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A humanised murine monoclonal antibody protects mice from Venezuelan equine encephalitis virus, Everglades virus and Mucambo virus when administered up to 48 h after airborne challenge

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ABSTRACT

Currently there are no licensed antiviral treatments for the *Alphaviruses* Venezuelan equine encephalitis virus (VEEV), Everglades virus and Mucambo virus. We previously developed a humanised version of the mouse monoclonal antibody 1A3B-7 (Hu1A3B-7) which exhibited a wide range of reactivity *in vitro* and was able to protect mice from infection with VEEV. Continued work with the humanised antibody has now demonstrated that it has the potential to be a new human therapeutic. Hu1A3B-7 successfully protected mice from infection with multiple *Alphaviruses*. The effectiveness of the humanisation process was determined by assessing proliferation responses in human T-cells to peptides derived from the murine and humanised versions of the V_H and V_L domains. This analysis showed that the number of human T-cell epitopes within the humanised antibody had been substantially reduced, indicating that Hu1A3B-7 may have reduced immunogenicity *in vivo*.

Introduction

As members of the *Alphavirus* genus, Venezuelan equine encephalitis virus (VEEV), Everglades virus and Mucambo virus are single-stranded, positive-sense RNA viruses that share a high level of genetic homology. Indeed, Everglades virus and Mucambo virus were formerly classified as VEEV subtypes II and IIIA respectively but they are now considered distinct species (http://ictvonline.org). VEEV is currently classified as a complex of viruses containing a number of different variants within a single subtype (IA/B, IC, ID, IE, IF). VEEV, Everglades virus and Mucambo virus cause a similar human disease which is generally characterised by a febrile illness but may lead to encephalitis and death in a small proportion of cases (Johnson and Martin, 1974).

VEEV, Everglades virus and Mucambo virus are considered to have potential as biological warfare agents due to attributes such as high infectivity, consistent disease induction, airborne transmissibility, stability and ease of production (Steele and Twenhafel, 2010). Indeed, VEEV was developed as a biological weapon by the former Soviet Union and the U.S.A. in the last century (Bronze et al., 2002). Two live, attenuated vaccines (TC-83 (Berge et al., 1961) and V3526 (Pratt et al., 2003)) were developed to prevent disease caused by VEEV, Everglades virus and Mucambo virus (Martin et al., 2010 and references within; Phillpotts and Wright, 1999) but both formulations caused unacceptable levels of reactogenicity to allow for general licensure (Alevizatos et al., 1967; Casamassima et al., 1987; Martin et al., 2010; Rayfield et al., 1976). In the absence of a vaccine, antiviral drugs would be beneficial but, unfortunately, there are no licensed antiviral therapies currently available for treating infection with VEEV, Everglades virus or Mucambo virus.

The potential of monoclonal antibodies as antiviral treatments in humans has been recognised (Breedveld, 2000; Marasco and Sui, 2007). Murine monoclonal antibodies have previously shown utility as therapeutics in mouse models of VEEV disease (Phillpotts, 2006; Phillpotts et al., 2002). However, administering murine antibodies to humans is inadvisable as they may be recognised as foreign by the human immune system, resulting in an anti-antibody response which may lead to anaphylactic shock and/or immune complex disease (Breedveld, 2000; Schroff et al., 1985). They may also interact incorrectly with human Fc receptors and/or human complement, resulting in a lack of appropriate effector functions (Tabrizi et al., 2006). The use of chimeric human/mouse antibodies or humanised murine antibodies or even fully human antibodies is therefore a better prospect for a licensed therapy.

Humanisation approaches have generally been taken in order to develop an antibody therapy for VEEV, Everglades virus and Mucambo virus, although a human antibody with specificity for multiple subtypes has been derived from bone marrow donors immunised with TC-83 (Hunt et al., 2010). This fully human antibody showed protection from disease with VEEV subtype IA/B in mouse models (Hunt et al., 2011). To date, three murine antibodies have been successfully humanised: 3B4C-4 (Hunt et al., 2006), 1A4A-1 (Hu et al., 2010) and 1A3B-7 (Goodchild et al., 2011). All three constructs were able to protect mice

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from challenge with VEEV subtype IA/B when administered up to 24 h post-exposure.

