Development of Therapeutic Antibodies as Medical Countermeasures to Biological Agents in Defence Research and Development Canada, Suffield Research Centre

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ABSTRACT

Antibodies are naturally produced in the body as highly versatile defence molecules to infectious agents and toxins, and have great potential for countering biological agents not addressed by current medical countermeasures. DRDC, Suffield Research Centre has a significant strength in research and development of therapeutic antibodies against biological agents with a state-of-the-art humanization strategy for converting mouse antibodies to human antibodies for clinical applications. Using this platform, a number of therapeutic antibodies against various biological agents have been developed. The most recently developed therapeutic anti-ricin antibody exhibits the highest efficacy among the reported anti-ricin antibodies. A tiny dose of 5 µg per mouse of this humanized anti-ricin antibody, when administered to mice 20 hours after an intranasal challenge with 2×LD50 of ricin, was sufficient to rescue all the mice. Unfortunately, therapeutic antibodies are among the most expensive drugs. In order to reduce the cost, plants are being used for the Good Manufacturing Practices (GMP) production of our therapeutic antibodies in preclinical stage, supported by Defense Threat Reduction Agency (DTRA), Canadian Biological Warfare Threat Medical Countermeasure (BWTMCM) Project and Preclinical-Funded Service of American National Institutes of Health (NIH). The plant-derived anti-ricin antibody will be evaluated to ensure it is indistinguishable from its conventional mammalian-derived counterpart. Meanwhile, therapeutic antibody transgene delivery by human mesenchymal stromal cells (MSCs) has been evaluated to explore the feasibility of sustaining production of therapeutic antibodies directly in the host for medical countermeasures against biological agents. Our study has showed that mice were fully protected against exposure to a lethal dose of Venezuelan encephalitis equine virus (VEEV) by a pre-treatment with engineered MSCs encoding one of our developed therapeutic antibodies, which targets VEEV 24 hours or 10 days before exposure as compared to the protection of only 2-3 days by the direct administration of anti-VEEV antibody. It demonstrated both rapid and prolonged immune protection that was superior to the direct antibody administration. This cutting-edge breakthrough has broad applications in prophylaxis and therapy against biological agents. By providing significant improvements over the direct antibody administration approach, it is expected that there will be decreased cost, single and simple administration, and prolonged immunity that translates to enhanced medical countermeasures against biological agents.

1.0 INTRODUCTION

Antibodies, naturally produced in the body as part of the immune response to infectious agents and toxins,
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can also be introduced artificially to treat many diseases. Advances in biotechnology in the last decades have made monoclonal antibodies (mAbs) as therapeutics possible. These mAbs currently enjoy unprecedented success and recognition of their potential. Unlike vaccines, therapeutic mAbs can confer an instant and consistent protection against biological agents when administered regardless of the recipient’s immune status. Therapeutic mAbs can be administered in higher levels than those elicited by vaccines, and thus provide a higher level of protection or treatment that is necessary in a biological agent attack with a higher level exposure of agents than found in nature. Furthermore, therapeutic mAbs have substantial advantages over antimicrobial drugs, such as high specificity, low systemic toxicity, and long serum half-life (around 20 days). Therapeutic mAbs can be used for both pre- and post-exposure protections in addition to therapeutic value for biodefence. With continuous advances in the technology of antibody and understanding of the infectivity and intoxication of biological agents, the new generation of mAbs with extraordinary efficacy will likely be developed as effective prophylactic and therapeutic products against unpredictable and uncertain future biological agents for biodefence.

2.0 STATE-OF-THE-ART HUMANIZATION OF MABS AGAINST BIOLOGICAL AGENTS

In 1975, the development of mAbs by murine hybridoma technology opened a new era in antibody therapy. It was widely believed that mAbs would be the magic bullets for therapy. However, the early excitement was rapidly replaced by disappointment when it was found that mAbs like animal plasma-derived antibodies have a serious side effect in humans, which is serum sickness due to foreignness to humans [1]. All therapeutic settings using antibody-based drugs require high and multiple doses and hence, animal antibody’s immunogenicity in humans is a critical concern [2, 3]. Repeat administration of these mAbs result in rapid clearance of the animal antibodies and anaphylaxis, which can sometimes be fatal. Despite the development of molecular display technologies and transgenic animals for the generation of fully human antibodies, humanization, a process to decrease the content of murine residues in mAbs to reduce murine mAb immunogenicity for clinical uses still remains an attractive and proven strategy for overcoming therapeutic deficiencies of murine mAbs [4-8]. For example, as of March 2012, there had been 28 antibodies (3 murine, 5 chimeric, 11 humanized, and 9 fully human) in the European Union or the US for therapeutic application [9]. Among these, humanized antibodies are the most. The “humanized” antibodies contain 85-95% human sequences. Numerous clinical studies have confirmed that humanized antibodies are less immunogenic and more therapeutic than murine or chimeric antibodies in humans [3, 10].

