Function and glycosylation of plant-derived antiviral monoclonal antibody

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Plant genetic engineering led to the production of plant-derived mAb (mAb^P), which provides a safe and economically feasible alternative to the current methods of antibody production in animal systems. In this study, the heavy and light chains of human anti-rabies mAb were expressed and assembled *in planta* under the control of two strong constitutive promoters. An alfalfa mosaic virus untranslated leader sequence and Lys-Asp-Glu-Leu (KDEL) endoplasmic reticulum retention signal were linked at the N and C terminus of the heavy chain, respectively. mAb^P was as effective at neutralizing the activity of the rabies virus as the mammalian-derived antibody (mAb^M) or human rabies Ig (HRIG). The mAb^P contained mainly oligomannose type N-glycans (90%) and had no potentially antigenic α(1,3)-linked fucose residues. mAb^P had a shorter half-life than mAb^M. The mAb^P was as efficient as HRIG for post-exposure prophylaxis against rabies virus in hamsters, indicating that differences in N-glycosylation do not affect the efficacy of the antibody in this model.

More than 10 million people annually receive rabies virus prophylaxis in the form of equine anti-rabies immunoglobluin (ERIG) or human anti-rabies Ig (HRIG), together with rabies vaccine (1). However, the world shortage of these immunoglobulins, as well as the risk of both adverse reactions associated with ERIG and the high cost of HRIG, has hampered global efforts to provide a post-exposure prophylaxis against rabies (2).

Transgenic plants have proven to be an efficient production system for the expression of functional therapeutic proteins (3). Plant-derived monoclonal antibodies (mAb^P) have the same advantages, namely, the lack of animal pathogenic contaminants, low cost of production, and ease of agricultural scale-up compared with the conventional fermentation methods. Since the initial report of functional mAbs expressed in transgenic plants (4), therapeutic and diagnostic $mAbs^P$ have been successfully produced in transgenic tobacco, soybean, alfalfa (5–8), and other plants (3). Two $mAbs^P$ have recently been used for topical passive immunization against *Streptococcus mutans* and herpes simplex virus in animals (5, 6). To date, no study has reported the use of systemic administration of mAb^P to provide immunoprotection.

Differences in posttranslational modifications, such as glycosylation, have been shown to influence the properties of plant-derived proteins (3, 9, 10). In plants, N-linked glycans may contain antigenic (11) and/or allergenic (12) $\beta(1,2)$-xylose (Xyl) residues attached to the β-linked mannose (Man) of the glycan core and α(1,3)-fucose (Fuc) residues linked to the proximal GlcNAc that are not present on mammalian glycans. Plant glycans do not contain sialic acid residues, and $mAbs^P$ do not require these residues for successful topical passive immunization (5, 6).

Glycosylation processing in the endoplasmic reticulum (ER) is conserved amongst almost all species and restricted to oligomannose ($Man_{5-9}GlcNAc_2$) type N-glycans, whereas the Golgi-generated processing to hybrid and complex type glycans is highly diverse (13). When attached to the C terminus, the ER retrieval motif KDEL (Lys-Asp-Glu-Leu) allows glycoproteins to be retained in, or returned to, the ER. Although there are exceptions (14), in general, glycans attached to proteins containing a C-terminal KDEL sequence would be expected to be restricted mainly to the oligomannose type (13, 15, 16). ER retention of proteins in transgenic plants usually improves the production levels (9, 17). However, because glycan processing can affect the stability of antibodies (18), it is unclear whether an mAb^P with modified glycan structures would be active and able to confer effective systemic post-exposure prophylaxis.

Here, we have expressed and characterized the human anti-rabies mAb derived from transgenic tobacco plants and demonstrated its effectiveness *in vivo*. The mAb^P was compared with the human mAb SO57 (mAb^M) expressed in murine/human hybridoma cell lines (19, 20) and/or commercial HRIG for rabies virus neutralization activity, protein stability, N-glycan processing, and the efficacy of rabies virus post-exposure prophylaxis in exposed animals.

