Antiviral Research 90 (2011) 1–8

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

A humanised murine monoclonal antibody with broad serogroup specificity protects mice from challenge with Venezuelan equine encephalitis virus

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ARTICLE INFO

Article history:
Received 20 September 2010
Received in revised form 31 January 2011
Accepted 31 January 2011
Available online 15 February 2011

Keywords:
Venezuelan equine encephalitis virus
Monoclonal antibody
Immunotherapy
Humanisation

ABSTRACT

In murine models of Venezuelan equine encephalitis virus (VEEV) infection, the neutralising monoclonal antibody 1A3B-7 has been shown to be effective in passive protection from challenge by the aerosol route with serogroups I, II and Mucambo virus (formally VEE complex subtype IIIA). This antibody is able to bind to all serogroups of the VEEV complex when used in ELISA and therefore is an excellent candidate for protein engineering in order to derive a humanised molecule suitable for therapeutic use in humans. A Complementarity Determining Region (CDR) grafting approach using human germline IgG frameworks was used to produce a panel of humanised variants of 1A3B-7, from which a single candidate molecule with retained binding specificity was identified. Evaluation of humanised 1A3B-7 (Hu1A3B-7) in in vitro studies indicated that Hu1A3B-7 retained both broad specificity and neutralising activity. Furthermore, in vivo experiments showed that Hu1A3B-7 successfully protected mice against lethal subcutaneous and aerosol challenges with VEEV strain TrD (serogroup I). Hu1A3B-7 is therefore a promising candidate for the future development of a broad-spectrum antiviral therapy to treat VEEV disease in humans.

1. Introduction

As part of the *Alphavirus* genus in the family Togaviridae, members of the Venezuelan equine encephalitis virus (VEEV) complex are single stranded, positive-sense RNA viruses. These viruses are maintained in nature in a cycle between small rodents and mosquitoes. Six serogroups (I–VI) are currently recognised within the VEEV complex which can be further divided into two types: epizootic (serogroups IA, IB and IC), and enzootic (II–VI, ID, IE and IF) including Mucambo virus species (formally VEEV subtype IIIA) (Weaver et al., 2004; http://www.ictvdb.org/index.htm). While enzootic strains are avirulent in equines (Wang et al., 2001; Walton et al., 1973), epizootic strains of VEEV have been responsible for widespread outbreaks of infection that can involve hundreds of thousands of equine and human cases (Paessler and Weaver, 2009; Rivas et al., 1997). Both epizootic and enzootic strains of VEEV are also highly infectious for humans by the airborne route and have been responsible for over 150 laboratory acquired infections that had no perforating injury associated with them (Paessler and Weaver, 2009). VEEV was developed as a biological weapon by both the former Soviet Union and the USA and is currently an agent with potential for bioterrorist use (Hawley and Eitzen, 2001; Hilleman, 2002).

Despite continued effort, there are currently no licensed vaccines available for immunisation of humans against VEEV (Paessler and Weaver, 2009). In the absence of appropriate vaccines, there is increased emphasis on the development of suitable antiviral candidates for prophylaxis and treatment of VEEV disease. In murine models of VEEV infection, monoclonal antibody therapy is an effective treatment against lethal challenge with virus when delivered prophylactically or up to 24 h post-exposure (Hunt et al., 2006; O'Brien et al., 2009; Phillpotts et al., 2002; Phillpotts, 2006; Hu et al., 2010). On this basis, it is possible that immunotherapeutic treatment would represent a useful means of intervention in human cases where early treatment is possible due to a known exposure to virus, e.g. laboratory containment failure or deliberate environmental release (Phillpotts et al., 2002; Hilleman, 2002). In this work, to underpin immunotherapy usage in such scenarios, and based on known protective efficacy of monoclonal antibody therapy from murine models, a theoretical assessment of the potential utility of monoclonal antibody therapy was undertaken. This has been based on the known protective efficacy of monoclonal antibody therapy in murine models extrapolated to human populations.

The potential for direct application of murine antibodies for human antiviral therapy may be problematic. Antibodies from different animal species may not interact properly with Fc receptors and/or complement leading to a lack of appropriate down-stream

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0166-3542/$ – see front matter.
doi:10.1016/j.antiviral.2011.01.010

effector functions and rapid clearance from the body (Clark et al., 1983; Tabrizi et al., 2006). This could be of specific importance in the use of antibodies targeted against VEEV where limited protective utility of antibody fragments devoid of Fc regions suggests that downstream immune effector functions are important in protective efficacy (Mathews et al., 1985). In addition, non-human antibodies can be recognised as "foreign" by the human immune system resulting in anti-antibody responses (AAR). Such responses can limit the efficacy of an immuno-therapy and raise the possibility of development of a severe immunogenic response that could include anaphylactic shock (Schroff et al., 1985). Humanisation techniques that reduce the non-human content of antibodies to a minimal level have therefore been developed in order to improve the clinical characteristics of antibodies for therapy (Hwang and Foote, 2005).