In this work we have built on our previous studies with humanised 1A3B-7 (Goodchild et al., 2011) to provide further evidence of the suitability of this construct for human therapy. The effects of administering the antibody at later time-points after challenge have been assessed and the ability of humanised 1A3B-7 to protect against disease induced by VEEV, Everglades virus and Mucambo virus has been determined. Additionally, an *in vitro* T-cell proliferation assay has been utilised with peptides derived from the murine and humanised V_H and V_L domains to provide an indication of immunogenicity *in vivo*.

Results

Therapeutic activity of Hu1A3B-7

Previously, Hu1A3B-7 was shown to protect mice from an aerosolised challenge with VEEV strain TrD (subtype IA/B) when administered up to 24 h post-exposure (Goodchild et al., 2011). In order to assess if the antibody was protective when it was administered at later time points, mice were treated with Hu1A3B-7 24 h, 48 h and 72 h after airborne exposure to VEEV strain TrD (Fig. 1). Control mice that received human IgG 24 h post-challenge did not survive and the median time to death was six days. In contrast, statistically significant levels of survival were observed when mice were treated with Hu1A3B-7 24 h or 48 h after challenge ($P \leq 0.01$ and $P \leq 0.001$respectively) but not when treatment was delayed until 72 h after challenge ($P \geq 0.05$). All surviving mice exhibited clinical scores of zero at the end of the challenge period (day 14), indicating that a full recovery had been achieved and that the infection with VEEV had been completely cleared.

Of the three murine antibodies that have previously been humanised (Goodchild et al., 2011; Hu et al., 2010; Hunt et al., 2006), Hu1A3B-7 has the widest range of cross-reactivity *in vitro* (Goodchild et al., 2011). The ability of Hu1A3B-7 to protect against disease caused by additional strains of VEEV, Everglades virus or Mucambo virus was therefore determined (Fig. 2). Mice were exposed to VEEV strain Mena II (subtype IE), Everglades virus strain Fe37c and Mucambo virus strain BeAn8 by the airborne route. Control or therapeutic antibody was delivered 24 h or 48 h after challenge with the various viruses. Hu1A3B-7 offered high levels of protection (80–100%) from all of the strains when administered at either time-point ($P \leq 0.001$ in all cases except against Mena II virus at 48 h, $P \geq 0.05$. The lack of statistical significance in this instance is due to the early death of a single mouse three days after the challenge dose). In contrast, only one mouse treated with human IgG survived the viral challenges. The clinical scores of mice surviving to day 14 were again zero suggesting that Hu1A3B-7 is capable of inducing complete viral clearance.

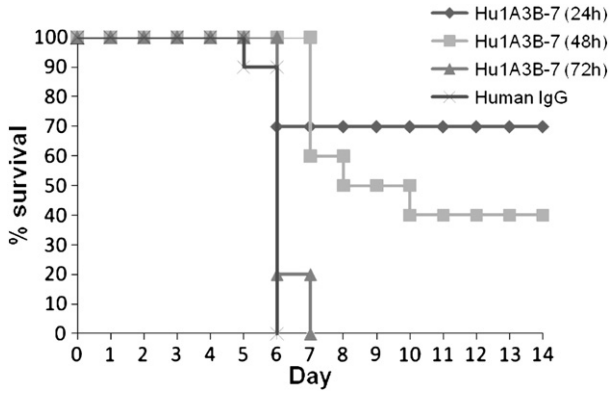

Fig. 1. Therapeutic activity of Hu1A3B-7 against airborne challenge. Groups of Balb/c mice (n = 10) were exposed to approximately 300LD_{50} VEEV strain TrD by the airborne route. One group of mice received human IgG intraperitoneally 24 h post-challenge. The remaining groups were treated intraperitoneally with Hu1A3B-7 24 h, 48 h or 72 h post-challenge. Mice were observed twice daily for clinical signs of infection after challenge and were culled when appropriate using humane endpoints.

