

Engineering, Expression in Transgenic Plants and Characterisation of E559, a Rabies Virus-Neutralising Monoclonal Antibody

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Rabies post-exposure prophylaxis (PEP) currently comprises administration of rabies vaccine together with rabies immunoglobulin (RIG) of either equine or human origin. In the developing world, RIG preparations are expensive, often in short supply, and of variable efficacy. Therefore, we are seeking to develop a monoclonal antibody cocktail to replace RIG. Here, we describe the cloning, engineering and production in plants of a candidate monoclonal antibody (E559) for inclusion in such a cocktail. The murine constant domains of E559 were replaced with human IgG1κ constant domains and the resulting chimeric mouse-human genes were cloned into plant expression vectors for stable nuclear transformation of *Nicotiana tabacum*. The plant-expressed, chimeric antibody was purified and biochemically characterized, was demonstrated to neutralize rabies virus in a fluorescent antibody virus neutralization assay, and conferred protection in a hamster challenge model.

Keywords. rabies; post-exposure prophylaxis; RIG; monoclonal antibody; *Nicotiana tabacum*.

Rabies is a zoonotic disease caused by rabies virus (RABV), the type member of the *Lyssavirus* genus, and is responsible for >55 000 deaths per annum [1] largely in the developing world [2–4], where transmission usually occurs following the bite of an infected dog. If left untreated, the virus progressively infects surrounding neurons and propagates in the central nervous system leading, almost invariably, to death. The disease can be prevented by post-exposure prophylaxis (PEP), which consists of administration of

inactivated RABV vaccine together with passive antibody therapy [5–7]. In passive antibody therapy, rabies immunoglobulin (RIG), derived either from immunized human (HRIG) or equine (ERIG) sources [8–11], is infiltrated into the wound site.

However, in the developing world, these serum-derived antibodies often suffer from drawbacks including limited availability, batch-to-batch variation, high cost, contamination with blood-borne adventitious agents, and/or risk of adverse reactions [12]; for these reasons, the World Health Organization (WHO) encourages the development and evaluation of alternative biologics for RIG replacement [13]. One such alternative is offered by monoclonal antibodies (mAbs) that are capable of neutralizing a wide range of RABV isolates [12, 14–18]. Rabies neutralizing antibodies are directed against the viral glycoprotein, and several studies have demonstrated that rabies-specific mAbs can protect rodents after RABV challenge [18–23].

However, given the unique epitope specificity of individual mAbs compared to polyclonal antiserum, any mAb-based product designed to replace RIG would

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ideally comprise a defined cocktail of RABV-neutralizing mAbs that would provide coverage against a broad range of RABV isolates, minimize the potential for viral escape and have a potency comparable to that of RIG. The low production costs, ability of plants to assemble and modify multimeric proteins such as mAbs, and ease of scalability make plants a viable platform for production of mAbs to replace RIG [24, 25].

Several groups have characterized RABV-neutralizing mAbs [14, 17, 25–30], and the World Health Organization Rabies Collaborating Centers (WHO RCCs) identified 5 murine mAbs [15], with 4 (E559.9.14, M727-5-1, M777-16-3 and 1112-1) recognizing antigenic site II of the glycoprotein and 1 (62-71-3) recognizing antigenic site I [31].

Amongst the mAbs identified by the WHO RCCs that recognize antigenic site II, E559 exhibited the broadest virus neutralization spectrum and greatest potency [15, 32] and therefore represents an important candidate mAb for inclusion in a RIG-replacement cocktail. In this study, we describe the cloning and sequences of the murine E559 antibody heavy and light chains, engineering of a chimeric mouse-human version of E559, expression in tobacco, and characterization of the purified, tobacco-derived, chimeric mAb in terms of in vitro virus neutralization and in vivo protection.

MATERIALS AND METHODS

Cell Lines, Viruses and Plasmids

Hybridoma cell line E559.9.14 [15, 32], expressing murine IgG1 κ mAb E559, was kindly provided by Dr Thomas Müller (WHO Collaborating Centre for Rabies Surveillance and Research, Friedrich-Loeffler-Institute, Germany). Cells were cultured at 37°C, under a 5% CO₂ atmosphere in CD hybridoma medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated, fetal bovine serum (Life Technologies) and 2 mM L-glutamine (Sigma, UK). For mAb production, the cells were adapted to serum-free conditions.

Lyssavirus strains used included challenge virus standard (CVS) [ATCC VR-959], derived from the original Pasteur virus [33] and animal-derived isolates, as well as RV61, isolated from a person bitten by a dog.

The pL32 and pTRAK.2 plasmids used for plant transformation are described in detail in the online [Supplementary Materials](#).

Agrobacterium tumefaciens strain LBA4404 was purchased from Invitrogen UK. *A. tumefaciens* strain GV3101::pMP90RK was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Leibniz Institute, Germany).

Cloning of Full-length Murine E559 IgG

Total RNA from hybridoma cell line E559.9.14 was isolated from 1 × 10⁶ cells using the RNeasy Mini kit (Qiagen). First strand complementary DNA (cDNA) was prepared using the Omniscript RT kit (Qiagen) with oligo-(dT)₁₅ as the primer.

Using the first strand cDNA as template, the murine γ 1 heavy chain gene was amplified using primers FR1 γ and 932 (see online [Supplementary Table 1](#) for a description of oligonucleotide primers). The murine κ light chain gene was amplified using primers FR1 κ and 933. The murine γ 1 heavy chain and κ light chain amplicons were digested with *Xho*I and *Eco*RI and ligated into binary vector pL32 restricted with the same enzymes.

Cloning of Chimeric Mouse-human E559 IgG

The cloning of the chimeric (mouse-human) heavy (χ E559H) and light (χ E559L) chain genes, and the codon-optimised versions of these genes, is described in detail in the online [Supplementary Materials](#).