Humanisation approaches have previously been applied to two murine antibodies, 1A4A-1 (Hu et al., 2007) and 3B4C-4 (Hunt et al., 2006), which are specific for the E2 glycoprotein of VEEV. Within this protein six epitopes ($E2^{c\text{-}h}$) that form a critical neutralisation domain have been defined through antibody binding studies (Roehrig et al., 1988; Roehrig and Mathews, 1985). Both 1A4A-1 and 3B4C-4 bind to the E2 glycoprotein at the $E2^{c}$ epitope and exhibit effective virus neutralisation characteristics based on blocking of virus binding to cells (Hunt et al., 1990; Roehrig et al., 1988; Roehrig and Mathews, 1985). The humanised variants of these antibodies retain the majority of binding and neutralisation activity of the parent murine antibodies in vitro (Hu et al., 2007; Hunt et al., 2006). Furthermore they exhibit protective effects in vivo when delivered 24 h prior to or 24 h after lethal challenge with VEEV (Hu et al., 2010; Hunt et al., 2006). However, both 1A4A-1 and 3B4C-4 have narrow binding specificities limited to only some of the subgroups (1A/B-D, II) within the VEEV complex (Roehrig et al., 1982; Roehrig and Mathews, 1985; Phillpotts, 2006). To be of greatest relevance as a therapeutic candidate for VEEV, an antibody molecule should ideally be able to target all serogroups present in the VEEV complex. On this basis CUF-372a, a murine antibody with broad serogroup reactivity derived from a phage display library represents an attractive candidate for humanisation (O'Brien et al., 2009). However, this antibody does not neutralise infective virus. While not essential for providing protection in vivo, neutralisation activity could be considered a desirable characteristic for future antiviral therapies (O'Brien et al., 2009; Phillpotts, 2006). One antibody, 1A3B-7, offers both broad specificity and neutralisation activity in vitro (Rico-Hesse et al., 1988; Roehrig and Mathews, 1985; Roehrig and Bolin, 1997). This antibody binds to the $E2^{h}$ epitope within the E2 glycoprotein targeting a different area of the critical neutralisation domain of VEEV than other antibodies used in humanisation studies (Roehrig et al., 1988; Roehrig and Mathews, 1985). This antibody also has proven protective effects in a murine model against aerosol exposure to strains of VEEV from the serotypes I, II and IIIA (Phillpotts, 2006). We therefore describe the humanisation of 1A3B-7 using a CDR grafting approach (Jones et al., 1986) with a view to generating a protective humanised molecule with the broadest spectrum of activity of any anti-VEEV therapeutic antibody candidate.

2. Materials and methods

2.1. Cell lines and viruses

L929 (murine fibroblast) and CHO DG44 (Chinese Hamster Ovary) cell lines (European Collection of Animal Cell Cultures, UK) were propagated by standard methods using the recommended culture media. Stocks of VEEV vaccine strain TC-83 were propagated from a vial of vaccine originally prepared for human use (National Drug Company, Philadelphia, USA). Strains of VEEV from serogroups IA/B (Trinidad donkey; TrD), IC (P676), ID (3880), IE (Mena II), IF (78V), II (Fe37c), IV (Pixuna), V (CaAr508), VI (AG80) and Mucambo virus (BeAn8), were kindly supplied by Dr. B. Shope (Arbovirus Research Unit, University of Texas, USA). Virulent virus stocks were prepared and the titre determined as described by Phillpotts (2006). All work with virulent VEEV was carried out under UK Advisory Committee on Dangerous Pathogens Level 3 containment.

2.2. Theoretical estimation of the efficacy of antibody in protection of a human population exposed to VEEV

A probit slope was generated from protective efficacy data of the anti-VEEV antibody 1A3A-9 in a murine model when delivered between 2 and 48 h after aerosol exposure with VEEV (Phillpotts et al., 2002). ID^{50}/pfu/ml figures from this probit slope were then applied to two illustrative theoretical scenarios using the Hazard Prediction and Assessment Capability (HPAC) system (Version 5 Service Pack 1, Defence Threat Reduction Agency (DTRA)). Scenario one consisted of a covert release of 1 kg VEEV, 3 km upwind of a battle group headquarters situated in a Deployed Operating Base (1500 personnel in an area of 2 km by 2 km). The second scenario investigated the release of 1 kg of VEEV, 1 km upwind of a stadium (0.5 km × 0.5 km) containing 43,760 spectators using the Urban Dispersion Model (UDM). In both scenarios the release was assumed to be an instantaneous release at night (15 °C, 50% humidity). Meteorological effects were investigated by inclusion of the following Pasquill-Gifford stability categories; Stability Category D, wind speed 4 m s^{-1}, Stability Category D, wind speed 7 m s^{-1}, Stability Category F, wind speed 2 m s^{-1}, Stability Category B, wind speed 1 m s^{-1} (Pasquill, 1961).

