Plant Physiol. (1994) 104: 699–710

Generation of Monoclonal Antibodies against Plant Cell-Wall Polysaccharides[1]

I. Characterization of a Monoclonal Antibody to a Terminal α-(1→2)-Linked Fucosyl-Containing Epitope

Jörg Puhlmann[2], Eva Bucheli[3], Michael J. Swain, Nancy Dunning, Peter Albersheim, Alan G. Darvill, and Michael G. Hahn*

The University of Georgia, Complex Carbohydrate Research Center (J.P., E.B., M.J.S., N.D., P.A., A.G.D., M.G.H.), and Departments of Botany (N.D., M.G.H.) and Biochemistry (P.A., A.G.D.), 220 Riverbend Road, Athens, Georgia 30602–4712

Monoclonal antibodies (McAbs) generated against rhamnogalacturonan I (RG-I) purified from suspension-cultured sycamore maple (*Acer pseudoplatanus*) cells fall into three recognition groups. Four McAbs (group I) recognize an epitope that appears to be immunodominant and is present on RG-I from maize and sycamore maple, pectin and polygalacturonic acid from citrus, gum tragacanth, and membrane glycoproteins from suspension-cultured cells of maize, tobacco, parsley, bean, and sycamore maple. A second set of McAbs (group II) recognizes an epitope present in sycamore maple RG-I but does not bind to any of the other polysaccharides or glycoproteins recognized by group I. Lastly, one McAb, CCRC-M1 (group III), binds to RG-I and more strongly to xyloglucan (XG) from sycamore maple but not to maize RG-I, citrus polygalacturonic acid, or to the plant membrane glycoproteins recognized by group I. The epitope to which CCRC-M1 binds has been examined in detail. Ligand competition assays using a series of oligosaccharides derived from or related to sycamore maple XG demonstrated that a terminal α-(1→2)-linked fucosyl residue constitutes an essential part of the epitope recognized by CCRC-M1. Oligosaccharides containing this structural motif compete with intact sycamore maple XG for binding to the antibody, whereas structurally related oligosaccharides, which do not contain terminal fucosyl residues or in which the terminal fucosyl residue is linked α-(1→3) to the adjacent glycosyl residue, do not compete for the antibody binding site. The ligand binding assays also indicate that CCRC-M1 binds to a conformationally dependent structure of the polysaccharide. Other results of this study establish that some of the carbohydrate epitopes of the plant extracellular matrix are shared among different macromolecules.

The plant extracellular matrix comprises a complex array of polysaccharides, glycoproteins, phenolics, and possibly other macromolecules (see reviews by McNeil et al. [1984] and Carpita and Gibeaut [1993]). It has become increasingly clear that this macromolecular complex is a dynamic structure that plays important roles in plant growth and development (Roberts, 1990) and in the interactions of plants with other organisms and the environment (Hahn et al., 1989). Thus, it is important not only to elucidate the chemical structures of the macromolecular components of the plant extracellular matrix, but also to determine how these components are organized within the matrix and how their structures and interrelationships change with time and in response to different stimuli. Molecular probes of known specificity are powerful tools with which to address these questions.

The number and diversity of molecular probes available for studies of the plant extracellular matrix are limited (reviewed by Hoson [1991] and Knox [1992]). Lectins and glycosylhydrolases have been of some value in localizing components in the extracellular matrix. However, the variety and specificity of such probes have been insufficient to localize all molecules present in plant cell walls. The potential of antibodies as specific molecular probes for analyzing the plant extracellular matrix has long been recognized (Roberts et al., 1985). A number of antibody probes have been utilized recently in plant studies to demonstrate cell type- and developmental stage-specific changes in the distribution in plant tissues of the epitopes recognized by these antibodies (Staffstrom and Staehelin, 1988; Knox et al., 1989, 1990, 1991;

[1] This work was supported by grants from the U.S. Department of Energy to M.G.H. (DE-AC05–87ER80507) and to A.G.D. (DE-FG09–85ER13426). Also supported in part by the U.S. Department of Energy-funded Center for Plant and Microbial Complex Carbohydrates (DE-FG09–87ER13810). The Monoclonal Antibody Facility in the Department of Botany at the University of Georgia was funded in part by a grant from the U.S. National Science Foundation (PCM-8315882). J.P. was the recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (Bonn, Germany).

[2] Present address: Hoechst Veterinär GmbH, Feldstrasse 1a, D-85716 Unterschleissheim, Germany.

[3] Present address: Ch. de la Verne 1, CH-1073 Savigny, Switzerland.

* Corresponding author; fax 1–706–542–4412.

Abbreviations: DP, degree of polymerization; Fab, monovalent antibody fragment; IC_{50}, concentration of a competitor required to inhibit the binding of an antibody to its ligand by 50%; McAb, monoclonal antibody; MeBSA, methylated bovine serum albumin; RG-I, rhamnogalacturonan I; TBS, 50 mM Tris-HCl, pH 7.6, containing 500 mM NaCl; XG, xyloglucan.

Stacey et al., 1990; Pennell et al., 1991, 1992; Lynch and Staehelin, 1992; Vian et al., 1992). Antibodies have also been used to observe these epitopes in the biosynthetic and secretory machinery of plant cells (Moore and Staehelin, 1988; Northcote et al., 1989; Moore et al., 1991; Zhang and Staehelin, 1992). The interpretation of these localization studies is limited by the extent to which the specificities of the antibodies have been characterized.

Knowledge of the structures in plant complex carbohydrates that are recognized by the antibodies generated thus far is limited. The binding specificities of polyclonal sera (Danielson and Gray, 1986; Moore et al., 1986; Hoson and Nevins, 1989; Northcote et al., 1989; Sone et al., 1989a, 1989b; Vian et al., 1992; Kikuchi et al., 1993) are inherently difficult to define due to the heterogeneous nature of the antisera. Most of the McAbs generated against plant extracellular matrix components bind the immunodominant arabinogalactan and/or arabinogalactan glycoprotein epitopes (Anderson et al., 1984; Knox et al., 1989, 1991; Pennell et al., 1989, 1991). The epitope structures recognized by these antibodies have not been determined in detail (Anderson et al., 1984; Brewin et al., 1985; Norman et al., 1986; Villanueva et al., 1986; Hahn et al., 1987; Knox et al., 1989, 1991; Pennell et al., 1989, 1991; Horsley et al., 1993). McAbs have also been generated against homogalacturonans (Liners et al., 1989; Knox et al., 1990), callose [(1→3)-β-glucan)] (Meikle et al., 1991), and a mixed linkage glucan (Meikle et al., 1994). There remains a need for well-characterized McAbs directed against components of the plant extracellular matrix.

We have initiated a research program to generate a library of McAbs that recognize diverse epitopes in plant cell-wall polysaccharides and to characterize, in as much detail as possible, the epitope structures recognized by those antibodies. The studies reported here focus on the pectic polysaccharide RG-I. RG-I is a large polysaccharide (M_r around 200,000) present in the primary cell walls of many plants (McNeil et al., 1980), and is probably a component of the walls of all higher plants. RG-I has a backbone consisting of alternating α-2-linked L-rhamnopyranosyl and α-4-linked D-galactopyranosyluronic acid residues (Lau et al., 1985). Some of the rhamnosyl residues have side chains of diverse structures attached to O-4 (McNeil et al., 1982; Lau et al., 1985, 1987; Ishii et al., 1989; Thomas et al., 1989).

RG-I has been used previously to generate a polyclonal antiserum, albeit of low titer (Moore et al., 1986). Those studies demonstrated that RG-I is immunogenic. We report here the generation of the first stable hybridoma cell lines that secrete McAbs against RG-I. The reactivities of these McAbs with several different plant cell-wall polysaccharides are described. In addition, we describe the characterization of the epitope structure recognized by CCRC-M1, an anti-RG-I McAb that binds even more strongly to XG, a hemicellulosic polysaccharide present in primary plant cell walls.

MATERIALS AND METHODS

Oligo- and Polysaccharides

RG-I, purified as described (Lau et al., 1985; Lerouge et al., 1993) from the mixture of pectic polysaccharides released by endopolygalacturonase from the cell walls of suspension-cultured sycamore maple (*Acer pseudoplatanus*) cells, was provided by D. Gollin and A. Whitcombe of this laboratory. XGs, prepared as described (York et al., 1990) from tamarind (*Tamarindus indicus*) seed and rapeseed (*Brassica napus*), were donated by W.S. York of this laboratory. XG from sycamore maple was purified as described (York et al., 1985) from the mixture of extracellular polysaccharides present in the medium of suspension cultures of these cells. RG-II, prepared as described (York et al., 1985; Marfá et al., 1991) from the mixture of pectic polysaccharides released by endopolygalacturonase from the cell walls of suspension-cultured sycamore maple cells, was obtained from A. Whitcombe (of this laboratory) and covalently coupled, as described (Smith and Ginsburg, 1980), to ovalbumin by W. Steffan of this laboratory. Arabinoxylan from wheat flour was obtained from Megazyme (North Rocks, Australia). Larchwood arabinogalactan, gum arabic from *Acacia senegal*, gum tragacanth from *Astragalus gummifer*, and polygalacturonic acid (sodium salt) were purchased from Sigma. A mixture of β-glucan fragments ("Void glucan" elicitor) was prepared by E. Bucheli of our laboratory from acid hydrolysates of the mycelial walls of *Phytophthora megasperma* f. sp. *glycinea* (Hahn et al., 1992). The mol wt estimates used to calculate molar concentrations of the above polysaccharides are: XG = 100,000; RG-I = 200,000; fungal β-glucan = 100,000; and wheat arabinoxylan = 100,000.

