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A High Proportion of Hybridomas Raised to a Plant Extract Secrete Antibody to Arabinose or Galactose

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ABSTRACT

A high proportion of hybridomas, obtained from mice immunized with style extracts prepared from mature flowers of an ornamental tobacco, *Nicotiana alata*, secrete antibody to arabinogalactan protein (AGP). The specificity of the antibodies secreted by three cloned cell lines is primarily directed to β-D-galactopyranose and α-L-arabinofuranose; antibodies from two cell lines preferentially bind β-D-galactopyranose residues and antibodies from the other cell line preferentially bind α-L-arabinofuranose. As AGPs are components of most plant tissues and exudates, it is likely that attempts to raise monoclonal antibodies to other plant extracts will result in hybridomas producing antibodies to AGPs.

We have raised monoclonal antibodies to style extracts prepared from mature flowers of an ornamental tobacco (*Nicotiana alata*). These experiments represent an approach to establishing the components of the female sexual tissues which may be involved in controlling fertilization in higher plants. The style is the female sexual organ through which pollen tubes carrying the sperm grow to the ovary where fertilization may occur. Our primary goal was to produce a monoclonal antibody directed to the product of the *S* (self-incompatibility) gene which acts to prevent inbreeding by arresting growth of pollen tubes of the same *S*-genotype within the style (5, 13). Material corresponding to *S*-genotye is a major antigenic component of style extracts from a number of self-incompatible species including *Nicotiana alata* (3) (for summary, see 15). We therefore immunized mice with extracts of mature styles; these extracts contain a number of proteins and glycoproteins, including those corresponding to *S*-genotype, as well as a class of proteoglycans, the AGPs[1], which account for a major proportion of the total soluble carbohydrate (7). Proteoglycans of this type are present in many plant tissues and secretions (4, 9); in particular, they are present in extracts of styles from many flowering plant families (14). Proteoglycans are generally considered to be weakly antigenic, and we were surprised to find that a high proportion of the hybridomas secreted antibody directed to the AGP.

MATERIALS AND METHODS

Materials. *Nicotiana alata* genotype S_1S_3 seeds were a generous gift of Dr. K. K. Pandey, DSIR, Grasslands Division, New Zealand. Samples of barley β-glucan, arabinoxylan, and wheat AGP were a gift from Professor B. A. Stone, LaTrobe University, Bundoora, Australia, and *Datura* lectin was a gift from Dr. A. K. Allen, Charing Cross Hospital, U.K. *N. alata* AGP was isolated from styles of mature flowers genotype S_1S_3 by affinity chromatography on J539-Sepharose 4B (7). BSA, potato lectin, larch AGP, pectin, D-galactose, β-D-Gal*p*-(1→3)-D-Ara*f*, β-D-Gal*p*-(1→4)-D-Glc*p*, α-D-Gal*p*-(1→6)-D-Glc*p*, L-fucose, and methyl-β-L-arabinopyranoside (Me-β-L-Ara*p*) were from Sigma Chemical Co.). D-Galacturonic acid and D-glucose were from BDH, Poole, U.K.; methyl-β-D-galactopyranoside, methyl-β-D-glucopyranoside, and methyl-β-D-xylopyranoside were from Pfanstiel Laboratories, Waukegan, IL, and D-xylose was from Calbiochem, Kingsgrove, N.S.W., Australia. Urease-conjugated rabbit anti-mouse $F(ab')_2$ immunoglobulin and urease substrate solution were from Commonwealth Serum Laboratories, Melbourne, Australia. Microtiter trays were from Costar, Cambridge, MA, and Sephadex G-25 was from Pharmacia, Uppsala, Sweden. All other materials were of reagent grade.

Style Extracts. Flowers from *N. alata* (genotype S_1S_3) were emasculated at the onset of petal coloration. Two d later, the fully mature styles were removed and either stored at −70°C or extracted immediately. (Styles refer to the style and stigma which were removed together; ovary is not included.) Styles (1 g) were ground to a fine powder in liquid nitrogen using a mortar and pestle; this was followed by further grinding in 10 ml of extracting buffer (50 mM Tris-HCl [pH 8.5], 1 mM $CaCl_2$, 10 mM NaCl, 1 mM DTT, 1% w/v insoluble PVP [Polyclar], 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged (12,000*g*, 15 min) and the supernatant was passed through a Sephadex G-25 column equilibrated with 50 mM Tris-HCl (pH 8.5) and 10 mM NaCl and used to immunize mice.