2.3. Harvesting of genes encoding the V_H and V_L chain domains of anti-VEEV antibody 1A3B-7

The hybridoma cell line 1A3B-7 (a kind gift of Dr. J.T. Roehrig, Division of Vector-Borne Infectious Diseases, CDC, Fort Collins, Colorado, USA) was grown in Dulbecco's modification of Eagle's medium supplemented with 10% foetal calf serum (DF10) plus pen/strep (1 mM) and sodium pyruvate (1 mM) (Gibco BRL). Samples of the media containing antibody were analysed using a murine monoclonal isotype analysis (IsoStrip Mouse Monoclonal Antibody Isotyping Kit, Roche) to confirm that the cell line produced a murine immunoglobulin of IgG2a/kappa isotype. RNA was extracted from cells and oligodT was used to prime cDNA synthesis (Superscript reverse transcriptase, Invitrogen). The resultant first strand cDNA was then used in PCR to amplify DNA fragments encoding the V_L and V_H regions of the 1A3B-7 antibody (Mouse Ig-Primer Set, Novagen). The gene fragments were then cloned and submitted for sequencing analysis.

2.4. Analysis of murine sequences and humanisation of antibody 1A3B-7

DNA sequences encoding antibody gene fragments were analysed using either DNA for windows software or DNASTar (DNASTAR, Incorporated. 3801 Regent Street Madison, WI 53705 USA). Assignment of kabat numbering to the V_L and V_H chains of 1A3B-7 was performed using Kabat sequence analysis tools (http://www.bioinf.org.uk/abs/simkab.html). Alignment of the sequences for the V_H and V_L to potential human germline candidates for humanisation was performed using NCBI IgBLAST tools (http://www.ncbi.nlm.nih.gov/igblast/). Genes encoding the humanised 1A3B-7 fragments were constructed using overlapping oligonucleotides in overlap extension PCR (Kobayashi et al., 1997). The resultant amplicons were cloned into T vector (Promega) and

submitted for sequencing to confirm the integrity of the sequences generated.

2.5. Production and purification of recombinant chimeric and humanised 1A3B-7 in mammalian cell culture

The murine and humanised versions of the V_H and V_L domains from 1A3B-7 were fused to human IgG1 or κ constant regions respectively in the eukaryotic expression vector pCMVScript (Stratagene) or pHEE (Wyeth) to generate full length chimeric (Ch1A3B-7) and humanised (Hu1A3B-7) molecules. Vectors harbouring constructs were transfected into CHO DG44 cells that had been cultured as adherent monolayers in Iscove's Modified Dulbecco's Media (IMDM) with 10% (v/v) Foetal Bovine Serum (FBS) (Gibco BRL) and hypoxanthine (0.1 mM) and thymidine (16 μM) (HT Supplement, Invitrogen). Prior to transfection, cells were recovered from two 90% confluent flasks, counted, diluted to 3×10^5 cells/ml and plated out in six-well plates (2.5 ml per well) Cells were incubated overnight then transfected with plasmid vector DNA encoding recombinant humanised or chimeric antibodies. Transfection of cells with plasmid DNA was performed using Lipofectamine 2000 at a ratio of 2.5 μl of Lipofectamine per μg of DNA in accordance with manufacturer's instructions (Invitrogen). Stable cell lines for bulk production of Hu1A3B-7 were generated using Geneticin (Gibco BRL) at 400 μg per ml, which were then propagated in IMDM (Gibco BRL) with 10% (v/v) FBS, 1% antimycotic, 25 mg Gentamycin, 1 mM sodium pyruvate, 2 mM glutamine, 1% (v/v) pen/strep, 1% (v/v) non-essential and 1% (v/v) essential amino acids (Gibco) and 10 mM methotrexate (Sigma). Cell lines secreting antibody were expanded and the highest producers selected. Humanised antibody was purified via protein A affinity chromatography using Prosep®-A (Millipore) and dialysed into Phosphate Buffered Saline (PBS). Samples of protein were analysed on denaturing SDS–PAGE gels to confirm the presence of heavy and light chains of the antibody molecules prior to quantification by capture ELISA and in vitro activity assays.

2.6. Quantification of recombinant humanised and chimeric 1A3B-7 by capture ELISA

Appropriate anti-species (anti-human kappa or anti-mouse Fc region (both Sigma) for the Hu1A3B-7 or Ch1A3B-7 and Mu1A3B-7, respectively) were diluted 1 in 1000 with sodium bicarbonate coating buffer (Sigma) and used to coat ELISA plates (Immulon 4, Nunc). Samples of antibody were then added to the plate and serially diluted. Appropriate human or murine standard antibodies at known concentrations (maximum of 1 μg/ml) were also added to provide means of calculating concentrations of the antibody samples. Bound antibody was then detected using appropriate secondary antibodies (Anti-human IgG lambda Horseradish peroxidase or anti-mouse IgG (Fc specific)–Horseradish peroxidise (both Sigma) for Hu1A3B-7 or Ch1A3B-7 and Mu1A3B-7, respectively) at a 1 in 1000 dilution.

