A Human Anti-*Pseudomonas aeruginosa* Serotype O6ad Immunoglobulin G1 Expressed in Transgenic Tobacco Is Capable of Recruiting Immune System Effector Function In Vitro[∇]

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The production of a recombinant human IgG1 in transgenic tobacco was examined to determine whether a plant-derived antibody could recruit immune system effector function against a bacterial pathogen. A plant transformation vector was engineered to contain genes for a human kappa light chain and a human gamma-1 heavy chain with V_H and V_L sequences from a previously identified human IgG2 monoclonal antibody (MAb) that specifically binds to and opsonizes *Pseudomonas aeruginosa* serotype O6ad. Unique NcoI and NotI restriction sites were incorporated to flank these variable sequences, resulting in a plant transformation vector that could be engineered for expression of any other human IgG1 antibody, requiring only the substitution of other V_H and V_L antigen-binding coding sequences. The plant-produced IgG1 was determined to have high-mannose glycan content and to be capable of mediating opsonophagocytosis of *P. aeruginosa* serotype O6ad in vitro using human complement and human polymorphonuclear leukocytes. Thus, MAbs produced in plants from this vector could provide human IgG1 MAbs for targeting other pathogens that require the recruitment of immune system effector functions.

Recombinant DNA technology applied to the production of antibody molecules makes possible their selection, maturation, improvement and expression in heterologous systems for large-scale preparation and purification. Recombinant antibody fragments, such as single-chain variable fragments and variable domains of heavy-chain camelid antibodies, can be produced in inexpensive bioreactors utilizing microbial expression hosts (4). However, full-length tetrameric monoclonal antibodies (MAbs) cannot be produced in this way and are thus typically produced for the pharmaceutical industry in bioreactors utilizing mammalian cell lines, such as Chinese hamster ovary (CHO) cells (55). The expense and lengthy time requirements for development of new production facilities for mammalian cell expression systems may limit their use in meeting the growing demand for MAb therapeutics. Therefore, other less expensive production systems capable of producing full-length multimeric MAbs are being actively investigated to complement these bioreactor production facilities (3, 53).

Transgenic plants offer an attractive alternative for recombinant MAb production. Tobacco, for example, offers ease of genetic manipulation, large biomass production (up to 200 metric tons per hectare), and lower probability of contaminating the food chain than other transgenic plants, as tobacco is not a food crop (1). Purification protocols for plant-produced MAbs are constantly being improved so that drug manufactur-

ers can take advantage of transgenic crops for the production of pharmaceuticals (49). Thus, plant-produced MAbs could offer an economical alternative to mammalian bioreactor-produced MAbs.

Glycosylation of the constant fragment (Fc) of an immunoglobulin G (IgG) is essential for the deposition of complement and interaction with Fc receptors to mediate immune system effector functions, such as complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity (54). Plant glycosylations differ from mammalian glycosylations in both sugar content and pattern, as they lack sialylations (47) and residues, such as $\beta(1,4)$ -Gal and $\alpha(2,6)$ -NeuAc, that are often found on mammalian glycans (17, 19). MAbs expressed in transgenic tobacco plants are typically glycosylated with high-mannose carbohydrates (10, 17, 29, 48, 51); thus, a major concern regarding plant-produced MAbs is whether they can support the recruitment of effector functions required for therapeutic efficacy against pathogen targets, such as bacteria.

To examine the capability of transgenic plants to produce MAbs with potential for immune effector function recruitment, we produced stable transgenic tobacco expressing an endoplasmic reticulum (ER)-targeted full-length human IgG1. Coding sequences for the antigen-binding component of this MAb were derived from a previously characterized human IgG2 shown to be a potent opsonin with specificity for the polysaccharide (PS) portion of the *Pseudomonas aeruginosa* serotype O6ad PS O side chain (22). This antigen is comprised of tetrasaccharide repeating units of L-rhamnose, N-acetyl-D-quinovosamine, N-formyl-D-galactosaminuramide, and N-acetyl-D-galactosaminuramide (27). The Fc component coding sequence was derived from a human gamma-1 heavy-chain (HC) polypeptide. HC and light-chain (LC) genes coding for

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this chimeric yet fully human IgG1 were assembled in a plant expression vector that would allow future subcloning of other antigen-binding coding sequences with different specificities while keeping the same constant regions. This tobacco-expressed IgG1 was determined to have high-mannose glycan content and was capable of supporting opsonization and phagocytosis of *P. aeruginosa* serotype O6ad in vitro using human complement and human polymorphonuclear leukocytes (PMN) as effector cell populations.

