A recombinant humanized monoclonal antibody completely protects mice against lethal challenge with Venezuelan equine encephalitis virus

Wei-Gang Hu [a]*, Amanda L. Phelps [b], Scott Jager [a], Damon Chau [a], Charles C. Hu [c], Lyn M. O'Brien [b], Stuart D. Perkins [b], Amanda J. Gates [b], Robert J. Phillpotts [b], and Les P. Nagata [a]

[a] Defence Research and Development Canada - Suffield, Box 4000, Station Main, Medicine Hat, Alberta, Canada T1A 8K6

[b] Defence Science and Technology Laboratory, Porton Down, Salisbury, United Kingdom, SP4 0JQ

[c] Faculty of Science, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1

*Corresponding author: wei-gang.hu@drdc-rddc.gc.ca

ABSTRACT

A recombinant humanized antibody to Venezuelan equine encephalitis virus (VEEV) was constructed in a monocistronic adenoviral expression vector with a foot-and-mouth-disease virus-derived 2A self-cleavage oligopeptide inserted between the antibody heavy and light chains. After expression in mammalian cells, the heavy and light chains of the humanized antibody (hu1A4A1IgG1-2A) were completely cleaved and properly dimerized. The purified hu1A4A1IgG1-2A retained VEEV binding affinity and neutralizing activity similar to its parental murine antibody. The half-life of hu1A4A1IgG1-2A in mice was approximately 2 days. Passive immunization of hu1A4A1IgG1-2A in mice (50 μg/mouse) 24 hr before or after virulent VEEV challenge provided complete protection, indicating that hu1A4A1IgG1-2A has potent prophylactic and therapeutic effects against VEEV infection.

Key Words: Recombinant humanized antibody, Venezuelan equine encephalitis virus, Prophylactic and therapeutic effects

Running Title: Humanized antibody completely protects mice from lethal challenge with VEEV

1. Introduction

Venezuelan equine encephalitis virus (VEEV), a single-stranded and positive-sense RNA alphavirus, is an important mosquito-borne pathogen in humans and equines [1]. VEEV causes a spectrum of human disease which includes acute encephalitis. Neurological disease appears in ~5 % of cases, and mortality is <1 % in adults [2]. The incidence of human infection during equine epizootics may be up to 30 %. Mortality associated with encephalitis in children is as high as 35 %. The outbreaks in Venezuela and Colombia in 1995 affected around 75,000 people including some 3,000 with neurological involvement and more than 300 fatal encephalitis cases [3]. Furthermore, VEEV is a potential biological warfare agent [4]. At present however, treatment for established VEEV infections is supportive care only, and no licensed vaccine or antiviral exists for human use.

Passive immunization with neutralizing antibodies may provide an alternative approach for both short-term prophylaxis and therapy against VEEV infections. Several antibodies have been approved by the US Food and Drug Administration for passive administration to prevent or treat various viral diseases including hepatitis, cytomegalovirus infection, rabies, respiratory syncytial virus infection, and varicella [5]. Like other alphaviruses, the VEEV virion is enveloped, and consists of three major structural proteins: capsid, E1 and E2. Of these, E2 protein is the major protective antigen [6, 7].

Monoclonal antibody (mAb) 1A4A1 is specific for E2 [8]. This mAb is efficient in protecting animals from a lethal peripheral challenge with virulent VEEV [8-10]. However,

it cannot be applied as a therapeutic agent in humans due to its murine origin which may trigger a human anti-mouse immune response. To overcome this hurdle, in our previous study [11], the heavy and light chain variable domains (VH, VL) of mAb 1A4A1 were successfully humanized and the humanized VH and VL were further respectively grafted onto human gamma 1 heavy chain constant domains and kappa 1 light chain constant domain to assemble into a full-length humanized antibody. Additionally, a furin-cleavable linker was inserted between the heavy chain (HC) and light chain (LC) genes, which were in one single open reading frame (ORF) driven by a single promoter within an adenoviral expression vector. However, the cleavage linker was not cleaved by furin after the humanized antibody was expressed in mammalian cells. Although the resultant non-cleaved humanized antibody still retained neutralizing activity, the activity was much lower as compared with parental murine mAb 1A4A1.

In this study, a DNA sequence encoding a foot-and-mouth-disease virus (FMDV)-derived 2A self-cleavage oligopeptide was applied to replace the furin linker in a monocistronic expression vector. After expression in mammalian cells, hu1A4A1IgG1-2A was demonstrated to retain antigen-binding specificity and neutralizing activity similar to the parental murine mAb 1A4A1. Passive immunization in mice provided 100 % protection against virulent VEEV challenge, indicating that hu1A4A1IgG1-2A has substantial prophylactic and therapeutic effects against VEEV infections.