2.7. Activity of humanised 1A3B-7 in ELISA and virus neutralisation assays

The ability of antibodies (1 μg/ml) to recognise a variety of VEEV strains and BPL-inactivated TC83 vaccine strain (Phillpotts et al., 2005) was tested by ELISA using sucrose density gradient-purified antigen (Phillpotts, 2006). So that the reactivity could be meaningfully compared, the VEEV antigens used in the ELISA were first examined by SDS–PAGE and scanning densitometry. Each antigen was diluted in coating buffer to contain an equivalent amount of virus glycoprotein. The ability of the antibody to neutralise virus infectivity was determined by mixing appropriate amounts of antibody (final concentration 50 μg/ml) with VEEV strains TrD, Fe37c or Mucambo virus strain BeAn8 (approximately 100 pfu) and incubating at 4 °C overnight. Residual infectious virus was estimated by plaque assay in L929 cells.

2.8. Persistence of humanised 1A3B-7 in serum of mice

To evaluate the persistence of antibody levels in sera of mice, groups of 5 age-matched Balb/c mice (Charles River Laboratories, UK) were injected intraperitoneally (i.p.) with 100 μg/mouse of Hu1A3B-7 or Mu1A3B-7 antibody in 100 μl PBS. Control mice did not receive any treatment. All mice were bled from a superficial tail vein at 1, 3, 5, 10 and 15 days post-treatment. Sera were then assayed by standard ELISA techniques to determine the levels of recombinant antibody using the Multiscan Ascent V1.22 software for data analysis. A linear regression model was fitted to the logged data to estimate the rate of change over time (slope of the line) and hence the half-life. The fitted regression model provides an estimate of the slope and the standard error of the estimate which can be used to calculate confidence intervals. To relate the model parameters to half-life, a change of 0.30101 units on the log scale equates to a 50% reduction of antibody concentration. This value is divided by the estimated model slope to obtain the half-life.

2.9. In vivo characterisation of protective effects of humanised 1A3B-7

The ability of Hu1A3B-7 to protect against a subcutaneous challenge dose of 100 LD_{50} (approximately 30–50 pfu) virulent VEEV strain TrD (subtype IA/B) was tested. Groups of Balb/c mice (7–9 weeks old, Charles River, UK) remained untreated or were injected intraperitoneally with 25, 50, 75 or 100 μg of antibody in 50–100 μl of PBS, 4 h prior to challenge. To evaluate the protective characteristics of Hu1A3B-7 in a post-exposure context against an aerosol challenge, mice were exposed for 10 min via the airborne route to a polydisperse aerosol of VEEV strain TrD generated by a Collison nebuliser within a Henderson apparatus as described previously (Druett, 1969; May, 1973). Sampling from the impinger allowed calculation of virus dose assuming a respiratory minute volume for mice of 2 ml/g. Mice were treated with 100 μg of Hu1A3B-7 by the intraperitoneal route at 6 or 24 h post-challenge. In both experiments, after challenge, mice were observed twice daily for clinical signs of infection by an independent observer. Humane endpoints were used and these experiments therefore record the occurrence of severe disease rather than mortality. Even though it is rare for animals infected with virulent VEEV and showing signs of severe illness to survive, our use of humane endpoints should be considered when interpreting any virus dose expressed here as LD50.

2.10. Statistical methods

Statistical analysis of survival using the Mantel-Haenszel logrank test was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com).

3. Results

3.1. Theoretical analysis of the utility of an anti-VEEV antibody therapy in protection of a human population from a deliberate release of VEEV

The window of opportunity for treatment of an established VEEV infection with antibody therapies is likely to be short. This is based on murine studies showing antibodies were only effective when administered 24 h and 48 h after infection (Phillpotts et al.,

Table 1
Probability of infection (%) in the absence of therapeutic countermeasure (no intervention) in comparison to antibody countermeasure delivered at time points after a known exposure event. All values are normalised from the baseline case with no intervention, to demonstrate the effect of the countermeasure. The values are provided with three times the standard deviation.

	No intervention	2 h	6 h	24 h	48 h
Deployed operating base	100 ± 18.0	25.8 ± 21.3**	38.2 ± 20.2**	70.8 ± 14.6*	88.8 ± 15.7§
Stadium	100 ± 12.2	44.9 ± 11.2**	59.2 ± 13.3*	77.6 ± 11.2*	95.9 ± 12.3§

* $p < 0.01$.
** $p < 0.001$.
§ Statistically indistinguishable from the baseline case, using a standard *T* test.