MATERIALS AND METHODS

Gene syntheses, binary-vector construction, plant transformation, and characterization. The V_H and V_L sequences of the S20 IgG2 (22), i.e., the first 119 and 113 amino acids of GenBank accession numbers AAK39434 and AAK39435, respectively, were combined in silico with the gamma-1 and kappa constant-region sequences, i.e., the entire 330 and 106 amino acids of GenBank accession numbers P01857 (18) and P01834 (20), respectively. Complete coding sequences for both HC and LC polypeptides were optimized for expression in transgenic tobacco and synthesized according to the method of Olea-Popelka et al. (36) and Almquist et al. (1). The gamma-1 sequence had six-His and KDEL ER retrieval signal (43) motifs added to its carboxyl terminus. V_H and V_L coding sequences were synthesized as NcoI-NotI DNA fragments and kappa and gamma-1 coding sequences as NotI-SacII fragments. Complete HC and LC genes were assembled in pBS-SK⁺ (Stratagene, La Jolla, CA) by introducing each coding sequence as an NcoI-SacII fragment behind the *Arabidopsis* basic chitinase signal sequence (40) and between the doubled-enhancer 35S promoter and 19S terminator of the cauliflower mosaic virus (50). The HC and LC genes were consecutively subcloned into pRD400 (15) as BamHI-SacI and XhoI-Sall fragments, respectively. A clone, referred to as pMM29, that contained both HC and LC genes oriented in the same transcriptional direction was selected for plant transformation. DNA syntheses, plasmid subcloning, and sequencing of entire HC and LC genes in the pRD400 vector were performed by the PBI/NRC DNA/Peptide Synthesis Laboratory of the National Research Council of Canada in Saskatoon, Saskatchewan, Canada. Fig. 1 shows a diagram of the anti-*P. aeruginosa* serotype O6ad human IgG1 expression construct assembly.

Agrobacterium-mediated tobacco transformations were performed as described in our previous publications (1, 2, 36) using *Nicotiana tabacum* variety 81V9 (34) and standard *Agrobacterium* procedures (42). Screening of primary transgenic (T_0) plants was done according to the method of Almquist et al. (1, 2), except that both reducing and nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses were performed, in which 2-mercaptoethanol was omitted from the gel-loading buffer for nonreducing gels. As in our previous studies, protein bands were detected with an anti-polyhistidine MAb (Penta-His; QIAGEN Inc., Mississauga, Ontario, Canada), followed by goat anti-mouse IgG conjugated to alkaline phosphatase (Pierce Biotechnology Inc., Rockford, IL) and developed in 1-Step nitroblue tetrazolium/BCIP (5-bromo-4-chloro-3-indolylphosphate) developing solution (Pierce).

Anti-*P. aeruginosa* serotype O6ad IgG purification and characterization. Antibody purification was done essentially according to the method of Almquist et al. (1, 2), although a final purification step involved Protein G Sepharose 4 Fast Flow (GE Healthcare, Baie d'Urfe, Quebec, Canada) affinity chromatography (25). IgG was subsequently eluted with 0.1 M glycine (pH 2.4) and then neutralized with 1.0 M Tris (pH 8), concentrated, and desalted in 1× phosphate-buffered saline using two Centricon Plus-20 centrifugal filter units (10-kDa nominal molecular mass limit; Millipore; Cambridge, Ontario, Canada) prior to storage at 4°C.