Generation and Screening of Transgenic *Nicotiana tabacum* Plants

The generation of transgenic plants is described in the online [Supplementary Materials](#). For screening of plants by Western blotting and enzyme-linked immunosorbent assay (ELISA), leaf discs were excised from leaves using the lid of a 1.5 mL Eppendorf tube as a punch. Leaf discs were homogenized using a plastic pestle in 300 μ L of PBS, centrifuged at 20 000 × *g* for 3 minutes, and the supernatant collected for analysis. Total soluble protein content of the supernatant was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, UK).

Purification of mAbs

For purification of the hybridoma-derived mAb (E559^{Hyb}), hybridoma E559.9.14 cells were grown for 7 days in serum-free conditions, centrifuged (1000 × *g*, 10 minutes, 4°C) to pellet the cells, and the supernatant filtered (0.2 μ m) and applied to an anti-mouse IgG1 (heavy chain specific)-agarose (Sigma, UK) affinity column.

The plant-expressed chimeric antibody (χ E559^P) was purified using Protein A/G agarose as described elsewhere [34]. In the case of the plant-expressed murine E559 (μ E559^P), an anti-mouse IgG1 (heavy chain specific)-agarose (Sigma, UK) affinity column was used instead.

Column fractions were analyzed on Coomassie stained SDS-PAGE gels. Fractions containing the antibody were pooled, dialyzed against phosphate-buffered saline (PBS), and stored in aliquots at –20°C. Dialyzed material was analyzed by ELISA and SDS-PAGE to determine the concentration, purity, and integrity of the mAb.

Samples destined for animal challenge studies were purified using MabSelect SuRe protein A chromatography on a 5 mL Hi-Trap column (GE Healthcare). In addition to affinity purification, samples were further purified using Capto Q (GE Healthcare) in flow through mode and polished using ceramic hydroxyapatite (CHT; BioRad Laboratories). All chromatography steps were conducted on an Akta Avant 150 operated via Unicorn 6.0 software.

Antibody concentrations were determined using a sandwich ELISA, by capturing samples with a heavy-chain specific reagent and detection with a light chain specific reagent. Commercially available human IgG1 κ (The Binding Site, UK) and mouse IgG1 κ (Sigma, UK) were used as concentration standards.

Deglycosylation Using PNGaseF

The deglycosylation protocol using PNGaseF is described in detail in the online [Supplementary Materials](#).

Glycan Analysis of the Plant-derived mAb E559

A glycoproteomic analysis was undertaken by in-gel digestion of S-carbamidomethylated sample and analysis by reverse-phase electrospray ionization mass spectrometry (RP-ESI-MS), as described elsewhere [35]. Tandem MS results were also subjected to Mascot MS/MS ion search (Matrix Science Ltd, London, UK; available at: <http://www.matrixscience.com>).

Enzyme-Linked Immunosorbent Assay

ELISA for detection of antibody heavy or light chains is described in detail in the online [Supplementary Materials](#).

SDS-PAGE and Western Blotting

Polyacrylamide gel electrophoresis (PAGE) and Western blotting protocols are described in detail in the online [Supplementary Materials](#).

Modified Fluorescent Antibody Virus Neutralization (mFAVN) Assay

Live virus experiments were performed using a modified form of the fluorescent antibody virus neutralization (FAVN) assay described for CVS-11 [36, 37] and described in more detail in the online [Supplementary Materials](#). OIE positive (OIE+) and OIE negative (OIE-) reference sera were included as controls. Virus was considered neutralized if the neutralization titer was >0.5 IU/mL [36].

Hamster Challenge Studies

Four groups of Syrian hamsters were included in the experiment. The challenge and treatment schedule was as follows: Group 1 (uninfected control) comprised 4 hamsters that did not receive any viral inoculum or biologics treatment. Group 2 (4 animals) and groups 3 and 4 (each comprising 9 animals) were all inoculated with 50 μ L of 1×10^6 TCID₅₀/mL of a RABV laboratory strain, Challenge Virus Standard CVS (at day 0) intramuscularly and treated subsequently (at day 1) with either PBS (group 2), or with 22.5 IU/kg of either undiluted commercial HRIG (Rabigam [150 IU/mL], National Bioproducts Institute, Pinetown, South Africa) (group 3) or χ E559^P mAb (group 4). Biologics (groups 3 and 4) and PBS (group 2) were administered in the gastrocnemius muscle in 50 μ L volumes to simulate passive immunization in PEP

treatment. No rabies vaccine was administered. The hamsters were observed twice daily over 28 days for any symptoms associated with RABV infection. Brain tissues were collected from animals to confirm rabies virus infection for all those hamsters that succumbed during the observation period and assessed for the presence of lyssavirus antigen using the fluorescent antibody test (FAT) [38]. All hamsters surviving for up to 28 days post-infection were killed with isoflurane and tested for rabies as described above. The animal experimental protocols, animal caging and care, as well as end point for the experiments were approved by the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures, and product development (Agricultural Research Council-Onderstepoort Veterinary Institute, South Africa).

RESULTS

Cloning of Antibody Heavy and Light Chain Genes From Hybridoma E559.9.14

The murine immunoglobulin γ 1 heavy and κ light chain genes expressed by the E559.9.14 hybridoma were amplified by polymerase chain reaction, using first strand cDNA as template. The deduced amino acid sequences of the E559 heavy and light chain genes are presented in Figure 1. Highlighted are important features, such as the complementarity determining regions [39] and the presence of potential N-linked glycosylation sites within the C_H2 domain and the light chain V_L domain.