2. Materials and methods

2.1. Virus production

VEEV TC-83 (attenuated strain) was obtained from American Type Culture Collections (ATCC) (Manassas, VA). VEEV TC83 stocks were made by inoculation of Vero cells (ATCC) in minimal essential media (Invitrogen, Burlington, ON) containing 5 % fetal calf serum (FBS, Invitrogen) with virus suspensions at a multiplicity of infection of less than 0.1. The supernatants were clarified by centrifugation at 10,000×*g* for 15 min, aliquotted and stored at -70^{o}C. Virulent wild-type VEEV strain Trinidad donkey (TrD) was kindly supplied by Dr. R. Shope (Yale Arbovirus Research Unit, University of Texas). This virus had been passaged 7 times in sucking mouse brain (SMB[7], 21.8.74). In order to retain the virulence of the virus and prepare stocks, a further passage was performed in suckling mice infected intracerebrally with a 1:1,000 dilution of the supplied virus. Infected brains were harvested at 24 hr post infection, prepared as 10 % tissue suspensions in Leibovitz L-15 medium (Sigma-Aldrich, UK) and clarified by centrifugation at 10,000×*g* for 15 min. All work with virulent TrD was carried out under UK Advisory Committee on Dangerous Pathogens, containment Level 3.

2.2. Construction, expression and purification of hu1A4A1IgG1-2A

Briefly, the furin cleavage linker between HC and LC in pUC57-hu1A4A1IgG1 [11] was replaced by a 2A self-cleavage linker encoding APVKQTLNFDLLKLAGDVESNPGP,

followed by a human antibody gamma 1 HC leader sequence synthesized by GenScript Corporation (Scotch Plaines, NJ), resulting in pUC57-hu1A4A1IgG1-2A. A recombinant adenovirus vector expressing hu1A4A1IgG1-2A was constructed using the AdEasy system (Qbiogene, Carlsbad, CA) according to the manufacturer's protocol. Briefly, hu1A4A1IgG-2A DNA fragment was ligated to pShuttle vector and the resulting pShuttle construct was co-transformed with the pAdEasy-1 vector into *E. coli* BJ5183 cells to produce a recombinant adenoviral construct, pAd-hu1A4A1IgG1-2A. It was then transfected into HEK 293 cells (American Type Culture Collection, Manassas, VA) cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 5 % FBS for amplification.

To express hu1A4A1IgG1-2A, HEK 293 cells were infected with the recombinant adenovirus pAd-hu1A4A1IgG1-2A at a multiplicity of infection of 1. The infected cells were cultured for one week and culture supernatant was harvested. The expressed hu1A4A1IgG1-2A was purified using ImmunoPure Protein (L) agarose gel from Pierce (Brockville, ON). Briefly, culture supernatant was dialyzed against phosphate buffer saline (PBS) (Sigma-Aldrich, Oakville, ON) for 12 hr and then concentrated using polyethylene glycol (Sigma-Aldrich) to less than 50 ml. The concentrated sample was incubated with 2 ml protein L agarose gel at 4 °C for 1 hr. The gel and supernatant mixture was then loaded onto an empty column, which was subsequently washed with binding buffer. Bound hu1A4A1IgG1-2A was eluted with elution buffer. The eluted antibody was further desalted using excellulose column (Pierce) and then concentrated by Centricon YM-30 (Millipore Corp., Bedford, MA).

2.3. Sodium dodcyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of hu1A4A1IgG1-2A

Antibodies were separated by 10% SDS-PAGE gels using a Mini-PROTEAN II apparatus (Bio-Rad Laboratories, Mississauga, ON). The bands were visualized by SimplyBlue Safestain staining (Invitrogen) and the molecular weights of samples were estimated by comparison to the relative mobility values of standards of known molecular weight. The image of the stained SDS-PAGE gel was recorded using Geliance 600 imagining system (PerkinElmer Life and Analytical Sciences, Woodbridge, ON).

2.4. Antigen binding specificity of hu1A4A1IgG1-2A

The reactivity of hu1A4A1IgG1-2A or parental murine mAb 1A4A1 to VEEV E2 antigen was determined by Enzyme-linked immunosorbent assay (ELISA)[11]. Briefly, various concentrations of hu1A4A1IgG1-2A or 1A4A1 diluted in PBS containing 0.05 % tween-20 (PBST) were added to the wells coated with bacterially-expressed recombinant VEEV E2 antigen (10 μg/ml). After 5 washes with PBST, the plates were incubated for 2 hr at room temperature with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG or anti-mouse IgG fragment crystallizable portion (Fc) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:5,000 in PBST. Finally, after washing, the plates were developed for 20 min at room temperature with a 3,3',5,5'-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories). The reactions were read at an absorbance of 615 nm by a microplate autoreader (Molecular Devices, Sunnyvale, CA).