Purified IgG was characterized by functional enzyme-linked immunosorbent assay, which was performed according to the method of Schreiber et al. (44, 45). PSs from *P. aeruginosa* strain IT-2 and serotype O6ad were prepared as previously described (21).

Opsonophagocytosis assays. Assays for the demonstration of opsonization and associated phagocytosis were performed according to previous publications from the Schreiber laboratory (6, 22, 30, 44, 46). Briefly, 10^4 CFU of *P. aeruginosa* serotype O6ad (International Typing System 011) were mixed with 10^6 fresh human PMN (44) in RPMI medium containing either tobacco-derived anti-*P. aeruginosa* serotype O6ad IgG (2.5, 12.5, or 25 µg/ml), irrelevant control IgG (25 µg/ml), or no antibody in 400-µl final volumes. A small amount of agammaglobulinemic human serum (1.7% final concentration) was used as a source of complement in these assays. After incubation at 37°C for 90 min, treatments

were put on ice and then diluted and spread on tryptic soy agar plates (Becton Dickinson Company, Spark, MD) for overnight incubation at 37°C (46). The percent opsonophagocytosis was calculated by using the following formula: $100 \times [(\text{number of CFU in the assay without antibody} - \text{number of CFU in a given assay}) / \text{number of CFU in the assay without antibody}]$. All assays were performed in quadruplicate.

Glycosylation analyses. Glycosylation analyses were performed at Prozyme, Inc. (San Leandro, CA). Purified plant IgG was diluted to 1.0 mg/ml in 20 mM sodium phosphate, and two 100-µl aliquots were treated twice, 4 h apart, with 2 µl *N*-glycosidase F (PNGase F; N-Glycanase-PLUS; Prozyme) and incubated at 37°C for 16 h. The released oligosaccharides were recovered by cold ethanol precipitation and dried by centrifugal evaporation.

Glycans released from one digestion tube were labeled with sodium-8-amino-1,3,6-naphthalene trisulfonate (ANTS) for fluorophore-assisted carbohydrate electrophoresis (FACE) N-linked oligosaccharide gel analysis. Relevant glycan standards (E3 Oligo Ladder; product GK50206; Prozyme) were also labeled with ANTS for comparison. A sample aliquot (4 µl of 16 µl total) of the ANTS-labeled plant antibody glycans from one PNGase F digestion was electrophoresed in a gel containing oligomannose ladder standards (oligomannose-9 [Man-9], Man-8, Man-7, Man-6, and Man-5), oligosaccharides released from serum-derived human IgG (product GKLB-005; Prozyme), and E5 Quantification Control (50 pmol maltotetraose; WS0016; Prozyme). The remaining 12-µl sample was analyzed on a second FACE oligosaccharide profiling gel, along with E3 Oligo Ladder and E5 Quantification Control. The gels were analyzed on a FACE Imager using FACE analytical software (ProZyme) with background correction. All methods were in accordance with those available from Prozyme (FACE oligosaccharide profiling kit booklet GK90000).

For confirmation purposes, glycans from the second digestion sample were labeled with 2-aminobenzamide (2-AB) for high-performance liquid chromatography (HPLC) analysis according to procedures outlined by Prozyme (Signal 2-AB labeling kit booklet GKK-404). 2-AB-labeled glycans were desalted using GlycoClean S cartridges (ProZyme). Glucose homopolymer (GKSB-503; ProZyme) was used as a standard to calculate the glucose unit (GU) values of the labeled glycans. Chromatography was performed on a GlycoSep N HPLC column (GKI-4728; ProZyme) using an acetonitrile and 250 mM ammonium formate, pH 4.4, gradient system.

Nucleotide sequence accession numbers. The complete HC and LC coding sequences are available as GenBank accession numbers AY829476 and AY829477, respectively.