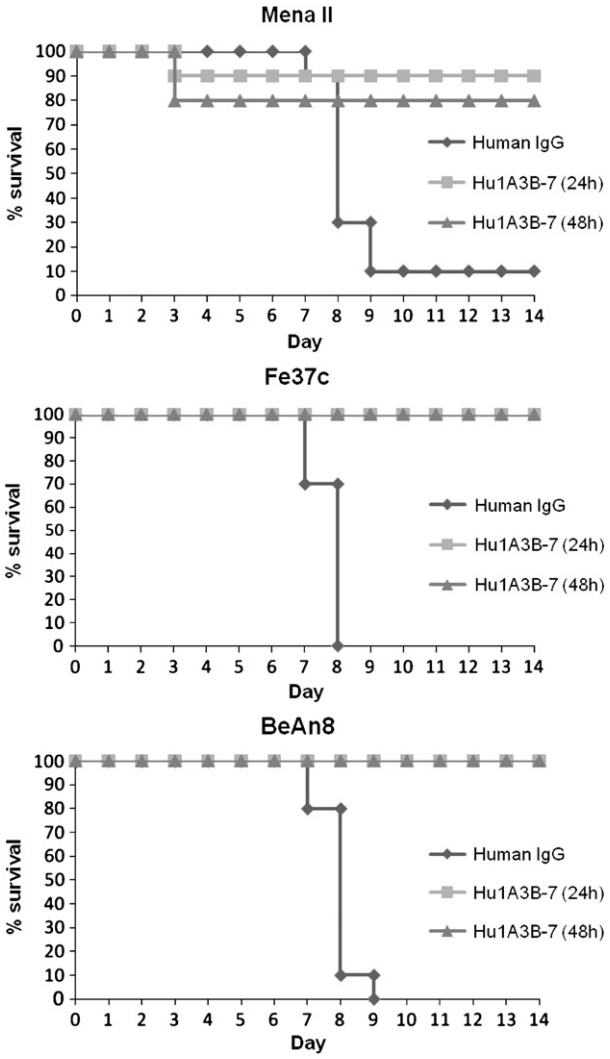

Fig. 2. Treatment with Hu1A3B-7 up to 48 h after airborne challenge provides cross-protection. Balb/c mice were challenged by the airborne route with VEEV strain Mena II, Everglades virus strain Fe37c or Mucambo virus strain BeAn8. Control mice (n = 10) received an intraperitoneal injection of human IgG 24 h post-exposure. Hu1A3B-7 was administered to the remaining mice by the intraperitoneal route either 24 h (n = 10) or 48 h (n = 5) post-exposure. Mice were observed twice daily for clinical signs of infection and were culled when appropriate using humane endpoints.

Identification of potential $CD4^+$ T-cell epitopes within Ch1A3B-7 and Hu1A3B-7

One of the aims of constructing a chimeric and humanised version of the mouse monoclonal antibody 1A3B-7 was to minimise antigenicity in humans. Thus, prior to considering clinical trials, attempts were made to assess the immunogenicity of the two constructs in *in vitro* assays. In this work, a T-cell proliferation assay was employed to identify potential $CD4^+$ T-cell epitopes within the protein sequences of the murine and humanised variable domains used to create Ch1A3B-7 and Hu1A3B-7 respectively. A panel of synthetic overlapping 15-mer peptides, derived from the V_H and V_L domains of each antibody, was incubated with antigen presenting cells and CFSE-labelled $CD4^+$ T-cells from healthy human donors. Any peptides in the variable domains of Ch1A3B-7 and Hu1A3B-7 that were