The process of humanization of murine antibodies is challenging. Humanization may result in a loss of antibody antigen-binding activity. To date, there have been some strategies for humanization of xenogeneic (commonly rodent) antibodies, including complementarity determining region (CDR)-grafting [4, 11, 12], resurfacing [5, 13], de-immunization [14, 15], and specificity-determining residues (SDR) grafting [8, 16]. However, it is unlikely to determine which one is the best, due to sparse data of the immunogenicity of humanized antibodies (only 11 humanized antibodies on the market). Nevertheless, CDR-grafting seems likely most popular.

The antibody is a "Y" shaped, having two branches attached to a single stem as showed in Fig. 1. CDR grafting sounds pretty straightforward, grafting the murine CDRs (responsible for the desired antigen-binding properties) into human donor antibody framework regions (FRs), responsible for scaffolding as showed in Fig. 2. However, simply grafting does not always reconstitute the binding affinity and specificity of the original murine antibody. As a matter of fact, this often results in partial or complete loss of antigen-binding affinity due to incompatibilities between murine CDRs and human donor FRs. In order to retain a comparable antigen-binding affinity and specificity after CDR-grafting, the murine CDR conformations have to be preserved in the humanized antibody. How to preserve the murine CDR
conformation in the humanized antibody becomes the critical process in reproducing the function of the original murine antibody, which usually includes two steps: the selection of appropriate human antibody FRs as donor, and the substitution of some key residues from human donor FRs into the murine original

Figure 1. Antibody structure. One antibody molecule consists of four polypeptide chains; two identical heavy chains (HCs) and two identical light chains (LCs) connected by disulfide bonds in black. Each chain is composed of two regions: variable region (V) and constant region (C). The V is responsible for antigen-binding, while the C determines the mechanism used to destroy the antigen. The V can be further subdivided into CDRs in red and FRs in blue. There are three CDRs and four FRs in each chain. The CDRs are the most important part for binding to antigens with a high variety while the FR regions which have more stable amino acids sequences separate the CDRs and serves as a scaffold to hold the CDRs in position to contact the antigen.

Figure 2. CDR grafting humanization of murine mAb by grafting the murine CDRs (responsible for the desired antigen-
FRs (backmutation) to restore antigen-binding affinity. The number of back mutations, the position of residues, and the type of residues to restore antigen-binding affinity are different from antibody to antibody. This requires a trial-and-error iterative procedure to determine backmutation sites in order to correct the structurally distorting residues and to reconstitute the original antigen-binding affinity of the original murine antibody. In short, although CDR-grafting is now well-established and most popular, it generally needs multiple design cycles, which are time-consuming. A novel CDR-grafting approach developed in DRDC-Suffield Research Centre, described here with anti-ricin mAb, D9 [17] as an example, is based on a comprehensive analysis of the antibody sequence and three-dimensional structure from molecular modeling to identify the critical residues in the given murine antibody, which guides to finish CDR-grafting in a single cycle. The single-cycle structure-based method is a considerable improvement over the standard CDR-grafting humanization approach.

2.1 Sequence and Structural Analysis of Murine Antibody Variable Domain (Fv)

Fv consists of heavy chain variable region (VH) and light chain variable region (VL). Most of FR residues of Fv do not participate in antigen-binding, but some of them directly or indirectly do. These FR residues can be called key FR residues. That is why simple CDR-grafting humanization without consideration of the key FR residues often results in somewhat loss of antigen-binding affinity. The most common approach to restoring high affinity binding is to keep the key murine FR residues in the humanized antibodies. These key FR residues are different from antibody to antibody. They should be determined on a case-by-case basis before humanization.