The structures of the oligosaccharides used in this study are shown in Figure 1. Oligosaccharides **1** and **2**, and a mixture of octasaccharides **3** and **4**, which were purified from endoglucanase digests of rapeseed and tamarind seed XGs (York et al., 1990), were the gift of W.S. York of this laboratory. Compounds **5** and **6** were synthesized (Sakai et al., 1990) and donated by T. Ogawa and his colleagues (RIKEN, Laboratory of Synthetic Cellular Chemistry, Wako, Japan). Oligosaccharide **7** was synthesized and donated by P. Sinaÿ and his colleagues (École Normale Supérieure, Laboratoire de Chimie, Paris, France). Trisaccharide **8**, L-Fuc, L-rhamnose, D-Gal, 2-deoxy-D-Gal, and β-lactose were purchased from Sigma. Trisaccharides **9** and **10** and disaccharide **11** were obtained from BioCarb Chemicals (Lund, Sweden).

Chemicals and Other Supplies

All reagents used were of the highest grade available. *p*-Nitrophenyl phosphate and 3,3′,5,5′-tetramethylbenzidene were from Pierce (Rockford, IL). 4-Chloro-1-naphthol, poly-L-Lys (M_r 70,000–150,000), ovalbumin, BSA (fatty acid free), and MeBSA were purchased from Sigma. Horse serum was from HyClone Laboratories (Logan, UT). Flat-bottom microtiter plates, either polystyrene (Nunc) or Immulon 2 (Dynatech), were obtained from VWR Scientific (Marietta, GA) and Fisher Scientific (Pittsburgh, PA), respectively. Hybond-N^+ nylon membranes were purchased from Amersham. Nitrocellulose (BA85) was purchased from Schleicher & Schuell.

Plant Materials

Bean (*Phaseolus vulgaris* L. cv Pinto) seeds, obtained from a local seed merchant, were planted and grown as described

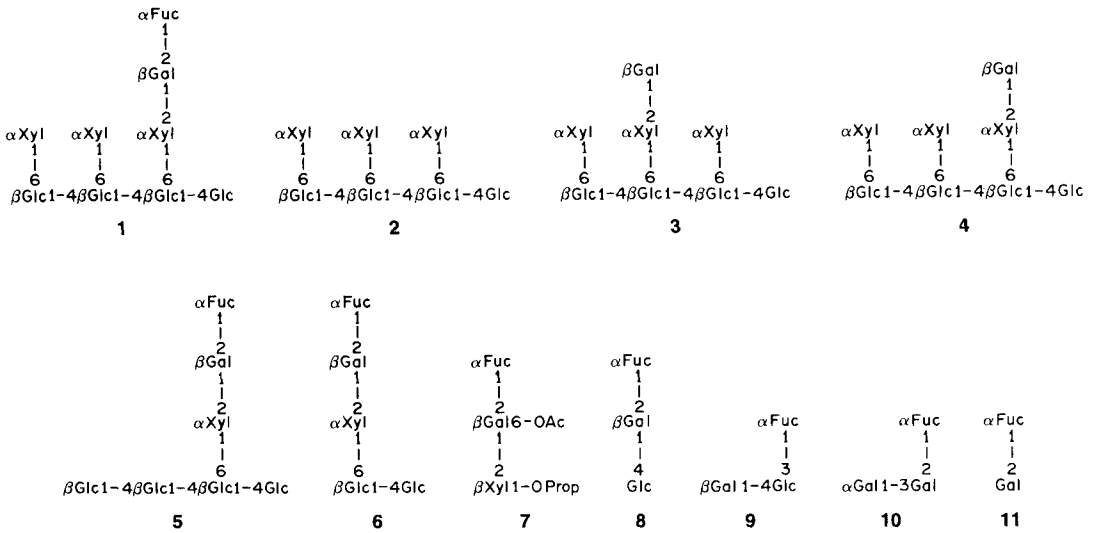

Figure 1. Structures of oligosaccharides used as competitive ligands for CCRC-M1.

(De Lorenzo et al., 1990), except that the plants were grown in continuous darkness for 6 d. Bean hypocotyls were harvested, sliced into small sections (1 cm), frozen in liquid nitrogen, and stored at −70°C.

Liquid cell-suspension cultures of maize (*Zea mays* L. cv Black Mexican Sweet), rice (*Oryza sativa* L. cv Yamabiko), and sycamore maple (*Acer pseudoplatanus* L.), which had been grown as described (Doares et al., 1989), were obtained from S. Doares of this laboratory. A liquid cell-suspension culture of parsley (*Petroselinum hortense* L.), which had been grown as described (Ragg et al., 1981), was obtained from J.-J. Cheong of this laboratory. A cell-suspension culture of tobacco (*Nicotiana tabacum* L.) was obtained from C.J. Lamb (Salk Institute, La Jolla, CA) and was maintained as described (Norman et al., 1986). Cells from actively dividing cultures were harvested by filtration on a porous glass filter, air dried for 2 min under suction on the filter, and frozen in liquid nitrogen. Frozen cells were stored at −70°C until needed.

Membrane Preparation

Total membrane fractions were prepared from frozen bean hypocotyl sections or suspension-cultured cells of various plants by grinding frozen tissue to a powder in liquid nitrogen using a mortar and pestle. Homogenization buffer (25 mM Tris-HCl, pH 7.4, containing 250 mM Suc, 3 mM EDTA, and 5 mM DTT) was added to the frozen cell powder (3 mL/g fresh weight of cells) and the resultant slush was allowed to come to 4°C. All subsequent steps were carried out at 4°C. The cell homogenate was then filtered through a double layer of Miracloth and the filtrate was centrifuged at 2,000*g* for 10 min. The pellet resulting from the low-speed centrifugation of bean hypocotyl extracts was retained and stored at −70°C ("bean cell wall"). The supernatants from the low-speed centrifugations were decanted and centrifuged at 100,000*g* for 1 h. The supernatants were discarded and the membrane pellets were resuspended in homogenization buffer and stored at −70°C until needed. Protein concentrations of the membrane preparations were estimated by the Bio-Rad Protein Assay based on the method of Bradford (1976) using bovine γ-globulin as the standard.

Glycosyl Residue Composition Analysis

Glycosyl residue composition analyses were performed using the alditol acetate method as described (York et al., 1985).

Antibodies

The generation and characterization of McAbs MH 3.2B4, MH 4.2A5, MH 4.3E5, and MH 4.4E4 have been described elsewhere (Hahn et al., 1987). McAbs PN 16.1B3 and PN 16.1B4, originally generated from mice immunized with membranes prepared from suspension-cultured cells of *Nicotiana glutinosa* (Norman et al., 1986), were obtained from C.J. Lamb (Salk Institute, La Jolla, CA). Goat anti-mouse Ig conjugated to horseradish peroxidase was purchased either from Bio-Rad or from Pierce. Goat anti-mouse Ig conjugated to alkaline phosphatase was from Pierce. Goat anti-mouse κ light chain-specific antibody coupled to horseradish peroxidase was purchased from Fisher Scientific. Antibody-enzyme conjugates were used in solution as supplied or reconstituted with deionized water according to the suppliers' instructions.

Generation of Hybridomas

Immunogen was prepared essentially following a protocol that was first used to generate antibodies against acidic algal polysaccharides (Vreeland, 1970) and that had also been used to generate a polyclonal antiserum against RG-I (Moore et al., 1986). The RG-I was mixed with an equal amount (weight basis) of MeBSA in distilled water. The polysaccharide/MeBSA mixtures were injected into female Balb/c mice (obtained from Harlan-Sprague Dawley, Indianapolis, IN) according to one of the three immunization schedules described below.

Schedule A (three mice) consisted of one intraperitoneal injection of a mixture of 100 μL containing 100 μg each of RG-I and MeBSA and 100 μL of complete Freund's adjuvant (Freund, 1956). The initial injection was followed 4 weeks later by an intraperitoneal injection of the same amount of immunogen with incomplete Freund's adjuvant (Freund, 1956). A final injection of 50 μg of RG-I and 50 μg of MeBSA in 50 μL of sterile water was given intravenously 4 d later. Splenectomy was performed 3 d after the final immunization.