RESULTS

Purification and characterization of anti-*P. aeruginosa* serotype O6ad IgG1 from transgenic tobacco. Forty-two T_0 plants were produced, 35 of which were determined by reducing and nonreducing SDS-PAGE and immunoblot analyses to express IgG1 (not shown). Anti-*P. aeruginosa* serotype O6ad IgG1 was purified from 500-g lots of transgenic tobacco leaf samples from T_0 plants no. 7, 16, 22, 23, 25, and 29, according to the flowchart in Fig. 2. Supernatants from centrifugation-cleared homogenate (lane 1), expanded-bed adsorption immobilized-metal affinity chromatography flowthrough (lane 2), imidazole eluant (lane 3), protein G affinity chromatography flowthrough (lane 4), and purified IgG (lane 5) from one preparation are shown in the SDS-PAGE gel in Fig. 2. The average yields per preparation using this protocol were approximately 4.0 mg/kg of fresh tobacco leaves. Lane 5 of Fig. 2 shows only the purified LC and HC polypeptides, which were predicted to be 24.9 and 51.4 kDa, respectively, after in vivo cleavages of the *Arabidopsis* basic chitinase SS (40).

A competitive inhibition enzyme-linked immunosorbent assay demonstrated that the anti-*P. aeruginosa* serotype O6ad IgG1 was functional and specific. Purified IgG1 was specifically inhibited from binding to solid-phase-immobilized *P. aeruginosa* serotype O6ad O side chain PS by preincubation with serotype O6ad PS, but not with control PS from *P. aeruginosa* serotype 02 (i.e., IT-2) (Fig. 3).

FIG. 1. Plant transformation and expression vector assembly. Complete LC and HC coding sequences were synthesized as XbaI-SacI fragments and subcloned between cauliflower mosaic virus promoter and terminator elements in separate Bluescript plasmids (middle). The entire HC and LC genes were subcloned into pRD400 (15) as BamHI-SacI and XhoI-SalI fragments, respectively (bottom). For assembly of other IgG1 genes, V_H and V_L domain coding sequences should be prepared as NcoI-NotI fragments, as shown at the top, and subcloned into the respective Bluescript plasmids (middle). See Materials and Methods for details. AcaClone software was used to draw the plasmid at the bottom of the figure.

Tobacco-derived anti-*P. aeruginosa* serotype O6ad IgG1 is capable of mediating opsonophagocytosis. Purified anti-*P. aeruginosa* serotype O6ad IgG1 from transgenic tobacco was capable of recruiting immune system effector function in vitro, as measured by an opsonophagocytosis assay (22, 30) using human PMN as a source of effector cells. Figure 4 shows the

percentage of *P. aeruginosa* serotype O6ad CFU that were phagocytosed after 90-minute incubation with MAb, 10⁶ human PMN, and human complement. Approximately 50% of the bacterial cells were phagocytosed in assays with 2.5 µg/ml tobacco IgG; approximately 80% were phagocytosed with 25 µg/ml. An irrelevant MAb (anti-IT-2 [30]) did not cause sig-

FIG. 2. Purification of human anti-*P. aeruginosa* serotype O6ad IgG1 from transgenic tobacco leaves. On the left is the purification scheme; on the right is a Coomassie blue-stained SDS-PAGE gel with samples taken as indicated in the purification scheme (lanes 1 to 5). The electrophoretic mobilities of the molecular mass markers, in kDa, are along the left side of the gel, and the top of the gel indicates the samples as molecular mass markers, supernatant (lane 1), flowthrough from nickel column (lane 2), 250 mM imidazole eluant (lane 3), flowthrough from protein G column (lane 4), and low-pH eluant (lane 5). LC and HC polypeptides in lane 5 were predicted to be 24.9 and 51.4 kDa, respectively, and are indicated by the arrows along the right side of the gel.

nificant uptake at 25 µg/ml, which indicated that specific binding to *P. aeruginosa* serotype O6ad cells was required; pairwise *t* tests (performed as described previously [30]) determined this result to be statistically different from the three other antibody treatments ($P \leq 0.006$) (data not shown). Berger et al., Hemachandra et al., Lai et al., and Schreiber and colleagues have previously shown that bacterial killing correlates with uptake of opsonized bacteria by PMN in this assay (6, 22, 30, 44).