2.1.1 Unusual FR Residue Determination

The rare FR residues could result from somatic hypermutation and may contribute to antigen-binding affinity or may not. Careful consideration should be given when murine CDRs are grafted on human donor antibody FRs. If the rare FR residues are near the CDRs as determined from the molecular modeling, they may possibly contact the antigen and therefore should be kept in the humanized antibody. If they are not close to the CDR, then they should be humanized because they may represent immunogenic epitopes in the humanized antibody. The unusual FR residues can be determined by comparison with the Kabat subgroup using the online free program “AbCheck - Antibody Sequence Test” (http://www.bioinf.org.uk/abs/seqtest.html). In terms of D9, VH44-N, VH33-I, VL31-A, and VL106a-K were identified as unusual FR residues.

2.1.2 Molecular Modeling

The molecular model of D9 Fv was established through PIGS (http://www.biocomputing.it/pigs) based on the most homologous antibody VH (2NR6), sharing 86% identity and VL (1MLB), sharing 70% identity with the corresponding VH and VL of D9 in the database of known antibody structure. 3D structure of D9 was then visualized using a protein data bank (PDB) molecular visualisation program, Deepview, as shown in Fig.3.

2.1.2.1 Prediction of Potential N-glycosylation Sites

Glycosylation sites may occur as part of the germline or arise through somatic hypermutation in the CDRs or FRs. Those carbohydrates may affect positioning of the antigen in the binding pocket. Therefore, their positions should be checked by molecular modeling to ensure they do not interfere with the CDRs. N-glycosylation sites can be predicted using the online free program “NetNGlyc” (http://www.cbs.dtu.dk/services/NetNGlyc). There was not any glycosylation site found in D9 Fv.
2.1.2.2. Determination of Vernier Zone (VZ) Residues

Figure 3. A structural representation of murine Fv structure predicted from molecular modeling. CDRs are colored (CDR-L1, green; CDR-L2, blue; CDR-L3, deep blue; CDR-H1, orange; CDR-H2, pink; CDR3-H3, red). FRs are in black for VL and in grey for VH.

Figure 4. Residues in VZ in yellow identified from a molecular modeling of a murine Fv. CDRs are colored (CDR-L1, green; CDR-L2, blue; CDR-L3, deep blue; CDR-H1, orange; CDR-H2, pink; CDR3-H3, red). FWs are in black for VL and in grey for VH.

The VZ residues can be identified by Fv molecular modeling by defining FR residues within 5 Å of CDR residues. VZ is a platform of FR residues directly locates under the CDRs to support CDR conformation. The VZ residues can be predicted by molecular modeling. D9 VZ residues are shown in Fig. 4.

2.1.2.3 Determination of Interface Residues

Residues buried at VL/VH packing interface may affect the relative disposition of CDRs and then affect the antigen-binding function [31,32]. These interface residues can be identified by defining residues in one chain within 5 Å of the other chain using molecular modeling. D9 VL/VH interface residues are shown in Fig. 5.

2.1.3 Key FR Residue Determination

As for D9, key FR residues were identified as abovementioned approaches and marked with “*” in Fig. 6.
2.2 Selection of Human Donor Antibody FRs

There are two sources of human antibody Fv sequences: mature and germline. Mature sequences result from the recombination of germline genes V, D, and J for VH or V and J for VL [18]. The germline sequence has two advantages over the mature sequences as FR donors for murine CDR grafting. One is that these are less immunogenic, unlike the mature sequences that carry somatic mutations for affinity maturation generated by random processes, resulting in potential immunogenicity [19]. The other is increasingly flexible [20, 21], resulting in more compatibility between murine CDRs and human donor FRs. Therefore, human germline sequences have increasingly been utilized as a source of FR donors. Since the murine antibodies are made of 95% κ-type light chains, IGLV and IGLJ are seldom used in humanization protocols. In addition, the D gene only encodes part of CDR3 for VH without any involvement of FR coding, so it is not taken into account in the selection of germline genes as FR donors for humanization of the murine VH.