Materials and Methods

Construction of Plant Transformation Binary Vector. cDNA fragments encoding for mAb SO57 light chain (LC, 729 bp; GenBank accession no. AY172960) and heavy chain (HC, 1,431 bp; GenBank accession no. AY172957) (20) were arranged into a pBI121 binary vector (CLONTECH) as follows. The HC was amplified with primers containing *Nco*I and *Xba*I sites (5′-cgccatggactggacctggaggttc-3′ and: 5′-gctctagattagagctcatctttgtgat-ggtgatggtgatgtttacccggggacagggag-3′, containing the KDEL ER retention signal) and placed with the alfalfa mosaic virus untranslated leader sequence (AMV) of RNA4 (21) under the control of the cauliflower mosaic virus 35S promoter with duplicated upstream B domains (Ca2p) (22) into the pUC9-based vector. The LC was amplified by using primers containing *Bam*HI and *Pst*I sites (5′-cgggatccatgagtgtccccaccatggcc-3′ and 5′-cgctgcagctatgaacattctgtaggggc-3′) and subcloned into the corresponding sites of pGEM T vector (Promega) between potato proteinase inhibitor II promoter (*Pin2*p) and its terminator (23). The HC and LC expression cassettes were consequently transferred as *Hin*dIII-*Eco*RI fragment (HC) and *Hin*dIII fragment (LC) into the plant binary vector pBI121 to yield pBIRA-57 (Fig. 1*A*).

Plant Transformation. Tobacco leaf explants (*Nicotiana tabacum* cv. *Xanthi*) were used for Agrobacterium-mediated transforma-

Abbreviations: mAb^P, plant-derived mAb; mAb^M, mammalian-derived mAb; KDEL, Lys-Asp-Glu-Leu; HRIG, human rabies Ig; HC, heavy chain; LC, light chain; Xyl, xylose; Fuc, fucose; ER, endoplasmic reticulum; *Pin2*p, proteinase inhibitor II promoter; MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight; HDCV, human diploid cell culture rabies virus; PNGase, peptide *N*-glycosidase; Man, mannose.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY172957 (mAb SO57 heavy chain) and AY172960 (mAb SO57 light chain)].

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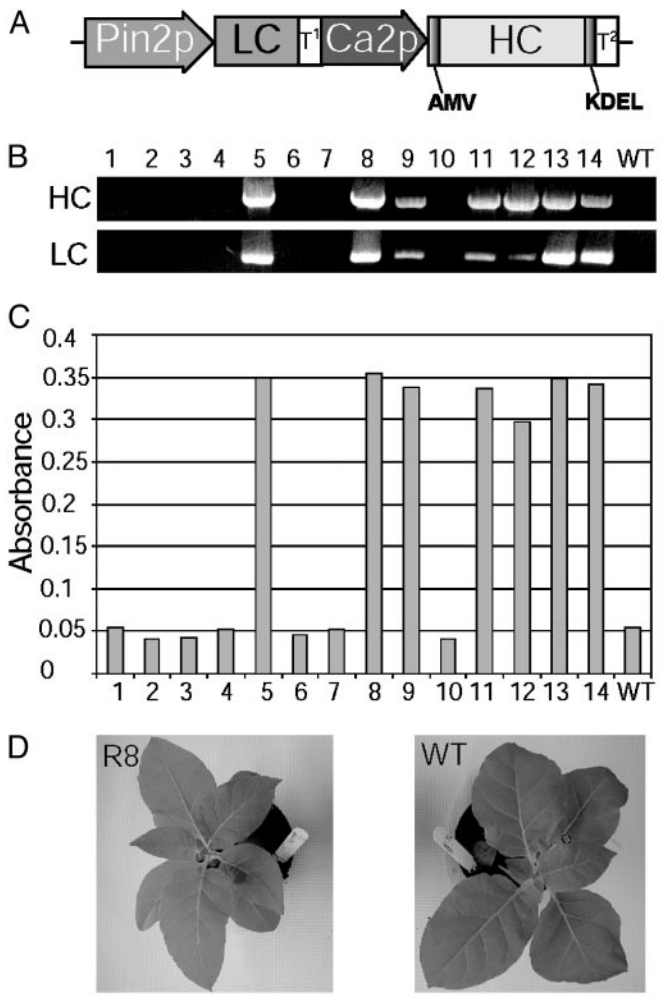

Fig. 1. Generation of transgenic tobacco plants expressing human mAb. (*A*) Schematic diagram of human mAb SO57 LC and HC arrangement in a binary vector (pBIRA-57) used for plant transformation. LC and modified HC were placed codirectionally under the control of the promoters *Pin2*p and Ca2p, respectively. AMV is alfalfa mosaic virus untranslated leader sequence of RNA 4, and KDEL is the 3′ ER retention motif. T^1 is the *Pin2* terminator and T^2 is the *NOS3* terminator. (*B*) PCR analysis of transgenic lines (nos. 1–14) for the presence of both mAb SO57 HC and LC in the plant genomic DNA. WT, wild-type plant. (*C*) ELISA analysis of the same transgenic lines for mAb SO57 protein expression. (*D*) Transgenic plant with high level of transgene expression (line R8) compared with WT.

tion (*A. tumefaciens* EHA105; ref. 23). Transgenic plants were selected on kanamycin (100 μg/ml; ref. 4). Transgenic tobacco lines were then maintained in soil, and subsequent generations were obtained.