Schedule B (three mice) consisted of one intraperitoneal injection of a mixture of 100 μL containing 100 μg each of RG-I and MeBSA and 100 μL of complete Freund's adjuvant. The initial injection was followed by four weekly intraperitoneal injections of the same amount of immunogen with incomplete Freund's adjuvant. A final injection of 50 μg of RG-I and 50 μg of MeBSA in 50 μL of sterile water was given intravenously 3 d prior to splenectomy (32 d after initial injection).

Schedule C (two mice) consisted of one intraperitoneal injection of a mixture of 500 μL of PBS (10 mM sodium phosphate, pH 7.2, containing 150 mM NaCl) containing 70 μg each of RG-I and MeBSA and 500 μL of complete Freund's adjuvant. The initial injection was followed 2 weeks later by an intraperitoneal injection of the same amount of immunogen with incomplete Freund's adjuvant. A third intraperitoneal immunization with the same amount of immunogen in 1 mL of PBS, but without adjuvant, followed 10 weeks later. Another injection with 25 μg of RG-I and 25 μg of MeBSA in 1 mL of PBS was given 2 weeks later. This injection was repeated twice more at 2-week intervals and splenectomy was performed 4 d after the final immunization.

Fusions were performed using freshly isolated splenic lymphocytes from one mouse each from the schedule A and B immunizations, and two mice from the schedule C immunizations. Lymphocytes were fused with a murine myeloma cell line (SP2/0-Ag14 [Shulman et al., 1978] for A and B, and X63-Ag8.653 [Kearney et al., 1979] for C) and the resulting hybridomas were grown as described (Pratt, 1984; Zola, 1987; Harlow and Lane, 1988). Hybridoma culture supernatants from initial hybridoma colonies were screened for antibodies against sycamore maple RG-I using a dot-blot immunoassay. Positive cell lines were expanded, cloned by limiting dilution, cryopreserved, and at least 1 L of their culture supernatants was collected for further analyses.

Immunoassays

Dot-Blot Assay

A piece of nitrocellulose membrane was prewetted in TBS and placed in a dot-blot manifold (Bio-Rad). Polysaccharide antigen solutions (50 μL), made up in deionized water, were applied to the membrane (20 μg/dot). The antigen solutions and all subsequent solutions, with the exception of the washes, were allowed to filter through the membrane by gravity flow. After washing the dots with TBS (200 μL), nonspecific antibody-binding sites were blocked with 5% (v/v) horse serum in TBS (200 μL). The dots were then washed with TBS containing 0.1% (v/v) horse serum. Hybridoma culture supernatants (50 μL) were applied to the dots. After the antibody incubation, the dots were washed with TBS containing 0.1% horse serum (3 × 200 μL) and then treated with goat anti-mouse Ig-horseradish peroxidase conjugate (diluted 1:3000 in TBS containing 0.1% horse serum). After another washing cycle with TBS containing 0.1% horse serum, the membrane was removed from the manifold and bound antibodies were visualized with 4-chloro-1-naphthol as described (Hahn et al., 1987). Color development was stopped by washing the membrane in deionized water.

Competitive dot-blot immunoassays with CCRC-M1 were carried out as described above except for the following modifications. Hybond-N^+ nylon membranes were used as the solid support. Sycamore maple XG (about 0.25 pmol/dot [25 ng/dot]) was used as the immobilized antigen. CCRC-M1 hybridoma culture supernatant (100 μL) was preincubated with various concentrations of the oligosaccharide or polysaccharide competitors in TBS for 4 h at 37°C prior to application to the dots. Control hybridoma culture supernatant was preincubated under the same conditions in the absence of competitors. Dot intensities were quantified with two-dimensional densitometric scans using a Bio-Rad video densitometer and software provided by the manufacturer. Background readings for dots without sycamore maple XG that had been incubated with untreated CCRC-M1 hybridoma supernatant were subtracted from all values. Competitor inhibition of antibody binding to the immobilized XG is reported as a percentage of the signal obtained with the antibody in the absence of competitors.

ELISA

Solutions (50 μL) of polysaccharides were prepared in 20 mM potassium phosphate, pH 7.8, unless otherwise indicated, and applied to the wells of a flat-bottom microtiter plate. The polysaccharide solutions were allowed to evaporate to dryness overnight at 37°C. All subsequent operations were carried out at room temperature. Nonspecific antibody binding sites were blocked by incubation for at least 1 h with 1% (w/v) ovalbumin in TBS (200 μL). Solutions were removed from the wells at the end of incubations by flicking the plate over a sink and blotting residual solution droplets adhering to the plate on a paper towel. Hybridoma culture supernatant solutions (50 μL) were added to the wells and incubated for 1 h. The wells were then washed with TBS containing 0.1% (w/v) ovalbumin (3 × 200 μL). McAb binding was detected using one of the following two secondary antibody-enzyme conjugates. Wells were incubated for 1 h with 50 μL of goat anti-mouse Ig-alkaline phosphatase conjugate (diluted 1:1000 in TBS) and then washed with TBS (5 × 200 μL). The alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as substrate (50 μL of a 1 mg/mL solution in 100 mM sodium bicarbonate, pH 9.5, containing 1 mM $MgCl_2$). The alkaline phosphatase was inactivated after 30 min by addition of 3 N NaOH (50 μL). The *p*-nitrophenol liberated was quantified by measuring the difference in A_{405} versus A_{492} using a microplate reader (Flow Laboratories, McLean, VA) interfaced with a computer (software supplied by Lee Pratt, Department of Botany, University of Georgia). Alternatively, goat anti-mouse Ig-horseradish peroxidase

conjugate (1:5000 dilution in TBS) was used. The wells were washed as above. Peroxidase was assayed with 3,3′,5,5′ tetramethylbenzidene (100 μL of a 0.1 mg/mL solution in 50 mM sodium phosphate, pH 5.0, containing 24 mM citric acid and 0.03% H_2O_2) as substrate. The peroxidase was inactivated after 4 min by addition of 0.5 N H_2SO_4 (100 μL) and the difference in A_{450} versus A_{620} was measured as above.

Competitive ELISAs were carried out as above except for the following modifications. Sycamore maple XG (about 0.25 pmol [25 ng]/well) was immobilized on the bottom of poly-L-Lys-coated microtiter plate wells as described (Ohbayashi et al., 1989). CCRC-M1 hybridoma culture supernatants were preincubated with different concentrations of oligo- or polysaccharides in TBS for 4 h at 37°C prior to application to the wells. Calculations for competitor activities were analogous to those described above for the competitive dot-blot assay.

Western Blots

Total cellular membranes (1 mL, containing 6–12 mg of protein) were diluted with 2 mL of 50 mM Tris-HCl, pH 7.4, containing 500 mM NaCl and 1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and the resulting suspension was gently stirred for 1 h at 4°C. Insoluble membrane debris was pelleted by centrifugation at 100,000*g* for 1 h. Solubilized membrane proteins (40 μg) were applied to a discontinuous SDS-polyacrylamide gel (5% acrylamide stacking gel, 10% acrylamide running gel) and electrophoresis was carried out in the presence of 0.1% (w/v) SDS as described (Laemmli, 1970). A sample of prestained mol wt standards (Bio-Rad) was also run on the same gel. Immunoblotting of the separated proteins was carried out as described (Towbin et al., 1979; Hahn et al., 1987).

Isotyping of McAbs

The immunoglobin classes to which the various McAbs belonged were determined using the HyClone Mouse Monoclonal Sub-isotyping kit (HyClone Labs) according the instructions supplied with the kit.

Detection of Periodate-Sensitive Epitopes

The periodate sensitivity of epitopes in RG-I recognized by various McAbs was determined as described (Woodward et al., 1985), except that the polysaccharide was attached to the microtiter plates and the ELISA was carried out as described above.

Preparation of CCRC-M1 Fab

CCRC-M1 was purified from hybridoma culture supernatants using a Quick Mab antibody purification column (Sterogene Bioseparations, Inc., Arcadia, CA) according to the manufacturer's instructions. Purified CCRC-M1 antibody (72 μg) in 300 μL of digestion buffer (100 mM sodium acetate, pH 5.5, containing 50 mM Cys and 1 mM EDTA) was mixed with 100 μL of a 50% (v/v) suspension of immobilized papain (Pierce) in digestion buffer and incubated overnight with constant mixing at 37°C. Proteolytic digestion was stopped by centrifuging the reaction mixture in a tabletop centrifuge and decanting the supernatant. The degree of hydrolysis of CCRC-M1 was determined by SDS-PAGE using a PhastGel gradient gel (10–15% acrylamide, Pharmacia) according to the supplier's instructions. Protein bands were visualized by silver staining as described (Blum et al., 1987). No intact heavy chain was observed on the gel (data not shown), indicating that formation of the Fab had gone to completion.

RESULTS

Selection and Characterization of Hybridoma Cell Lines Secreting Antibodies against RG-I

Dot-blot immunoassays were used to screen hybridoma culture supernatants for antibodies reactive with sycamore maple RG-I. A total of 2085 hybridoma lines were screened, yielding 68 positive lines. Twelve of these 68 lines were cloned by limiting dilution. Ten of these 12 lines came from a single mouse from immunization schedule C. Of these 12 lines, 7 were stable through cryopreservation, thawing, and recloning. The antibodies secreted by the hybridoma lines belong to one of two isotype classes: IgM (CCRC-M2, CCRC-M8, CCRC-M9) and IgG_1 (CCRC-M1, CCRC-M5, CCRC-M7, CCRC-M12). All light chains are of the κ class.

