of cells that excluded trypan blue or formed visible colonies after 10 days of incubation at 37°C (Table 1). The failure of a 2-h exposure to WGA to reduce the number of colonies formed by a population of suspended L cells is further evidence that they were not aggregated by such treatment. When WGA was left in the medium for the entire 10-day incubation, 10 μg/ml reduced the number of visible colonies by only 50%.

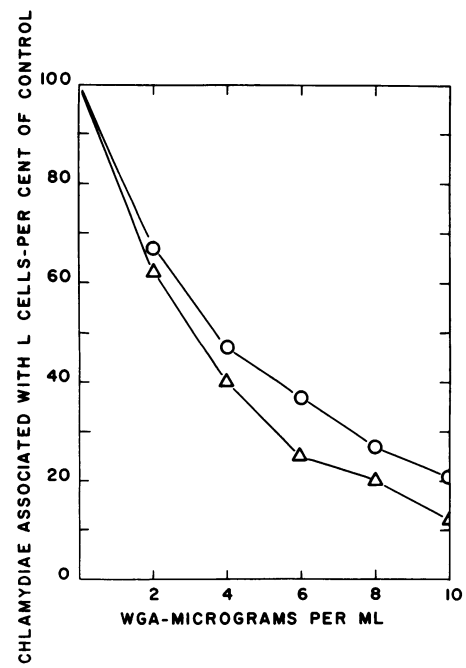

FIG. 2. *Inhibition of the attachment of C. psittaci and C. trachomatis to L cells in suspension by low levels of WGA. The experimental protocol was the same as for Fig. 1. (△) Inhibition of attachment of ^{14}C-labeled C. psittaci. The labeled C. psittaci preparation was the same as for Fig. 1, and the multiplicity of infection was again 1 ID_{50} per L cell. (○) Inhibition of attachment of ^{14}C-labeled C. trachomatis. The multiplicity of infection for C. trachomatis was 100 ID_{50} per host cell, and 5 × 10^6 cells without WGA attached 2,000 cpm of labeled chlamydiae. See the text for details of experimental procedures.*

The extent to which WGA inhibits chlamydial attachment depends on factors other than lectin concentration. L cells in monolayer were less susceptible to WGA inhibition of *C. psittaci* attachment than were L cells in suspension. Figure 1 illustrates the magnitude of this difference when inhibition was assayed in terms of association of ^{14}C-labeled chlamydiae with host cells. A similar difference was observed when inhibition of inclusion formation was compared in suspension and in monolayer.

Maximum blockage of chlamydial attachment was obtained when WGA was incubated with L cells before addition of *C. psittaci*. Much greater inhibitions were observed when WGA was added 1 h before the L cells were inoculated with chlamydiae than when the two reagents were added at the same time. For example, 10 μg of WGA per ml inhibited association of ^{14}C-labeled chlamydiae with L cells by 88% when the lectin was added 1 h before *C. psittaci* and by only 36% when WGA and *C. psittaci* were added at the same time.

When *C. psittaci* was added to L cells that had already been incubated for 1 h with WGA, the observed inhibition of attachment decreased at each successive interval of measurement (Fig. 3). That is, the longer the chlamydiae were incubated with WGA-treated host cells, the less the interference with their attachment. Although greater inhibitions could be obtained with shorter periods of incubation of ^{14}C-labeled

TABLE 1. *Effect of WGA on the cloning efficiency of L cells*

Concn of WGA (μg/ml)	Visible colonies formed by 100 L cells after 10 days of incubation	
	WGA removed after 2 h	WGA present for 10 days
0	60	71
10	54	36
50	55	0
200	45	—[a]

[a] —, Not done.

C. psittaci with WGA-treated L cells, 1 h was taken as the standard assay period for convenience. Also, during this 1-h contact between chlamydiae and host cells, the ^{14}C counts that became associated with L cells were proportional to the time of contact (Fig. 3).

One factor that did not materially affect the degree of inhibition was multiplicity of infection. At a constant WGA concentration, a fourfold variation in multiplicity of infection did not significantly alter the ability of the lectin to prevent association of chlamydiae with L cells (Table 2). In other experiments, the multiplicity of infection was raised as high as 100 ID_{50} per L cell without materially reducing the inhibition produced by a given concentration of WGA. Also, in the range used in these experiments, the association of ^{14}C counts with L cells was proportional to the multiplicity of infection (Table 2).