Molecular Characterization of Transgenic Plants. PCR amplification of genomic DNA for the presence of mAb SO57 HC and LC was performed by using primers described above. mAb SO57 expression levels and assembly were further analyzed by ELISA. 96-well MaxiSorp Surface plates (Nunc) were coated with rabies virus strain CVS-11. Plates were loaded with soluble protein leaf extracts (24) and with dilutions of 2 μg/ml mAb SO57 purified from the hybridoma supernatant (mAb^M; ref. 20) as a positive control. Goat anti-human horseradish peroxidase conjugate (Jackson ImmunoResearch) was detected by *O*-phenylenediamine dihydrochloride (Sigma). Absorbance (490 nm) was read on a SPECTRAmax 340PC Microplate Spectrophotometer (Molecular Devices).

SDS/PAGE and Protein Blot Analysis. One gram of leaf tissues was ground in liquid nitrogen with 100 μl of extraction buffer (50 mM Tris, pH 7.5, 250 mM sucrose) containing Complete inhibitor mixture (Roche Diagnostics). Forty micrograms of soluble protein was resolved on 12% SDS/PAGE and transferred to Immobilon-P (Millipore) by using a miniProtean II system (Bio-Rad). Goat anti-human antibody [Fcγ and F(ab′)$_2$ fragment-specific] conjugated to horseradish peroxidase (Jackson ImmunoResearch) was used to detect HC and LC, respectively, by using SuperSignal (Pierce). mAb^M was used as a positive control (20).

Purification of Plant-Derived Antibodies. Soluble protein extracts were one-step affinity purified by using protein A agarose matrix (Invitrogen) overnight (4°C) and either applied for HPLC analysis of N-linked glycans or an extra purification step was carried out on protein G column (Pierce) according to the manufacturer's recommendations, dialyzed in 1× PBS buffer and brought to the appropriate concentrations with Millipore spin-columns [10,000 nominal molecular weight limit (NMWL)]. Preparations were either used immediately or stored at −80°C as aliquots.

***In Vitro* Rabies Virus Neutralization Assay.** The rapid fluorescent focus inhibition test (RFFIT) was carried out with some modifications (19, 25). Three-fold serial dilutions of mAb^P, mAb^M, and HRIG (Imogam Rabies-HT, Aventis Pasteur, Swiftwater, PA) were incubated with rabies virus cell culture adapted strains (CVS-11 and CVS-N2c) and dog street rabies virus (DRV-4) for 60 min at 37°C. The mixture of antibody and virus was used to infect baby hamster kidney (BHK-21) cells. After incubation in a 96-well flat-bottom plate for 40 h at 37°C, the cells were washed, fixed, and stained with the FITC-labeled anti-rabies reagent and examined under a fluorescence microscope (25).

HPLC Analysis of N-Linked Glycans. The HC and LC of mAb^P and mAb^M proteins were separated on an SDS/PAGE. N-linked glycans were released from gel slices by incubation with peptide *N*-glycosidase (PNGase) F and PNGase A (14, 26). Labeling, HPLC, and simultaneous exoglycosidase sequencing of the released glycan pool were performed (27–32). Exoglycosidases were used at the following concentrations: *Arthrobacter ureafaciens* sialidase (ABS, EC 3.2.1.18), 1–2 units/ml; almond meal α-fucosidase (AMF, EC 3.2.1.111), 3 milliunits/ml; bovine testes β-galactosidase (BTG, EC 3.2.1.23), 1–2 units/ml; *Streptococcus pneumoniae* α-hexosaminidase (SPH, EC 3.2.1.30), 120 units/ml; and Jack Bean α-mannosidase (JBM, EC 3.2.1.24), 100 milliunits/ml. Glycopro Glucosaminidase (GluH, Prozyme, San Leandro, CA) was used as recommended by the manufacturer.