Effect of Periodate Treatment on the Binding of McAbs to Sycamore Maple RG-I

Pretreatment of sycamore maple RG-I with 10 mM periodate under mildly acidic conditions abolishes more than 90% of the binding of six of the seven antibodies generated in this study, and 73% of the seventh (CCRC-M9). Under acidic conditions, periodate cleaves vicinal hydroxyl groups (Bobbitt, 1956), such as those found in many carbohydrate structures. The results of the periodate treatment provide evidence that the epitopes in RG-I recognized by the antibodies generated in this study are at least in part carbohydrate in nature.

Binding of the McAbs to Plant Cell-Wall Polysaccharides

The ability of the antibodies obtained in this study to bind to plant cell-wall polysaccharides was tested in ELISAs. Several McAbs previously generated against *N. tabacum* mesophyll protoplasts (MH series) (Hahn et al., 1987) or *N. glutinosa* membranes (PN series) (Norman et al., 1986) were also included in the screens. The seven antibodies generated in the present study fall into three groups based on their abilities to bind to different plant cell-wall polysaccharides (Fig. 2). Group I antibodies bind to RG-I from sycamore maple and maize and to gum tragacanth (Fig. 2A). The MH series McAbs and PN 16.1B3 also belong to this group. Group II antibodies bind only to sycamore maple RG-I (Fig. 2B). The group III antibody binds strongly to XG and RG-I from sycamore maple (Fig. 2C). PN 16.4B4, a McAb that binds to an arabinogalactan glycoprotein in *N. glutinosa* (Norman et al., 1990), binds, in our studies, only to gum arabic (data not shown).

Binding of the McAbs to Detergent-Solubilized Plant Membrane Proteins

The ability of one representative McAb from each group (see Fig. 2) to bind to proteins solubilized from plant mem-

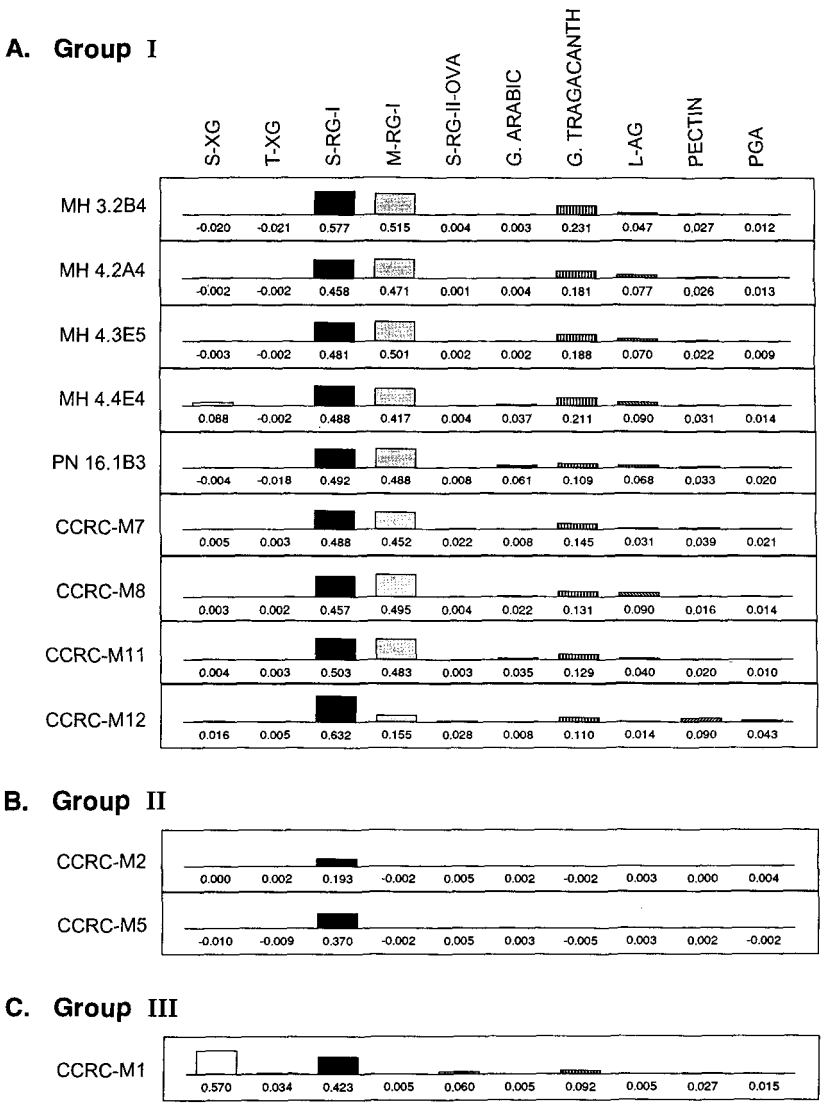

Figure 2. Abilities of McAbs to bind to a variety of plant cell-wall polysaccharides in ELISAs. Polysaccharides tested were: sycamore maple XG (S-XG); tamarind seed XG (T-XG); sycamore maple RG-I (S-RG-I); maize RG-I (M-RG-I); sycamore maple RG-II-ovalbumin conjugate (S-RG-II-OVA); gum arabic; gum tragacanth; larchwood arabinogalactan (L-AG); citrus pectin; and polygalacturonic acid (PGA). Polysaccharides (50 ng/well) were applied to Immulon 2 microtiter plates and ELISAs were performed as described in "Materials and Methods." All antibodies were tested at the same time and the assay was repeated three times. A representative assay is shown. The numbers below each bar are the A_{405} to A_{620} corrected for the background reading obtained without antigen.

brane preparations was determined in protein immunoblots. The antibodies in groups II and III (represented by CCRC-M2 and CCRC-M1, respectively) do not bind to the solubilized membrane proteins (Fig. 3). In contrast, CCRC-M7, a representative of group I, binds polydisperse species from several plants with M_r's between 39,000 and 130,000. ELISAs confirmed that the McAbs in group I bind to epitopes present in the membranes of several species, whereas the group II and group III antibodies do not (data not shown).

Fuc Content of Cell-Wall Polysaccharides

The Fuc contents of several cell-wall polysaccharides, given in Table I, correlate with the ability of CCRC-M1 to bind to the polysaccharides (see below).

Binding of CCRC-M1 to Cell-Wall Polysaccharides

The binding of CCRC-M1 to XGs from several sources and to sycamore maple RG-I was determined first in indirect ELISAs. CCRC-M1 binds most efficiently to XGs from rapeseed and sycamore maple (data not shown). The detection range for rapeseed XG is between 0.03 and 20 ng/well, and for sycamore maple XG it is between 0.08 and 20 ng/well (half-maximum binding for both at 0.5 ng/well). Sycamore maple RG-I, the immunogen used to generate CCRC-M1, is recognized less efficiently by the antibody, having a detection range about 100 times higher (8–4000 ng/well). In contrast, the XG isolated from tamarind seed is very weakly detected by CCRC-M1, binding maximally only about 10% as much of CCRC-M1 as the XGs from the other plants, and that only when at least 1000 times more tamarind seed XG is applied to the microtiter plate.

The differences observed in indirect ELISAs in the binding of CCRC-M1 to the different polysaccharides could potentially have arisen from a differential ability of the polysaccharides to adhere to the microtiter plates. As a control for this possibility, competitive ELISAs were performed in which sycamore maple XG was used as the immobilized antigen and various polysaccharides were used to competitively inhibit the binding of CCRC-M1 to the immobilized XG. All

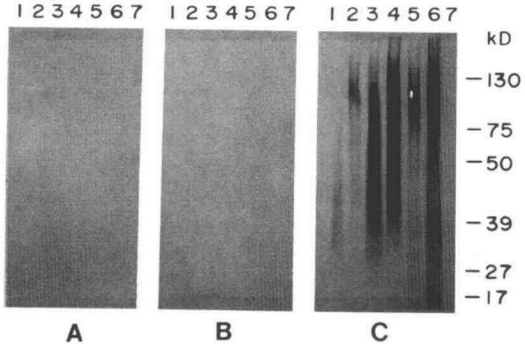

Figure 3. Western immunoassay of binding of McAbs to detergent-solubilized plant membrane proteins. Detergent-solubilized proteins (14 μg/lane for tobacco, 40 μg/lane for all others) were separated by SDS-PAGE, and immunoblotting of the separated proteins was carried out as described in "Materials and Methods." Samples loaded were: lanes 1, tobacco; lanes 2, sycamore maple; lanes 3, rice; lanes 4, parsley; lanes 5, bean cell wall; lanes 6, bean; lanes 7, molecular mass standards. McAbs used as primary antibodies were CCRC-M2 (A), CCRC-M1 (B), and CCRC-M7 (C). Positions of molecular mass markers are indicated.

three of the XGs tested, as well as sycamore maple RG-I, competitively inhibit the binding of CCRC-M1 to immobilized sycamore maple XG, albeit with different efficiencies (Fig. 4). The most effective competitor is rapeseed XG. Sycamore maple XG is 2-fold less effective, whereas sycamore maple RG-I and tamarind seed XG are approximately 100- and 1000-fold less effective as competitors, respectively (Table I). An arabinoxylan from wheat and a fungal β-glucan preparation from *P. megasperma*, at the highest concentrations tested (10 and 100 mg/mL, respectively), do not compete with sycamore maple XG for binding to CCRC-M1 (Table I).