identical in sequence were only tested as part of the peptide library derived from Ch1A3B-7. Thus, light chain peptides 9, 19–21 and 34 and heavy chain peptides 51–52 and 65–69 were excluded from the Hu1A3B-7 peptide library. The immunogenicity of each peptide was indicated both by the extent of proliferation of labelled cells, which was measured by a decrease in CFSE-intensity, and by the number of donors making a proliferative response. A cell division index (CDI) was determined for each individual donor by dividing the proportion of $CD4^+$, CFSE-dim cells after antigen stimulation by the proportion of $CD4^+$, CFSE-dim cells without antigen stimulation. A response that had a CDI ≥ 2, and was also two standard deviations above background, was deemed to be positive. The % antigenicity

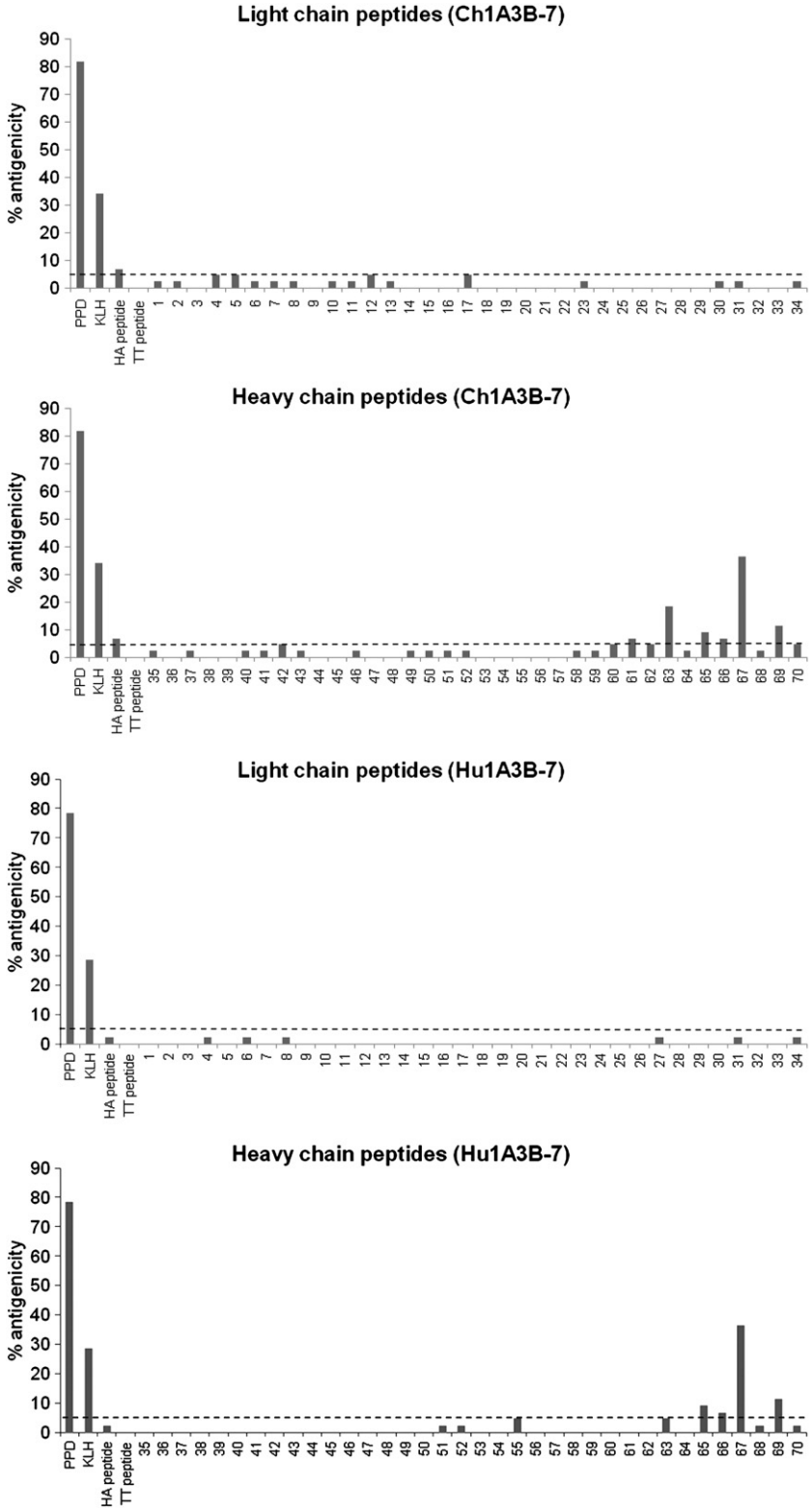

Fig. 3. % antigenicity of reference antigens and test peptides derived from the V_H and V_L domains of Ch1A3B-7 and Hu1A3B-7. % antigenicity was calculated by dividing the number of positive responses (defined as CDI ≥ 2 and two standard deviations above background) by the total number of donors assayed. Peptides stimulating responses at or above the threshold antigenicity (shown as a dashed line) may contain $CD4^+$ T-cell epitopes.

was expressed as the number of positive responses divided by the total number of donors tested. A CDI≥2 in two or more independent donors was considered indicative of a potential $CD4^+$ T-cell epitope. For Ch1A3B-7 and Hu1A3B-7, this equated to a threshold of 4.55% and 4.76% antigenicity respectively for any given peptide. Fig. 3 shows the % antigenicity of the reference antigens used in the assay and of each peptide in the panels derived from the V_H and V_L domains of Ch1A3B-7 and Hu1A3B-7 (for completeness, identical peptides that were only tested as part of the Ch1A3B-7 peptide library are also shown in the results for the Hu1A3B-7 peptide library). Peptides derived from the murine variable domains stimulated the proliferation of human $CD4^+$ T-cells from a larger number of donors than those derived from the humanised domains. Indeed, human $CD4^+$ T-cells rarely responded to the peptides derived from the V_H and V_L regions of Hu1A3B-7. Significant levels of antigenicity were only recorded with peptides 65–67 and 69, all peptides that are identical in sequence between Ch1A3B-7 and Hu1A3B-7.