Matrix-Assisted Laser Desorption Ionization–Time-of-Flight (MALDI-TOF) Mass Spectrometry of Released Glycans. After removing traces of gel (C18 column), underivatized glycans were purified by using a Nafion 117 membrane (Aldrich) and examined by MALDI mass spectrometer by using a positive mode with a Micromass TOFSpec 2E reflectron-TOF instrument (Micromass, Manchester, U.K.). Samples (0.3 μl in water) were mixed with a saturated solution of 2,5-dihydroxybenzoic acid on the MALDI target, allowed to dry at room temperature, and then recrystallized from the ethanol. Operating conditions for the mass spectrometer were as follows: acceleration voltage, 20 kV; pulse voltage, 3,200 V; and the delayed extraction ion source was 500 ns. The instrument was calibrated with dextran oligomers. Monoisotopic masses of the $[M + Na]^+$ ions were within 0.1 mass units of the calculated values. Multiple-stage MALDI fragmentation spectra were acquired on an AXIMA-QIT MALDI quadrupole ion trap TOF instrument controlled by Kratos LAUNCHPAD software (Kratos Analytical Instru-

ments). The nitrogen laser (337 nm, 3 ns pulse width) pulse rate was 10 Hz, and a small bias voltage (2.5–30 V) was applied to the MALDI sample plate, depending on the mass of the analyte under investigation. After ionization, ions were extracted by a negative potential (−10 kV); trapped by application of a retarding potential to the end-cap and an rf potential (500 kHz) to the ring electrode of the ion trap; and cooled by using helium (6×10^{-3} Torr). Fragmentation was induced by resonant excitation after application of a supplementary AC potential to the end cap electrodes. Product ions from the molecular or fragment ions were extracted into the TOF analyzer with an accelerating voltage of 10 kV. The TOF was externally calibrated by using fullerite deposited directly onto the sample stage. Glycans, subjected to fragmentation, were previously digested with *A. ureafaciens* sialidase, and the sample target was prepared as above.

***In Vivo* Half-Life of mAbs.** Five micrograms of mAb^P or mAb^M in 100 μl of 1× PBS buffer were injected i.p. into 10 and 7 BALB/c mice (female, 6–8 wk; The Jackson Laboratory), respectively. Blood samples were collected from the orbital sinus on days 1, 2, 3, 4, 7, 8, and 10 after injection; each mouse was bled twice during the entire time period. Serum levels of mAb^P and mAb^M were detected by ELISA as described above.

***In Vivo* Protection Assay Against Rabies Virus.** Two month-old (100 g) female Syrian hamsters (Harlan–Sprague–Dawley) were inoculated with 50 μl of a homogenate [$\approx 10^{6.8}$ mouse intracerebral lethal dose $(MICLD)_{50}$/ml of a Texas coyote rabies virus in circulation at the United States–Mexico border] of salivary gland tissue from a naturally infected rabid coyote (33). The post-exposure prophylaxis protocol was initiated 4 h after intramuscular inoculation of the rabies virus in the left gastrocnemius muscle. The trial consisted of nine groups (nine hamsters each) and untreated controls. Each treated group i.p. received HRIG [2 international units (IU) per animal; Bayrab, Bayer, Elkhart, IN], and mAb^P at 0.4, 0.7, and 3 IU per animal with or without human diploid cell culture rabies virus (HDCV) vaccine (Imovax, Aventis Pasteur), respectively. The HRIG or mAb^P was inoculated in the left gastrocnemius muscle. A tenth group of three hamsters received only the HDCV vaccine. The HDCV vaccine was administered in the right muscle at a volume of 50 μl undiluted from the vial on days 0, 3, 7, and 14. Animals were observed daily and were sedated with ketamine hydrochloride and then killed by CO_2 intoxication on the first day that clinical signs of rabies became evident.

Results

Tobacco transgenic lines were generated by Agrobacterium-mediated transformation with a binary vector carrying both the heavy chain (HC) and light chain (LC) of human mAb SO57 (Fig. 1). Fig. 1*A* shows the construct arrangement. The HC cDNA was fused with AMV at its 5′ end and the KDEL retention signal at its 3′ end and placed downstream of the enhanced cauliflower mosaic virus 35S promoter (Ca2p). The LC was placed under the control of the strong *Pin2* promoter. PCR analysis of transgenic plants generated from independent transformation events revealed the presence of both HC and LC in seven transgenic lines (R5, R8, R9, R11, R12, R13, and R14; Fig. 1*B*). Protein expression analysis by ELISA confirmed that human mAb SO57 is expressed in the same transgenic lines (Fig. 1*C*). Transgenic line R8, with the highest absorbance level (>0.35), was selected for further studies. Transgenic plant R8 did not differ morphologically from the WT plant (Fig. 1*D*) and retained the same level of protein expression over several generations (data not shown).