Ligand Competition Immunoassays Using Oligo- and Monosaccharides

The epitope on XG recognized by CCRC-M1 was further characterized using the collection of oligosaccharides shown in Figure 1. Since attempts to bind the oligosaccharides directly to insoluble supports (either nylon membrane or plastic microtiter plate) failed, the relative affinity of CCRC-M1 for the various oligosaccharides was investigated in competitive ligand binding studies against sycamore maple XG. Of the tested oligosaccharides released from XG by endoglucanase digestion (compounds **1**-**4**), only nonasaccharide **1** competes with intact XG for antibody binding (Fig. 5). In fact, nonasaccharide **1** is the most effective competitor of all the oligosaccharides tested (IC_{50} = 13 μM) (Table II). Neither the heptasaccharide (compound **2**) nor a mixture of two isomeric octasaccharides (compounds **3** and **4**), which all lack the terminal fucosyl residue, measurably inhibit binding of CCRC-M1 to intact XG, even at the highest concentration tested (500 μM). These results confirm that the terminal fucosyl residue is an essential structural element of the epitope recognized by CCRC-M1.

A number of synthetic or commercially available Fuc-containing oligosaccharides structurally related to XG were tested for their ability to compete with intact XG for binding to CCRC-M1 (Fig. 5). Those oligosaccharides that contain a terminal fucosyl residue linked α-(1→2) to a galactosyl residue compete with intact XG in proportion to the size of the oligosaccharide. Thus, hepta- and pentasaccharides **5** and **6**, which contain the complete Fuc-containing side chain of XG plus four and two backbone glucosyl residues, are 2- and 3-fold less efficient competitors than nonasaccharide **1** (Table II). Trisaccharides **7**, **8**, and **10** are less effective competitors, each having an IC_{50} about 100-fold higher than the nonasaccharide. Disaccharide **11** has an IC_{50} about 500 times higher than the nonasaccharide **1**. The only oligosaccharide examined that contains a terminal fucosyl residue but does not competitively inhibit CCRC-M1 binding to intact XG over the concentration range tested (≤10 mM) is trisaccharide **9**

Table I. *Comparison of the Fuc content of selected polysaccharides with their relative abilities to inhibit the binding of CCRC-M1 to sycamore maple XG*

Polysaccharide	IC_{50}[a]	Fuc[b]
	nM	*mol %*
Rapeseed XG	0.49 ± 0.27	16
Sycamore maple XG	1.0 ± 0.36	15
Sycamore maple RG-I	54 ± 4.1	3.3
Tamarind seed XG	630 ± 44	0
Fungal β-glucan	$>10^6$	0
Wheat arabinoxylan	$>10^5$	n.d.

[a] The IC_{50} is defined as the concentration of the polysaccharide required to give 50% inhibition of the binding, in competitive ELISAs, of CCRC-M1 to immobilized sycamore maple XG (see Fig. 3). Data are the averages of three replicates ± SD. [b] Fuc content determined using the alditol acetate method as described in the text. n.d., Not determined.

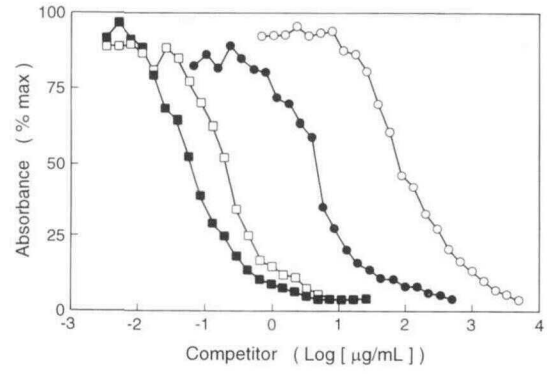

Figure 4. Competitive inhibition of the binding of CCRC-M1 to sycamore maple XG by plant cell-wall polysaccharides. Sycamore maple XG (5 nmol/well) was immobilized to the bottom of polylysine-coated flat-bottom microtiter plates and nonspecific antibody binding sites were blocked with BSA as described in "Materials and Methods." CCRC-M1 hybridoma culture supernatants were preincubated for 4 h at 37°C with various concentrations of another polysaccharide prior to application to the plates. The ELISAs were carried out as described in "Materials and Methods." The polysaccharides tested as competitors were: rapeseed XG (■), sycamore maple XG (□), tamarind seed XG (○), sycamore maple RG-I (●).

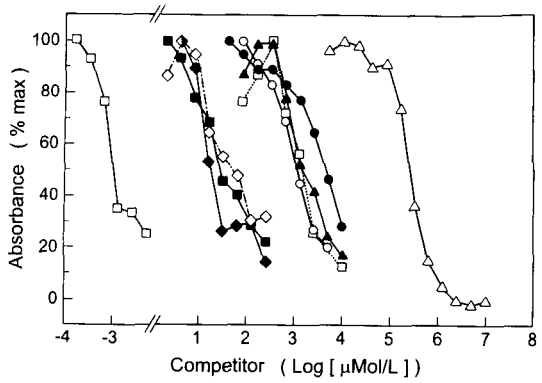

Figure 5. Competitive inhibition by Fuc-containing oligosaccharides of binding of CCRC-M1 to sycamore maple XG. Sycamore maple XG (5 nmol/dot) was applied to a sheet of Hybond-N+ membrane and nonspecific antibody binding sites were blocked with horse serum as described in "Materials and Methods." CCRC-M1 hybridoma culture supernatants were preincubated with various concentrations of an oligosaccharide for 4 h at 37°C prior to application to the dots. The dot-blot assay was carried out as described in "Materials and Methods." Competitors used were: nonasaccharide **1** (—♦—), heptasaccharide **5** (—■—), pentasaccharide **6** (·—◇—·), trisaccharide **7** (···□···), trisaccharide **8** (—▲—), trisaccharide **10** (—○—), disaccharide **11** (—●—), and Fuc (—△—). The structures of the numbered oligosaccharides are shown in Figure 1. The competition curve for sycamore maple XG (—□—) is shown for reference.

(Table II), which contains a terminal fucosyl residue linked α-(1→3) to a glucosyl residue (Fig. 1). Lactose [β-Gal-(1→4)-Glu] is not recognized by CCRC-M1, even at a concentration of 2.5 M (Table II).

Several monosaccharides were tested for their ability to inhibit binding of CCRC-M1 to intact XG. L-Fuc is the only monosaccharide tested that is able to inhibit binding of CCRC-M1 to XG (Fig. 5), although its IC_{50} of 170 mM makes it roughly 10^5 times less effective than nonasaccharide **1** (Table II).

Comparison of the Abilities of CCRC-M1 Fab and CCRC-M1 to Bind to Sycamore Maple XG

The abilities of sycamore maple XG and XG nonasaccharide (compound **1**) to prevent the binding of CCRC-M1 and the Fab derived from CCRC-M1 to immobilized sycamore maple XG were compared to estimate the binding constant for the CCRC-M1 binding site. CCRC-M1 and its Fab are equally inhibited from binding to immobilized XG by sycamore maple XG (Fig. 6). The apparent K_d for the binding of CCRC-M1 to XG is about 5 nM. Nonasaccharide **1** (Fig. 1) is about 10-fold more effective in inhibiting binding of the CCRC-M1 Fab to immobilized sycamore maple XG than in inhibiting binding of the intact antibody.

DISCUSSION

The McAbs described in this report fall into three groups, each with a distinct pattern of recognition of different plant cell-wall polysaccharides. Two of these groups (groups II and

Table II. *Comparison of the relative abilities of mono- and oligosaccharides and sycamore maple XG to inhibit the binding of CCRC-M1 to immobilized sycamore maple XG*

Compound[a]	IC_{50}[b]
	mM
Sycamore maple XG	0.000001 ± 0.00000036
XG oligosaccharides (DPs 17–22)[c]	0.0063 ± 0.0003
Nonasaccharide **1**	0.013 ± 0.012
Heptasaccharide **5**	0.027 ± 0.023
Pentasaccharide **6**	0.036 ± 0.034
Heptasaccharide **2**	>0.5
Mixture of octasaccharides **3** and **4**	>0.5
Trisaccharide **10**	1
Trisaccharide **7**	2 ± 0.2
Trisaccharide **8**	2 ± 0.2
Disaccharide **11**	6.3 ± 0.69
Trisaccharide **9**	>10
L-Fuc	170 ± 40
Lactose	>2,500
2-Deoxy-D-Gal	>3,300
L-Rhamnose	>10,000
D-Gal	>10,000

[a] Compound numbers refer to oligosaccharides whose structures are given in Figure 1. [b] The IC_{50} is defined as the concentration of a mono- or oligosaccharide required to give 50% inhibition of the binding of CCRC-M1 to immobilized sycamore maple XG (see Fig. 5). Competitive dot-blot assays were carried out as described in "Materials and Methods." Data are the averages of three independent assays ± SD, with the exception of compound **10**, which was only assayed twice. [c] Mixture of XG oligosaccharides released from sycamore maple XG by endoglucanase digestion (Hisamatsu et al., 1992).