2.5. Determination of antigen binding affinity of hu1A4A1IgG1-2A

The affinity of hu1A4A1IgG1-2A or 1A4A1 binding to VEEV E2 expressed as the dissociation constant (K_d) was measured by ELISA [12]. Briefly, Nunc maxisorp™ flat bottomed 96 well plates were coated overnight at 4 °C with VEEV E2 antigen at a concentration of 10 nM in carbonate bicarbonate buffer, pH 9.6. Also, VEEV E2 antigen at various concentrations (320, 160, 80, 40, 20, 10, 5 and 2.5 nM) was incubated with 2.5 nM of hu1A4A1IgG1-2A or 1A4A1 overnight at room temperature to reach equilibrium. To determine the concentration of free antibody in the equilibrium mixture, 100 µl was transferred to the VEEV E2-coated plate for ELISA test, performed similarly as described above except primary antibody incubation time of 1 hr at room temperature. The K_d was deduced from the Klotz slope, by plotting the inverse value of the fraction of bound antibody against the inverse value of total antigen.

2.6. Neutralization assay in vitro for hu1A4A1IgG1-2A

Neutralizing activity of hu1A4A1IgG1-2A or parental murine mAb 1A4A1 against VEEV TC-83 was analyzed by plaque reduction assay. Briefly, hu1A4A1IgG1-2A or 1A4A1 was serially two-fold diluted and mixed with an equal volume containing 50 plaque forming units (PFU) of virus per 100 µl. After mixtures were incubated for 1 hr at room temperature, 200 µl of the mixture was inoculated in duplicate into wells of 6-well plates containing confluent Vero cell monolayers and incubated at 37 °C for 1 hr. At the end of

the incubation, the virus/antibody mixtures were removed and the wells overlaid with tragacanth gum (Sigma-Aldrich) diluted in maintenance medium and then incubated for 2 days at 37 °C. The wells were stained with 0.3% crystal violet (Sigma-Aldrich) and plaques were counted. Neutralization titer was expressed as the highest antibody dilution in ng/ml that inhibited 50 % of virus plaques.

2.7. Determination of hu1A4A1IgG1-2A half-life in serum

To evaluate the antibody levels in sera of mice treated with hu1A4A1IgG1-2A, groups of 4-5 age-matched Balb/c mice (Charles River Laboratories, UK) were injected intraperitoneally (i.p.) with 50 or 100 µg/mouse of antibody in 100 µl PBS, or did not receive any treatment, and were bled from a superficial tail vein at 1, 2, 3, 5, 7, 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. Briefly, pooled sera samples diluted 1:3 in PBST (0.01 % tween-20) were added to the wells of 96 well plates coated with virus antigen (VEEV TC-83, inactivated with beta propiolactone and incubated for 2 hr at room temperature. After three washes with PBST, plates were incubated for 2 hr at room temperature with HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich) diluted 1:2,000 in PBST. Finally, after washing, the plates were developed for 15 min at room temperature with 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich). Stop reagent was added and absorbance values obtained at 450 nm using Labsystems Multiskan plate reader. 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 we note that a change of 0.30101 units on the log scale equates to a 50 percent reduction of the data on the original scale. This value is divided by the estimated model slope to obtain the half-life.

2.8. Passive immunization with hu1A4A1IgG1-2A

For prophylactic studies, groups of 8 age-matched Balb/c mice were injected i.p with 50 µg/mouse of hu1A4A1IgG1-2A or control human IgG (Sigma-Aldrich) in 100 µl PBS, or did not receive any treatment 24 hr prior to VEEV challenge. The use of human IgG antibody was to demonstrate the specificity of hu1A4A1IgG-2A.

For therapeutic studies, the mice were injected i.p. with 50 µg/mouse of hu1A4A1IgG1-2A in 100 µl PBS 24 hr or 72 hr after VEEV challenge, or did not receive any treatment.

2.9. VEEV challenge

Each Balb/c mouse was challenged subcutaneously (s.c.) with 30-50 PFU of virulent wild-type VEEV TrD in 100 µl of Leibovitz L15 maintenance medium. This challenge dose approximated to 100 × 50 % lethal dose (LD_{50}). Mice were examined twice daily for clinical signs of infection for 14 days (piloerection, hunching, inactivity, excitability and

paralysis), and humane endpoints were used [13]. In previous studies their association with VEEV infection as the cause of death has been established by the isolation of virus from the brain and other internal organs [9]. The severity of clinical signs was measured on a truncated scale of 1 (clearly observable) to 2 (pronounced), 0 indicating absence of the clinical sign. Any animal scoring 2 for any two clinical signs was humanely culled. In addition, any animal found paralysed was also humanely culled in accordance with the UK Animals (Scientific Procedures) Act 1986. These experiments therefore recorded the occurrence of severe disease rather than mortality.

3. Results

3.1 Construction, expression, purification, and SDS-PAGE analysis of hu1A4A1IgG1

In order to obtain an equi-molar expression of antibody HL and LC in a single ORF driven by one single promoter, a 2A self-cleavage linker encoding a 24-reside-oligopeptide, was introduced to replace the furin cleavage linker between HC and LC genes in pUC57-hu1A4A1IgG1. In addition, a HC leader sequence was added upstream from the HC, downstream from the 2A self-cleavage linker as shown in Fig.1. The resultant pUC57-hu1A4A1IgG1-2A was co-transformed with the pAdEasy-1 vector into *E. coli* BJ5183 cells to produce recombinant adenoviral genomic construct, pAdhu1A4A1IgG1-2A.