Glycosylation analyses of anti-*P. aeruginosa* serotype O6ad IgG1 from transgenic tobacco. Plant-made MAbs, compared to mammalian antibodies, possess atypical glycosylations due to differences in the biosynthesis of N-linked glycans between plants and mammals (19). The 339-amino-acid sequence of the Fc of the tobacco-expressed anti-*P. aeruginosa* serotype O6ad IgG1 was identical, except for 3 amino acids (not shown), to the Fc of the SO57 MAb expressed in tobacco by Ko et al. (29), which was determined to contain mainly oligomannose-type N glycans. Glycan analyses of purified IgG1 were therefore

modeled on that paper and performed at ProZyme, Inc. (San Leandro, CA). Intact N-linked oligosaccharides were enzymatically released using PNGase F, and the reducing ends of the released glycans were stoichiometrically labeled with ANTS. The ANTS label imparted a negative charge that allowed FACE separations by size, glycan charge, and linkage differences. A FACE profiling gel (Fig. 5) shows PNGase F released oligosaccharides from the plant IgG1, along with the E3 Oligo Ladder and E5 Quantification Control. Table 1 lists the percentage composition of predicted glycan structures on this IgG1 as determined by FACE. To corroborate these results, 2-AB-labeled glycans from a parallel PNGase F digestion were subjected to GlycoSep N HPLC separation for GU value calculation (see Materials and Methods). Due to overlapping GU and degree of polymerization values for various structures, precise identification could not be made based on migration patterns alone; thus, Table 1 lists the best possible candidate glycans based on known migration patterns of expected glycans associated with this type of MAb (19, 29).

The results of both assays are in good agreement, with the exception of a few minor peaks, and showed that the major N-linked glycans of the IgG1 were Man-7 (at least 64%) and Man-8 (at least 18%) (Table 1). Small amounts of Man-5 and

FIG. 3. Inhibition of binding of purified human anti-*P. aeruginosa* serotype O6ad IgG1 MAb to solid-phase *P. aeruginosa* serotype O6ad PS by preincubation with either serotype O6ad PS (diamonds) or control PS (squares) from serotype O2 (IT-2). The *x* axis shows the concentration of serotype O6ad or control PS added to each well. The *y* axis shows the percent reduction of absorbance obtained with each PS inhibitor. The data represent means from three separate assays, each run in duplicate, and standard errors of the means (SEMs). Data points without apparent SEM bars have SEMs smaller than the symbol.

FIG. 4. Phagocytosis of *P. aeruginosa* serotype O6ad cells by tobacco-derived human IgG1 in the presence of human PMN and complement. The *y* axis depicts percent phagocytosis of the inoculum of 10^4 CFU of *P. aeruginosa* serotype O6ad after 90 min of incubation with MAb, 10^6 human PMN, and human complement. The error bars indicate standard errors of the mean from quadruplicate assays; percent phagocytosis by control antibody is statistically different from the three treatments with the tobacco-derived IgG1 (see the text).

FIG. 5. N-linked glycan analysis. Oligosaccharides released from tobacco-derived human IgG1 by PNGase F were separated through a FACE profiling gel at Prozyme, Inc. (San Leandro, CA). The three samples, labeled at the top of the gel, were E3 oligosaccharide standard ladder (lane 1), released IgG1 oligosaccharides (lane 2), and 50 pmol of maltotetraose (E5 quantitation standard) (lane 3). Sample 1 contained a mixture of glucose polymers; the 10 fastest-migrating oligosaccharide bands, from the bottom, were G3 through G12. The two most intense oligosaccharide bands in the IgG1 profile were Man-7 (electrophoretic mobility between standards G7 and G8) and Man-8 (electrophoretic mobility between G8 and G9). IgG1 oligosaccharide identities were corroborated by HPLC analysis.

Man-9 were also detected, as well as other N-linked glycans (Table 1). There was no evidence of sialylated structures typically found on mammalian glycoproteins (39, 52). High-mannose glycans are known to be assembled in the ER lumen of plant cells (26) and are transferred to Asn residues of Asn-X-Ser/Thr acceptor sequences (16, 23). The plant-produced HC polypeptide has one such acceptor sequence (GenBank AY829476), which is the likely position of glycosylation of this plant MAb.