Inhibition of *C. psittaci* attachment to L cells by WGA is specific. Two lectins with specificities for sugars common to cell surfaces but different from that of WGA were also examined. Figure 4 shows the inhibition of chlamydial attachment to L cells obtained with the

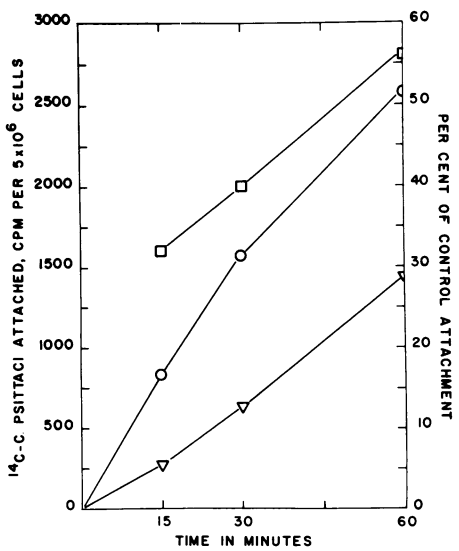

FIG. 3. *Decrease in WGA inhibition of attachment of C. psittaci to L cells with time of incubation. L cells were incubated in suspension with 6 μg of WGA per ml at 37°C. At 1 h, ^{14}C-labeled C. psittaci was added, and attachment to host cells was measured at intervals thereafter. The labeled C. psittaci preparation was the same as for Fig. 1. (○) Counts attached to L cells in the absence of WGA. (△) Counts attached in the presence of WGA. (□) Counts attached in the presence of WGA as the percentage of counts attached in its absence. See the text for details of experimental procedures.*

TABLE 2. *Effect of multiplicity of infection on WGA inhibition of attachment of ^{14}C-labeled C. psittaci to L cells*

Multiplicity of infection (ID_{50}/L cell)	*C. psittaci* associated with L cells		
	No WGA (A) (cpm/5 × 10^6 L cells)	5 μg of WGA per ml (B) (cpm/5 × 10^6 L cells)	Inhibition by WGA (B/A) (% of control)
0.5	1,919	1,304	68
1	4,490	2,486	56
2	8,633	4,737	55

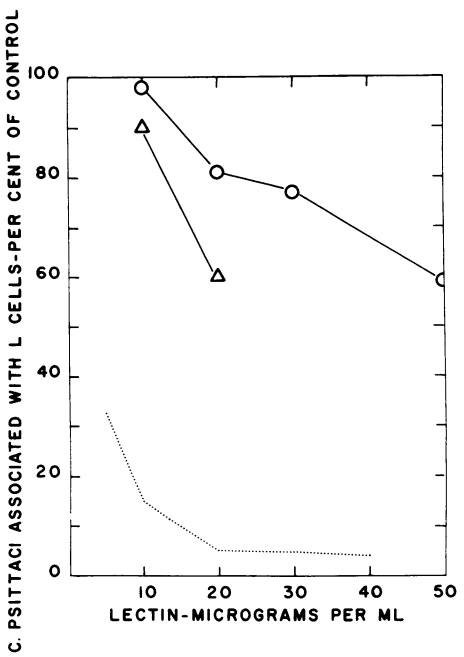

FIG. 4. *Inhibition of the attachment of C. psittaci to suspended L cells by other lectins. The experimental protocol and the labeled C. psittaci preparation were the same as for Fig. 1. Inhibition of attachment of ^{14}C-labeled C. psittaci to L cells treated with R. communis lectin (△) or concanavalin A (○). L cells were agglutinated by R. communis lectin concentrations greater than 20 μg/per ml. The comparable WGA curve from Fig. 1 is reproduced as a dotted line. See the text for details of experimental procedures.*

N-acetyl-D-galactosamine-specific lectin from castor beans (*R. communis*) (14) and with the α-mannopyranoside-specific lectin from jack beans (*Canavalia ensiformis*), concanavalin A (14). As compared with that for WGA, much higher concentrations of these lectins were required to give measurable inhibition of chlamydial attachment, and the extent of inhibition

never approached that obtained with WGA.