After expression in HEK-293 cells, hu1A4A1IgG1-2A was purified using an ImmunoPure Protein (L) agarose column. The purified product was subjected to 10 % SDS-PAGE, and one obvious band of ~150 kDa in non-reducing conditions and two clear bands of ~50 kDa and ~25 kDa in reducing conditions (cleavage of disulfide bridges) were observed (Fig.2).

3.2. Antigen binding specificity of hu1A4A1IgG1-2A

The specific binding reactivity of hu1A4A1IgG1-2A to VEEV E2 antigen was examined by ELISA. When the plates were coated with a fixed concentration of VEEV E2 (10 μg/ml), hu1A4A1IgG1-2A bound to VEEV E2 in a dose-dependent manner, similar to

that of parental murine mAb 1A4A1 binding to VEEV E2 (Fig. 3) and did not bind to western equine encephalitis virus, one other member of alphavirus genus of the family Togaviridae (data not shown).

3.3. Antigen binding affinity of hu1A4A1IgG1-2A

In order to compare the antigen-binding affinity between hu1A4A1IgG1-2A and parental murine mAb 1A4A1, the measurements of affinity constant (*Kd*) for antibodies binding to VEEV E2 were performed by ELISA. VEEV E2 was incubated in excess with antibodies in solution overnight. After the equilibrium was reached, the free antibody remaining in solution was measured by ELISA, where absorbance changed linearly with the amount of antibody. A Klotz plot was constructed by plotting the inverse value of the fraction of bound antibody against the inverse value of total antigen as showed in Fig. 4. As calculated from the slope of straight lines in the figure, the *Kd* values for hu1A4A1IgG1-2A and the parental murine mAb 1A4A1 are 3.9 nM (R^2=0.976) and 3.2 nM (R^2=0.971) respectively.

3.4. Neutralization analysis of hu1A4A1IgG1-2A

To assess the neutralization potential of hu1A4A1IgG1-2A, an *in vitro* plaque reduction assay was performed. Both hu1A4A1IgG1-2A and the parental murine mAb 1A4A1 had a similar neutralizing titer of ~30 ng/ml (data not shown).

3.5. Half-life of hu1A4A1IgG1-2A in serum

To determine the half-life of hu1A4A1IgG1-2A in mouse serum, mice were injected i.p. with 50 and 100 μg of hu1A4A1IgG1-2A in 100 μl PBS or did not receive any treatment. Mice were then bled from a superficial tail vein at specified time points. The quantity of antibody present in serum samples was determined by immunoassay (Fig. 5).

The half-life was estimated as 2.25 days with a 95% confidence interval of (1.94, 2.69) days when mice were treated with 50 μg/mouse, and 2.13 days with a 95% confidence interval of (1.97, 2.32) days when mice were treated with 100 μg/mouse (Fig. 5). These intervals overlap so there is no evidence of a difference in half-life for the two sets of data. Previous studies have established the half-life of murine antibodies evaluated in mice to be between 5.8 days and 10 days (1A3A-9 and 1A4A1)[9]. It is understandable that we have a higher decay rate given this antibody contains significant portions of non-mouse molecule.

3.6 Prophylactic and therapeutic efficacy of hu1A4A1IgG1-2A

To evaluate the prophylactic efficacy of hu1A4A1IgG1-2A, mice were injected i.p. with antibodies 24 hr prior to lethal s.c. virus challenge. All hu1A4A1IgG1-2A treated mice survived the lethal VEEV challenge without any clinical signs of infection throughout the 14 day observation period. In contrast, none of the control human IgG treated mice or untreated mice survived (Fig. 6a). The total mean clinical scores are also shown in figure

6a and demonstrated the rapid onset of disease in this model system, with increasing clinical score correlating with a decrease in survival.

To further evaluate the therapeutic efficacy of hu1A4A1IgG1-2A, mice were treated with 50 μg/mouse i.p., 24 hr or 72 hr after lethal s.c. VEEV challenge. As shown in figure 6b, mice treated with hu1A4A1IgG1-2A 24 hr post-challenge were fully protected. All mice treated with hu1A4A1IgG1-2A 72 hr post-challenge succumbed to VEEV infection and were clinically indistinguishable from the untreated control mice. This suggests that the therapeutic window for hu1A4A1IgG1-2A is <72 hrs. The total mean clinical scores indicate a rapid and severe onset of disease in untreated controls and in those mice treated 72 hr post-challenge. Mice treated with hu1A4A1IgG1-2A 24 hr post challenge exhibited only minor clinical signs of disease, with a maximum mean score of <1 on day 5 post-challenge. These minor clinical signs subsequently resolved by day 10 post-challenge.