2.2.1 Selection of Germline Human Donor V Gene

The key for humanized antibodies by CDR-grafting to retain the original murine antibody antigen-binding affinity relies on the preservation of the murine CDR conformation in the humanized antibody FRs. The CDR conformation is mainly dependent on CDR canonical structures. In order to keep murine CDR conformation unchanged in the human donor antibody FRs, the human donor antibody FRs should have the same CDR canonical structures as the given murine antibody. The canonical structures can be identified for CDRs 1, 2, and 3 of VL (CDR-L1, CDR-L2 and CDR-L3), and CDRs 1 and 2 of VH (CDR-H1 and CDR-H2) using the online free program “AbCheck - Antibody Sequence Test” (http://www.bioinf.org.uk/abs/seqtest.html) by a few canonical conserved residues located in CDRs and FRs [27,28]. As for D9, the CDR canonical structures for VH and VL were determined as 2-1 and 1-1-2. In order to select human germline V gene candidates as FR donors for murine D9 antibody humanization, based on D9 CDR canonical structures, a shortlist of germline human antibody V gene candidates for VH or VL sharing the same canonical structures can be retrieved from germline human antibody V gene tables [6] to ensure that the human germline donor antibody V gene FRs support the murine CDR canonical structures. Next, within the shortlist of human germline V genes, those having the highest similarity in CDRs (CDRs 1, 2 for HC or CDRs 1 to 3 for LC) and key FRs 1 to 3 to the original murine antibody’s, can be selected as FRs 1-3 donors. In order to select human germline antibody V and J genes as FR donors for D9 humanization, a shortlist of human germline antibody HC’ or and LC V gene candidates with the same CDR canonical structure as the D9 counterpart was then formed to ensure that the human antibody donor FRs support the mouse CDR canonical structures. Within the shortlist of human germline V genes, those with the highest similarity in CDRs (CDRs 1, 2 for HC and CDRs 1 to 3 for LC) and key residues of FRs 1 to 3 to the D9 counterparts were chosen as FRs 1-3 donors. As a result, human germline genes 1-18
and O12 were chosen for HC and LC respectively.

2.2.2 Selection of Germline Human Donor J Gene

There are only 6 human heavy chain germline J genes and 5 human K chain germline J genes available for selection of human donors to provide FR4 for murine antibody humanization [6]. The similarity of each human candidate sequences to the original murine antibody, regarding CDR3 and key FR4 residues, should be analyzed and scored. The J gene with highest similarity to the original murine antibody should be selected as the donor of FR4. Human JH6 and Jk4 genes were chosen based on the highest similarity in CDR3 and key residues in FR4 to those of D9.

2.2.3 Human Donor Antibody FRs for D9 FV Humanization

Consequently, human HC germline genes 1-18 and JH6 were selected as FR donors for humanization of D9 VH; human LC germline genes O12 and JK4 were selected as FR donors for humanization of D9 VL.

2.3 Selection of Back-Mutation Sites

Finally, key residues can be identified and marked with star as showed in Fig. 6 for D9.

![Figure 6. Humanization of D9 Fv by CDR-grafting. Residues are numbered according to Kabat. CDRs are marked with boxes and FRs are between boxes. Key FR residues are marked with *. Two key FR residues in D9 VH, which were kept in humanized D9 (hD9) VH are marked with green.](image)

Those non-identical or non-conserved residues at the key FR positions in the selected human donor V and J genes should be analyzed one by one carefully. The analysis should be focused on the size, charge, hydro, accessibility, and neighboring circumstances. If the substitution is favorable or neutral, the backmutation is not necessary. However, if the substitution is not favorable, the backmutation should be considered. In other words, this key FR residue should be kept in humanized antibody.

Most of the key FR residues of D9 were the same or conserved as human donor antibodies. Only 2 residues were not conserved, VH44 (mouse N versus human G) and VH82a (mouse L versus human R). VH44-N was an unusual interchain packing residue, located in the VH-VL interface, interacting with VL87-Y, VL98-F, VL99-G, and VL100-A as shown in Fig. 7. Only 0.3% VH have N in the position 44, indicating it came from somatic mutation, which might indirectly enhance antibody binding. VH82a-L was a Vernier Zone residue to support VH CDR2 by interacting with VH65-D in VH CDR2 as shown in Fig. 8. The substitution at both positions might significantly alter CDR’s conformation. As a result, these murine residues were retained. Advantageously, molecular modeling revealed both of these as not solvent accessible, indicating that these are not located on the surface of Fv and are unlikely to elicit an immune response in humans. Therefore, when the CDRs of D9 were grafted onto the donor human antibody FRs,
VH44-N and VH82a-L were kept in hD9 Fv.

Figure 7. VH44-N interaction with four VL residues, VL87-Y, VL98-F, VL99-G, and VL100-A in VH-VL interface.