DISCUSSION

We set out to produce a fully human IgG antibody in transgenic tobacco and to determine whether it was capable of recruiting immune system effector function. Fully human (29) and humanized (56) MAbs have been produced in plants and have been shown to have neutralizing activities against specific viral targets, but because antibody binding can prevent viral entry into cells by steric hindrance (9), it is possible that the efficacies of these MAbs did not require effector function. Murine MAbs have been produced by plants and shown to be functional against human tumor antigens (7, 28) and *Streptococcus mutans* (i.e., Guy's 13 MAb) (10, 11, 31, 35). The Fv region of our MAb was taken from a human IgG2 MAb, S20, produced via XenoMouse immunization (22, 33), which had specificity for the bacterial pathogen *P. aeruginosa* serotype

O6ad. This and nine other anti-*P. aeruginosa* serotype-specific MAbs have been shown to be successful at specific opsonophagocytosis in vitro and to protect mice in vivo from specific bacterial challenge (22, 30).

All IgG subclasses have the ability to recognize foreign antigens, but they differ in their abilities to trigger immune system function and in their serum persistences. We chose to test the functionality of a plant-produced human IgG1 because this subclass can induce strong antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity effector functions (8, 14, 38, 41), and it has longer serum persistence than the other human IgG subclasses (32). Therefore, we switched the subclass of the S20 MAb (22) by combining its Fv region with human gamma-1 (HC) and kappa (LC) constant regions.

There are a number of other differences between the S20 MAb and the tobacco-produced IgG1. The plant MAb has NcoI and NotI sites engineered to flank both the V_H and V_L sequences, resulting in two and three additional alanines, respectively, between the V_H and V_L and their respective constant regions. Also, the plant MAb has a six-His epitope and a KDEL ER retrieval signal on the C terminus of its HC. Insertion of these extra amino acids did not appear to adversely affect the binding specificity (Fig. 3) or effector function recruitment (Fig. 4). The KDEL sequence was incorporated because ER retention is known to increase antibody expression in plants (43); it appeared to be functional, as glycosylation analyses (Table 1) revealed high-mannose modifications consistent with ER localization (10, 26, 29, 51). High mannose content is known to be essential for mediating effector function (54). Whether the high mannose content of this plant MAb would reduce its serum persistence compared with native IgG1 antibodies is not known, but the Guy's 13 MAb, also produced in tobacco, has high mannose content (10) and was shown to be nonimmunogenic in mice after subcutaneous administration (11).

Production of this plant MAb resulted in the development of a human IgG1 expression vector containing gamma-1 and kappa constant region coding sequences that were synthesized with tobacco-preferred codons. This vector could be used to produce other human or humanized (12) IgG1 MAbs from plants that are capable of recruiting immune system effector function against any pathogen by replacement of NcoI-NotI fragments with new V_H and V_L sequences. If further reduction of the differences from human IgG1 antibodies is required, deletion of the C-terminal epitopes encoded on the HC (i.e.,

TABLE 1. Glycan composition of tobacco-expressed human anti-*P. aeruginosa* O6ad IgG1

Predicted glycan structure	% Composition	
	FACE	HPLC
M3N2 or M3N2F	0.6	1.4
NGA2	0.9	4.7
NGA2F	2.6	3.4
Man-5	0.9	1.0
Man-6	2.7	3.8
Man-7	71.0	64.1
Man-8	20.3	18.9
Man-9	1.0	2.5

KDEL and six-His) and expression in plants bioengineered to be capable of mammalian-type glycosylations (5, 13, 24, 37) should be performed. Although deletion of the KDEL ER retrieval signal would likely reduce the amount of MAb produced in planta, we have recently shown that protein G affinity is sufficient for purification of this IgG1 to a quality similar to that shown in Fig. 2 (unpublished data).

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