

Gold-Conjugated Reagents for the Labelling of Carbohydrate-Recognition Domains and Glycoconjugates on Nematode Surfaces¹

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Abstract: Various fluorescent conjugated lectins have been used for the detection of glycoconjugates on nematode surfaces under light microscopy. Several problems have been experienced with these reagents including penetration of the cuticle by fluorescent lectins, non-glycoconjugate specificity, strong nematode autofluorescence at the emission wavelength of the fluorescent dye, and prevention of persistent visualization due to rapid quenching of the fluorescent components. Gold-conjugated reagents combined with silver enhancement alleviated these difficulties when working with three phytonematode species (*Heterodera avenae*, *H. latipons*, and *Meloidogyne javanica*) and two entomopathogenic species (*Steinernema carpocapsae* and *S. glaseri*) under light microscopy visualization of binding by fluorescent lectins and neoglycoproteins. Moreover, gold-conjugated reagents resulted in stable bindings that enabled long-term observations.

Key words: concanavalin A, fluorescent, glycoconjugate, *Heterodera avenae*, *Heterodera latipons*, lectin, *Meloidogyne javanica*, nematode, neoglycoprotein, *Steinernema carpocapsae*, *Steinernema glaseri*, wheat germ agglutinin.

Various probes applied to intact or sectioned nematode cuticles have been used to characterize and map glycoconjugates on nematode surfaces (1,8,9,11). Most of these probes were fluorescent. Fluorescein isothiocyanate-conjugated lectins or tetramethylrhodamine isothiocyanate-conjugated lectins were used in light microscopy, and ferritin-conjugated lectins were used for electron microscopy. Some of these lectins bound to the head region or the tail region, or both, and rarely to other body areas (2-4,6-8). The occurrence and distribution of several lectin binding sites on outer surfaces of plant-parasitic forms have depended on the nematode's parasitic habit and stage. For example, pre-parasitic second-stage juveniles (J2) of *Anguina tritici*, a seed gall

nematode, showed more affinity for lectins than did adults (10), whereas a greater diversity of lectins bound to females of the root-inhabiting nematodes *Meloidogyne incognita* (10) and *M. javanica* (10) than to J2 of these species.

In addition to lectins, rhodamine and fluorescein hydrazides (for light microscopy) and ferritin hydrazide (for electron microscopy) were synthesized and used to detect glycoconjugates on nematode surfaces. Glycoconjugates were oxidized with sodium metaperiodate or galactose oxidase to form aldehyde groups, which reacted with the respective hydrazides (9,11,12).

We describe the use of two probes, combined with silver enhancement: I. gold-conjugated lectin to detect glycoconjugates; and II. gold-conjugated neoglycoprotein (a saccharide residue linked to bovine serum albumin) to detect carbohydrate-recognition domains using light microscopy.

MATERIALS AND METHODS

Abbreviations: Bovine serum albumin (BSA); concanavalin A (Con A); N-acetylglucosamine (GlcNAc); fluorescein

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isothiocyanate (FITC); phosphate buffered saline, pH 7.4 (PBS); room temperature (RT); second-stage juveniles (J2); tetramethylrhodamine isothiocyanate (TRITC); wheat germ agglutinin (WGA).

Nematodes: *Meloidogyne javanica* was propagated on tomato (*Lycopersicon esculentum* Mill cv. Hosen Eilon) in the greenhouse. Eggs were separated from egg masses with sodium hypochlorite (0.5%, 1 minute) and hatched in PBS to obtain infective J2. *Heterodera avenae* J2 and *H. latipons* J2 were hatched from brown cysts separated from naturally infested soil by sieving and decanting. *Steinernema carpocapsae* and *S. glaseri* were grown on *Galleria mellonella* as described by Dutky et al. (5).

Labelling with FITC- or TRITC-conjugated lectins and neoglycoproteins: Nematodes were washed with PBS and incubated with either Con A and WGA, each conjugated with TRITC (*Steinernema* spp.) or FITC (*Heterodera* and *Meloidogyne* spp.) as described previously (4,10). The conjugated lectins were obtained from Sigma (Rehovot, Israel). Binding specificity was confirmed by preincubation of the lectins, for 30 minutes at RT, with 0.05 M of the following haptens: mannose (Con A) and N-acetylglucosamine or chitobiose (WGA). Protocols for fluorescence microscopy were described previously (4,10).

Neoglycoproteins were used to detect carbohydrate binding sites. *Meloidogyne javanica* J2 were first blocked for 30 minutes with 1% BSA in PBS, washed with 0.1% BSA in PBS, and then incubated for 1 hour with one of the following 0.1-mg/ml FITC-conjugated reagents (Sigma): BSA-FITC (as control), BSA- α (or β)-D-galactopyranosyl-phenylisothiocyanate, BSA- α -L-fucopyranosyl-phenylisothiocyanate, and BSA- α -D-mannopyranosyl-phenylisothiocyanate.

Labelling with gold-conjugated lectins and neoglycoproteins: Juveniles for these experiments were hatched in the presence of Ca^{++} ions. Nematodes were blocked and washed as described above and incubated for 30 minutes at RT in PBS solutions containing 0.1% BSA and 2.5 $\mu\text{g}/\text{ml}$ of gold

(10 nm)-conjugated Con A or WGA in the presence of Ca^{++} , Mg^{++} , and Mn^{++} ions. Protocols for specificity of lectin binding were used as described above. Nematodes were then washed with PBS, 0.1% BSA, and finally with distilled water before incubation for 5–15 minutes with silver enhancing solution (BioCell Cardiff, UK) until labelling could be visualized by light microscopy. Further enhancement was prevented by washing with distilled water. Nematodes treated with the enhancing solution alone served as controls.

