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Wheat Germ Agglutinin Bound to the Outer Cuticle of the Seed Gall Nematodes *Anguina agrostis* and *A. tritici*[1]

YITZHAK SPIEGEL,[2] MICHAEL A. MCCLURE,[3] ITZHAK KAHANE,[4]
WALTER M. ROBERTSON,[5] AND RAFFI SALOMON[6]

Abstract: The presence of wheat germ agglutinin (WGA) on the cuticular surface of the seed gall nematodes *Anguina agrostis* and *Anguina tritici* was demonstrated, and the nature of its binding was examined. Crude extracts from the cuticles of *A. tritici* agglutinated human red blood cells, and only N-acetylglucosamine (GlucNAc) inhibited the agglutination. Distribution of the lectin was visualized by treating live infective juveniles (J2) with rabbit anti-WGA antibody and staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG. The lectin bound to the outer cuticular surface of the whole body wall. Pretreatment with GlucNAc oligomers did not reduce the fluorescence created by the anti-WGA-WGA binding, indicating at least a partial nonspecific adhesion of the WGA to the nematode surface. Proteolytic enzyme pretreatments diminished the fluorescence, whereas lipase and periodate pretreatments increased the fluorescence. Adult females and males were labeled only on the head and tail, whereas eggs were not labeled at all. It was concluded that the WGA on the J2 cuticle originates from the host.
Key words: *Anguina agrostis, Anguina tritici,* cuticle, lectin, nematode, wheat germ agglutinin.

Anguina agrostis and *Anguina tritici* are phytophagous nematodes specifically parasitic on ryegrass (*Lolium rigidum*), wheat (*Triticum aestivum*), and certain other grasses. The high degree of host specificity exhibited by these nematodes, and their critical dependency on host plant development, suggests that recognition phenomena may play an important role in host-parasite compatibility. Several recent studies (9,15) have implicated nematode surface glycans as determinants in pathogenic relationships. Carbohydrate binding sites for a lectin, wheat germ agglutinin (WGA), occur on the cuticular surface of the infective juveniles (J2) of *A. agrostis* (3,4) and *A. tritici*, where they occur only in the cephalic region (18).

Like 80 other grass species, the respective hosts of *A. agrostis* and *A. tritici*, ryegrass and wheat, contain immunochemically identical lectins with similar specificities (10). The most prominent one is WGA (11,12). Wheat and ryegrass lectins are synthesized specifically during seed formation, and their accumulation coincides with the development of both the primary axes (10) and maturation of *Anguina* in the seed (2).

These properties of lectins have led us to investigate the possible occurrence and origin of WGA on the cuticular surface of *A. agrostis* and *A. tritici*.

MATERIALS AND METHODS

Infective J2 of *A. agrostis* and *A. tritici* were extracted from seed galls of their respective hosts by dissection in phosphate-buffered saline (PBS), pH 7.2. The J2 were allowed to migrate through a 10-μm-pore nylon cloth to remove any plant tissue and then were washed thoroughly with distilled water and PBS. Eggs, J2, and adults of *A. tritici* were obtained from 'Tonichi' wheat plants sown in pasteurized sandy-loam soil and inoculated with seed galls containing infective J2 (8). Green galls that developed on the inflorescence were harvested, and the nematodes they contained were dis-

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[2] Department of Nematology, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.
[3] Department of Plant Pathology, University of Arizona, Tucson, AZ 85721.
[4] Department of Membrane and Ultrastructure Research, The Hebrew University, Haddasah Medical School, Jerusalem, Israel.
[5] Department of Zoology, Scottish Crop Research Institute, Invergowrie, Dundee, Scotland.
[6] Department of Virology, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

sected in PBS and washed three times with the same buffer.

Agglutination assays: Agglutination titers (performed only with *A. tritici* J2) were determined with fresh human red blood cells (group A). Agglutination assays were conducted in multi-well plates (Becton Dickinson Labware, Oxnard, CA) in a final volume of 0.1 ml containing 80 μl of a 1% suspension of red blood cells and 20 μl of cuticular crude extract (5), diluted serially in twofold increments. Agglutination after 10 minutes at 23 C was determined by phase-contrast microscopy. The specificity of WGA lectin was determined with a series of several carbohydrates (Sigma Chemical, St. Louis, MO)—arabinose, fucose, galactose, galactosamine, glucose, glucosamine, mannose, N-acetylgalactosamine, GlucNAc, oligomers of GlucNAc, and trehalose—at final concentrations of 0.1 M.