Of the seventy peptides derived from the murine variable domains of Ch1A3B-7, fourteen elicited positive responses (CDI≥2) in two or more independent donors (Table 1). As the peptides overlap in sequence, positive responses should be observed with adjacent peptides for a region to be considered a T-cell epitope. Thus, a single discrete epitope was identified in framework region 1 of the V_L chain (spanning peptides 4 and 5) whilst an epitope cluster, likely to be at least two interleaving epitopes, was identified that spanned much of framework 3, CDR3 and the J region of the V_H chain (peptides 60 to 70). Fig. 4 illustrates the location of potential $CD4^+$ T-cell epitopes within the variable domains of Ch1A3B-7. In contrast, far fewer peptides from the Hu1A3B-7 library elicited positive responses (CDI≥2) in two or more independent donors and no additional epitopes were identified. The area corresponding to the epitope cluster in the V_H chain of Ch1A3B-7 appears to have lost antigenicity in Hu1A3B-7, as only peptides 63 and 65–69 elicited positive responses. It was therefore concluded that the $CD4^+$ T-cell epitope content of the V_H and V_L chains of Hu1A3B-7 had been considerably diminished as a result of the humanisation process.

Discussion

Antiviral treatments for VEEV, Everglades virus and Mucambo virus are required due to the lack of a suitable vaccine. Here, we have extended our earlier work with Hu1A3B-7 (Goodchild et al., 2011) and shown the potential of a humanised monoclonal antibody to be a generic antiviral suitable for treating humans infected with multiple VEEV subtypes, Everglades virus or Mucambo virus. As a more likely route of infection in laboratory accidents or biowarfare and bioterrorist incidents is by aerosol exposure to virus, we have focussed exclusively in this work on the ability of Hu1A3B-7 to provide protection against airborne challenges.