4. Discussion

Antibodies are large molecules composed of two chains that need to be assembled into a four subunit structure to fully exert their functions. To produce recombinant humanized antibodies in mammalian cells, consideration must be given to the possible expression vectors. A number of strategies may be employed to express antibody genes. The expression in cells of two chains by two vectors could result in an imbalance in production of HC and LC. Unpaired chains usually pile up in the rough endoplasmic reticulum, resulting in inhibition of protein production including antibodies. That is why extra chain production might be toxic to the antibody-expressing cells and thus affect the efficiency of antibody expression. This explains the previously observed low yield of antibodies expressed in mammalian cells using the two-vector approach observed in our laboratory. A second approach is to insert an internal ribosomal entry site (IRES) between the heavy and light chain genes in a bicistronic vector, under a single promoter [14]. Transcription from this bicistronic vector produces a single mRNA molecule encoding both HC and LC. The IRES enables the ribosome to bind to the initiation site of the second gene. In this way, the HC and LC are translated separately from the same mRNA molecule and expression levels of both chains are assumed to be equal. However, the two gene products are not always expressed equally [15] possibly as a result of variability in ribosome recruitment of the two genes. A third approach uses a bidirectional cassette, consisting of two promoters orientated in opposite directions, driving the expression of both products but this approach has not been widely tested [16]. Finally the HC and LC genes may be expressed in a monocistronic vector, with a cleavable linker between them. HC and LC are translated as a

single polyprotein that is subsequently cleaved within the linker, so that the HC and LC are separated. This has proven to be an effective route to produce a balance of HC and LC [17].

In our previous study, a 6-residue furin cleavage sequence, RGRKRR containing the recognition site for the protease furin, was incorporated between HC and LC of the humanized anti-VEEV antibody. However, after expression in mammalian cells, HC and LC were not cleaved. The same furin cleavage sequence was cleaved in another Fab construct expressed in a mammalian system [18], indicating that the conformation of the non-cleaved humanized anti-VEEV antibody may have made the furin cleavage sequence inaccessible. Alternatively the sequence surrounding the linker may have inhibited furin cleavage by another mechanism.

Although this non-cleaved antibody still neutralized VEEV, the activity was much lower than parental murine 1A4A1. In order to improve VEEV binding affinity, a FMDV-derived 2A self-cleavage linker was introduced to replace the furin cleavage linker between the HC and LC genes of the humanized antibody. The 2A oligopeptide sequence was expected to undergo self-cleavage to generate separate HC and LC after translation. The exact mechanism of 2A self-cleavage is still unknown. It has been hypothesized that the 2A sequence impairs peptide bond formation between 2A glycine and 2B proline through a ribosomal skip mechanism [19]. Previously the 2A expression system has been successfully used to express multi-proteins in a single ORF including antibody HC and LC [17].

After the recombinant 1A4A1IgG1-2A was expressed in mammalian cells, HC and LC appeared completely cleaved without detectable unpaired chains (Fig. 2). The cleavage is designed to occur at the C-terminus of the 2A sequence, leaving 23 residues of 2A sequence fused to the end of LC and adding one residue to the N-terminus of the leader sequence of HC. Since the leader sequence is immediately cleaved from the HC once it has been translocated into the endoplasmic reticulum, the one extra residue would be removed with the leader sequence, leaving the HC without any extra residues. A potential drawback of this 2A expression system is that the small, 2A tag (23 residues) left at the end of the C-terminus of LC might affect antibody function or contribute to the antigenicity of antibodies. However, these problems have not been observed [20]. In this study, the recombinant hu1A4A1IgG1-2A was properly dimerized and retained VEEV binding affinity and neutralizing activity, similar to the parental murine mAb 1A4A1.

Passive transfer of hu1A4A1IgG1-2A fully protected mice against a lethal s.c. VEEV challenge when administered 24 hr either prior to or post-challenge. However there was no therapeutic effect when antibody was administered 72 hr post-challenge. Infection in mice s.c. with VEEV results in high viral titres in the skin and lymphoid tissues, with a resulting viremia, which then disseminates virus to the brain through the peripheral nervous system either via the olfactory or dentate nerves [21-23]. VEEV infection of the central nervous system causes significant cerebral oedema (dependant upon virus phenotype) and is established within 72 hr post-challenge [24]. Administration of hu1A4A1IgG1-2A 24 hr prior to or post-challenge may prevent the spread of virus to the brain and allow the host's immune response to control infection, as antibody to VEEV was detected around 5 days

post-infection [25, 26]. Delayed mAb treatment is unlikely to have any therapeutic effect once virus has reached the brain, since antibodies are not transported across the blood-brain barrier [27]. Timely administration of mAbs is crucial to their success as medical countermeasures to VEEV infection. In other studies, the optimal therapeutic effect of murine 1A4A1 was 24 hr prior to infection, or within 24 hr post-infection [9]. The protective efficacy seen here with hu1A4A1IgG1-2A is comparable to the parent 1A4A1, against a lethal systemic VEEV challenge in mice.