Analysis of Hybridoma-derived E559

Analysis of the purified murine hybridoma-derived E559 (E559^{Hyb}) by SDS-PAGE under reducing conditions, followed by Coomassie staining, showed the presence of 3 bands with molecular weights of 50 kDa, 27 kDa and 25 kDa (Figure 2A). Western blotting confirmed previous findings [15] that the 50 kDa band corresponded to the heavy chain (Figure 2B), and that the 2 lower molecular weight bands were murine light chains (Figure 2C). The 2 lower molecular weight bands were excised from the gel, treated with trypsin, and analyzed by LC-MS. The panel of peptides generated from each band were nearly identical and in accord with the sequence deduced from the cloned light chain gene (see Figure 1), indicating that these 2 bands are murine κ light chain isoforms. Minor differences in the identified peptides are likely due to differences in the extent of trypsin digestion between the 2 samples. The identification of a potential N-glycosylation site within the V_L domain of the light chain (Figure 1) suggested that the difference between the light chain isoforms might be due to the presence of N-linked glycans. E559^{Hyb} was deglycosylated by treatment with PNGaseF. Blotting under reducing conditions shows that after treatment with PNGaseF, the 27 kDa band is lost, leaving only a single band at 25 kDa (Figure 2D), providing evidence that the

E559 V_H domain

V_H: ***QVKLQES***GSVLVRRPGASVKLSCKASGYIF***T******SFWMQ***WARQRPGQALEWIG***QIHPNSDY******TNYNEKFKG***KATLTVDTSSSTAYVDSLRLTSEDSAVYYCAR***EIWDGGFDY***WGQGSTLTVSS

E559 C_H region

C_{H1}: AKTTPPSVYPLAPGSAATNSMVTLGCLVKGYFPEPVTVTWSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWVPSSETVTCNVAHPASSTKVDKKI

Hinge: VPRDCGCKPCICT

C_{H2}: VPEVSSVFIFFPKPKDVLITITLTPKVTQVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQF***NSTFR***SVSELPIMHQDWLNGKEFKCRVNSAAPPAPIEKTISKTK

C_{H3}: GRPKAPQVYTIFFPKQMAKDVKVSLTCMITDFFPEDITVEWQNGQPAENYKNTQPIMDTDGYSYFVYSKLVNQKSNWEAGNTFTCSVLHEGLHNHHTKSLSHSP***GK***

E559 V_L domain

V_L: ***DI******VL******TQ***SPSSLSASLGERVSL***TC******RANQDIGNSLN***WLQRE***PNG***TIKRL***Y******ATFSLDS***GVPKRFSGSRSGSDYSLTISSLESEDFVDY***C******LQYATSPYT***FGGGTKLEIK

27 kDa band: ANQDIGNSLNWLQR RLIYATFSLDSGVPKR

25 kDa band: ANQDIGNSLNWLQR LIYATFSLDSGVPKR

E559 C_L domain

C_L: RADAAPTIVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSRQNGVLNSWTDQDSKDYSTYSMSSTLTLLTKDEYERHNSYTCCEAHTKSTSPIVKSFNRN***EC***

27 kDa band: ADAAPTIVSIFPPSSEQLTSGGASVVCFLNNFYPK WKIDGSRQNGVLNSWTDQDSKDYSTYSMSSTLTLLTKDEYER TSTSPIVK

25 kDa band: ADAAPTIVSIFPPSSEQLTSGGASVVCFLNNFYPK WKIDGSRQNGVLNSWTDQDSKDYSTYSMSSTLTLLTK TSTSPIVK

Figure 1. Sequences and mass spectrometry analysis of E559. Deduced amino acid sequences of the heavy chain variable domain (V_H), the heavy chain constant region domains (C_{H1}, Hinge, C_{H2}, and C_{H3}), the light chain variable domain (V_L), and the light chain constant domain (C_L) of E559. Complementarity determining regions (CDRs), as defined by Kabat et al [39], are highlighted in bold and underlined. Amino acids encoded by the primers used for amplification are shown in bold italics. Potential N-linked glycosylation sites are double-underlined. Peptides identified by mass spectrometry analysis of the 25 kDa and 27 kDa isoforms of the E559^{Hyb} light chain are shown aligned below the corresponding V_L and C_L sequences (see text).

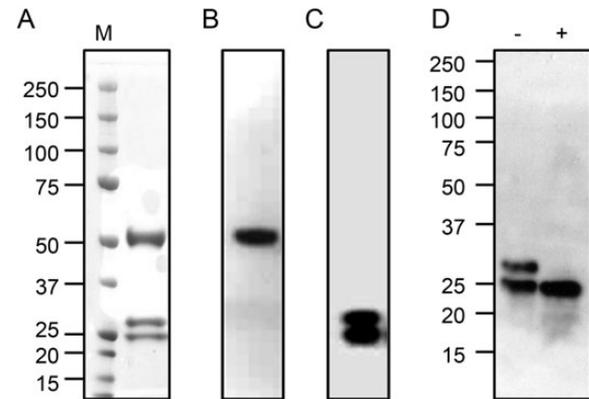


Figure 2. Analysis of hybridoma-derived E559. Hybridoma-derived E559 (E559^{Hyb}) was purified by affinity chromatography and analyzed by SDS-PAGE under reducing conditions, followed by staining with Coomassie Brilliant Blue (A) or blotted to nitrocellulose and probed with HRP-labeled antisera specific for murine γ 1 heavy chains (B) or murine κ light chains (C). Purified E559^{Hyb} was also treated with PNGaseF, and proteins were separated by SDS-PAGE under reducing conditions, blotted to nitrocellulose and probed with HRP-labeled light chain-specific antiserum (D). Lane M: molecular weight standards; (-): untreated E559^{Hyb}; (+): PNGaseF-treated E559^{Hyb}. Abbreviation: HRP, horseradish peroxidase.

27 kDa species is a glycosylated form of the light chain and the 25 kDa band is the aglycosylated species.

Characterization of Plant-derived E559

Murine and chimeric (mouse-human) heavy and light chain genes were cloned into the binary vector pL32 and transformed into *Agrobacterium tumefaciens*. Co-cultivation of *Nicotiana tabacum* leaf discs with *A. tumefaciens* strains harboring the recombinant pL32 binary vectors was used to generate transgenic tobacco lines expressing murine heavy (pL32-muE559H), chimeric heavy (pL32- χ E559H), murine light (pL32-muE559L), or chimeric light (pL32- χ E559L) chains. Several independent plants lines derived from each transformation were screened by ELISA to identify transgenic plants expressing each antibody chain.