Human (Hunt et al., 2011) and humanised (Goodchild et al., 2011; Hu et al., 2010; Hunt et al., 2006) antibodies have previously exhibited therapeutic activity in mice when administered up to 24 h after exposure to VEEV subtype IA/B. However, this is the first time that significant levels of protection against disease have been observed when treatment was delayed until 48 h after challenge. Unfortunately, like the humanised version of 1A4A-1 (Hu et al., 2010), Hu1A3B-7 was not able to protect mice when administration of the antibody occurred 72 h post-exposure indicating that antibody therapy has little effect on an established infection. Since VEEV causes a breakdown in the blood–brain barrier within two days of infection (Schäfer et al., 2011), antibody that is delivered at later time-points should be able to access the brain but it appears that it is still unable to prevent the development of encephalitis. These results illustrate the narrow time-frame that is available for treating VEEV, Everglades virus and Mucambo virus once an exposure has occurred. However, we have previously used computer models of theoretical scenarios to show that even a short treatment window is of benefit in protecting humans against an environmental release of VEEV (Goodchild et al., 2011).

This work is also the first demonstration of a humanised or human antibody having therapeutic activity against disease induced by multiple VEEV subtypes, Everglades virus or Mucambo virus. The humanised murine antibodies generated by Hu et al. (2010) and Hunt et al. (2006) possess little cross-reactivity against diverse strains *in vitro* and would therefore not be expected to offer wide-ranging protection *in vivo*. The human antibody developed by Hunt et al. (2010) exhibits comparable levels of cross-reactivity *in vitro* to Hu1A3B-7 (Goodchild et al., 2011) which suggests that this antibody may also be capable of preventing disease caused by multiple subtypes of VEEV, Everglades virus or Mucambo virus. Utilising a cross-reactive antibody as a therapeutic has the great advantage that the viral strain does not have to be identified prior to treatment.

It is extremely difficult to predict what immunological effects a chimeric or humanised antibody may have *in vivo*. Even though it was reported that the degree of humanisation of a protein sequence correlated with reduced antigenicity *in vivo* (Hwang and Foote, 2005), it should not be assumed that more human-like sequences are inherently less immunogenic. Removing T-cell epitopes has long been recognised as being beneficial for the reduction of antigenicity (Holgate and Baker, 2009; Tangri et al., 2005) so the discovery that Hu1A3B-7 possessed substantially lower numbers of $CD4^+$ T-cell epitopes than Ch1A3B-7 was highly encouraging for this antibody, in terms of future use in humans. As there are so few *in vitro* assays capable of predicting *in vivo* immunogenicity, a true reflection of the antigenicity of a therapeutic molecule may only be revealed during clinical trials. It is probable that a fully human antibody, such as that derived by Hunt et al. (2010, 2011) would have no immunological effects *in vivo* but this could only be confirmed by administering it to humans.

The work described here has demonstrated that Hu1A3B-7 is a highly promising candidate for a future antiviral treatment to combat infection with VEEV subtypes, Everglades virus and Mucambo virus. The humanised antibody should now be assessed in non-human primate models of disease prior to evaluation in human clinical trials.

Table 1
$CD4^+$ T-cell responses to peptides derived from the murine V_H and V_L domains of Ch1A3B-7.

Domain	Peptide	Peptide sequence	Epitope-containing sequence stretch	% antigenicity
V_L	4	SLAVSLGQRATISCR	SLAVSLGQRATISCRASQ	4.55
	5	VSLGQRATISCRASQ		4.55
	12	YVYMHWYRQKPGQPP		4.55
	17	KLLIKYSSNLESGVP		4.55
V_H	42	CTVVGFNIKGTYIHW		4.55
	60	SSTAYLHLSSLTSED	SSTAYLHLSSLTSEDTAVY	4.55
	61	AYLHLSSLTSEDTAV	YCAISEGYGNFPFAYWGQ	6.82
	62	HLSSLTSEDTAVYYC	GTLVTVSA	4.55
	63	SLTSEDTAVYYCAIS		18.18
	64	SEDTAVYYCAISEGY		2.27
	65	TAVYYCAISEGYGNF		9.09
	66	YYCAISEGYGNFPFA		6.82
	67	AISEGYGNFPFAYWG		36.36
	68	EGYGNFPFAYWGQGT		2.27
	69	GNFPFAYWGQGTLVT		11.36
	70	PFAYWGQGTLVTVSA		4.55