Both HC and LC were identified in the soluble protein extracts from the transgenic plant R8 leaf tissue as major bands migrating

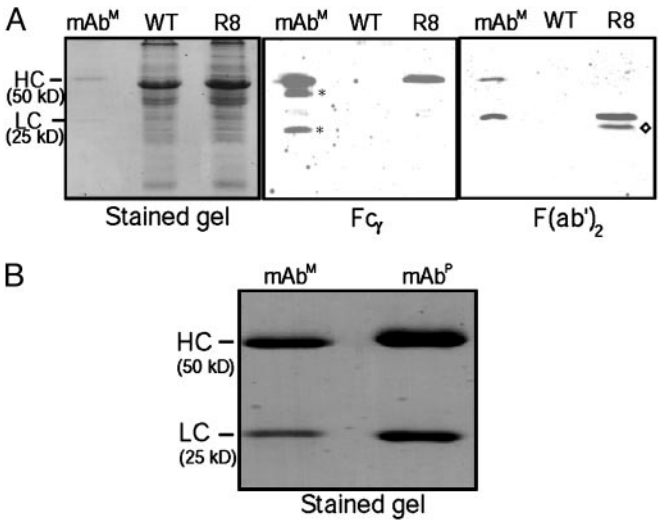

Fig. 2. Analysis of mAb protein expression in transgenic plants by SDS/PAGE. (*A*) HC (50 kDa) and LC (25 kDa) of mAb^M and total protein of R8 were resolved under denaturing conditions and either stained (*Left*) or blotted for detection with goat anti-human Fcγ (*Center*) or $F(ab')_2$ (*Right*) specific antibody conjugated with horseradish peroxidase. Purified mAb^M loaded at 30 ng per lane. Soluble proteins from leaf tissue of WT and transgenic plant R8 loaded at 40 μg per lane. Asterisks and diamond (*Center* and *Right*) indicate additional bands recognized by Fcγ or $F(ab')_2$ antibodies, respectively. (*B*) Affinity-purified mAbs from mammalian and plant expression systems resolved on SDS/PAGE at 2.5 and 3.5 μg per lane, respectively.

at the expected molecular masses, 50 kDa and 25 kDa, respectively (Fig. 2*A*). HC was readily detected by anti-human Fcγ fragment-specific antibody in both mAb^M and R8 (Fig. 2*A Center*). Two lower molecular mass bands observed in mAb^M (Fig. 2*A*, asterisks) are most likely to be HC proteolytic degradation products. LC was detected by anti-human $F(ab')_2$ fragment-specific antibody. The HC band was detected in mAb^M, whereas only LC was detected in R8 together with a lower molecular mass band probably resulting from proteolytic degradation (Fig. 2*A Right*, diamond). The expression level of mAb^P in R8 plants was calculated as 3 μg/g of fresh leaf weight (0.07% of total soluble protein), consistent with ELISA results (data not shown). Further purification of antibody derived from plant leaf extracts (mAb^P) and from hybridoma supernatants (mAb^M) using a single- and/or double-step purification with protein A and protein G yielded high quality protein products (HC and LC only). Fig. 2*B* shows the results of the single-step (protein A) purification.

In vitro comparison of the neutralizing activity of mAb^P, mAb^M, and HRIG against cell culture-adapted and a street rabies virus (Table 1) indicated that mAb^P was as active against

Table 1. Comparison of virus-neutralizing activity of mAb^P, mAb^M, and HRIG against different rabies viruses

	Results of VNA*, IU/ml		
	Antibody		
Rabies viruses used†	mAb^P	mAb^M	HRIG
CVS-11	162	162	162
CVS-N2c	108	108	54
DRV-4	81	54	27

*Virus-neutralizing antibody (VNA) titer was determined as described (35).
†CVS-11 and CVS-N2c are cell culture-adapted virus strains, and DRV-4 is a dog street rabies virus.