NAG, the monosaccharide specific for WGA (17), prevented WGA from inhibiting the association of chlamydiae with host cells when first the sugar was incubated with WGA, and then the NAG-WGA mixture was incubated with L cells, and finally ^{14}C-labeled *C. psittaci* was added. Figure 5 shows the results of increasing NAG concentrations on the effect of 10 μg of WGA per ml on attachment of chlamydiae to L cells. A 500-fold excess of NAG blocked the inhibitory action of WGA by more than 70%. In comparable experiments, increasing the concentration of WGA reduced the blocking action of any given concentration of NAG.

No evidence for combination of NAG with the chlamydial ligand that binds to host cells (6) was obtained. When NAG was incubated with *C. psittaci* before inoculation of L cells, attachment proceeded normally.

N-Acetyl-D-mannosamine, *N*-acetyl-D-galactosamine, and *N*-acetylneuraminic acid, monosaccharides related to NAG, did not block the action of WGA when tested in 500-fold excess under the conditions described in the legend of Fig. 5.

Exposure of L cells to trypsin and to *C. psittaci* reduces their ability to associate with WGA. Cell-associated, ^{3}H-labeled WGA was estimated by an assay that, like the one for cell-associated ^{14}C-labeled *C. psittaci*, measured the sum of surface-attached plus interiorized WGA. Over the range of WGA concentrations used to study blockage of chlamydial attachment, suspended L cells firmly associated with an approximately constant proportion of added ^{3}H-labeled WGA (Fig. 6). At the concentrations of lectin that maximally inhibited association of chlamydiae with host cells, 5 to 10 μg/ml, only a fraction of the total available ^{3}H-labeled WGA-binding capacity of the L cells had been used up.

Because Byrne (4) has shown that trypsin destroys the ability of L cells to attach *C. psittaci*, a comparable experiment was performed with ^{3}H-labeled WGA (Fig. 7). Incubation of L cells with trypsin before addition of labeled WGA reduced its association with L cells by about half. Prior incubation of L cells with a very high multiplicity of *C. psittaci* reduced the

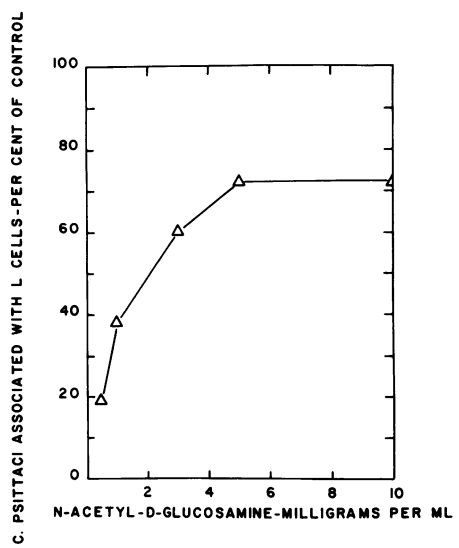

FIG. 5. *Reversal of WGA inhibition of attachment of ^{14}C-labeled C. psittaci to L cells by increasing concentrations of NAG. Increasing concentrations of NAG were incubated with a constant concentration of WGA. After 1 h at 37°C, the mixtures were added to L cells to yield 10 μg of WGA per ml and the indicated concentrations of NAG. One hour later, ^{14}C-labeled C. psittaci was added to the treated cells, and the interaction between host cells and chlamydiae was measured after an additional hour at 37°C. Attachment was expressed as the percentage of the control, with the attachment in the absence of both WGA and NAG taken as 100%. The C. psittaci preparation was the same as for Fig. 1. See the text for details of experimental procedures.*

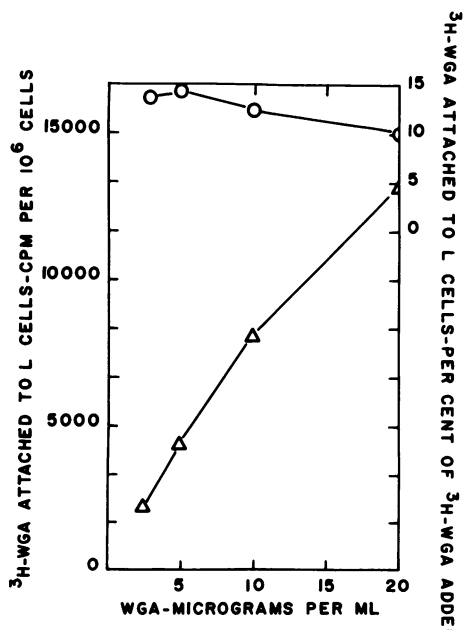

FIG. 6. *Binding of increasing concentrations of ^{3}H-labeled WGA by L cells. Indicated concentrations of ^{3}H-labeled WGA were added to L cells at 37°C. After 1 h, the extent of binding of labeled WGA to the L cells was measured. Counts associated with L cells: (△) cpm per 10^{6} L cells; (○) percentage of total ^{3}H-labeled WGA counts added. See the text for details of experimental procedures.*