Fluorescent labeling: Nematodes rinsed with PBS were treated with 1.0 mg/ml rabbit anti-WGA IgG (Sigma) in 0.2 ml PBS for 60 minutes at 23 C and then centrifuged at 300 *g*. The nematode pellet was rinsed three times in PBS and incubated with 1.0 mg/ml fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Sigma) in 0.2 ml PBS at 23 C for 60 minutes. After a final rinse with PBS, up to 200 specimens were examined by fluorescence microscopy and rated qualitatively for intensity of fluorescence (9). To verify the specificity and to assess the nature of the observed binding, several modifications of the above procedure were performed: 1) Rabbit anti-WGA was excluded. 2) Rabbit anti-Concanavalin A IgG (Sigma) was used instead of anti-WGA. 3) Goat anti-mouse FITC (Sigma) was used instead of goat anti-rabbit FITC. 4) Anti-WGA was pretreated with 200 μg/ml WGA (BioMakor, Rehovot, Israel) for 2 hours at 23 C. 5) Juveniles were pretreated with a mixture of anti-WGA and WGA for 2 hours at 23 C. 6) Juveniles were pretreated with a 0.2 M mixture of N-acetyl glucosamine oligomers for 2, 20, and 60 hours at 23 C. 7) Juveniles were pretreated with 2.0% SDS or 1% cetyltrimethylammonium bromide (CTAB) for 1 and 3 hours at 23 C. 8) Juveniles were pretreated by boiling in 2.0% SDS or 1% CTAB for 4 minutes. 9) Juveniles were pretreated with 2.0 M, 3.0 M, or 5.0 M NaCl solution for 2, 3, or 24 hours at 23 C. 10) Juveniles were pretreated with 10 mM freshly made sodium periodate in pH 4.5 sodium acetate buffer in the dark for 4 hours at 4 C. 11) Juveniles were pretreated with enzymes. Proteolytic digestions were accomplished at 37 C for 3 hours, with 5 mg (10,000 units) per ml pepsin (Calbiochem, San Diego, CA) in sodium acetate buffer, pH 4.0, or with 5 mg (100 units) and 10 mg (100,000 units) per ml proteinase K (Calbiochem) or trypsin (Calbiochem), respectively, in PBS. Lipase treatment methods have been reported elsewhere (16).

Adult females and males of *A. tritici* were treated with rabbit anti-WGA IgG and then with FITC-conjugated anti-rabbit IgG. Specificity of the reaction was verified by modifications 1, 2, and 3 detailed in the preceding paragraph.

The following experiments were performed in order to test the origin of WGA lectin on the cuticle of *A. tritici* J2: 1) Eggs were collected from green seed galls, rinsed three times with PBS and the resulting suspension passed through a 75-μm-pore sieve to remove plant materials and J2. Eggs that passed through the sieve were rinsed again and incubated at 23 C for 24 hours in PBS. The newly hatched J2 were collected, washed with PBS, and labeled with anti-WGA and anti-rabbit FITC as described. 2) Washed eggs were incubated for 60 minutes with 100 μg/ml WGA-FITC (Sigma) at 23 C, washed again with PBS, transferred through a 75-μm-pore sieve to remove previously hatched J2, and then incubated overnight in the dark. After 18 hours, eggs (most containing unhatched J2) were washed and prepared for fluorescence microscopy. 3) Freshly hatched J2 were carefully washed with PBS and treated with FITC solution in PBS (21 μg/ml; same concentration as in WGA-FITC preparation) and with anti-rabbit-FITC, for