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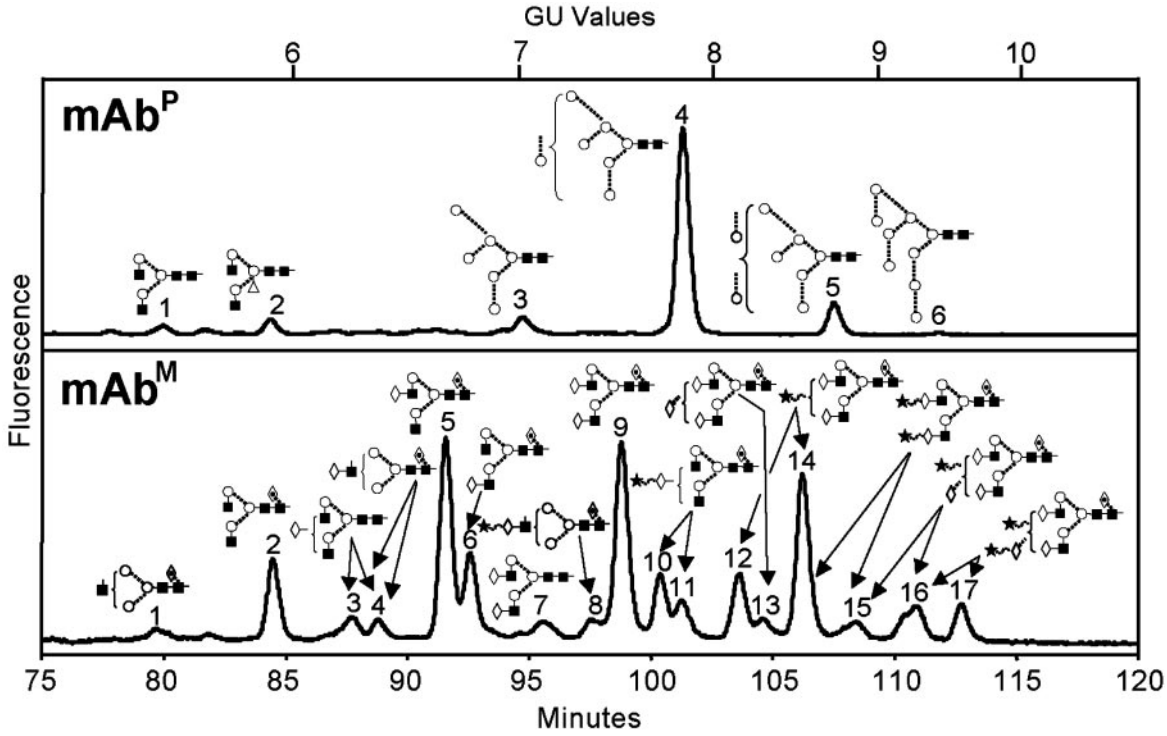

Fig. 3. NP-HPLC chromatograms of 2-AB-labeled N-glycans released enzymatically with PNGase F from HCs of mAb^P and mAb^M. The schematic glycan structures of the main peaks found in N-glycan pools from mAb^P (*Upper*) and mAb^M (*Lower*) are indicated. All peaks were numbered, and corresponding glycans were assigned by using an array of exoglycosidase enzymes and confirmed by MALDI-TOF mass spectrometry. The symbols of the glycan structures are: filled square, GlcNAc; open circle, mannose; open diamond, galactose; diamond with a dot, fucose; filled star, sialic acid; open triangle, xylose; dotted line, α-linkage; solid line, β-linkage; |, 1–2 linkage; /, 1–3 linkage; –, 1–4 linkage; \, 1–6 linkage; ~, undetermined linkage.

the cell culture-adapted virus strain CVS-11 as mAb^M and HRIG, and more active than HRIG against the CVS-N2c strain. MAb^P had stronger activity compared with mAb^M and HRIG against the street virus DRV-4. Together, these data demonstrate that the rabies virus-neutralizing activity of mAb^P is as high as that of mAb^M and similar to that of HRIG.

Structural differences in proteins expressed in heterologous systems are known to arise from posttranslational modifications, mostly from glycosylation. Human mAb SO57 has a conserved N-linked glycosylation site in each HC. HCs of mAb^P and mAb^M were isolated on SDS/PAGE, and the glycans were released directly from the gel bands with two different endoglycosidases: PNGase F, which releases N-glycans that do not contain an α(1,3)-Fuc residue linked to the core GlcNAc that is proximal to the Asn residue, and PNGase A, which liberates all N-glycans. No significant differences were found between the glycan pools obtained by these different enzymatic releases (data not shown), indicating that no α(1,3)-Fuc was present in the mAb^P. The HPLC profiles of the fluorescently labeled N-glycan pools, released by PNGase F from mAb^P and mAb^M, are shown in Fig. 3. Preliminary structures assigned to the glycans in the 6 peaks in the mAb^P profile and 17 peaks in the mAb^M profile (Fig. 3) were confirmed by exoglycosidase digestions and were consistent with MALDI-TOF mass spectrometry results (data not shown).