Sexual crossing was used to produce plants lines expressing the fully assembled chimeric E559 (pL32- χ E559) or fully assembled murine E559 (pL32-muE559). Plants were analyzed by ELISA for antibody assembly and expression levels. The results from a selected set of plants provide evidence that both the chimeric (Figure 3A) and murine (Figure 3B) antibodies are assembled. Control plants expressing only the heavy

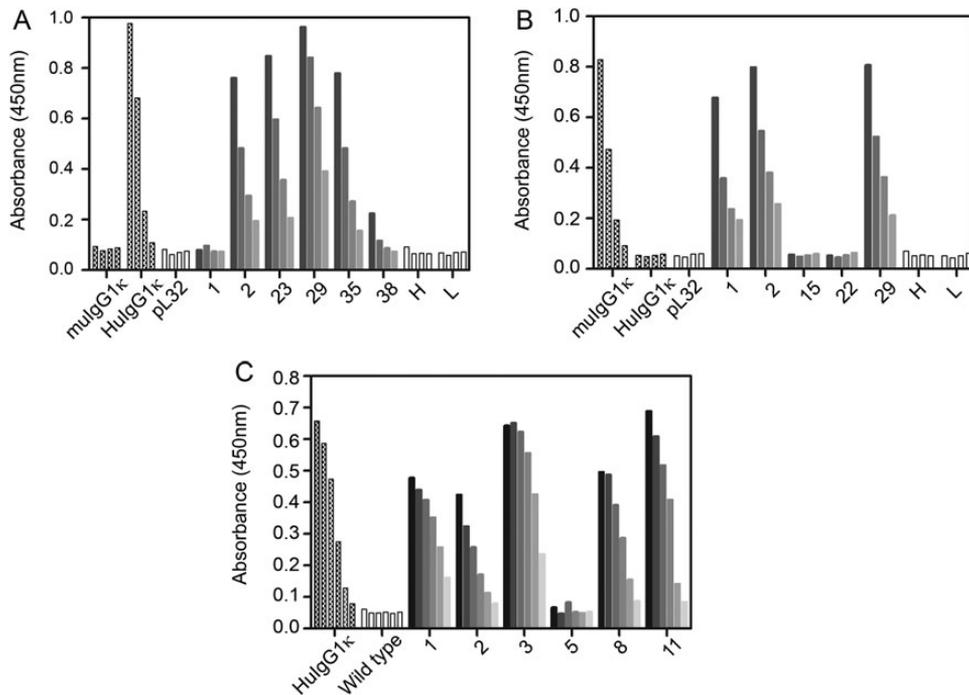


Figure 3. ELISA analysis of transgenic plants expressing fully assembled E559 monoclonal antibodies. Leaf discs from selected independent plant lines expressing either (A) chimeric E559 (pL32- χ E559), (B) murine E559 (pL32-muE559), or (C) codon-optimized, chimeric E559 (pTRAK- χ E559), were extracted in PBS and loaded onto ELISA plates coated with either sheep anti-human IgG1 (panels A and C) or sheep anti-mouse IgG1 (panel B) antisera. Bound antibodies were detected with HRP-labeled antibodies specific for either human κ light chains (A and C) or murine κ light chains (B). Control samples were: isotype-matched, commercially available human IgG1 κ (HulG1 κ) or mouse IgG1 κ (mulgG1 κ) antibodies; samples from transgenic plant lines expressing only the heavy or light chains of the chimeric E559 (H or L, respectively in panel A); samples from transgenic plant lines expressing only the heavy or light chains of the murine E559 (H or L, respectively, in panel B); a plant line transformed with nonrecombinant binary vector (pL32); and a wild-type (nontransgenic) plant. For panels A and B, plant samples were serially diluted 2-fold, whereas the isotype-matched controls were serially diluted 5-fold. In panel C, all samples and controls were serially diluted 4-fold. Abbreviations: ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

chain (pL32- χ E559H or pL32-muE559H) or the light chain (pL32- χ E559L or pL32-muE559L) did not produce any signal above that of the nonrecombinant pL32 control. In sum, 5 of the 6 plants shown in Figure 3A expressed the chimeric antibody, whereas 3 of the 5 plants shown in Figure 3B expressed the murine antibody.

Using a commercially available human IgG1 κ as an ELISA standard, the best expression level of the chimeric E559 (χ E559^P) was calculated as 1.8 mg/kg of fresh leaf weight (0.04% of total soluble protein), whereas the best yield achieved from the plant-derived, murine E559 (muE559^P) was 1.2 mg/kg of fresh leaf weight (0.03% of total soluble protein).

As an alternative expression strategy, codon-optimized versions of the chimeric E559 heavy and light chain genes were cloned into expression cassettes arranged in tandem (head-to-tail orientation) in plant transformation vector pTRAK2. Co-cultivation of *N. tabacum* leaf discs with an *A. tumefaciens* strain harboring the recombinant pTRAK2 was used to generate transgenic tobacco lines, pTRAK- χ E559, which were analyzed by ELISA for antibody assembly and yield (Figure 3C).

The best yield of plant-derived chimeric E559 (χ E559^P) was determined to be 280 mg/kg of fresh leaf weight, approximately 150-fold greater than the nonoptimized, chimeric antibody expressed using the pL32 vector.

The purified χ E559^P was analyzed by Coomassie staining under nonreducing and reducing conditions. A nonreducing gel (Figure 4A) showed a predominant high molecular weight band (indicated by the asterisk) at the expected size for the fully assembled antibody and, despite the presence of some minor low molecular weight bands, indicates a high degree of purity was achieved using the single-step (protein A/G) purification. The reducing gel (Figure 4B) shows the heavy chain (indicated by H) migrating at the expected position. As previously observed for the hybridoma-derived E559 (Figure 2A), the plant-derived χ E559^P also comprises 2 isoforms of the light chain (indicated by L1 and L2). Additional higher molecular weight species in the reducing gel most likely represent incompletely reduced antibody.

