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...
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 IgG1κ 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) 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 γ1 heavy chain gene was amplified using primers FR1γ and 932 (see online Supplementary Table 1 for a description of oligonucleotide primers). The murine κ light chain gene was amplified using primers FR1κ and 933. The murine γ1 heavy chain and κ light chain amplicons were digested with XhoI and EcoRI 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 (κE559H) and light (χE559L) 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 µL 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 (E559Hyb), 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 µm) and applied to an anti-mouse IgG1 (heavy chain specific)-agarose (Sigma, UK) affinity column.

The plant-expressed chimeric antibody (χE559P) was purified using Protein A/G agarose as described elsewhere [34]. In the case of the plant-expressed murine E559 (muE559P), 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. Dialedyzed 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 HiTrap 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 hydroxypatite (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 IgG1κ (The Binding Site, UK) and mouse IgG1κ (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 µL 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 χE559  mAb (group 4). Biologics (groups 3 and 4) and PBS (group 2) were administered in the gastrocnemius muscle in 50 µL 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 γ1 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_{H}2 domain and the light chain V_{L} domain.

**Analysis of Hybridoma-derived E559**
Analysis of the purified murine hybridoma-derived E559 (E559Hyb) 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. E559Hyb 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\textsubscript{H} domain

\begin{align*}
V\textsubscript{H} = & \quad OVQLQESVLYVPASGLFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLSSFLS
chain (pL32-χE559 or pL32-muE559), or the light chain (pL32-χE559L 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 mu-rine antibody.

Using a commercially available human IgG1κ as an ELISA standard, the best expression level of the chimeric E559 (χE559) 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) was 1.2 mg/kg of fresh leaf weight (0.03% of total soluble protein).

As an alternative expression strategy, codon-optimised 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 pTRAk.2. Co-cultivation of N. tabacum leaf discs with an A. tumefaciens strain harboring the recombinant pTRAk.2 was used to generate transgenic tobacco lines, pTRAk-χE559, which were analyzed by ELISA for antibody assembly and yield (Figure 3C). The best yield of plant-derived chimeric E559 (χE559) 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 χE559 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 χE559 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 χE559 was also analyzed by Western blotting, alongside purified E559Hyb. Figure 4C shows the results of a
nonreducing blot, detected with antisera specific for human (lane 1) or mouse (lane 2) heavy chains. Both \( \chi E559^p \) and E559Hyb 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.

Blotting of the light chains under nonreducing conditions (Figure 4E) revealed the fully assembled antibody for both \( \chi E559^p \) (lane 1) and E559Hyb (lane 2) as well as some additional lower molecular weight species. Under reducing conditions (Figure 4F), E559Hyb (lane 2) showed 2 bands, corresponding to the 2 glycoform variants, and \( \chi E559^p \) (lane 1) also showed 2 light chain species. The lower band in \( \chi E559^p \) corresponded in size to the lower band in E559Hyb, indicating that this is also an aglycosylated form of the light chain. The higher band in \( \chi E559^p \) had a slightly faster mobility compared to the glycosylated isoform of E559Hyb, indicating that this is also an aglycosylated form of the light chain. The higher band in \( \chi E559^p \) had a slightly faster mobility compared to the glycosylated isoform of E559Hyb, 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...
the Fc glycosylation site [EQFNSTFR](Figure 5A) and the V_{L} glycosylation site [EPNGTIK](Figure 5B) were identified (N-linked glycosylation sites are underlined). The glycan analysis revealed that $\chi_{E559P}$ 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 $\beta$1,2-linked xylose residues attached to the $\beta$-linked mannose and the $\alpha$1,3-fucose residue linked to the Asn-linked N-acetyl-glucosaminе. The light chain glycosylation pattern (MGNX and MMX) was also largely typical of plant glycoproteins, except for the lack of the $\alpha$1,3-fucose residue linked to the Asn-linked N-acetyl-glucosaminе. Tandem MS results were submitted to Mascot MS/MS ion search, which confirmed the sample to contain essentially mAb E559.

In vitro Neutralization

The hybridoma-derived E559 ($E559^{Hb}$) and both plant-derived antibodies (muE559 $P$ and $\chi_{E559P}$) 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).

In vivo Challenge Studies

The efficacy of the $\chi_{E559P}$ 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 were neutralized by the antibodies. All groups of hamsters that received vaccine as part of the PEP regimen. The data show that the $\chi_{E559P}$ antibody is at least as effective as the HRIG.

**Table 1. Virus Neutralizing Activity of Plant-derived Antibodies**

| Phylogroup | Lyssavirus Species (Genotype) | Virus Reference No. | Animal of Origin | Country of Origin | OIE+ | muE559 $P$ | $\chi_{E559P}$ | E559 $Hb$ |
|------------|-------------------------------|---------------------|-----------------|------------------|-----|-----------|------------|----------|
| 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 (E. helvum) | Nigeria          | –   | –         | –          | –        |
|            | MOK (3)                       | RV4                 | Shrew (Crocidura spp.) | Nigeria | –   | –         | –          | –        |
| I          | DUVV (4)                      | RV131               | Bat (N. thebaica) | Zimbabwe         | –   | +         | +          | +        |
|            | EBLV1 (5)                     | RV9                 | Bat (E. serotinus) | Germany          | +   | +         | +          | +        |
|            | EBLV2 (6)                     | RV1781              | Bat (M. daubentonii) | 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 ($\chi_{E559P}$), plant-derived murine E559 (muE559 $P$), hybridoma-derived murine E559 ($E559^{Hb}$) 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.

**Table 2. In vivo Efficacy of $\chi_{E559P}$ for Postexposure Prophylaxis**

| Group (Treatment) | No. Animal of Origin | Country of Origin |
|-------------------|----------------------|-------------------|
| Group 1 (Uninfected control) | 14 d | 28 d |
| Group 2 (PBS) | 4/4 | 4/4 |
| Group 3 (HRIG) | 0/4 | 0/4 |
| Group 4 (E559 $Hb$) | 0/9 | 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 $\chi_{E559P}$ 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.
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 (E559Hyb) with the same murine antibody produced in N. tabacum (muE559P), as well as a mouse-human chimeric version (χE559P), 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 V1 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 χE559P. The effect of the V1 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 χE559P 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 E559Hyb and χE559P will need to be addressed in human trials.

Functionally, χE559P retained neutralization activity and had the same breadth of lyssavirus coverage as E559Hyb. 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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