Materials and methods

Antibodies and reagents

Chimeric 1A3B-7 (consisting of the murine V_H and V_L domains of 1A3B-7 fused to human IgG1 constant regions; Ch1A3B-7) and humanised 1A3B-7 (Hu1A3B-7) were produced as described by Goodchild et al. (2011). All other reagents were supplied by Sigma (U.K.) unless otherwise indicated.

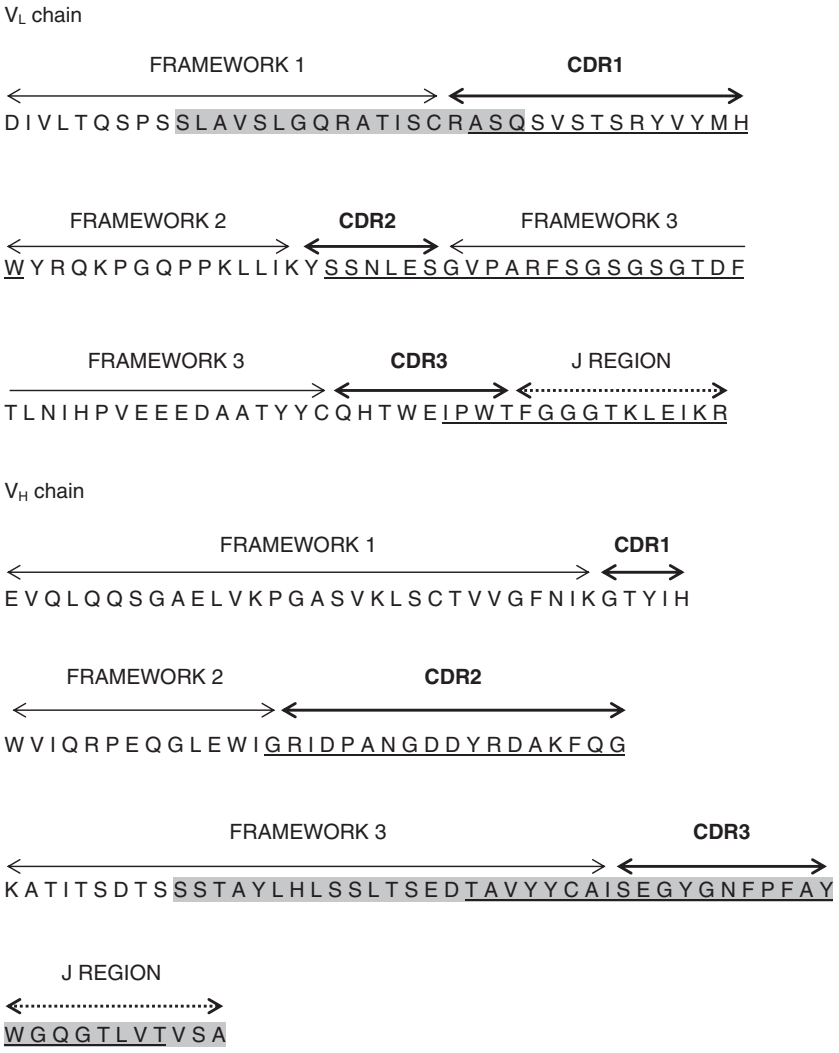

Fig. 4. Annotated amino acid sequence of the V_L and V_H domains of Ch1A3B-7. Nucleotide sequences were edited and translated using Lasergene software. Potential $CD4^+$ T-cell epitopes are indicated with shading. Areas of sequence that are identical between the human and chimeric constructs are underlined.