For mAb^P (Fig. 3 *Upper*), 90% of the total glycan pool consisted of oligomannose-type oligosaccharides (peaks 3–6 contained $Man_{6-9}GlcNAc_2$, respectively). The remaining two peaks (1 and 2) corresponded to $GlcNAc_2Man_3GlcNAc_2$ (4.3%) and $GlcNAc_2Man_3(Xyl)GlcNAc_2$ (5.7%). No Fuc or Gal residues were found on any plant-derived glycan structures. In contrast, the mAb^M (Fig. 3 *Lower*) displayed a range of complex glycans, most of which (95.4%) contained a core α(1,6)-Fuc and outer-arm Gal and about half contained sialic acid (peaks 7–12 and 14–17). Twelve percent of glycans (within peaks 13 and 15–17) contained additional Gal residues. This additional Gal was identified as the terminal monosaccharide in Galα1–3Galβ1–4GlcNAc-R, because it was digested with coffee bean α-galactosidase that releases only α-Gal-linked monosaccharides. Consistent with the HPLC elution position and the exoglycosidase digestions, MALDI mass spectrometry using a tandem ion trap-TOF instrument also located the extra Gal to the nonreducing terminus of one of the antennae of the glycan with the composition $Hex_6HexNAc_4dHex_1$ ($Gal_3GlcNAc_4Man_3Fuc_1$, $[M + Na]^+$ at m/z 1971.7). Two successive stages of fragmentation (MS^3 spectrum) indicated that the structure of the ion at m/z 550.2, derived from this antenna, was Hex-Hex-HexNAc (data not shown). This Gal residue is therefore the commonly known α-Gal epitope of murine origin that is antigenic to humans (34).

In vivo stability of mAb^P and mAb^M was analyzed in a comparative clearance test in which mice were injected i.p. and blood samples were collected for up to 10 days. In an ELISA to determine the levels of these antibodies in serum from mice, mAb^P was barely detectable 10 days after injection, whereas mAb^M was still abundant (Fig. 4).

The efficacy of mAb^P in post-exposure prophylaxis was examined in hamsters injected with rabies virus of coyote origin (Table 2). In this *in vivo* protection assay, all untreated control hamsters succumbed to fatal rabies virus encephalitis, as did those animals that received HDCV vaccine only. The survival rate for hamsters that received 3 IU of mAb^P or 2 IU of HRIG without administration of HDCV vaccine was ≈50%, whereas the rate was decreased in hamsters receiving lower mAb^P doses of 0.4 and 0.7 IU alone. The survival rate

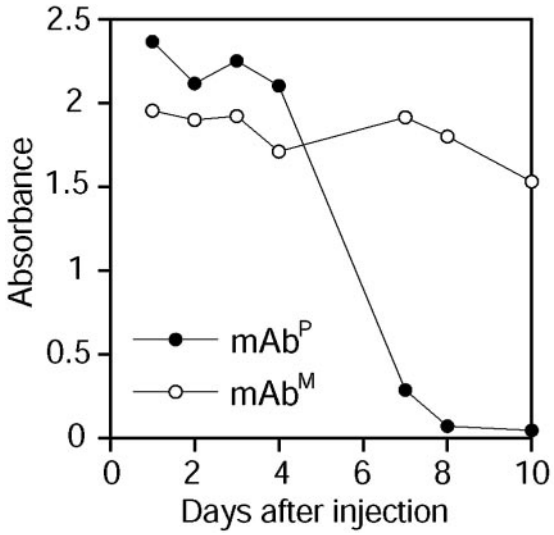

Fig. 4. Stability of plant- and mammalian-derived mAb in mice. The presence of antibody in serum from BALB/c mice injected i.p. with mAb^P or mAb^M was determined by ELISA. Samples were collected and analyzed against CVS-11 rabies virus strain.

remained high (8 of 9) when mAb^P at any concentration level was administered together with HDCV vaccine (Table 2).

Discussion

Our present data demonstrate that the plant-expressed human mAb (mAb^P), containing mainly oligomannose type N-glycans, has anti-rabies virus-neutralizing activity comparable to that of its mammalian-derived counterpart, and an efficacy in rabies post-exposure prophylaxis comparable with that of HRIG. The expression level and appropriate posttranslational events (correct folding, glycosylation, and subcellular targeting) are important factors for the effectiveness of antibodies produced in plants (7, 10, 17, 35). In our study, these factors were optimized by the combination of gene regulatory elements in a plant binary vector. Two different strong constitutive promoters (*Pin2*p and Ca2p) controlling the expression of LC and HC of the human antibody, respectively, and a translation AMV activator and ER retention signal KDEL, fused to HC, were combined to avoid homology-based gene silencing (36), aimed to increase HC expression levels (23) and to induce compartment-specific accumulation, respectively (5, 6, 8). The human antibodies were expressed (0.07% of total soluble protein) and fully assembled in plants without any gene silencing through two generations of the R8 transgenic line with single gene integration (data not shown). These data indicate the feasibility of the construct arrangements to express and assemble functional mAbs in plants.