Figure 8. VH82a-L interaction with VH65-D in VH CDR2.

2.4 Construction, Expression, and Purification of Full-Length Humanized Antibody

2.4.1 Construction of Full-Length Humanized Antibody Gene

In order to express the full-length humanized antibody, the humanized VH and VL need to be respectively grafted onto human antibody constant regions, CH and CL. As for humanized D9 (hD9), the VH of hD9 was further grafted onto the human gamma 1 HC CHs to form a complete HC, while the VL was grafted onto the human kappa 1 LC CL to form a whole LC.

2.4.2 Expression of Full-Length Humanized Antibody Gene in Mammalian Cells

Antibodies are large molecules composed of two chains. In order to obtain an equi-molar expression of antibody HC and LC in a single open reading frame (ORF) driven by one single promoter, a 2A self-cleavage linker encoding a 24-residue-oligopeptide, was introduced between HC and LC DNA sequences to express a full-length antibody from a ORF driven by a single promoter in an adenoviral vector [29,30]. To get the expressed hD9 to be secreted to culture media, a leader sequence was added upstream of the HC and LC, respectively. The whole DNA sequence including the human antibody kappa LC O12 leader sequence, the humanized LC (VL+CL), 2A linker, 1-18 HC leader sequence, and humanized HC (VH+CH1+CH2+CH3), around 2 kb was synthesized and then cloned into an adenoviral vector for expression. The recombinant hD9 was expressed in HEK-293 cells and purified using an ImmunoPure
2.5 Evaluation of Humanized Antibody

2.5.1 Antigen-Binding Affinity Analysis of Humanized Antibody

In order to compare the antigen-binding affinity between humanized antibody and its parental murine antibody, measurements of affinity constant (KD) for antibodies binding to antigen should be performed by Surface Plasmon Resonance (SPR) biosensor. The kinetics of association and dissociation can be recorded in SPR sensorgram. The kinetic rate constants kon and koff can be calculated from the ascending rate of resonance units during association and the descending rate during dissociation. The KD of antibody for the antigen can be determined from the ratio of koff/kon. As shown in Fig. 9, hD9 had higher affinity to ricin with KD of 1.63 nM, comparable to than D9 (2.55 nM), indicating humanization of hD9 is successful.

![Figure 9. SPR sensorgrams of the kinetics of association and dissociation of a range of concentrations from 0 to 500 nm of hD9 (A) or D9 (B) to immobilized ricin.](image)

2.5.2 Therapeutic Efficacy Evaluation of Humanized Antibody in vivo

To evaluate the therapeutic efficacy of hD9, a mouse model was used. Ricin was given at the dose of 2×LD50 to mice by intranasal (i.n.) route and hD9 at the dose of 5 µg was administered by intramuscular injection 20 or 24 hours after ricin challenge. As shown in Fig.10, hD9 could rescue 100% of the mice 20 hours after ricin challenge.

3.0 MESENCHYMAL STROMAL CELLS (MSCs) AS GENE DELIVERY VEHICLES FOR THERAPEUTIC ANTIBODIES AGAINST BIOLOGICAL AGENTS

Direct administration of therapeutic antibodies can confer immediate immunity to the host for prophylaxis and therapy against biological agents. However, the provided immunity is short (minutes to weeks) and antibody production is lengthy, laborious and expensive. Additionally, to achieve a therapeutic plasma concentration, antibodies have to be administered in large doses, which requires intravenous administrations via hour-long infusions and must be repeated over a long period of time in a specific hospital environment [22]. As a result, this approach becomes impractical when large populations are exposed to a biological agent attack.

An alternative approach is to take advantage of the body’s natural ability to express transgenes to produce therapeutic antibodies within the host [23]. Currently, there are two kinds of vectors for gene delivery:
viral and non-viral [24]. In terms of transfection efficiency \textit{in vivo}, viral vectors are superior to non-viral vectors [25]. However, most viral vectors are inappropriate for gene delivery in humans, since their unpredictable chromosomal integrations often lead to tumorigenecity[26]. The adenoviral vector is exceptional [27] in that it does not integrate into the host genome but remains as an isolated plasmid. Consequently, adenoviral vectors are favored and extensively employed in gene delivery systems. One challenge in using adenoviral vectors for gene therapy is its potential inactivation by the immune system of hosts with pre-existing immunity to human adenovirus [28]. Many people already have antibodies against the adenovirus from earlier infection. These pre-existing antibodies can inactivate the recombinant adenovirus at the outset and attenuate transgene expression in the host. One promising method to circumvent this problem is the use of MSCs as cellular vehicles to deliver recombinant adenovirus into the host. MSCs have a low immunogenic profile [29] and can be easily uploaded with a genetic payload, persist within the host for months, and have limited tumorigenic potential because they lack telomerase [7, 30]. As such, MSCs may provide an ideal shield to protect the recombinant adenovirus from immune responses within the host and facilitate successful gene transfer.