Viruses

Strains of VEEV from serogroups IA/B (Trinidad donkey; TrD) and IE (Mena II), Everglades virus (strain Fe37c) and Mucambo virus (strain BeAn8) were kindly supplied by Dr. R.E. Shope (University of Texas Medical Branch, U.S.A.). Virus stocks were prepared and the titre determined as described by Phillpotts (2006). All work with VEEV, Everglades virus and Mucambo virus was carried out under U.K. Advisory Committee on Dangerous Pathogens Level 3 containment.

Viral challenge of mice

The protective capacity of Hu1A3B-7 against airborne challenge with VEEV strains TrD and Mena II, Everglades virus strain Fe37c and Mucambo virus strain BeAn8 was tested. Balb/c mice (7–9 weeks old, Charles River, U.K.) were exposed to aerosolised virus as described by Goodchild et al. (2011). A challenge dose of $300LD_{50}$ was employed with VEEV strain TrD. The LD_{50} for the remaining viral strains has not been established in Balb/c mice but preliminary experiments ascertained that titres of 5×10^7pfu/ml Mena II virus, 5×10^6pfu/ml Fe37c virus and 1×10^7pfu/ml BeAn8 virus were required in the Collison reservoir of the Henderson apparatus for 100% mortality to be reliably achieved. The mean dose of challenge virus per mouse was calculated as 8 pfu, 3 pfu and 3 pfu for Mena II virus, Fe37c virus and BeAn8 virus respectively when the above viral concentrations were used in this set of experiments. Mice were subsequently observed twice daily for clinical signs of infection by an independent observer (Phillpotts et al., 2005). Humane endpoints were used and these experiments therefore record the occurrence of severe disease rather than mortality (Wright and Phillpotts, 1998). Mice showing signs of severe illness from infection with VEEV, Everglades virus or Mucambo virus rarely survive but the use of humane endpoints should be considered when interpreting any virus dose expressed here as 50% lethal doses (LD_{50}). At the appropriate time after airborne challenge, groups of mice were treated with 100 μg of human IgG or Hu1A3B-7 in 100 μl PBS by the intraperitoneal route.

T-cell epitope detection

Assays to identify potential $CD4^+$ T-cell epitopes within the variable domains of Ch1A3B-7 and Hu1A3B-7 were performed by ProImmune Ltd. (U.K.) using the ProImmune REVEAL™ T-cell assay. A library of 95% pure 15-mer synthetic peptides, overlapping by 12 residues and covering the V_H and V_L chains of each antibody, was incubated with $CD8^+$ T-cell-depleted peripheral blood mononuclear cells (PBMC) from a minimum of 40 different healthy human donors for 7 days. Each PBMC sample was HLA-typed and a panel was selected that represented MHC class II allele frequency distribution in the

global population. PBMC samples were labelled with carboxyfluorescein succinimidyl ester (CFSE) prior to incubation with peptides and were a source of both antigen presenting cells and $CD4^+$ T-cells within the assay. Peptides were used at a working concentration of 5 μM. Proliferation of $CD4^+$ T-cells was used as an indication of peptide immunogenicity and was detected by measuring a decrease in CFSE intensity using a flow cytometer. Tuberculin purified protein derivative (PPD), Keyhole Limpet hemocyanin (KLH), Influenza A hemagglutinin (HA) peptide and tetanus toxoid (TT) peptide were utilised as reference antigens within the assay as they are known to contain $CD4^+$ T-cell epitopes. The assays were performed in sextuplicate for each peptide. The highest and lowest values for each peptide were discarded and the four central values were averaged and used for subsequent analysis.

Statistical methods

Statistical analysis was performed with GraphPad Prism software and survival curves were analysed using the Logrank (Mantel-Cox) test.

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