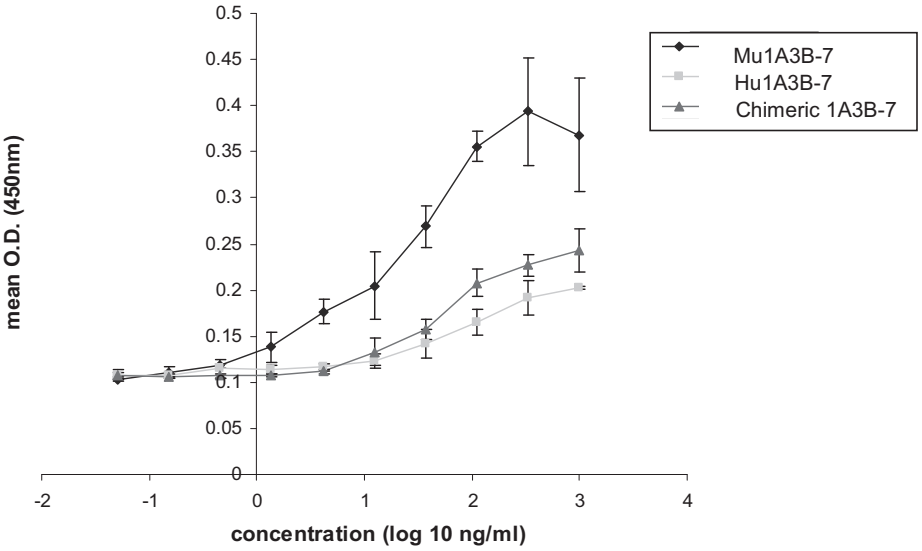

Fig. 1. Analysis of the binding activity of Hu1A3B-7 towards inactivated TC-83 VEEV in ELISA in comparison to parental Mu1A3B-7 and chimeric 1A3B-7 (murine variable and human constant domains). Binding profiles of the murine molecule with the humanised and chimeric molecules are not comparable across the dilution series due the necessity to use different anti-species detection regents within this ELISA. $n = 3$ for all data points; 95% confidence limits are shown.

2002; Hunt et al., 2006) but not at 72 h (Phillpotts et al., 2002). To illustrate whether or not this short treatment window would be sufficient to provide benefit in human protection, a theoretical assessment of the impact of availability of an antibody therapy against environmental release of VEEV was performed utilising the HPAC modelling system. Two scenarios were modelled to mimic either a bio-warfare or bio-terrorism release of VEEV near either a deployed operating base or a stadium, respectively. Table 1 shows the probability of infection for personnel in both scenarios in the absence of therapy in comparison to antibody therapy administered at intervals from 2 h up to 48 h after exposure. The uncertainty values result from averaging the exposure over personnel (in different locations) and differences in the four meteorological conditions used for each scenario. Statistically significant reductions in infection in comparison to the baseline (no intervention) were observed in both scenarios if a candidate antibody therapy was administered up to 24 h after a known exposure event (Table 1). This analysis indicates that the availability of an efficacious antibody therapy could have an impact that might be clinically important compared to unprotected personnel. When considering a release of an unknown serogroup of VEEV, antibody therapy capable of generic VEEV protection would clearly be advantageous if this could be delivered rapidly. This illustrative assessment therefore substantiates the aim of this study, to produce additional humanised antibodies that are capable of broad effective protection against VEEV serogroups.

3.2. Humanisation of anti-VEEV antibody 1A3B-7 by CDR grafting

The variable domains from murine 1A3B-7 were subjected to a process of humanisation utilising a CDR grafting approach. Human germline sequences appropriate for supporting the murine CDR regions from 1A3B-7 were selected according to homology within the framework regions. To mitigate the risk of loss of antibody function as a result of the humanisation process, a panel of three heavy chain and three light chain sequences was generated. These variants could then be assessed in all nine V_H/V_L combinations to select the most promising humanised domains to take forward for further evaluation.

Variable light chain sequences selected for humanisation of 1A3B-7 were B1, A26 and L6. No unusual amino acids were identified in the light chain framework regions and these humanised genes were therefore constructed by conventional CDR grafting with no other amendments to the human frameworks. Of note however, is that the 1A3B-7 murine V_L domain possesses an unusually long CDR1 domain (15 amino acids). The B1 germline sequence is also unusual in that it naturally supports a CDR1 sequence of the same size and therefore has an additional advantageous characteristic for the humanisation process further to overall sequence homology.

Murine V_H CDRs were grafted onto framework regions from human V_H domains DP-1 and DP-75. The analysis of the sequence of the murine antibody domains highlighted the presence of an unusual isoleucine residue at position 94 (Kabat et al., 1991) in the Framework 3 region of the murine heavy chain. The close proximity of this isoleucine residue to CDR 3 of the heavy chain suggested that it may be important in the binding of the antibody to target antigen (Foote and Winter, 1992). To take account of this characteristic of the murine antibody, this amino acid was retained in the third version of the humanised V_H gene using the DP-75 framework with a substitution from arginine to isoleucine at position 94.