Meloidogyne javanica J2 were incubated in 1 $\mu\text{g}/\text{ml}$ gold (10 nm)-conjugated neoglycoproteins, including fucosylated-BSA or mannosylated-BSA or glucosylated-BSA or galNAc-beta-(1,4)gal-beta-O-BSA (E.Y. Laboratories, San Mateo, CA), in PBS containing 0.1% BSA and Ca^{++} and Mg^{++} ions. Preincubation with 0.1 M of fucose, α -methyl mannoside, glucose, and galNAc or galactose was used to verify the specificity. Gold-conjugated BSA was used as a negative control. Washed nematodes were then exposed to silver enhancing solution as described above.

RESULTS AND DISCUSSION

As reported previously (7, Spiegel, unpubl.), FITC-Con A and FITC-WGA labelled only the amphids of *M. javanica* J2. The entire cuticle of *H. avenae* was labelled with FITC-WGA, but no binding was recorded with *H. latipons* (Table 1). TRITC-Con A did not bind to either species of *Steinernema* tested. TRITC-WGA bound to the head region of *S. carpocapsae* and strongly to the amphids of *S. glaseri*.

Gold-Con A labelled the amphidial secretions of *M. javanica* as well as the transverse annulations and lateral alae over the entire nematode surface though less intensively in the head region (Fig. 1). Labelling was inhibited by the appropriate hapten. Gold-WGA labelled the amphidial secretions of *M. javanica*, but the entire nematode surface labelling was much weaker than that recorded with Con A (Table 1). No binding was recorded on *H. latipons*

TABLE 1. Fluorescent- and gold-lectin binding to cuticular surfaces of *Heterodera avenae* (H. a.), *H. latipons* (H.l.), *Meloidogyne javanica* (M.j.), *Steinernema carpocapsae* (S.c.) and *S. glaseri* (S.g.).

Lectin	H. a.	H. l.	M. j.	S. c.	S. g.
			Fluorescent lectin		
Con A	nt	nt	+++a;+b	—	—
WGA	++b;+a	—	++a	h;+l	+a
			Gold lectin		
Con A	nt	nt	+++l;+a	—	—
WGA	++++l	—	++l;+a	+++l	++l

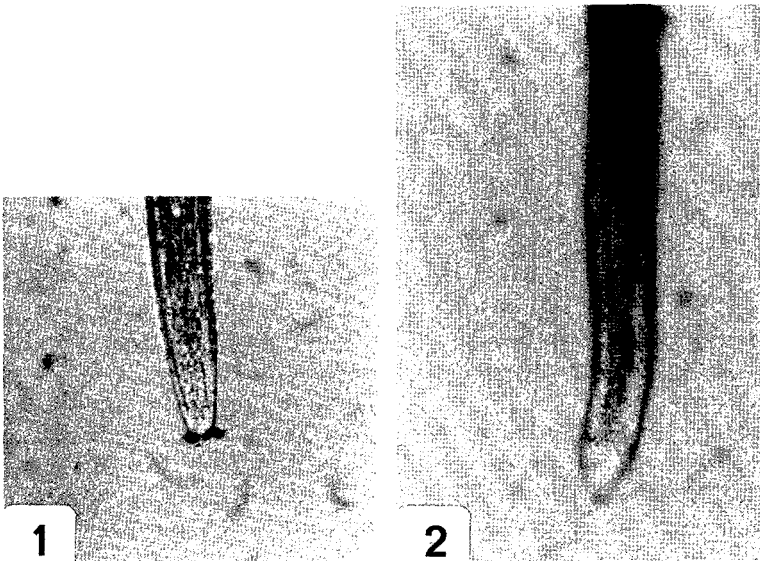
— = no labelling, +b; ++b; +++b = increasing intensities of labelling of entire cuticle, +a, ++a, +++a = increasing intensities of labelling to the amphids, h = labelling of the head area, +l; ++l; +++l; ++++l = increasing intensities of labelling of lateral alae and transverse annulation, nt = not tested.

with gold-WGA, while strong labelling was recorded on the entire cuticle of *H. avenae* with this reagent (Table 1). Binding of gold-WGA to *S. carpocapsae* and *S. glaseri* revealed clear and strong labelling of the transverse annulation and lateral alae, but not of the head region or of the amphidial openings of these species.

No binding was recorded with any of the FITC-conjugated neoglycoproteins tested with *M. javanica* J2. Gold-conjugated fucosylated- and mannosylated-BSA labelled the nematode surface with much the same pattern observed for Con A except that the head region was not labelled (Fig. 2). Fucose and α -methyl mannoside pretreatments reduced these labellings. The gal-

Nac- β -(1-4)gal-O-BSA labelled J2 in a similar pattern, but specificity could not be verified by pretreatment with the appropriate hapten.

Ideally, a cell surface reagent should possess the following properties: its molecules should be small but without the ability to penetrate into the cell, it should react under physiological conditions and form stable bonds, and it should be easily detectable. Fluorescent lectins do not comply perfectly with these requirements, and several problems have been experienced with these reagents: penetration of the cuticle by fluorescent lectins (Spiegel, unpubl.), non-glycoconjugate specificity (4,6), strong nematode autofluorescence at the same



FIGS. 1,2. Anterior end of *Meloidogyne javanica* second-stage juveniles. 1) Labelling with gold-Concanavaline A. 2) Labelling with gold-conjugated fucosylated-BSA (bovine serum albumin).

wavelength as the fluorescent emission of fluorescein or rhodamine (4,6,9), and prevention of persistent visualization due to rapid quenching of the fluorescent components.

The gold labelling method with silver enhancement offers a sensitive, usually specific, clear visualization of labelling. Moreover, it produces stable bindings and so enables long-term observations under light microscopy.

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