Table 2. *In vivo* efficacy of mAb^P for post-exposure prophylaxis of hamster injected with a lethal dose of coyote rabies street viruses

	Post-exposure treatment	
	Vaccine (HDCV)*	
Antibody (units per animal)	−	+
mAb^P (3)	5/9	9/9
mAb^P (0.7)	1/9	8/9
mAb^P (0.4)	2/9	8/9
HRIG (2)	4/9	8/9
Untreated control	0/9	0/3

Data are of surviving hamsters/number of hamsters tested.
*HDCV, commercial (Imovax, lot MO475) human diploid cell culture rabies virus vaccine. − and +, treatments without or with HDCV, respectively.

In contrast to mAb^M, which contains 17 complex N-glycans in the conserved glycosylation site on each HC, the mAb^P modified to contain a KDEL sequence displays mainly oligomannose-type N-glycans (90%). A previous report indicated that mAb^P (lacking an ER retention signal) contains a greater diversity of N-glycan structures (37). This result is predictable because, in the absence of a KDEL sequence, mAb^P would be expected to be transported to the Golgi apparatus where oligomannose-type glycans can be processed to complex type structures (13). The presence of $Man_{6-9}GlcNAc_2$ (90%), $GlcNAc_2Man_3GlcNAc_2$ (4.3%), and $GlcNAc_2(Xyl)Man_3GlcNAc_2$ (5.7%) glycans in mAb^P indicates that most (90%) of mAb^P/KDEL did not pass further along the secretory pathway than the cis-Golgi stack, from which it was probably retrieved and returned to the ER (15, 16). As a result, the modified mAb^P did not contain glycans with the plant-specific $\alpha(1,3)$-linked Fuc residues, minimizing the risk of immunogenic and allergenic reactions to this epitope in humans (11). The $\alpha(1,3)$-linked Fuc residue is recognized by both IgG and IgE (38). If present, the Xyl residue that is $\alpha(1,2)$-linked to the β-linked core Man of the sugars attached to mAb^P forms part of the anti-$\alpha(1,3)$-linked Fuc antibody epitope, but does not, on its own constitute a potent epitope (38). Moreover, the Xyl-containing glycans in mAb^P also contain an $\alpha(1,3)$-antenna, and, on these grounds too, the Xyl is unlikely to bind IgE (12). In contrast, α-Gal residues are potent antigens (34). The terminal α-Gal residues on the sugars attached to mAb^M are likely to be accessible even in the context of the IgG CH2 domains in which the glycans are sequestered. Interestingly, the detection of the α-Gal residue provides evidence that the hybridoma cell line had used the murine rather than the human glycosylation machinery.

mAb^P had similar *in vitro* neutralizing activity against cell culture-adapted virus strains and a street virus, compared with the mAb^M. Neutralization depends on blocking of binding sites on the virion and may be mostly mediated by steric hindrance resulting from the relatively large size of the antibody molecule (39). The altered glycosylation on the CH2 domain of antibodies does not affect their affinity for antigen (18, 40). The altered glycosylation of mAb^P with oligomannose N-glycans might reduce the affinity for an Fc receptor and be deficient in antibody-dependent cell-mediated cytotoxicity (41), which is involved in antiviral activity (40, 42). *In vivo* efficacy of mAb^P for post-exposure prophylaxis was similar to that of HRIG.

mAb^P with oligomannose-type N-glycans was rapidly cleared *in vivo* compared with mAb^M. The shorter half-life of mAb^P is predictable, because oligomannose-type N-glycan structures would be expected to lead to an increased clearance rate through binding of the antibodies to Man receptors such as the macrophage Man receptor in liver (41, 43). However, the shorter half-life of the mAb^P-containing oligomannose-type glycans did not adversely affect the immunological protection against rabies for post-exposure prophylaxis. The dual effect of rabies post-exposure treatment with both antibody and vaccine in mammals may occasionally lead to interference between passive and active immunization because of larger persistence of the antibody in the circulation (44–46). Thus, the shorter half-life of this mAb^P, if confirmed in humans, may offer a certain advantage to the current commercial antibody-vaccine prophylaxis because there will be less probability of interference between the passive and active immunity.

Our findings indicate that the modified N-glycosylation pattern of mAb^P does not affect the neutralizing and protective efficacy of post-exposure prophylaxis against rabies virus in hamsters. mAb^P was found to be as efficient as HRIG. The main

benefits of using plant as an expression system include, but are not limited to, the absence of antigenic and/or allergenic epitopes, and ample protein yield. Further studies are needed to determine the efficacy of the mAb^P against other lyssavirus candidate infections in different animal models, including non-human primates. There is a dramatic shortage of rabies antibodies worldwide; hence, production of inexpensive and safe plant-derived mAb should be useful in post-exposure prophylaxis.

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