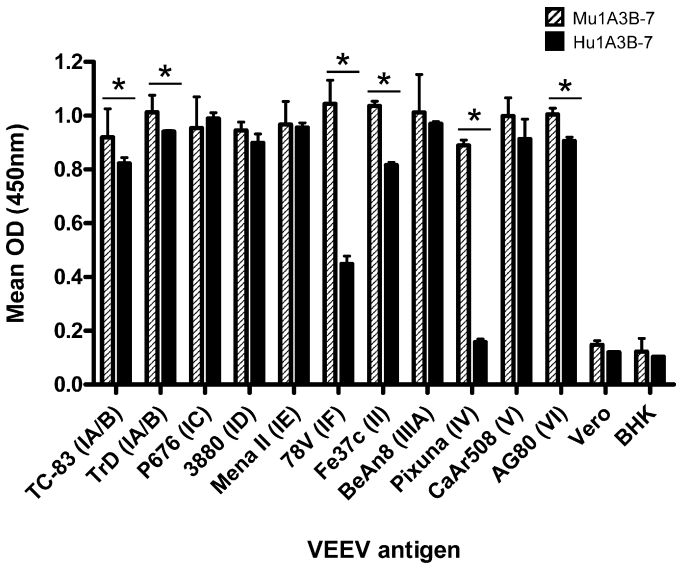

Fig. 2. Comparison of the relative binding efficiency of Hu1A3B-7 to a range of VEEV strains and Mucambo virus strain BeAn8 in comparison to the parental Mu1A3B-7 antibody. Negative control antigen was prepared from cells that had been mock infected. $n = 6$ for all data points, 95% confidence intervals are shown (*significantly different $p < 0.05$).

3.3. Production of humanised 1A3B-7 variants and assessment of activity in ELISA and neutralisation assays

Samples of the nine humanised 1A3B-7 candidates produced by combining the three V_L and three V_H domains in all combinations were expressed in mammalian cell culture (CHO DG44 cells). Six constructs successfully produced antibody. No expression could be observed for any of the constructs utilising the DP 1 heavy chain variant. Further work with these constructs was therefore halted. Antibody samples were used in ELISA to determine binding to BPL-inactivated TC-83 VEEV in comparison to chimeric 1A3B-7. One combination of humanised heavy and light chain successfully produced an active molecule (Fig. 1). This molecule (Hu1A3B-7) utilised the human germline heavy chain template DP-75 incorporating the isoleucine variation at H94 in combination with the kappa light chain B1.

To ensure that the broad-spectrum of VEEV reactivity had been retained during the humanisation process, the antibody was tested in comparison to the murine 1A3B-7 in an ELISA using antigens from multiple strains (Fig. 2). Comparable levels of reactivity for both the murine and humanised versions of 1A3B-7 were observed for most strains tested, with the exception of strains 78V (IF); Pixuna (IV) and Fe37c (II) where the humanised version showed substantially reduced binding activity. The ability of the Hu1A3B-7 to neutralise virus was also assessed in in vitro cell culture against three representative strains of VEEV. This analysis showed that the virus had retained the ability to neutralise VEEV from subtypes IA/B, II and Mucambo virus at a comparable level to that of the original antibody (Table 2).

3.4. Activity of humanised 1A3B-7 in protecting mice from lethal VEEV challenge

The successful humanisation of the broadly reactive anti-VEEV antibody in terms of retained breadth of serogroup reactivity in ELISA and in vitro neutralisation activity, encouraged further evaluation of this molecule in in vivo protection studies. Initially, we evaluated the half-life of the Hu1A3B-7 in a murine model in comparison to the parental Mu1A3B-7. The half-lives of Mu1A3B-7 and Hu1A3B-7 were estimated as 7.37 and 4.70 days, respectively, with 95% confidence intervals of (6.20–9.09) and (3.64–6.66) days, respectively (Fig. 3).

Table 2
Comparison of the relative neutralisation activity of Hu1A3B-7 to parental Mu1A3B-7 antibody for VEEV strains TrD (IA/B), Fe37c (II) and Mucambo virus BeAn8 (VEE complex subtype IIIA). Residual infectivity (pfu) of antibody-treated virus in plaque assay in L929 cells is shown, 95% confidence intervals are shown ($n = 3$). Results are representative of two experiments.

	Mean pfu recovered after incubation with		
	Media	Mu1A3B-7	Hu1A3B-7
TrD	74.33 ± 15.07	26.0 ± 5.03	27.0 ± 3.06
Fe37c	82.67 ± 6.96	47.67 ± 16.43	45.67 ± 10.48
BeAn8	132.0 ± 21.39	21.67 ± 9.40	17.0 ± 4.0

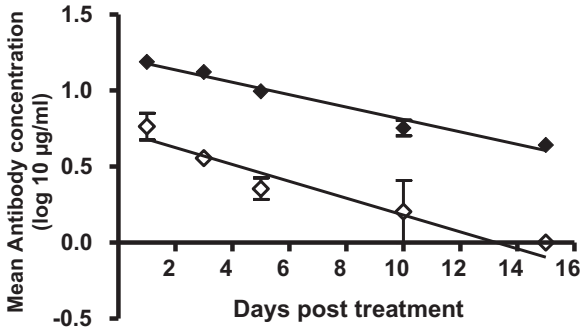

Fig. 3. Half-life of murine and humanised antibodies. A single dose of either Mu1A3B-7 (♦) or Hu1A3B-7 (◊) was administered to groups of 5 Balb/c mice by the i.p. route. Concentration of antibody was monitored for 15 days. Error bars indicate SEM.