The half-life of hu1A4A1IgG1-2A in mice was estimated to be just over 2 days, suggesting that this antibody could be an effective prophylactic against VEEV within a defined window. However the immunity provided by passive transfer of antibodies may not be sufficiently long lasting for all purposes. Furthermore, antibody production is a lengthy, labour intensive and expensive process. An alternative approach would be to take advantage of the body's natural ability to express transgenes to produce passive antibodies [28]. Since adenovirus expression vectors have been widely used in gene delivery *in vivo*, the pAdhu1A4A1IgG1-2A has the potential to be used in antibody-gene delivery to overcome the limitations of passive transfer.

In addition to 1A4A1, several other mAbs have potential for VEEV prophylaxis or treatment. The murine mAbs 1A3B7, 3B2A9 and 1A3A9 provided protection against a lethal VEEV challenge in mice [9, 10]. Hunt and colleagues [29] developed a humanized mAb to VEEV using combinatorial antibody libraries and phage-display technology that was able to confer effective protection against VEEV challenge in mice. Its murine parental

mAb is 3B4C-4, which is specific to the $E2^c$ epitope located in residues 182-209 [7, 29, 30]. 1A4A1 was assumed to be specific to $E2^c$ as well [8]. We tried to map the mAb 1A4A1's epitope using an approach of overlapping peptides and found 1A4A1 only slightly bound to the peptide 206-225 (data not shown). It would appear that 1A4A1's epitope is not linear, supporting previous data that the epitope is conformational [8]. Further study needs to be done to determine 1A4A1's epitope and to fully elucidate the relative properties of $E2^c$ reactive antibodies.

A cocktail of mAbs may prove more potent than a single antibody and reduce the potential for generation of escape mutants [31, 32]. However, synergy between different murine anti-VEEV mAbs has been shown *in vitro* in plaque-reduction neutralisation assays, but this did not transfer to the *in vivo* situation [9, 33]. A combination of murine mAbs 1A3A9 and 1A4A1 administered 24 hr post VEEV challenge, yielded indistinguishable levels of protection when compared to mAbs administered alone [9]. The emergence of antibody-resistant mutants has not been investigated here as full protective efficacy was observed. However, selection pressure is likely to drive 'neutralisation-escape' of the virus through point mutations, resulting in viral persistence *in vivo* [34, 35] and should not be discounted when considering the use of mAbs as therapeutics.

Acknowledgements

The excellent technical assistance of Mrs W. Butcher, Dr T. Laws and Mr R Mansson is recognised and appreciated, Defence Science and Technology Laboratory, Porton Down, Salisbury, United Kingdom, SP4 0JQ.

References

[1] Weaver SC, Ferro C, Barrera R, Boshell J, Navarro JC. Venezuelan equine encephalitis. Annu Rev Entomol 2004;49:141-74.

[2] Griffin DE. Alphaviruses. In: Knipe D.M., Howley PM, editors. Fields virology. 4th ed. Philadelphia, PA: Lippincott-Raven, 2001: 937-41.

[3] Rivas F, Diaz LA, Cardenas VM, Daza E, Bruzon L, Alcala A, et al. Epidemic Venezuelan equine encephalitis in La Guajira, Colombia, 1995. J Infect Dis 1997 Apr;175(4):828-32.

[4] Hawley RJ, Eitzen EM, Jr. Biological weapons--a primer for microbiologists. Annu Rev Microbiol 2001;55:235-53.

[5] Sawyer LA. Antibodies for the prevention and treatment of viral diseases. Antiviral Res 2000 Aug;47(2):57-77.

[6] France JK, Wyrick BC, Trent DW. Biochemical and antigenic comparison of the envelope glycoproteins of Venezuelan equine encephalomyelitis virus strains. J Gen Virol 1979 Sep;44(3):725-40.

[7] Roehrig JT, Day JW, Kinney RM. Antigenic analysis of the surface glycoproteins of a Venezuelan equine encephalomyelitis virus (TC-83) using monoclonal antibodies. Virology 1982 Apr 30;118(2):269-78.

[8] Roehrig JT, Mathews JH. The neutralization site on the E2 glycoprotein of Venezuelan equine encephalomyelitis (TC-83) virus is composed of multiple conformationally stable epitopes. Virology 1985 Apr 30;142(2):347-56.

[9] Phillpotts RJ, Jones LD, Howard SC. Monoclonal antibody protects mice against infection and disease when given either before or up to 24 h after airborne challenge with virulent Venezuelan equine encephalitis virus. Vaccine 2002 Feb 22;20(11-12):1497-504.

[10] Phillpotts RJ. Venezuelan equine encephalitis virus complex-specific monoclonal antibody provides broad protection, in murine models, against airborne challenge with viruses from serogroups I, II and III. Virus Res 2006 Sep;120(1-2):107-12.

[11] Hu WG, Chau D, Wu J, Jager S, Nagata LP. Humanization and mammalian expression of a murine monoclonal antibody against Venezuelan equine encephalitis virus. Vaccine 2007 Apr 20;25(16):3210-4.