The purified χ E559^P was also analyzed by Western blotting, alongside purified E559^{Hyb}. Figure 4C shows the results of a

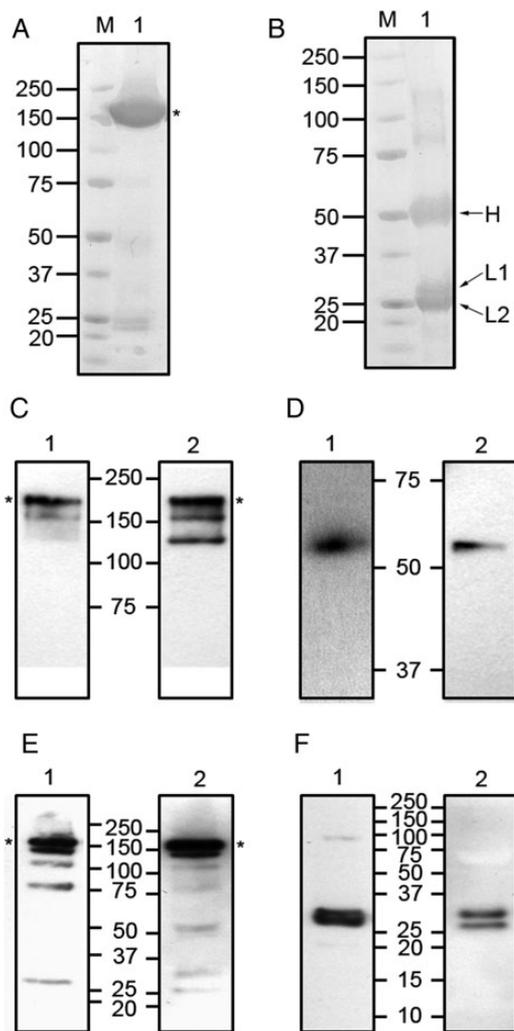


Figure 4. Gel and Western blotting analysis of purified χ E559^P. Purified, plant-derived chimeric E559 (χ E559^P) was analyzed by Coomassie staining of polyacrylamide gels under nonreducing (panel A) and reducing (panel B) conditions, and by Western blotting under nonreducing (panels C and E) and reducing (panels D and F) conditions. For Western blotting, the nitrocellulose membranes were probed with HRP-conjugated antibodies specific for heavy chains (panels C and D), or with HRP-conjugated antibodies specific for light chains (panels E and F). The χ E559^P samples (lane 1) were probed with human-specific reagents, whereas E559^{Hyb} samples (lane 2) were probed with murine-specific reagents. Abbreviations: HRP, horseradish peroxidase; M, molecular weight standards. Asterisks indicate the positions of the fully assembled antibodies.

nonreducing blot, detected with antisera specific for human (lane 1) or mouse (lane 2) heavy chains. Both χ E559^P and E559^{Hyb} samples have a high molecular weight band migrating at the expected position for the fully assembled antibody (indicated by the asterisk), with some additional lower molecular weight bands, representing either assembly intermediates or proteolytic fragments [34]. Under reducing conditions (Figure 4D), both samples showed a single band at approximately 55 kDa, corresponding to the expected size for free heavy chains.

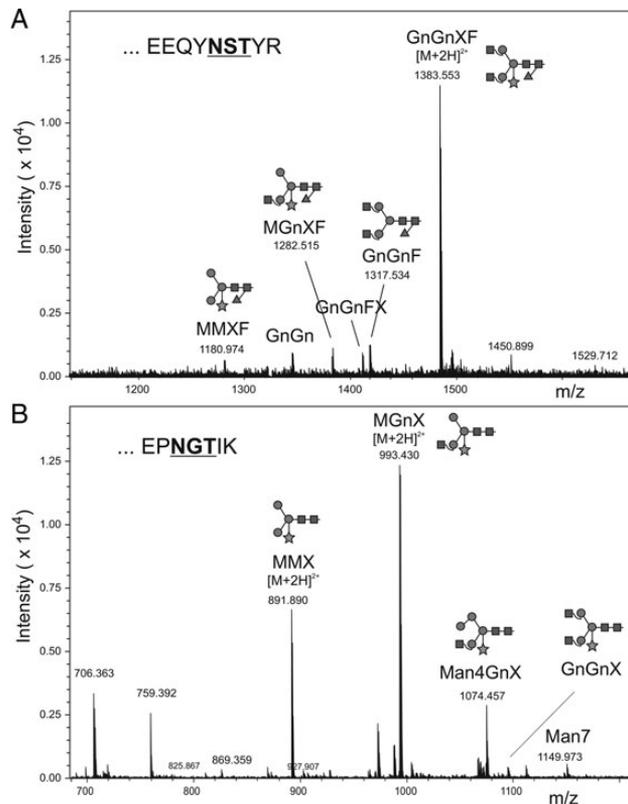


Figure 5. Glycan analysis of χ E559^P. Purified, plant-derived chimeric E559 was analyzed by in-gel digestion of *S*-carbamidomethylated sample and RP-ESI-MS. Deconvoluted spectra of the glycopeptide elution region of the Fc glycopeptide (A) and light chain glycopeptide (B). Masses correspond to oligomannosidic and complex-type structures. Abbreviations: F, fucose; Gn, *N*-acetylglucosamine; M, mannose; RP-ESI-MS, reverse-phase electrospray ionization mass spectrometry; X, xylose.

Blotting of the light chains under nonreducing conditions (Figure 4E) revealed the fully assembled antibody for both χ E559^P (lane 1) and E559^{Hyb} (lane 2) as well as some additional lower molecular weight species. Under reducing conditions (Figure 4F), E559^{Hyb} (lane 2) showed 2 bands, corresponding to the 2 glycoform variants, and χ E559^P (lane 1) also showed 2 light chain species. The lower band in χ E559^P corresponded in size to the lower band in E559^{Hyb}, indicating that this is also an aglycosylated form of the light chain. The higher band in χ E559^P had a slightly faster mobility compared to the glycosylated isoform of E559^{Hyb}, and this probably reflects differences in the *N*-linked glycan structures between plants and mammals.