Human umbilical cord perivascular cells (HUCPVCs) are a rich source of umbilical cord matrix-derived progenitor cells [31-35] (Fig.11) that exceed the minimum phenotypic criteria for MSCs [31-33]. HUCPVCs are isolated by plastic adherence, rapidly expand to clinically relevant numbers, and are similar to bone marrow-derived MSCs [31, 32, 36]. HUCPVCs are ideal for military applications since they are obtained non-invasively from tissue classified as medical waste, are amenable to stock-piling, and are suitable for administration to any individual. It was here investigated whether HUCPVCs could be engineered as stealth gene delivery vehicles to shield exogenous DNA from immune detection and sustain production of therapeutic antibodies \textit{in vivo} with humanized anti-Venezuelan Equine Encephalitis Virus (VEEV) antibody as an example.

3.1 Genetic Engineering of HUCPVCs by Transduction with Anti-VEEV Antibody Gene

The humanized anti-VEEV antibody gene was previously cloned into a single open reading frame to generate a recombinant adenovirus construct (pAd5-anti-VEEV) [7]. HUCPVCs were transduced with the recombinant pAd5-anti-VEEV at 200 MOI with no apparent toxicity.
3.2 Evaluation of Anti-VEEV Antibody Secretion by Engineered HUCPVCs \textit{in vitro}

To determine if engineered HUCPVCs can secrete the anti-VEEV antibody \textit{in vitro}, culture supernatant was harvested from engineered cells every 3-4 days and antibody levels quantified by functional enzyme linked immunosorbent assay (ELISA). Five days after transduction, one million engineered HUCPVCs secreted 5 µg of anti-VEEV immunoglobulin G (IgG) per day, increasing to approximately 23 µg per day by day 20 (Fig. 12). This rate could be maintained for 42 days in culture.

3.3 Evaluation of Anti-VEEV Antibody Secretion by Engineered HUCPVCs \textit{in vivo}
In vivo testing was performed using nude mice, since HUCPVCs are not suitable for xenogeneic grafting despite being non-alloreactive in humans [37]. Nude mice received an intramuscular injection of 2.5 million antibody-secreting HUCPVCs, 4 days after genetic modification. Control groups received 50 µg of purified anti-VEEV antibody intramuscularly. Serological profiles of circulating anti-VEEV were generated from blood samples taken at various time points. The purified anti-VEEV antibody administered by direct injection decayed exponentially at a rate of exhibited a serum half-life of 3.7 (+/- 0.5) days. The passively administered anti-VEEV immediately began to deteriorate (Fig. 13). In contrast, the serum antibody titers of mice receiving engineered HUCPVCs increased for up to 10 days after transplant. Predicted protective titers were maintained up to 38 days post-transplant in 40% of mice.

Figure 13. Administration of HUCPVCs, engineered with the pAd5-anti-VEEV transgene, can prolong therapeutic levels of circulating anti-VEEV antibody.

3.4 Evaluation of Efficacy against VEEV Challenge in Mice Pre-Treated with Engineered HUCPVCs

Mice were intranasally infected with a lethal dose of the highly virulent VEEV strain Trinidad donkey (TrD) in biosafety level 3 (BSL3) containment. Mice were treated with either 50 µg of purified antibody or 2.5 million antibody-secreting HUCPVCs, 24 hours or 10 days prior to challenge. Control groups were pre-treated with saline (untreated) or HUCPVCs transduced with pAd5-eGFP (sham HUCPVCs). Clinical signs of infection were assessed blind on a 5-point scale reflecting weight loss and clinical symptoms, and humanely euthanized if scored at 4. Efficacy of the humanized anti-VEEV antibody was previously demonstrated in wild-type mice receiving a low dose of 30-50 plaque-forming units (PFU) of VEEV TrD [38]. Here, mice were challenged with a 20-fold higher dose of 1,000 PFU per nude mouse. The dose increase was necessitated by the immune-compromised mouse model, in which the absence of T cells likely impedes the rapid hyper-inflammatory response that produces lethal encephalitis in wild-type mice.