[12] Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J Immunol Methods 1985 Mar 18;77(2):305-19.

[13] Wright AJ, Phillpotts RJ. Humane endpoints are an objective measure of morbidity in Venezuelan encephalomyelitis virus infection of mice. Arch Virol 1998;143(6):1155-62.

[14] Davies JS, Holter JL, Knight D, Beaucourt SM, Murphy D, Carter DA, et al. Manipulating sorting signals to generate co-expression of somatostatin and eGFP in the regulated secretory pathway from a monocistronic construct. J Mol Endocrinol 2004 Oct;33(2):523-32.

[15] Mizuguchi H, Xu Z, Ishii-Watabe A, Uchida E, Hayakawa T. IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. Mol Ther 2000 Apr;1(4):376-82.

[16] Jerecic J, Single F, Kruth U, Krestel H, Kolhekar R, Storck T, et al. Studies on conditional gene expression in the brain. Ann N Y Acad Sci 1999 Apr 30;868:27-37.
[17] Fang J, Qian JJ, Yi S, Harding TC, Tu GH, VanRoey M, et al. Stable antibody expression at therapeutic levels using the 2A peptide. Nat Biotechnol 2005 May;23(5):584-90.
[18] Reed CD, Rast H, Hu WG, Mah D, Nagata L, Masri SA. Expression of furin-linked Fab fragments against anthrax toxin in a single mammalian expression vector. Protein Expr Purif 2007 Aug;54(2):261-6.
[19] Donnelly ML, Luke G, Mehrotra A, Li X, Hughes LE, Gani D, et al. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. J Gen Virol 2001 May;82(Pt 5):1013-25.
[20] Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, et al. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol 2004 May;22(5):589-94.
[21] Charles PC, Walters E, Margolis F, Johnston RE. Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse. Virology 1995 Apr 20;208(2):662-71.
[22] Jackson AC, SenGupta SK, Smith JF. Pathogenesis of Venezuelan equine encephalitis virus infection in mice and hamsters. Vet Pathol 1991 Sep;28(5):410-8.
[23] Vogel P, Abplanalp D, Kell W, Ibrahim MS, Downs MB, Pratt WD, et al. Venezuelan equine encephalitis in BALB/c mice: kinetic analysis of central nervous system infection following aerosol or subcutaneous inoculation. Arch Pathol Lab Med 1996 Feb;120(2):164-72.
[24] Schoneboom BA, Catlin KM, Marty AM, Grieder FB. Inflammation is a component of neurodegeneration in response to Venezuelan equine encephalitis virus infection in mice. J Neuroimmunol 2000 Sep 22;109(2):132-46.
[25] Johnston R.E., Peters CJ. Alphaviruses. In: Fields BN, Knipe DM, Howley PM, editors. Fields virology, . 3rd ed. New York: Lippencott-Raven, 1995: 843-98.
[26] Phillpotts RJ, Wright AJ. TC-83 vaccine protects against airborne or subcutaneous challenge with heterologous mouse-virulent strains of Venezuelan equine encephalitis virus. Vaccine 1999 Feb 26;17(7-8):982-8.
[27] Davson H. Review lecture. The blood-brain barrier. J Physiol 1976 Feb;255(1):1-28.
[28] Hu WG, Nagata LP. Antibody gene-based prophylaxis and therapy for biodefence. Hum Vaccin 2008 Jan-Feb;4(1):74-8.
[29] Hunt AR, Frederickson S, Hinkel C, Bowdish KS, Roehrig JT. A humanized murine monoclonal antibody protects mice either before or after challenge with virulent Venezuelan equine encephalomyelitis virus. J Gen Virol 2006 Sep;87(Pt 9):2467-76.
[30] Johnson BJ, Brubaker JR, Roehrig JT, Trent DW. Variants of Venezuelan equine encephalitis virus that resist neutralization define a domain of the E2 glycoprotein. Virology 1990 Aug;177(2):676-83.
[31] Bakker AB, Marissen WE, Kramer RA, Rice AB, Weldon WC, Niezgoda M, et al. Novel human monoclonal antibody combination effectively neutralizing natural rabies virus variants and individual in vitro escape mutants. J Virol 2005 Jul;79(14):9062-8.

[32] Goudsmit J, Marissen WE, Weldon WC, Niezgoda M, Hanlon CA, Rice AB, et al. Comparison of an anti-rabies human monoclonal antibody combination with human polyclonal anti-rabies immune globulin. J Infect Dis 2006 Mar 15;193(6):796-801.
[33] Sanna PP, Ramiro-Ibanez F, De Logu A. Synergistic interactions of antibodies in rate of virus neutralization. Virology 2000 May 10;270(2):386-96.
[34] Ciurea A, Hunziker L, Zinkernagel RM, Hengartner H. Viral escape from the neutralizing antibody response: the lymphocytic choriomeningitis virus model. Immunogenetics 2001 Apr;53(3):185-9.
[35] Ciurea A, Klenerman P, Hunziker L, Horvath E, Senn BM, Ochsenbein AF, et al. Viral persistence in vivo through selection of neutralizing antibody-escape variants. Proc Natl Acad Sci U S A 2000 Mar 14;97(6):2749-54.