Glycoproteomic Analysis

Sequence analysis of heavy and light chains of mAb E559 predicted the presence of 2 potential *N*-linked glycosylation sites, a conserved site in the antibody Fc region, and one in the V_L domain. The plant-derived antibody was subjected to glycoproteomic analysis by RP-ESI-MS (Figure 5). Glycopeptides comprising

Table 1. Virus Neutralizing Activity of Plant-derived Antibodies

Phylogroup	Lyssavirus Species (Genotype)	Virus Reference No.	Animal of Origin	Country of Origin	OIE+	muE559 ^P	χE559 ^P	E559 ^{Hyb}
I	RABV (1)	CVS	Standard stock	n/a	+	+	+	+
		RV51	Fox	USA	+	+	+	+
		RV61	Human ex dog	UK (ex India)	+	+	+	+
		RV108	Bat	Chile	+	+	+	+
		RV410	Mongoose	South Africa	+	+	+	+
		RV437	Raccoon	Estonia	+	+	+	+
		RV1237	Deer	Yugoslavia	+	+	+	+
II	LBV (2)	RV1	Bat (<i>E. helvum</i>)	Nigeria	–	–	–	–
		MOK (3)	RV4	Shrew (<i>Crocidura spp.</i>)	Nigeria	–	–	–
I	DUVV (4)	RV131	Bat (<i>N. thebaica</i>)	Zimbabwe	–	+	+	+
		EBLV1 (5)	RV9	Bat (<i>E. serotinus</i>)	Germany	+	+	+
		EBLV2 (6)	RV1781	Bat (<i>M. daubentonii</i>)	UK	+	+	+
		ABLV (7)	RV634	Fruit bat	Australia	+	+	+

A modified fluorescent antibody virus neutralization (mFAVN) assay was used to compare the virus neutralizing activity of plant-derived chimeric E559 (χE559^P), plant-derived murine E559 (muE559^P), hybridoma-derived murine E559 (E559^{Hyb}) and pooled dog reference sera from immunized animals (OIE+) against different lyssaviruses. Virus abbreviations: ABLV, *Australian bat lyssavirus*; CVS, challenge virus standard; DUVV, *Duvenhage virus*; EBLV1, *European bat lyssavirus type 1*; EBLV2, *European bat lyssavirus type 2*; LBV, *Lagos bat virus*; MOK, *Mokola virus*. Virus was considered neutralized if the neutralization titer was >0.5 IU/mL [36]. (+) indicates neutralization, (–) indicates no neutralization.

the Fc glycosylation site EEQFNSTFR (Figure 5A) and the V_L glycosylation site EPNGTIK (Figure 5B) were identified (N-linked glycosylation sites are underlined). The glycan analysis revealed that χE559^P heavy chain displayed glycan compositions typical of plant glycoproteins, with predominantly complex type glycans containing xylose and fucose (GnGnXF), which are presumed to be the β1,2-linked xylose residues attached to the β-linked mannose and the α1,3-fucose residue linked to the Asn-linked *N*-acetyl-glucosamine. The light chain glycosylation pattern (MGnX and MMX) was also largely typical of plant glycoproteins, except for the lack of the α1,3-fucose residue linked to the Asn-linked *N*-acetyl-glucosamine. Tandem MS results were subjected to Mascot MS/MS ion search, which confirmed the sample to contain essentially mAb E559.

In vitro Neutralization

The hybridoma-derived E559 (E559^{Hyb}) and both plant-derived antibodies (muE559^P and χE559^P) were tested for neutralization of a diverse panel of lyssavirus species and strains using the mFAVN assay. The results (Table 1) show that both plant-derived antibodies mirrored the hybridoma-derived antibody in terms of breadth of neutralization. Representative viruses from phylogroups I and II [5, 40] were assayed for their ability to be neutralized by the antibodies. All tested phylogroup I viruses, covering the type species member (classical RABV), *Duvenhage virus*, *European bat lyssavirus* types 1 and 2, and *Australian bat lyssavirus*, were neutralized by all 3 antibodies and, except for *Duvenhage virus*, also by the OIE+ control. No neutralization was observed for the phylogroup II viruses tested (*Lagos bat virus* and *Mokola virus*).

Table 2. In vivo Efficacy of χE559^P for Postexposure Prophylaxis

Group (Treatment)	14 d	28 d
Group 1 (Uninfected control)	4/4	4/4
Group 2 (PBS)	0/4	0/4
Group 3 (HRIG)	5/9	0/9
Group 4 (χE559 ^P)	6/9	1/9

Four groups of Syrian hamsters were included in the experiment. Group 1 (uninfected control) animals did not receive any viral inoculum or biologics treatment. Groups 2, 3 and 4 were all inoculated with a genotype 1 RABV variant (at day 0) and treated subsequently (at day 1) with either PBS (group 2), HRIG (Rabigam) (group 3), or purified χE559^P mAb (group 4). Data are presented as the no. of surviving hamsters/no. of hamsters tested. Abbreviations: HRIG, human rabies immunoglobulin; PBS, phosphate-buffered saline.

In vivo Challenge Studies

The efficacy of the χE559^P in post-exposure prophylaxis was examined in hamsters injected with a lethal dose of a laboratory strain of RABV (CVS-11; Table 2). In this in vivo protection assay, all uninfected hamsters (group 1) survived. All hamsters that were infected with challenge virus and received mock PEP in the form of PBS (group 2) died after 14 days. The survival rates for hamsters that received PEP in the form of 22.5 IU/kg of either HRIG (group 3) or χE559^P (group 4) was >50% after 14 days, although after 28 days survival dropped to zero and 11% for HRIG and χE559^P groups, respectively. None of the groups received vaccine as part of the PEP regimen. The data show that the χE559^P antibody is at least as effective as the HRIG.

DISCUSSION

Current PEP for bites by rabid animals involves the use of blood-derived RIG, which can display batch-to-batch variation and may be of limited availability in case of sudden mass exposures. The concerns arising from the use of blood-derived products could be circumvented, and consistent batches of neutralizing antibodies could be produced in large quantities by adopting an approach based on a cocktail of rabies neutralizing mAbs. To this end, it is envisaged that RIG could be replaced by a mAb cocktail, produced using plants as the expression platform. Two different mAb production platforms in plants have already gained regulatory approval for human trials (Pharma-Planta Consortium, personal communication to J. Ma; [41]), demonstrating that plants are amenable to current Good Manufacturing Practice (cGMP) compliance [42].