Production of Hybridomas. Nine young female mice (Balb/c) were immunized intraperitoneally with style extract (0.1 ml, 0.62 mg protein/ml, 0.1 mg carbohydrate as galactose/ml of which 40% is AGP [7]); Freund's adjuvant was not used. The mice were boosted on day 14, and on day 28 serum samples were obtained and tested for the presence of antibodies to *N. alata* (S_1S_3) style extract using an ELISA assay. When diluted 1 in 80, sera from all mice gave complete color development in the assay within 10 min. There was no color development with a nonimmune mouse serum. The two mice bearing the sera which gave the most rapid color development (within 5 min) received booster injections intraperitoneally on day 28; 3 d later their spleens were removed and fused with NS-1 myeloma cells using PEG (16). Hybrids were selected in HAT medium and cloned by limiting dilution (one cell/10 wells; [16]). Supernatants from the hybridoma cell lines were screened for antibodies to unfractionated extracts from *N. alata* (S_1S_3) styles and AGP isolated from *N. alata* (S_1S_3) styles (7).

ELISA Assay for Screening Cell Supernatants. Style extract

[1] Abbreviations: AGP, arabinogalactan-protein; PBS, phosphate-buffered saline.

(50 μl; 50 μg protein/ml in PBS) or purified *N. alata* AGP (50 μl, 40 μg carbohydrate/ml in PBS) was added to the wells of a microtiter tray. After 16 h at 4°C, the solutions were removed and the wells washed three times in PBS followed by two washes with 1% BSA in PBS. Remaining binding sites were blocked by filling the wells with 1% BSA in PBS. After 60 min, the BSA was removed and the wells washed twice with 1% BSA in PBS. The plates were either used immediately or stored at 4°C after filling each well with 100 μl 0.2% NaN_3 in PBS.

For screening of cell supernatants, 50 μl of each supernatant was placed in the coated microtiter wells and incubated for 90 min at 37°C. The wells were then washed three times with 1% BSA. Bound antibody was detected by the addition of urease-conjugated rabbit anti-mouse $F(ab')_2$ immunoglobulin (50 μl, 1 in 900 dilution). After 30 min at 37°C, this solution was removed and the wells were washed three times using 1% BSA in PBS followed by seven rinses with filtered water (Millipore Q). Urease substrate solution (50 μl) was added and the plates incubated at 37°C for 15 to 30 min. Color development was measured using a multichannel colorimeter connected to a Titertek Multiscan MC (Flow Laboratories, McClean, VA). Results were expressed as a percentage of maximum color development.

RESULTS

In two separate fusions, about half the hybridomas secreted antibodies to *N. alata* style AGP (Table I). This AGP contains 68% carbohydrate of which arabinose and galactose are the major components. (Monosaccharide composition—Rha:Ara:Xyl:Gal:Glc in the ratio 1.5:30:1.5:63:4 [7]. A high proportion of the hybridomas (group 1) were apparently directed to both L-arabinose and D-galactose, as antibody binding to the isolated AGP was inhibited by either L-arabinose or D-galactose but not D-glucose (Table II). Hybridomas were also produced which secreted antibody with preference for L-arabinose compared with D-galactose (group 2) and with preference for D-galactose compared with L-arabinose (group 3) In addition, a high proportion were produced in which binding to the AGP was not inhibited by either L-arabinose or D-galactose when presented as the free monosaccharide (group 4).

We attempted to clone representative hybridomas of each of these four groups and succeeded in producing three cloned cell lines, PCBC 1, 2, and 3 from groups 1 and 2. PCBC 1 secretes an IgG2B and PCBC 1 secretes an IgG2A. The antibodies secreted by these cloned lines bound to isolated AGP; this binding was inhibited by the mono- and disaccharides shown in Table III and the polysaccharides shown in Table IV.

The specificity of the antibodies produced by the three cloned cell lines is primarily for the α-L-arabinofuranosyl and β-D-galactopyranosyl residues of the AGP. The evidence is that AGPs from other sources (wheat and larch) are effective inhibitors, but unrelated polysaccharides such as barley endosperm (1→3),(1→4)-β-D-glucan, and yeast mannan [(1→6)-α-D-mannan with side branches of (1→2) and (1→3)-linked mannosyl residues] are not inhibitors of the interaction between antibody and AGP (Table IV). The monosaccharides L-arabinose and D-galactose are effective inhibitors of the interaction, but unrelated monosaccharides such as D-xylose, D-glucose, D-mannose, and their derivatives Me-β-D-Xyl and Me-β-D-Glc, were not effective inhibitors (Table III). The D-galactose residues must be in the β-anomeric config-

Table I. *Number of Anti-Nicotiana alata (S_1S_3) Style and AGP Antibody Secreting Hybridomas Produced in Two Fusions*

	No. of Wells Seeded[a]	Hybridomas Secreting Antibody to Whole *N. alata* (S_1S_3) Style Exract[b]	Hybridomas[c] Secreting Antibody to Isolated *N. alata* (S_1S_3) AGP
Fusion 1	800	43	18
Fusion 2	800	87	38

[a] 800 wells were seeded but growth was found in only about 30% of the wells.
[b] Style refers to stigma plus style.
[c] Also reacted with whole style extract.