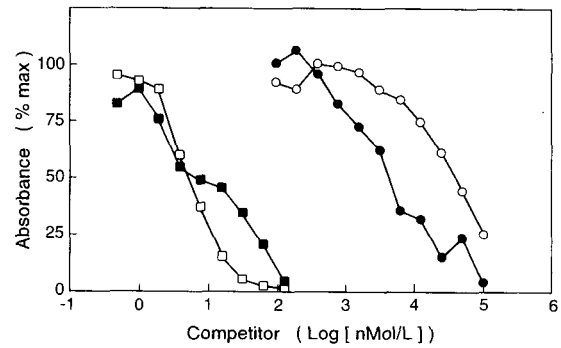

Figure 6. Competitive inhibition by sycamore maple XG and XG nonasaccharide of the binding of CCRC-M1 and its Fab to XG. Sycamore maple XG (50 ng/well) was immobilized on the bottom of poly-L-Lys-coated microtiter plate well as described in "Materials and Methods." Purified CCRC-M1 (38 ng/mL) (□, ○) and its Fab (380 ng/mL) (■, ●) were preincubated for 3 h at 37°C with either sycamore maple XG (■, □) or nonasaccharide **1** (●, ○) prior to application to the plate. Bound antibody and Fab were detected using goat anti-mouse κ light chain-horseradish peroxidase conjugate (1:2000 in TBS containing 0.2% ovalbumin) and tetramethylbenzidene as the substrate as described in the text. Data are reported as a percentage of controls that had been preincubated in the absence of competitors.

III) have a pattern of reactivity (Fig. 2) that differs from all McAbs against plant complex carbohydrates reported to date.

The epitope in plant arabinogalactans recognized by the group I antibodies differs from epitope(s) recognized by McAbs against arabinogalactans obtained in other studies (Anderson et al., 1984; Hahn et al., 1987; Knox et al., 1989, 1991; Pennell et al., 1989; Norman et al., 1990; Horsley et al., 1993). At least four of the previously generated anti-arabinogalactan antibodies bind to gum arabic (Knox et al., 1989; Pennell et al., 1989, 1991), a polysaccharide that does not bind significantly to any of the antibodies described in the present study (Fig. 2).

The group II McAbs (e.g. CCRC-M2 and CCRC-M5) are the first that bind to RG-I without binding to other pectic polysaccharides. There is only one known structural difference between the RG-Is of cereals and dicots: the absence of fucosyl residues in cereal RG-I (Thomas et al., 1989). However, the failure of the group II antibodies to bind other polysaccharides that have terminal fucosyl residues (Fig. 2B) makes it unlikely that the terminal fucosyl residues are a major part of the epitope recognized by these antibodies.

CCRC-M1 is the first McAb that binds an epitope in XG, a hemicellulosic polysaccharide. It is noteworthy that CCRC-M1 binds to XG from sycamore maple but not to tamarind seed XG. The only known structural difference between the two XGs is the absence of terminal fucosyl residues in tamarind seed XG (York et al., 1990). Terminal fucosyl residues are also present in sycamore maple RG-I (McNeil et al., 1980).

Several workers have reported the generation of polyclonal antisera against XG using, as immunogen, either the intact polysaccharide (Moore et al., 1986; Vian et al., 1992) or XG oligosaccharides covalently linked to protein (Sone et al., 1989a, 1989b). XG oligosaccharides have been used in competitive immunoassays to demonstrate the specificity of a polyclonal antiserum for xylose-containing epitopes in XG from tamarind seed (Sone et al., 1989a, 1989b). However, the ability of that antiserum to bind to other plant cell-wall polysaccharides was not examined. Another polyclonal antiserum against XG (Moore et al., 1986) binds to sycamore maple and tamarind XGs, to a graminaceous monocot β-(1→3)-(1→4)-linked glucan, and to solubilized but not to crystalline cellulose (Lynch and Staehelin, 1992). However, the epitopes recognized by the antiserum were not characterized in greater detail. Thus, CCRC-M1 is the first well-characterized McAb that binds to XG.

Ligand competition assays using defined oligosaccharides establish that a terminal fucosyl residue linked α-(1→2) to another glycosyl residue is an essential structural feature of the epitope recognized by CCRC-M1. All of the oligosaccharides tested that contain such a terminal fucosyl residue bind to the antibody (Fig. 5, Table II). Furthermore, free Fuc is also able to compete for the antibody combining site, albeit only at millimolar concentrations (Fig. 5, Table II). Oligosaccharides that lack a terminal fucosyl residue (i.e. compounds **2**, **3**, and **4**, and lactose) or in which the terminal fucosyl residue is linked differently to the adjacent glycosyl residue (compound **9**) are unable to compete for the antibody combining site (Table II). In addition, purified sycamore laccase, a glycoprotein whose oligosaccharide side chains contain terminal fucosyl residues linked α-(1→6) and α-(1→3) to *N*-acetylglucosaminyl residues (Takahashi et al., 1986), is not recognized by CCRC-M1 (our unpublished results).

Several lines of evidence demonstrate that, although the terminal fucosyl residue is a dominant part of the epitope recognized by CCRC-M1, it is not the only structural feature in XGs recognized by this McAb. First, the ability of Fuc-containing oligosaccharides to compete for the CCRC-M1 combining site increases over 2.5 orders of magnitude of concentration as the size of the oligosaccharide increases. Disaccharide **11** is the least effective competitor tested that inhibits binding of XG to CCRC-M1, whereas a mixture of XG oligosaccharides having DPs of 17 to 22 is the most effective oligosaccharide competitor tested (Table II). Such a size dependence would not be exhibited if the antibody were recognizing only the terminal fucosyl residue. Second, the best single oligosaccharide competitor tested, nonasaccharide **1**, is, nevertheless, about 10,000 times less effective than intact sycamore maple XG in competing for the antibody combining site. Even the mixture of larger XG oligosaccharides (DP 17–22) is 5000-fold less effective than the intact polysaccharide. Finally, tamarind seed XG, a polysaccharide that has no detectable Fuc (York et al., 1990) and confirmed here) is also a better competitor (20-fold) than nonasaccharide **1**.

Two possible explanations come to mind for the difference in the behavior of polymeric XG and XG oligosaccharides in the competitive binding assays. First, the superior ability of intact XG compared with XG oligosaccharides to compete for the antibody combining site could be a reflection of the antibody's avidity for a multivalent antigen. A single molecule of CCRC-M1 might bind to a single molecule of XG using both of its combining sites (CCRC-M1 is an IgG_1 and thus has two binding sites), since the Fuc-containing side chain is present many times in intact XG. If CCRC-M1 binds simultaneously to two epitopes, the effective binding constant (or avidity) for XG would be approximately equal to the square of the binding constant for a single antibody combining site binding to a single epitope. However, since the polysaccharide is a 1000-fold more effective competitor than the nonasaccharide for the single combining site of the Fab of CCRC-M1 (Fig. 6), the hypothesis that divalent binding sites account for the greater avidity of CCRC-M1 for the polysaccharide is ruled out.

An alternative explanation is that CCRC-M1 recognizes a conformation that is favored in polymeric XG but that is only infrequently adopted by XG oligosaccharides of the sizes tested in this report. A XG dimer, formed by hydrogen bonding between the glucan backbones, is one possible conformation of the polysaccharide that is not adopted by the oligosaccharides (Bauer et al., 1973; Valent and Albersheim, 1974). Indeed, the most energetically favored conformation of oligomers of the sizes tested here (DPs $\leq$ 21) is thought to be one in which the backbone adopts a twisted conformation (Levy et al., 1991), a conformation that is not conducive to the formation of hydrogen bonds between the backbone chains.

Evidence that XGs adopt different conformations as they increase in size is demonstrated by their ability to bind iodine once their mol wt increases to 20,000 (Hayashi et al., 1984). Thus, CCRC-M1 might identify the size of XGs at which the

iodine-binding conformational transition occurs. Such a conformational transition may be relevant to the role of XGs in plant cell wall assembly and growth.