We compared the murine hybridoma-derived E559 (E559^{Hyb}) with the same murine antibody produced in *N. tabacum* (muE559^P), as well as a mouse-human chimeric version (χ E559^P), also expressed in *N. tabacum*. In vitro testing of virus neutralization demonstrated that all 3 versions of E559 were equivalent, with all 3 neutralizing phylogroup I viruses but not the phylogroup II viruses. This is in accord with previous reports showing that neutralizing antibodies targeting phylogroup I viruses are not effective at neutralizing phylogroup II viruses [40, 43].

E559 has a predicted glycosylation site in the framework region of the V_L domain, which appears to be utilized, as 2 forms of the hybridoma-derived light chain (glycosylated and aglycosylated) are observed under reducing conditions, with the higher molecular weight form disappearing after treatment with PNGaseF. Two isoforms were also observed in the plant expressed χ E559^P. The effect of the V_L glycosylation is unknown, as both glycosylated and aglycosylated forms of the light chain were present in the hybridoma and plant preparations used for assessment of antibody functionality.

Purified χ E559^P was analyzed by mass spectrometry and was shown to be glycosylated with typical plant complex glycan structures. It is well established that plant N-linked glycosylation differs from murine glycosylation [44], due to differences in complex glycan processing in the Golgi compartment. Previous studies have shown that plant-derived mAbs can have different half-lives in animals, compared to mammalian-derived mAbs [25, 45]. Although these differences have been attributed to differences in glycosylation, a more recent study [46] found no difference in the clearance rates of a RABV-neutralizing human mAb expressed in hybridoma cells or plants. The impact on the in vivo half-life of the glycosylation differences between E559^{Hyb} and χ E559^P will need to be addressed in human trials.

Functionally, χ E559^P retained neutralization activity and had the same breadth of lyssavirus coverage as E559^{Hyb}. In vivo, the

chimeric antibody was as effective as a commercial HRIG product in a hamster challenge model.

The potential for viral escape, and the need to provide protection across a broad range of lyssaviruses, means that a single mAb will probably not be sufficient for a rabies PEP product, and this has been recognized by various groups [15, 17, 18, and 31]. However, the cost of mAbs produced in mammalian cell bioreactors is currently prohibitive for rabies products intended for use in resource-poor settings, so it seems unlikely that products combining 2 or more mAbs produced using such traditional platforms will be commercially viable outside the developed world. Production of RABV-neutralizing mAbs in plants raises hopes that these mAbs will be available in quantities sufficient to meet the needs for PEP in rabies-endemic areas, particularly across the developing world.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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References

1. World Health Organization. WHO Fact Sheet no. 99. Available at: <http://web.archive.org/web/20130310122553/http://www.who.int/mediacentre/factsheets/fs099/en/index.html>. Accessed 19 February 2014.
2. Hampson K, Dobson A, Kaare M, et al. Rabies exposures, post-exposure prophylaxis and deaths in a region of endemic canine rabies. *PLoS Negl Trop Dis* 2008; 2:e339.