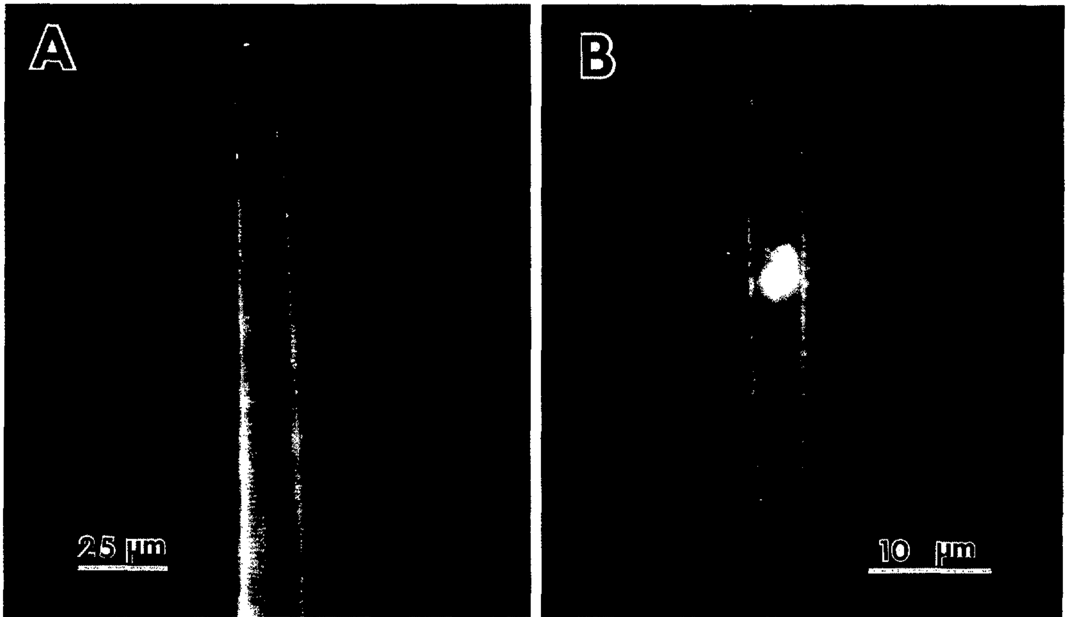

FIG. 1. *Anguina tritici* labeled with rabbit anti-WGA followed by goat anti-rabbit FITC. A) Anterior of J2. B) Lateral field of J2 pretreated with lipase.

60 minutes at 23 C. They were then washed and prepared for fluorescence microscopy. 4) Eggs, washed and free of plant material, were treated with 21 µg/ml FITC at 23 C for 60 minutes, washed again, and incubated overnight in the dark. After 18 hours, newly hatched J2 were washed in PBS and examined by fluorescence microscopy.

All experiments in this study were repeated three times with consistent results.

RESULTS AND DISCUSSION

Agglutination assays: Crude extracts of *A. tritici* cuticles agglutinated human red blood cells. Agglutination titers exceeded 32-fold dilution. Only GlucNAc inhibited the agglutination. Oligomers of this sugar were even more effective inhibitors; chitobiose or chitotriose at 200 µg/ml completely inhibited agglutination. The data suggest that lectin-like activity resembling that of WGA exists in the crude cuticular extract.

Fluorescent labeling: Anguina agrostis and *A. tritici* J2 were strongly labeled over the entire body length after reaction with rabbit anti-WGA (Fig. 1A). In some lots of nematodes, however, 10–20% of the J2 were not labeled anywhere on the body. Labeling of the adult females and males was restricted to the head and tail areas.

We believe that labeling of both J2 and adults probably resulted from specific interaction between the anti-WGA antibody and the WGA residues on the nematode surface, for the following reasons.

First, only sequential treatments of nematodes with rabbit anti-WGA and goat anti-rabbit FITC resulted in intensive fluorescence of the outer cuticle. Reversing the sequence resulted in no labeling.

Second, fluorescent labeling did not occur when nonhomologous, FITC goat anti-mouse IgG was substituted for FITC goat anti-rabbit IgG or when anti-Con A was used instead of anti-WGA.

Third, preincubating the anti-WGA with WGA reduced the fluorescene labeling, but pretreatment of J2 with a mixture of anti-WGA plus WGA did not, as WGA-receptors (GlucNAc residues) are native constituents of the J2 cuticle (18). Exogenously applied WGA would bind to these receptors, which, in turn, would bind anti-WGA. Although WGA does not bind its antibody on the same site that it binds its specific saccharide, we assume that preincubation

of anti-WGA with WGA causes steric hindrance, which reduces saccharide binding. Evidence for this assumption is lacking.

Fourth, a mixture of GlucNAc oligomers did not inhibit the binding of anti-WGA to WGA on the J2 cuticle, even after preincubation for 60 hours. Previous experiments (3,18) indicated that application of competitive GlucNAc oligomers completely prevented the binding of WGA applied exogenously to *Anguina* J2 cuticle. Therefore, we conclude that although WGA on the cuticular surface may be bound in part to native carbohydrate residues, it is primarily bound to the J2 cuticle by a nonspecific mechanism(s) and, consequently, is not displaced by haptenic saccharides. Such a mechanism, probably a hydrophobic ligand devoid of haptenic sugar, binds the lectin at a site distinct from the carbohydrate binding site. Nonspecific binding of WGA to *Heterodera schachtii* parasitic juveniles has been linked to fatty acids in the cuticular exudates (1).

Detergent treatments under mild conditions interfered only slightly with the binding of anti-WGA to the J2 cuticle (Table 1). Lectin was not stripped by SDS, whereas CTAB partially released the lectin after incubation for 24 hours. Only severe conditions, namely boiling with SDS for a period of 4 minutes, reduced subsequent fluorescence on *A. agrostis,* whereas fluorescence of *A. tritici* following that treatment remained the same. Boiling J2 with CTAB destroyed the cuticle of *A. agrostis* but not the cuticle of *A. tritici,* which still bound anti-WGA, indicating that the lectin was still present. Cationic detergents strip cuticle surface antigens from various nematodes more effectively than neutral and anionic detergents (7,13). However, the inability of CTAB to reduce labeling of *A. tritici* with anti-WGA indicates that mechanisms other than surface charge could be involved. The cuticular surface of *Xiphinema index* has a double outer membrane that stained for carbohydrate when treated with periodic acid and thiosemicarbazide followed by silver proteinate (17). It may be that *A. tritici* has a similar double layer, the outermost of which can be removed by CTAB.