The binding and ligand competition experiments may provide additional insight into the nature of the polysaccharide binding site on CCRC-M1. We propose that the binding site consists of a groove, into which the polysaccharide chain fits, with one or more pockets into which the terminal fucosyl residues fit. Groove- and pocket-type binding sites on antibodies have been previously proposed based on results from ligand binding and molecular modeling studies (Cisar et al., 1975; Glaudemans, 1987; Oomen et al., 1991). X-ray crystallographic evidence of a pocket-type site on a McAb against a bacterial *O*-antigen oligosaccharide has been obtained (Cygler et al., 1991). We hypothesize that XG oligosaccharides having the terminal α-(1→2)-linked fucosyl residue interact with both the pocket and groove, thereby interfering with binding of the polysaccharide. We propose that an oligosaccharide lacking the terminal fucosyl residue (e.g. oligosaccharides **2**, **3**, or **4**) cannot bind to the antibody combining site because the interactions with the pocket are absent and the number of interactions with the groove are insufficient to hold the oligosaccharide. On the other hand, a polysaccharide that lacks the terminal fucosyl residues, such as tamarind XG, is bound despite the absence of contributions from the pocket sites because of the larger number of interactions with the groove.

Knowledge of the epitope of CCRC-M1 will assist in the interpretation of studies directed toward the localization of this epitope on polysaccharides in plant cell walls and on biosynthetic intermediates of plant cell wall polysaccharides in Golgi cisternae (Zhang and Staehelin, 1992). However, it must be kept in mind that McAbs are epitope- and not necessarily antigen-specific, and that carbohydrate epitopes are frequently present on a spectrum of glycoconjugates. For example, CCRC-M1 can bind to both XG and RG-I in sycamore maple cells (Fig. 4), although RG-I is recognized about 50-fold less effectively by the antibody (Table I). Thus, the ligand specificity results reported here suggest caution in the interpretation of localization results.

The characterization of the specificities of the McAbs generated in this study have revealed some interesting relationships between different glycoconjugates present in the plant extracellular matrix. The cross-reactivity of the group I McAbs with plant cell-wall polysaccharides and plant membrane glycoproteins (Figs. 2 and 3) provides unambiguous evidence that these different types of macromolecular glycoconjugates, both of which are present at the plant cell surface, share carbohydrate structures in common. Furthermore, the cross-reactivity of CCRC-M1 with RG-I and XG (Figs. 2C and 4) confirms that these two polysaccharides, which differ significantly in structure and properties, have at least one structural feature in common, namely a terminal fucosyl residue linked α-(1→2) to a galactosyl residue (Lau et al., 1987; York et al., 1990). The occurrence of similar carbohydrate structures on different macromolecular glycoconjugates suggests that plant cells use a common biosynthetic pathway for the synthesis of these macromolecules (Driouich et al., 1993).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Mary Sledge (Department of Botany, University of Georgia) and Cindy Lloyd (Athens Research and Technology, Inc., Athens, GA), who performed all of the hybridoma tissue culture. The authors also thank William S. York and Dr. Giuseppe Impalomeni for generously providing us with purified XG oligosaccharides, and Dr. Pierre Sinaÿ and Dr. Tomoya Ogawa and their colleagues for the generous gifts of synthetic XG oligosaccharides. We also are grateful to Carol L. Gubbins Hahn for drawing the figures.

Received September 23, 1993; accepted November 1, 1993.
Copyright Clearance Center: 0032-0889/94/104/0699/12.

LITERATURE CITED

Anderson MA, Sandrin MS, Clarke AE (1984) A high proportion of hybridomas raised to a plant extract secrete antibody to arabinose or galactose. Plant Physiol **75:** 1013–1016

Bauer WD, Talmadge KW, Keegstra K, Albersheim P (1973) The structure of plant cell walls. II. The hemicellulose of the walls of suspension-cultured sycamore cells. Plant Physiol **51:** 174–187

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93–99

Bobbitt JM (1956) Periodate oxidation of carbohydrates. Adv Carbohydr Chem **11:** 1–41

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brewin NJ, Robertson JG, Wood EA, Wells B, Larkins AP, Galfre G, Butcher GW (1985) Monoclonal antibodies to antigens in the peribacteroid membrane from *Rhizobium*-induced root nodules of pea cross-react with plasma membranes and Golgi bodies. EMBO J **4:** 605–611

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J **3:** 1–30

Cisar J, Kabat EA, Dorner MM, Liao J (1975) Binding properties of immunoglobin combining sites specific for terminal or nonterminal antigenic determinants in dextran. J Exp Med **142:** 435–459

Cygler M, Rose DR, Bundle DR (1991) Recognition of a cell-surface oligosaccharide of pathogenic *Salmonella* by an antibody Fab fragment. Science **253:** 442–445

Danielson SJ, Gray GR (1986) Immunochemical characterization of polylysine conjugates containing reductively aminated cellulose oligosaccharides. Glycoconjugate J **3:** 363–377

De Lorenzo G, Ito Y, D'Ovidio R, Cervone F, Albersheim P, Darvill AG (1990) Host-pathogen interactions. XXXVII. Abilities of the polygalacturonase-inhibiting proteins from four cultivars of *Phaseolus vulgaris* to inhibit the *endo*polygalacturonases from three races of *Colletotrichum lindemuthianum*. Physiol Mol Plant Pathol **36:** 421–435

Doares SH, Bucheli P, Albersheim P, Darvill AG (1989) Host-pathogen interactions. XXXIV. A heat-labile activity secreted by a fungal phytopathogen releases fragments of plant cell walls that kill plant cells. Mol Plant Microbe Interact **2:** 346–353

Driouich A, Faye L, Staehelin LA (1993) The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. Trends Biochem Sci **18:** 210–214

Freund J (1956) The mode of action of immunologic adjuvants. Adv Tuberc Res **7:** 130–148

Glaudemans CPJ (1987) Seven structurally different murine monoclonal galactan-specific antibodies show identity in their galactosyl-binding subsite arrangements. Mol Immunol **24:** 371–377

Hahn MG, Bucheli P, Cervone F, Doares SH, O'Neill RA, Darvill A, Albersheim P (1989) Roles of cell wall constituents in plant-pathogen interactions. *In* T Kosuge, EW Nester, eds, Plant-Microbe Interactions. Molecular and Genetic Perspectives, Vol 3. McGraw Hill, New York, pp 131–181

Hahn MG, Darvill A, Albersheim P, Bergmann C, Cheong J-J, Koller A, Lò V-M (1992) Preparation and characterization of oligosaccharide elicitors of phytoalexin accumulation. *In* S Gurr, M McPherson, DJ Bowles, eds, Molecular Plant Pathology, Vol II: A Practical Approach. Oxford University Press, Oxford, UK, pp 103–147

Hahn MG, Lerner DR, Fitter MS, Norman PM, Lamb CJ (1987) Characterization of monoclonal antibodies to protoplast membranes of *Nicotiana tabacum* identified by an enzyme-linked immunosorbent assay. Planta **171:** 453–465

Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Hayashi T, Wong YS, Maclachlan G (1984) Pea xyloglucan and cellulose. II. Hydrolysis by pea endo-1,4-β-glucanases. Plant Physiol **75:** 605–610

Hisamatsu M, York WS, Darvill AG, Albersheim P (1992) Characterization of seven xyloglucan oligosaccharides containing from seventeen to twenty glycosyl residues. Carbohydr Res **227:** 45–71

Horsley D, Coleman J, Evans D, Crooks K, Peart J, Satiat-Jeunemaitre B, Hawes C (1993) A monoclonal antibody, JIM 84, recognizes the Golgi apparatus and plasma membrane in plant cells. J Exp Bot **44:** S-223–229

Hoson T (1991) Structure and function of plant cell walls: immunological approaches. Int Rev Cytol **130:** 233–268

Hoson T, Nevins DJ (1989) Antibodies as probes for the study of location and metabolism of (1→3),(1→4)-β-D-glucans. Physiol Plant **75:** 452–457

Ishii T, Thomas J, Darvill A, Albersheim P (1989) Structure of plant cell walls. XXVI. The walls of suspension-cultured sycamore cells contain a family of rhamnogalacturonan-I-like pectic polysaccharides. Plant Physiol **89:** 421–428

Kearney JF, Radbruch A, Liesengang B, Rajewsky K (1979) A new mouse myeloma cell line which has lost immunoglobin expression but permits the construction of antibody in secreting hybrid lines. J Immunol **123:** 1548–1550

Kikuchi S, Ohinata A, Tsumuraya Y, Hashimoto Y, Kaneko Y, Matsushima H (1993) Production and characterization of antibodies to the β-(1→6)-galactotetraosyl group and their interaction with arabinogalactan-proteins. Planta **190:** 525–535

Knox JP (1992) Molecular probes for the plant cell surface. Protoplasma **167:** 1–9

Knox JP, Day S, Roberts K (1989) A set of cell surface glycoproteins forms an early marker of cell position, but not cell type, in the root apical meristem of *Daucus carota* L. Development **106:** 47–56

Knox JP, Linstead PJ, King J, Cooper C, Roberts K (1990) Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. Planta **181:** 512–521

Knox JP, Linstead PJ, Peart J, Cooper C, Roberts K (1991) Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation. Plant J **1:** 317–326