Following this, Hu1A3B-7 was assessed for its ability to provide protection against a lethal challenge of virus in a small animal model of disease. Balb/c mice were pre-treated with a range of doses of Hu1A3B-7. 24 h later, the animals were challenged (s.c.) with 100 LD_{50} of VEEV (strain TrD, subtype IA/B) and monitored for 14 days. The results show that the humanised antibody provides levels of protection comparable to that provided by the original murine molecule (Table 3) (Phillpotts, 2006). To demonstrate that this antibody is capable of treating virus infection after exposure by the airborne route, mice were challenged with either a low (10 LD_{50}) or high challenge dose (100 LD_{50}) of VEEV strain TrD. Groups of mice were treated with a single dose (100 µg) of Hu1A3B-7 at either 6 or 24 h after exposure. All control mice at 10 and 100 LD_{50} challenge levels succumbed to infection by day 7. In comparison, 90% or 100% of mice survived following treatment with 100 µg of Hu1A3B-7 at 6 h or 24 h post exposure with 10 LD_{50} (Fig. 4A). At the higher challenge dose (100 LD_{50}), 70% and 90% of mice survived following treatment at 6 h or 24 h, respectively (Fig. 4B). In all cases, treatment offered a statistically significantly benefit ($p < 0.0001$) (Mantel-Haenszel logrank).

Table 3
Survival of Balb/c mice pre-treated with Hu1A3B-7 antibody 24 h before subcutaneous challenge with 100 LD_{50} of virulent VEEV. Figures show number of surviving mice/total number of mice challenged and percent survival in parentheses.

Antibody dose	Hu1A3B-7
0 µg	0 (0%)
5 µg	8/10 (80%)
25 µg	10/10 (100%)
50 µg	10/10 (100%)
75 µg	10/10 (100%)
100 µg	10/10 (100%)

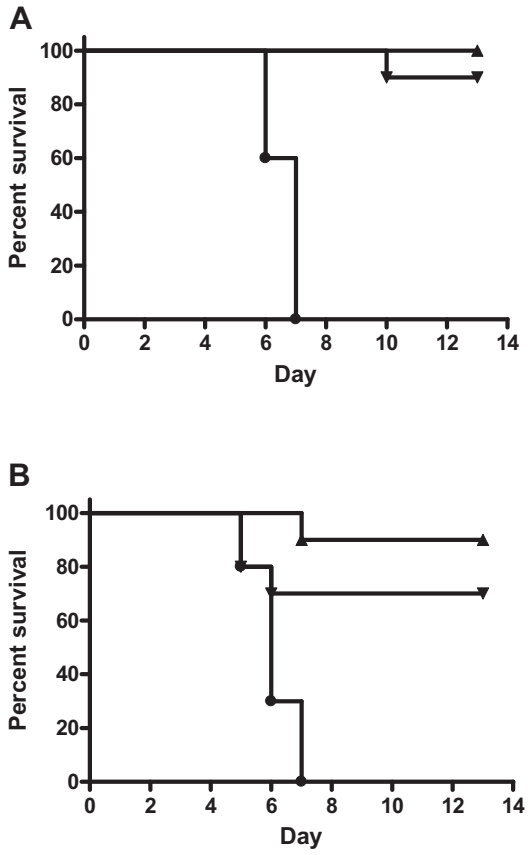

Fig. 4. Protection against aerosolized VEEV challenge. Mice ($n = 10$/group) were challenged with either 10 LD_{50} (A) or 100 LD_{50} (B) of virulent airborne VEEV serotype IA/B. Mice were either untreated (●), or given Hu1A3B-7 at 6 h (▼) or 24 h (▲) post-challenge.

4. Discussion

In the absence of appropriate vaccines, there is a requirement for development of therapies effective for prophylaxis and treatment of VEEV infections. Several approaches for the development of novel therapies for VEEV have focused on the non-structural virus-encoded mediators of replication and genetic targets (Reichert et al., 2009; Paessler et al., 2008; O'Brien, 2007). The administration of VEEV-specific monoclonal antibodies also offers a promising means of medical intervention for those exposed to VEEV (Phillpotts et al., 2002). Previous studies have shown that treatment with monoclonal antibody has a beneficial impact on existing VEEV infection in murine models (Phillpotts et al., 2002; Hunt et al., 2006). It is likely that antibody is able to prevent spread of the virus to the brain through control of the initial vireamia, giving the host immune system time to mount an effective immune response (Phillpotts et al., 2002; Hunt et al., 2006). Although these studies also indicate that the window in which antibody therapy is efficacious is small, monoclonal antibody therapy of VEEV using murine monoclonal antibodies in mouse models can demonstrate protective efficacies of 75% (Hunt et al., 2006) and 50% (Phillpotts et al., 2002) at 24 h post-exposure and 30% protective efficacy at 48 h post-exposure (Hunt et al., 2006). The mouse has been extensively used as a model for human disease and the pathogenesis of VEEV infection in this model has previously been characterised (Vogel et al., 1996; Charles et al., 1995). We are therefore able to infer that antibody therapy would need to be delivered rapidly to a human population to offer therapeutic effect. An understanding of the constraints that this short window of opportunity for treatment places on the utility of an antibody therapy is therefore useful to underpin further efforts to develop humanised variants.