Compared with control treatments, mild periodate and lipase pretreatments enhanced fluorescence of J2 treated with FITC-conjugated antibody (Table 1). Lipase pretreatment of *A. tritici* produced localized areas of intense fluorescence within the lateral field (Fig. 1B). Binding of WGA-FITC to J2 was also enhanced after periodate treatment (16). Increased intensity of labeling after periodate and lipase pretreatments might be explained by the stripping of cuticular components that mask the carbohydrate and the lectin binding sites of the surface coat or glycocalyx. Surface lipids have been detected on several animal-parasitic nematodes (14) and in the cuticular exudates of *Heterodera* species (1).

Juveniles (J2) that had hatched in vitro during the previous 24 hours were faintly, but uniformly, labeled with FITC-anti-WGA, whereas J2 extracted from green seed galls strongly bound FITC-anti-WGA along the entire cuticle. Although eggs were labeled with unconjugated FITC, no fluorescence occurred on freshly hatched J2 treated with FITC or anti-rabbit-FITC. However, embryonated eggs pretreated with WGA-FITC produced J2 strongly labeled over the entire cuticle. Because the surface of J2 hatched in vitro was not labeled (nor were the surfaces of J1 and J2 embryos from crushed eggs when the eggs were pretreated with FITC-WGA), we conclude that the WGA on the surface of J2 from seed galls originates from the plant and adheres to the surface of the J2 as it emerges from the egg.

Binding of FITC-anti-WGA to adults of *A. tritici* was restricted to the cuticle of the head and tail. A difference in the pattern of binding between J2 and adults suggests changes in the cuticular surface that probably occur during maturation, when WGA receptor sites on the cuticle could either be masked or reduced. Lack of fluorescence on females and males of *A. tritici* treated with WGA-FITC (16) may partially explain the absence of the lectin on adult cuticle in the current study.

TABLE 1. Effect of pretreatment of J2 on the binding of fluorescent (fluorescein isothiocyanate) anti-wheat germ agglutinin antibody to the outer cuticle of *Anguina agrostis* and *Anguina tritici* infective juveniles.

Treatment	Labeling intensity	
	A. agrostis	*A. tritici*
Untreated (control)	++++	++++
Boiled nematodes	++++	++++
Incubation with $(GlucNAc)_n$	++++	++++
2.0% SDS at 23 C for 1 or 3 hr	++++	++++
1.0% CTAB at 23 C for 3 hr	–	++ to +++
Boiling with 1.0% CTAB, 4 min	+ to +++	++++
Boiling with 2.0% SDS, 4 min	–	++ to ++++
2.0 M, 3.0 M, or 5.0 M NaCl for 2, 3, or 24 hr at 23 C	++++	++++
10 mM sodium periodate at 0 C	+++++	+++++
Pepsin	++++	++++
Proteinase K	+	+
Trypsin	+ to ++	++
Lipase	+++++	+++++

+ = detectable labeling (fluorescence). ++, +++, ++++, and +++++ = increasing intensities of labeling. – = no labeling of cuticle. SDS = sodium dodecyl sulphate. CTAB = cetyltrimethylammonium bromide.

A small fraction of J2 from galls failed to bind anti-WGA antibody. Should these J2 truly lack WGA, they could be used for studies in comparative pathogenicity, or to investigate mechanisms by which the Coryneform bacteria *Clavibacter* sp. (syn. = *Corynebacterium rathayi*) and *Clavibacter tritici* adhere, respectively, to the cuticles of *A. agrostis* and *A. tritici.* The involvement of a lectin–carbohydrate mediated process has been proposed for the binding of *C. rathayi* to *A. agrostis* (3), and the presence of GlucNAc residues on the *C. rathayi* capsule (unpubl.) apparently supports this hypothesis.

The biological significance of WGA on the surface of *A. agrostis* and *A. tritici* is problematic. Lectins might participate in the host-finding process (6,19), but it is not known how long WGA is retained on J2 in the soil or if absence of the lectin affects the ability of the nematode to detect stimuli emanating from host tissues. Alternatively, surface-bound lectins may serve as camouflage to deceive plant defense mechanisms by presenting a surface image similar to that of host plant cells.

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