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lau JM, McNeil M, Darvill AG, Albersheim P (1985) Structure of the backbone of rhamnogalacturonan I, a pectic polysaccharide in the primary cell walls of plants. Carbohydr Res **137:** 111–125

Lau JM, McNeil M, Darvill AG, Albersheim P (1987) Treatment of rhamnogalacturonan I with lithium in ethylenediamine. Carbohydr Res **168:** 245–274

Lerouge P, O'Neill MA, Darvill AG, Albersheim P (1993) Structural characterization of endoglycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. Carbohydr Res **243:** 359–371

Levy S, York WS, Stuike-Prill R, Meyer B, Staehelin LA (1991) Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated side chain in surface-specific side chain folding. Plant J **1:** 195–216

Liners F, Letesson J-J, Didembourg C, Van Cutsem P (1989) Monoclonal antibodies against pectin. Recognition of a conformation induced by calcium. Plant Physiol **91:** 1419–1424

Lynch MA, Staehelin LA (1992) Domain-specific and cell type-specific localization of two types of cell wall matrix polysaccharides in the clover root tip. J Cell Biol **118:** 467–479

Marfà V, Gollin DJ, Eberhard S, Mohnen D, Darvill A, Albersheim P (1991) Oligogalacturonides are able to induce flowers to form on tobacco explants. Plant J **1:** 217–225

McNeil M, Darvill AG, Albersheim P (1980) Structure of plant cell walls. X. Rhamnogalacturonan I, a structurally complex pectic polysaccharide in the walls of suspension-cultured sycamore cells. Plant Physiol **66:** 1128–1134

McNeil M, Darvill AG, Albersheim P (1982) Structure of plant cell walls. XII. Identification of seven differently linked glycosyl residues attached to O-4 of the 2,4-linked L-rhamnosyl residues of rhamnogalacturonan I. Plant Physiol **70:** 1586–1591

McNeil M, Darvill A, Fry SC, Albersheim P (1984) Structure and function of the primary cell walls of plants. Annu Rev Biochem **53:** 625–663

Meikle PJ, Bonig I, Hoogenraad NJ, Clarke AE, Stone BA (1991) The location of (1→3)-β-glucans in the walls of pollen tubes of *Nicotiana alata* using a (1→3)-β-glucan-specific monoclonal antibody. Planta **185:** 1–8

Meikle PJ, Hoogenraad NJ, Bonig I, Clarke AE, Stone BA (1994) A (1→3,1→4)-β-glucan specific monoclonal antibody and its use in the quantitation and immunocytochemical location of (1→3, 1→4)-β-glucans. Plant J (in press)

Moore PJ, Darvill AG, Albersheim P, Staehelin LA (1986) Immunogold localization of xyloglucan and rhamnogalacturonan I in the cell walls of suspension-cultured sycamore cells. Plant Physiol **82:** 787–794

Moore PJ, Staehelin LA (1988) Immunogold localization of the cell-wall-matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.: implication for secretory pathways. Planta **174:** 433–445

Moore PJ, Swords KMM, Lynch MA, Staehelin LA (1991) Spatial organization of the assembly pathways of glycoproteins and complex polysaccharides in the Golgi apparatus of plants. J Cell Biol **112:** 589–602

Norman PM, Kjellbom P, Bradley DJ, Hahn MG, Lamb CJ (1990) Immunoaffinity purification and biochemical characterization of plasma-membrane arabino-galactan-rich glycoproteins of *Nicotiana glutinosa*. Planta **181:** 365–373

Norman PM, Wingate VPM, Fitter MS, Lamb CJ (1986) Monoclonal antibodies to plant plasma-membrane antigens. Planta **167:** 452–459

Northcote DH, Davey R, Lay J (1989) Use of antisera to localize callose, xylan and arabinogalactan in the cell-plate, primary and secondary walls of plant cells. Planta **178:** 353–366

Ohbayashi H, Endo T, Yamashita K, Kuroki M, Matsuoka Y, Kobata A (1989) Novel methods to determine the epitopes on the asparagine-linked oligosaccharides of glycoproteins. Anal Biochem **182:** 200–206

Oomen RP, Young NM, Bundle DR (1991) Molecular modeling of antibody-antigen complexes between the *Brucella abortus* O-chain polysaccharide and a specific monoclonal antibody. Protein Eng **4:** 427–433

Pennell RI, Janniche L, Kjellbom P, Scofield GN, Peart JM, Roberts K (1991) Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. Plant Cell **3:** 1317–1326

Pennell RI, Janniche L, Scofield GN, Booij H, de Vries SC, Roberts K (1992) Identification of a transitional cell state in the developmental pathway to carrot somatic embryogenesis. J Cell Biol **119:** 1371–1380

Pennell RI, Knox JP, Scofield GN, Selvendran R, Roberts K (1989) A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. J Cell Biol **108:** 1967–1977

Pratt LH (1984) Phytochrome immunochemistry. *In* H Smith, MG Holmes, eds, Techniques in Photomorphogenesis. Academic Press, London, pp 201–226

Ragg H, Kuhn DN, Hahlbrock K (1981) Coordinated regulation of 4-coumarate:CoA ligase and phenylalanine ammonia-lyase mRNAs in cultured plant cells. J Biol Chem **256:** 10061–10065

Roberts K (1990) Structures at the plant cell surface. Curr Opin Cell Biol **2:** 920–928

Roberts K, Phillips J, Shaw P, Grief C, Smith E (1985) An immu-

nological approach to the plant cell wall. *In* CT Brett, ed, The Plant Cell Wall. Cambridge University Press, Cambridge, UK, pp 125–154

Sakai K, Nakahara Y, Ogawa T (1990) Total synthesis of nonasaccharide repeating unit of plant cell wall xyloglucan: an endogenous hormone which regulates cell growth. Tetrahedron Lett **31:** 3035–3038

Shulman M, Wilde CD, Köhler G (1978) A better cell line for making hybridomas secreting specific antibodies. Nature **276:** 269–270

Smith DF, Ginsburg V (1980) Antibodies against sialyloligosaccharides coupled to protein. J Biol Chem **255:** 55–59

Sone Y, Kuramae J, Shibata S, Misaki A (1989a) Immunochemical specificities of antibody to the heptasaccharide unit of plant xyloglucan. Agric Biol Chem **53:** 2821–2823

Sone Y, Misaki A, Shibata S (1989b) Preparation and characterization of antibodies against 6-*O*-α-D-xylopyranosyl-β-D-glucopyranose (β-isoprimeverose), the disaccharide unit of xyloglucan in plant cell-walls. Carbohydr Res **191:** 79–89

Stacey NJ, Roberts K, Knox JP (1990) Patterns of expression of the JIM4 arabinogalactan-protein epitope in cell cultures and during somatic embryogenesis in *Daucus carota* L. Planta **180:** 285–292

Stafstrom JP, Staehelin LA (1988) Antibody localization of extensin in cell walls of carrot storage roots. Planta **174:** 321–332

Takahashi N, Hotta T, Ishihara H, Mori M, Tejima S, Bligny R, Akazawa T, Endo S, Arata Y (1986) Xylose-containing common structural unit in N-linked oligosaccharides of laccase from sycamore cells. Biochemistry **25:** 388–395

Thomas JR, Darvill AG, Albersheim P (1989) Structure of plant cell walls. XXV. Rhamnogalacturonan I, a pectic polysaccharide that is a component of monocot cell walls. Carbohydr Res **185:** 279–305

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

Valent BS, Albersheim P (1974) The structure of plant cell walls. V. On the binding of xyloglucan to cellulose fibers. Plant Physiol **54:** 105–108

Vian B, Temsah M, Reis D, Roland JC (1992) Co-localization of the cellulose framework and cell-wall matrix in helicoidal constructions. J Microsc **166:** 111–122

Villanueva MA, Metcalf TN, Wang JL (1986) Monoclonal antibodies directed against protoplasts of soybean cells: generation of hybridomas and characterization of a monoclonal antibody reactive with the cell surface. Planta **168:** 503–511

Vreeland V (1970) Localization of a cell wall polysaccharide in a brown alga with labeled antibody. J Histochem Cytochem **18:** 371–373

Woodward MP, Young WW Jr, Bloodgood RA (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J Immunol Methods **78:** 143–153

York WS, Darvill AG, McNeil M, Stevenson TT, Albersheim P (1985) Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol **118:** 3–40

York WS, van Halbeek H, Darvill AG, Albersheim P (1990) Structural analysis of xyloglucan oligosaccharides by ^{1}H-n.m.r. spectroscopy and fast-atom-bombardment mass spectrometry. Carbohydr Res **200:** 9–31

Zhang GF, Staehelin LA (1992) Functional compartmentation of the Golgi apparatus of plant cells. Immunocytochemical analysis of high-pressure frozen- and freeze-substituted sycamore maple suspension culture cells. Plant Physiol **99:** 1070–1083

Zola H (1987) Monoclonal Antibodies: A Manual of Techniques. CRC Press, Boca Raton, FL