In this work, modelling data based on protective data generated in a mouse aerosol exposure model with the monoclonal antibody 1A3A-9 (Phillpotts et al., 2002) was used to illustrate the impact that a monoclonal antibody therapy could have in scenarios involving deliberate release of VEEV. In scenarios where release of VEEV was known, the impact of the availability of an anti-VEEV antibody therapy would be high if it could be delivered within 24 h of release. Availability of an efficacious antibody capable of treatment of all VEEV serogroups would therefore be advantageous, ensuring that therapy could be delivered rapidly with confidence that the VEEV complex would be covered by a single treatment. Consequentially, humanised variants generated from a broad-spectrum antibody such as 1A3B-7 provide excellent candidates for human therapy.

Three humanised variants of the V_H and V_L domains of 1A3B-7 were generated in this work and this provided a means to evaluate the impact of two of the unusual characteristics of the murine antibody. These were an unusually long CDR1 within the V_L domain and an atypical isoleucine at position 94 in the Framework 3 region of the variable heavy chain domain (Kabat et al., 1991). Through the assessment of each of these variants in each appropriate combination it was possible to show that both the $R^{94}I$ amino acid change and use of a framework capable of supporting a longer V_L CDR1 were required to retain biological activity in the humanised molecule. Evaluation of Hu1A3B-7 indicates that the key characteristics of the murine parental antibody namely breadth of activity and in vivo potency in protection of mice from lethal challenge with VEEV remain intact (Phillpotts, 2006). Differences were however observed regarding the serum half-life of the humanised antibody in comparison to the murine version (4.70 days and 7.37, respectively). The reasons for this much more rapid clearance of antibody are as yet not known, but it is likely that humanisation has rendered this antibody more immunogenic to the murine immune system, promoting a more rapid clearance of the antibody from the circulation.

In addition to the humanised 1A3B-7 molecule produced in this work, humanisation of murine antibodies has proven to be a fruitful way of generating antibodies with characteristics that may have application in human therapy (Hu et al., 2007, 2010; Hunt et al., 2006). However, in comparison to Hu1A3B-7, the narrow specificity of other therapeutic candidates for VEEV produced by humanisation may limit their application to treatment of specific VEEV serogroups (Hu et al., 2007; Hunt et al., 2006). In all cases, while development of a molecule that retains the biological activity of the parent antibody represents a successful outcome to the humanisation process, questions remain regarding how a more human-like protein sequence relates to immunogenicity in vivo. Studies on the relationship between "humanness" of antibodies by sequence and antigenicity in vivo indicate that there is little link between these two characteristics (Abhinandan and Martin, 2007) although humanisation does, in general, appear to correlate with reduced immunological effects in clinical trials (Hwang and Foote, 2005). Limited availability of sequence data correlated to the results from clinical trials means that a detailed investigation of sequence versus antigenicity is difficult to achieve. While techniques such as elimination of T-cell activation peptides are recognised as being beneficial for reduction of antigenicity of protein therapeutics (Tangri et al., 2002, 2005) and can be built into the humanisation process (Lazar et al., 2007) few in vitro assays are recognised as predictors of immunotoxicity of the therapeutic prior to clinical trials (Descotes, 2009).

Antibodies derived from human hosts may provide therapeutic molecules that could potentially circumvent this issue and be used without any further protein engineering. Fully human anti-

body fragments specific to VEEV have recently been isolated via phage-display from a naïve human single chain variable domain library (scFv) (Kirsch et al., 2008) and from a Fab antibody library generated from immune human tissue (Hunt et al., 2010). Antibodies with specificity to the E2 viral protein, derived from the Fab candidate molecules have been characterised and demonstrate both broad serogroup reactivity and neutralisation activity (Hunt et al., 2010). If these molecules are able to show protective effects in animal models, then these are exciting developments that may ultimately alleviate concerns about immunogenicity of mouse-derived molecules.

The work described here has successfully applied a CDR grafting technique to humanise the monoclonal antibody 1A3B-7 with proven broad-spectrum binding and neutralisation activity for VEEV (Phillpotts, 2006). The humanised variant retains broad serogroup binding activity and ability to neutralise VEEV strains from serogroups 1A/B (TrD), II (Fe37c) and Mucambo virus strain BeAn8. Furthermore, Hu1A3B-7 provides protection to mice against challenge with virulent VEEV when delivered prior to a subcutaneous infection and importantly also when delivered subsequent to an aerosol exposure of virus; a more likely route of infection in laboratory accidents or bio-warfare and bio-terrorist incidents. This has successfully generated a candidate molecule for consideration for human therapy, with a greater range of activity for the virulent strains of VEEV than any other molecule with in vivo efficacy. These data support further in vitro studies to demonstrate the range of serogroup neutralisation provided by this antibody in a variety of cell types and further in vivo studies to characterise the protective characteristics of this antibody against airborne challenge with other virulent VEEV serogroups.

Acknowledgments

The authors would like to thank A. Phelps, L. Eastaugh and A. Gates for their valuable contributions to this work. All work was funded by the Ministry of Defence, UK.

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