Although 50 µg of purified anti-VEEV fully protected mice against 30-50 PFU of VEEV [38], it was insufficient to protect against 1,000 PFU (Fig. 14). In contrast, pre-treatment with engineered HUCPVCs 24 hours or 10 days prior to exposure fully protected mice from intranasal VEEV infection. Only one mouse died (maroon) from causes likely unrelated to the study, as she never exhibited clinical signs of
infection. Notably, mice pre-treated with engineered HUCPVCs not only survived the challenge, but exhibited considerably less morbidity than mice pre-treated with the antibody alone.

Figure 14. Prophylactic treatment with engineered HUCPVCs confers protection against lethal exposure to VEEV.

The serological data suggest that mice pre-treated with engineered HUCPVCs may continue to be protected 21, 31 or 38 days after administration. To reduce the requirement for animals, this hypothesis was tested in vitro. The VEEV-neutralizing activity of pooled serum obtained from mice pre-treated with anti-VEEV-expressing HUCPVCs immediately prior to lethal challenge, or 21, 31, and 38 days after administration, was assayed by plaque reduction neutralization test (PRNT) [39]. VEEV-neutralizing antibody titers (PRNT50) are expressed as the highest dilution of serum producing a 50% or greater reduction in plaque count, as compared with negative controls in which virus was incubated with diluent. Average serum titers were calculated from individual serum titers obtained previously by functional ELISA. Serum from mice that remained asymptomatic in the lethal challenge study had considerably more neutralizing activity (PRNT50 5,120) than mice that exhibited clinical signs of infection but eventually recovered (PRNT50 3,840; Table 1). Serum obtained from unchallenged mice 21 days after administration of HUCPVCs had equivalent neutralizing activity to the challenged mice that recovered (Table 1) suggesting that mice would still be protected 21 days after engineered cell treatment. Although it was less potent significant, the neutralizing activity was still detected in serum obtained 31 and 38 days after transplant of HUCPVCs expressing anti-VEEV (Table 1). These data suggest that mice could be partially protected from lethal exposure to VEEV up to 38 days after prophylactic treatment with engineered HUCPVCs.

4.0 DEVELOPMENT OF PLANT-BASED THERAPEUTIC ANTIBODIES AGAINST BIOLOGICAL AGENTS

Therapeutic mAbs are among the most expensive drugs. The current production of therapeutic mAbs are involved in the use of very large cultures of mammalian cells followed by extensive purification steps, under Good Manufacturing Practice (GMP) conditions, leading to extremely high production costs ($2,000/gram). The high cost has dramatically affected the development of mAbs as therapeutics.
The cost might be reduced by certain new technologies, such as plant-based mAb production. Plants can be used as bioreactors for large-scale production of antibodies at a low cost. Plants are being evaluated for production of therapeutic mAbs [40, 41]. Significant progress has been made in the expression of mAbs in plants. In the first human trial of a mAb produced in transgenic tobacco plants, the mAb was demonstrated to provide protection against oral Streptococcus mutant colonization [42]. Preclinical studies of a humanized anti-herpes simplex virus (HSV) mAb produced in transgenic soybean plants showed that the mAb prevented transmission of HSV infection in mice [43]. These results suggest potential promise for the use of this technology.

Plants systems have several advantages over mammalian cell culture. They are fast, efficient, highly versatile (for new product development), and easily scalable with significantly reduced manufacturing costs. In addition, they are free from contamination by mammalian pathogens. However, before plant-produced therapeutic mAbs come to the market, the plant production system for therapeutic mAbs has to meet the rigorous regulatory requirements and standards for pharmaceutical products.

Currently, plants are being used for the GMP production of our humanized anti-VEEV antibody, supported by Defense Threat Reduction Agency (DTRA) and humanized anti-ricin antibody, hD9 in preclinical stage, supported by Canadian Biological Warfare Threat Medical Countermeasure (BWTMCM) Project and Preclinical-Funded Service of American National Institutes of Health (NIH). The plant-derived anti-ricin antibody will be evaluated to ensure it is indistinguishable from its conventional mammalian-derived counterpart.

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