Table II. *Monosaccharide Inhibition of Anti-AGP Antibody Secreted by Hybridomas Produced during Two Fusions*

Group	Effect of Monosaccharides[a]	No. of Hybridomas	
		Fusion 1	Fusion 2
1	Inhibition by either L-arabinose or D-galactose but not D-glucose	10	13
2	Inhibition by L-arabinose, less by D-galactose[b]	0	2
3	Inhibition by D-galactose, less by L-arabinose[b]	4	4
4	Inhibition by neither D-galactose nor L-arabinose	4	19
		18/43	38/87

[a] Antibody-containing supernatants were incubated for 2 h at room temperature with monosaccharide (100 mM) before application to AGP-coated plates; bound antibody was measured by the ELISA assay.
[b] Both monosaccharides affected binding, but one was dominant, causing about 90% inhibition, the other causing less than 50% inhibition.

Table III. *Inhibition of Monoclonal Antibody Interaction by Mono- and Disaccharides*

No inhibition at 100 mM by: Xyl, Glc, Man, Me-β-D-Xyl, Me-β-D-Arap, Me-β-L-Arap, Me-β-D-Glc. <, 100% inhibition at the concentration stated. >, No inhibition at the concentration stated.

	Concn. Required for 50% Inhibition		
	PCBC 1	PCBC 2	PCBC 3
		mM	
D-Gal	25	12.5	50
D-Gal A	12.5	12.5	50
Me-β-D-Gal	6.2	1.5	6.2
β-D-Gal*p*-(1→3)-D-Ara*f*	<1.5	<1.5	<1.5
β-D-Gal*p*-(1→4)-Glc*p*	50	25	25
α-D-Gal*p*-(1→6)-Glc*p*	>100	>100	>100
L-Ara	25	12.5	6.2
L-Rha	50	25	100
L-Fuc	100	50	25

Table IV. *Inhibition of AGP-Monoclonal Antibody Interaction by Polymeric Carbohydrates*

>, No inhibition at the concentration stated. Higher concentrations were difficult to obtain as the polysaccharides have a limited solubility.

	Conc. Required for 50% Inhibition		
	PCBC 1	PCBC 2	PCBC 3
		mg/ml	
Barley (1→3), (1→4)-β-D-glucan	>0.5	>0.5	>0.5
Yeast mannan	>0.5	>0.5	>0.5
Potato lectin	>5	>5	5
Datura lectin	>1	>1	>1
Pectin	0.5	0.1	0.5
Larch AGP	0.25	0.25	0.25
Wheat AGP	0.50	0.10	0.25
Arabinoxylan	>0.5	>0.5	0.25

uration to inhibit effectively; β-D-Gal*p*-(1→3)-D-Ara*f*, Me-β-D-Gal, and β-D-Gal*p*-(1→4)-D-Glc*p* gave 50% inhibition at concentrations of 50 mM or less, while α-D-Gal*p*-(1→6)-D-Glc*p* was not an inhibitor at 100 mM. The preferred configuration of arabinose is α-L-Ara*f* as neither Me-β-D-Ara*p* nor Me-β-L-Ara*p* are inhibitors (at 100 mM) (Table III), but L-arabinose as the free monosaccharide (Table III) and the polysaccharide arabinoxylan (Table IV) are effective inhibitors.

It is formally possible that nonspecific interactions between AGP and the carbohydrate moiety of the Fc fragment of IgG could contribute to the total AGP-antibody binding. This is not significant under the experimental conditions used as there was no color development within 10 min in the ELISA assay using nonimmune mouse serum (diluted 1 in 80) and plates coated with style extract (containing 40 μg AGP/ml). Further evidence is that half the hybridomas produced secrete antibodies which do not react with isolated AGP, although they give a positive ELISA assay with whole style extract. Similar observations are made using precipitin band formation as the assay: supernatant fluids from all three cloned cell lines (PCBC 1, PCBC 2, PCBC 3) give precipitin bands in double diffusion experiments with isolated AGP; however, neither nonimmune mouse serum nor supernatant fluids from cell lines which react with style extracts (but not AGP) give precipitin bands in double diffusion experiments with isolated AGP.