binding of ^{3}H-labeled WGA to about the same extent (Fig. 7). However, once the labeled WGA had become associated with L cells, subsequently added *C. psittaci* did not displace it (Fig. 7).

The effect of WGA on chlamydial attachment is a general phenomenon not limited to L cells and *C. psittaci*. When the effect of WGA on the attachment of a ^{14}C-labeled lymphogranuloma venereum strain (440L) of *C. trachomatis* to suspended L cells was determined in a manner identical to that used for *C. psittaci*, the curves relating inhibition of attachment of the two chlamydial strains to lectin concentration were almost superimposable (Fig. 2).

When other established cell lines of human, simian, and murine origin were tested in monolayer for the ability of WGA to prevent attachment of ^{14}C-labeled *C. psittaci*, the results were comparable to those obtained with monolayers of L cells (Fig. 8).

DISCUSSION

These investigations suggest that the monosaccharide NAG is an essential component of the structure on the surface of L cells that attaches to *C. psittaci* and facilitates its ingestion. Attachment of *C. psittaci* was almost completely blocked by low concentrations of WGA, a lectin specific for NAG (14), but not by lectins of other specificities, and inhibition of chlamydial attachment by WGA was antagonized by NAG but not by monosaccharides of related structure.

Although NAG appears to be the most important sugar residue in the binding of chlamydiae to the L-cell surface, other carbohydrates may also be involved. For optimal binding of WGA to its receptor, it has been reported that intact sialic acid residues are required (3). However, the one sialic acid tested, *N*-acetylneuraminic acid, did not antagonize the blocking action of WGA, and neuraminidase did not destroy the ability of L cells to attach *C. psittaci*. The small inhibitions of chlamydial attachment achieved by high concentrations of lectins specific for α-

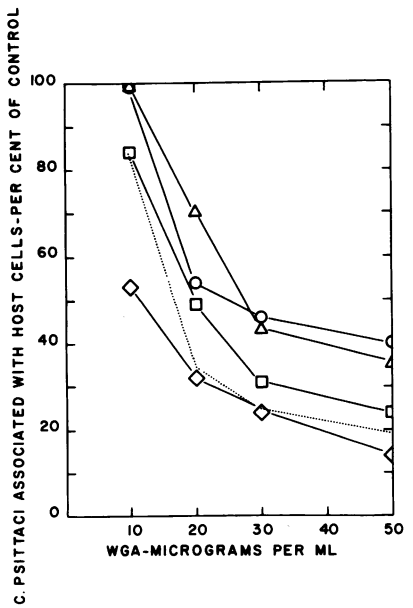

FIG. 8. *Inhibition of WGA of attachment of C. psittaci to other cells lines in monolayer. The experiments were conducted and the results were expressed as for Fig. 1. The other cell lines were grown in essentially the same way as L cells. The ^{14}C-labeled C. psittaci preparation was the same as for Fig. 1, and attachment to host cells in the absence of WGA is given below as counts per minute per 5 × 10^6 cells. The multiplicity of infection was approximately 1 L cell ID$_{50}$ per host cell. (△) HeLa cells, 1,485 cpm; (○) HEP-2 cells, 827 cpm; (□) Vero cells, 2,382 cpm; (△) McCoy cells, 2,926 cpm. Comparable data for an L-cell monolayer (Fig. 1) are shown as a dotted line. See text for details of experimental procedures.*