3. Dodet B. The fight against rabies in Africa: from recognition to action. *Vaccine* **2009**; 27:5027–32.
4. Fooks AR. Rabies remains a ‘neglected disease’. *Euro Surveill* **2005**; 10:211–2.
5. Both L, Banyard AC, van Dolleweerd C, Horton DL, Ma JK, Fooks AR. Passive immunity in the prevention of rabies. *Lancet Infect Dis* **2012**; 12:397–407.
6. Rupprecht CE, Hanlon CA, Hemachudha T. Rabies re-examined. *Lancet Infect Dis* **2002**; 2:327–43.
7. Hemachudha T, Laothamatas J, Rupprecht CE. Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges. *Lancet Neurol* **2002**; 1:101–9.
8. Ertl HC. Novel vaccines to human rabies. *PLoS Negl Trop Dis* **2009**; 3:e515.
9. Rupprecht CE, Gibbons RV. Clinical practice: prophylaxis against rabies. *N Engl J Med* **2004**; 351:2626–35.
10. Warrell M. Rabies and African bat lyssavirus encephalitis and its prevention. *Int J Antimicrob Agents* **2010**; 36(Suppl 1):S47–52.
11. Satpathy DM, Sahu T, Behera TR. Equine rabies immunoglobulin: a study on its clinical safety. *J Indian Med Assoc* **2005**; 103:238, 41–2.
12. World Health Organization. WHO consultation on a rabies monoclonal antibody cocktail for rabies post exposure treatment. Geneva: WHO, 23–24 May 2002. World Health Organization, Geneva, Switzerland, **2002**. Available at: http://web.archive.org/web/20100525173651/http://www.who.int/rabies/vaccines/en/mabs_final_report.pdf. Accessed 19 February 2014.
13. World Health Organization. WHO expert consultation on rabies (1st report). WHO technical report series 931. Geneva, Switzerland, **2005**.
14. Bakker AB, Marissen WE, Kramer RA, et al. Novel human monoclonal antibody combination effectively neutralizing natural rabies virus variants and individual in vitro escape mutants. *J Virol* **2005**; 79:9062–8.
15. Müller T, Dietzschold B, Ertl H, et al. Development of a mouse monoclonal antibody cocktail for post-exposure rabies prophylaxis in humans. *PLoS Negl Trop Dis* **2009**; 3:e542.
16. Both L, Banyard AC, van Dolleweerd C, Wright E, Ma JK, Fooks AR. Monoclonal antibodies for prophylactic and therapeutic use against viral infections. *Vaccine* **2013**; 31:1553–9.
17. Goudsmit J, Marissen WE, Weldon WC, et al. Comparison of an anti-rabies human monoclonal antibody combination with human polyclonal anti-rabies immune globulin. *J Infect Dis* **2006**; 193:796–801.
18. Prośniak M, Faber M, Hanlon CA, Rupprecht CE, Hooper DC, Dietzschold B. Development of a cocktail of recombinant-expressed human rabies virus-neutralizing monoclonal antibodies for postexposure prophylaxis of rabies. *J Infect Dis* **2003**; 188:53–6.
19. Dietzschold B, Gore M, Casali P, et al. Biological characterization of human monoclonal antibodies to rabies virus. *J Virol* **1990**; 64:3087–90.
20. Enssle K, Kurrle R, Kohler R, et al. A rabies-specific human monoclonal antibody that protects mice against lethal rabies. *Hybridoma* **1991**; 10:547–56.
21. Champoin JM, Kean RB, Rupprecht CE, et al. The development of monoclonal human rabies virus-neutralizing antibodies as a substitute for pooled human immune globulin in the prophylactic treatment of rabies virus exposure. *J Immunol Methods* **2000**; 235:81–90.
22. Hanlon CA, DeMattos CA, DeMattos CC, et al. Experimental utility of rabies virus-neutralizing human monoclonal antibodies in post-exposure prophylaxis. *Vaccine* **2001**; 19:3834–42.
23. Muhamuda K, Madhusudana SN, Ravi V. Use of neutralizing murine monoclonal antibodies to rabies glycoprotein in passive immunotherapy against rabies. *Hum Vaccin* **2007**; 3:192–5.
24. Paul M, van Dolleweerd C, Drake PM, et al. Molecular pharming: future targets and aspirations. *Hum Vaccin* **2011**; 7:375–82.
25. Ko KS, Tekoah Y, Rudd PM, et al. Function and glycosylation of plant-derived antiviral monoclonal antibody. *P Natl Acad Sci USA* **2003**; 100:8013–8.
26. Schumacher CL, Dietzschold B, Ertl HC, Niu HS, Rupprecht CE, Koprowski H. Use of mouse anti-rabies monoclonal antibodies in post-exposure treatment of rabies. *J Clin Invest* **1989**; 84:971–5.
27. de Kruijf J, Bakker AB, Marissen WE, et al. A human monoclonal antibody cocktail as a novel component of rabies postexposure prophylaxis. *Ann Rev Med* **2007**; 58:359–68.
28. Bakker AB, Python C, Kissling CJ, et al. First administration to humans of a monoclonal antibody cocktail against rabies virus: safety, tolerability, and neutralizing activity. *Vaccine* **2008**; 26:5922–7.
29. Sloan SE, Hanlon C, Weldon W, et al. Identification and characterization of a human monoclonal antibody that potentially neutralizes a broad panel of rabies virus isolates. *Vaccine* **2007**; 25:2800–10.
30. Gogtay N, Thatte U, Kshirsagar N, et al. Safety and pharmacokinetics of a human monoclonal antibody to rabies virus: a randomized, dose-escalation phase 1 study in adults. *Vaccine* **2012**; 30:7315–20.
31. Both L, van Dolleweerd C, Wright E, et al. Production, characterization, and antigen specificity of recombinant 62-71-3, a candidate monoclonal antibody for rabies prophylaxis in humans. *FASEB J* **2013**; 27:2055–65.
32. Schneider LG, Meyer S. Antigenic determinants of rabies virus as demonstrated by monoclonal antibody. In: Bishop DH, Compans RW, eds. *The Replication of Negative Strand Viruses: Developments in Cell Biology*. Vol. 7. North Holland: Elsevier, **1981**:947–83.
33. Kissling RE. Growth of rabies virus in non-nervous tissue culture. *Proc Soc Exp Biol Med* **1958**; 98:223–5.
34. Hehle VK, Paul MJ, Drake PM, Ma JK, van Dolleweerd CJ. Antibody degradation in tobacco plants: a predominantly apoplastic process. *BMC biotechnology* **2011**; 11:128.
35. Stadlmann J, Pabst M, Kolarich D, Kunert R, Altmann F. Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. *Proteomics* **2008**; 8:2858–71.
36. Cliquet F, Aubert M, Sagne L. Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *J Immunol Methods* **1998**; 212:79–87.
37. Brookes SM, Parsons G, Johnson N, McElhinney LM, Fooks AR. Rabies human diploid cell vaccine elicits cross-neutralising and cross-protecting immune responses against European and Australian bat lyssaviruses. *Vaccine* **2005**; 23:4101–9.
38. Dean DJ, Ableseth MK, Atanasiu P. The fluorescent antibody test. In: Meslin F-X, Kaplan MM, Koprowski H, eds. *Laboratory Techniques in Rabies*. 4th ed. Geneva: WHO, **1996**:88–95.
39. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. *Sequences of proteins of immunological interest*. 5th Edition ed. Bethesda, Maryland: National Institutes of Health, **1991**.
40. Badrane H, Bahloul C, Perrin P, Tordo N. Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. *J Virol* **2001**; 75:3268–76.
41. McCormick AA, Reddy S, Reil SJ, et al. Plant-produced idiotype vaccines for the treatment of non-Hodgkin’s lymphoma: safety and immunogenicity in a phase I clinical study. *Proc Natl Acad Sci U S A* **2008**; 105:10131–6.
42. Whaley KJ, Hiatt A, Zeitlin L. Emerging antibody products and *Nicotiana* manufacturing. *Hum Vaccines* **2011**; 7:349–56.
43. Horton DL, McElhinney LM, Marston DA, et al. Quantifying antigenic relationships among the lyssaviruses. *J Virol* **2010**; 84:11841–8.
44. Cabanes-Macheteau M, Fitchette-Laine AC, Loutelier-Bourhis C, et al. N-glycosylation of a mouse IgG expressed in transgenic tobacco plants. *Glycobiology* **1999**; 9:365–72.
45. Triguero A, Cabrera G, Rodriguez M, et al. Differential N-glycosylation of a monoclonal antibody expressed in tobacco leaves with and without endoplasmic reticulum retention signal apparently induces similar in vivo stability in mice. *Plant Biotechnol J* **2011**; 9:1120–30.
46. Lee JH, Park DY, Lee KJ, et al. Intracellular reprogramming of expression, glycosylation, and function of a plant-derived antiviral therapeutic monoclonal antibody. *PLoS one* **2013**; 8:e68772.