Although each of the three cloned lines has a primary specificity for β-D-Gal*p* and α-L-Ara*f*, there are differences in the detailed binding requirements. Antibody PCBC 3 has a preference for L-arabinose residues compared with antibodies PCBC 1 and PCBC 2; L-arabinose is an effective inhibitor of antibody PCBC 3 at 6.2 mM while 50 mM D-galactose is required for the same level of inhibition. In contrast, D-galactose and L-arabinose are equally effective inhibitors of the other two antibodies. This preference of antibody PCBC 3 for L-arabinosyl residues is consistent with the inhibition data from polymeric carbohydrates (Table IV). Arabinoxylan [(1→4)-β-D-xylan backbone, with single terminal α-L-Ara*f* side branches (7)] is a good inhibitor of antibody PCBC 3, but not of the antibodies PCBC 1 or PCBC 2. Potato and *Datura* lectins are poor inhibitors of all the antibodies, consistent with their known low content of D-galactose which is in the α-configuration (2, 6).These lectins have a high content of L-arabinose which is mainly present as tri- and tetra-arabinosyl side chains. The anomeric linkages are in the β-configuration except for the terminal nonreducing arabinosyl group of the tetra arabinosyl side chains which are in the α-configuration (2, 6).

Thus, the monoclonal antibodies generated are consistent with their being directed to terminal α-L-Ara*f* and β-D-Gal*p* residues of the style AGPs. It seems likely that the binding sites extend over several residues because of the greatly enhanced binding of β-D-Gal*p*-(1→3)-D-Ara*f* compared with β-D-Gal*p*-(1→4)-D-Glc*p*.

DISCUSSION

Immunological methods, and particularly monoclonal antibody technology are being applied to an increasing number of problems in plant biology. The data presented here indicate that attempts to raise monoclonal antibodies to whole plant extracts may result in hybridomas that produce antibodies to AGPs. The yield of hybridomas to particular protein antigens in an extract could be enhanced by, for example, subjecting the plant extract to affinity chromatography, gel filtration, or precipitation with the Yariv artificial carbohydrate antigen (for reviews, see 4, 9) to remove AGPs, before using it to immunize mice. An alternative approach to detect protein-directed antibodies produced from injection of a crude extract is to screen the hybridoma supernatants for the desired protein antigen-antibody interaction in the presence of the disaccharide β-D-Gal*p*-(1→3)-D-Ara*f*. Under these conditions, binding of antibodies directed to L-Ara and D-Gal would be inhibited allowing other protein-directed antibodies to be detected.

The data presented here also indicate that it is possible to produce monoclonal antibodies with a preference for particular plant monosaccharide residues held in specific configurations. This was foreshadowed by work with polyclonal antisera (11, 12) in which two distinct populations of antibodies, one directed against α-L-Ara*f* and the other directed against β-D-Gal*p*-(1→6)-D-Gal*p*, were detected in a rabbit antiserum raised to isolated AGP. The fact that these plant monosaccharide residues are effective antigens, in contrast to the monosaccharides of animal glycoproteins, may be related to 'foreigness' of the plant monosaccharides to the experimental animal; arabinose is, for example, not a component of most animal tissues, although it has been tentatively identified as a minor component in hyaluronic acid from bovine brain (17).

These monoclonal antibodies will be useful for measuring and localizing saccharide residues in plant tissues, although a limitation to their use is that they may detect particular saccharide residues or sequences in whatever polymer they occur—that is, they may not be polymer-specific. For example, the monoclonal antibody directed against α-L-Ara*f* residues will detect α-L-Ara*f* residues whether they occur in an arabinogalactan-protein or an arabinoxylan. Similarly, the β-D-Gal*p* directed monoclonal antibody will detect these groups, whether they occur in a proteoglycan, glycoprotein, or presumably a polysaccharide. This limitation may be overcome by a complete definition of the antibodies by measuring their affinity constants for a series of oligosaccharides and polysaccharides. It is likely, by analogy with other saccharide-directed antibodies and lectins, that the binding site will include a number of saccharide residues, for example, the most effective inhibitor of a mouse IgA myeloma which binds 1→6-linked β-D-galactosyl residues is the 1→6-linked β-D-galactotetraose (10). The difficulty with this approach is that few defined oligosaccharides are available commercially. However, if the relative affinities of the monoclonal antibodies for different but related saccharide sequences can be established, it would be possible by careful titering, to detect only a single saccharide sequence in a tissue.

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