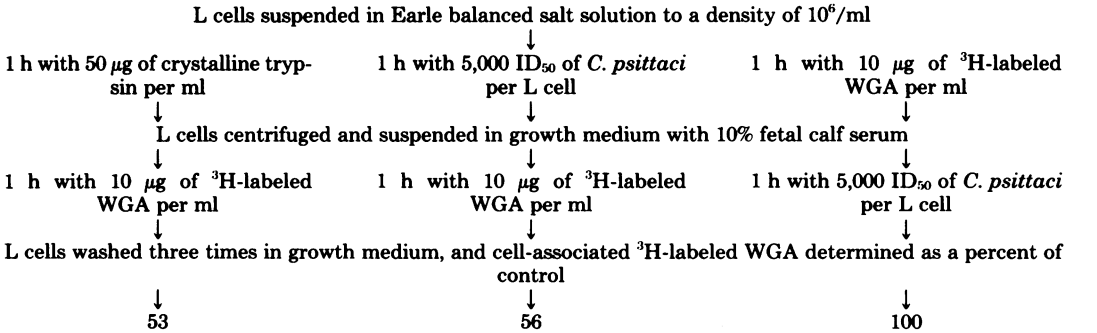

FIG. 7. *Effect of trypsin and C. psittaci on binding of ^{3}H-labeled WGA to L cells. All incubations were at 37°C. L cells not treated with either trypsin or C. psittaci bound 3,000 cpm of ^{3}H-labeled WGA per 10^6 cells. This value was taken as 100%. See text for details of experimental procedures.*

mannosides and *N*-acetyl-D-galactosamine suggest minor roles for these sugars as well.

The observations reported here help define the molecular nature of the structures on the surface of *C. psittaci* and the L cell that participate in the interaction responsible for attachment of the parasite to the host cell. Because tryptic digestion abolishes attachment of chlamydiae to host cells and releases chlamydiae already attached, Byrne (4) postulated that the L-cell sites which bind chlamydiae are protein or glycoprotein in nature. Trypsin, WGA, and *C. psittaci* may react with a common L-cell structure. Stanley and Carver (18) have demonstrated that cells have binding sites with high and low affinities for WGA. *C. psittaci* appears to react with high-affinity sites because its attachment to L cells was almost totally prevented by WGA concentrations considerably below those required to fill all of the available binding sites on the host cell surface. Exposure of L cells to either trypsin or very high multiplicities of *C. psittaci* removed about half of the sites that bound WGA. This agreement could be fortuitous, but it is of interest that, in fat cells, trypsin preferentially destroys binding sites with high affinity for WGA (7). The most uncomplicated picture of the L-cell receptor for *C. psittaci* is that of a NAG-containing, trypsin-sensitive glycoprotein that binds both chlamydiae and WGA with high affinity. However, the chlamydial receptor must be isolated before its structure is known with certainty. Glycolipids may also act as cell surface receptors (2). There is also the possibility that the binding sites for chlamydiae and WGA are not identical but are instead located very close to each other on the L-cell surface, so close that when one site is occupied, the other is rendered nonfunctional.

The results of this investigation suggest that the ligand on the surface of *C. psittaci* that attaches it to the L-cell host has some of the properties of a lectin. Lectins were originally found in plants but now have also been described for vertebrates, invertebrates, and bacteria (14). Attachment to cell surfaces is an important virulence factor in the pathogenesis of infectious diseases, and sugar residues are increasingly identified as parts of the cell surface receptor sites (15). For example, the binding of *E. coli* to human epithelial cells is reversed by D-mannose (16), and the adherence of *Vibrio cholera* to the rabbit intestinal brush border is inhibited by D-fucose and to a lesser extent by D-mannose (11). However, there are two important differences between the bacterial lectins responsible for these interactions and the chlamydial ligand. First, attachment of the chlamydial ligand to the host cell surface is not blocked by any known simple sugar; second, when the ligand binds chlamydiae to host cells, the interaction proceeds beyond attachment to ingestion, perhaps by the zipper mechanism proposed by Byrne and Moulder (6) and Griffin and his associates (9).

The nearly identical effect of WGA on the association of the 6BC strain of *C. psittaci* and the lymphogranuloma 440L strain of *C. trachomatis* with L cells adds to the previously reported (6), numerous similarities between phagocytosis of these two chlamydial strains. This similarity is remarkable because the two strains differ in many other phenotypic properties and show little or no genetic homology (13).

The similar dosage-response curves for blocking of the attachment of *C. psittaci* by WGA obtained with a number of established cell lines of murine, simian, and human origin suggest the widespread distribution of chlamydial receptors similar to those demonstrated for L cells.

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