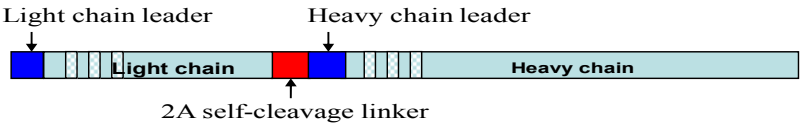

Fig.1. Schematic diagram of hu1A4A1IgG1-2A gene layout. The murine complemetarity determining regions in humanized antibody heavy and light chains are marked in shaded boxes.

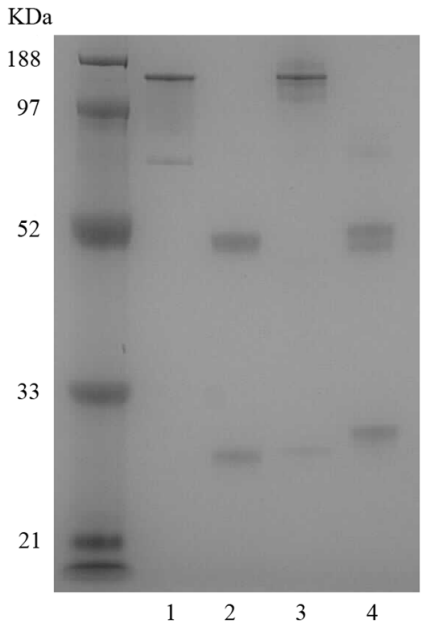

Fig.2. SDS-PAGE analysis of purified hu1A4A1IgG1-2A. Samples were resolved by SDS-PAGE. Lane 1 and 3, control human IgG1 and purified hu1A4A1IgG1-2A in non-reducing conditions; 2 and 4, control human IgG1 and purified hu1A4A1IgG1-2A in reducing conditions.

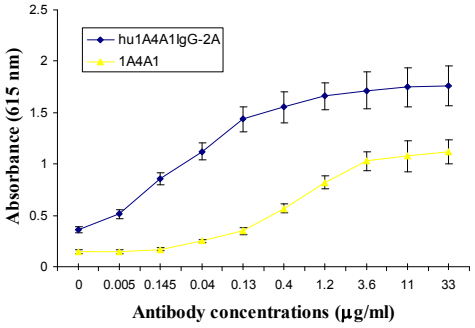

Fig.3. Binding of hu1A4A1IgG1-2A and its parental murine mAb, 1A4A1, to VEEV E2 by ELISA.

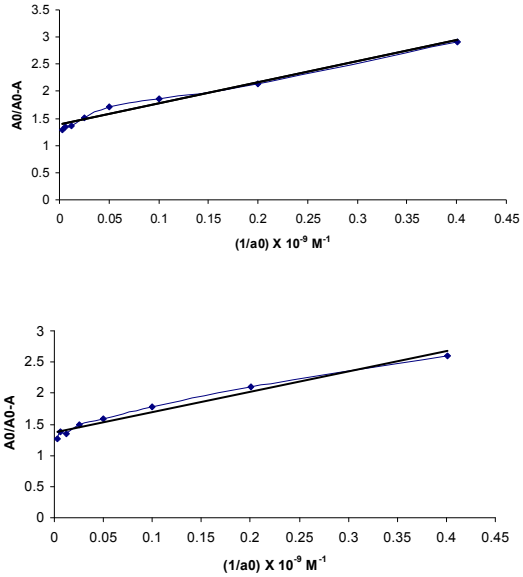

Fig.4. Klotz plot of hu1A4A1IgG1-2A (A) or parental murine mAb 1A4A1 (B) binding to VEEV E2 by ELISA. a_0: total VEEV E2 concentration; A_0: absorbance measured for antibodies in the absence of VEEV E2; A: absorbance measured for bound antibodies, hu1A4A1IgG1-2A or 1A4A1.

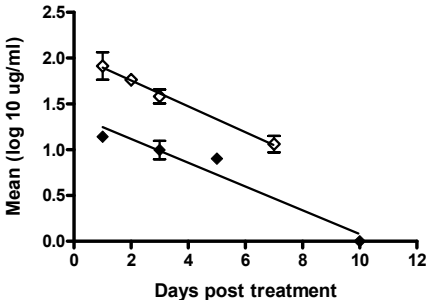

Fig.5. Half-life of hu1A4A1IgG1-2A in pooled serum after a single dose of 50 µg/100 µl (filled diamonds, n=4), or 100 µg/100 µl (empty diamonds, n=5), administered i.p. Replicates of 3 used in the immunoassay, error bars indicate 95 % confidence interval.

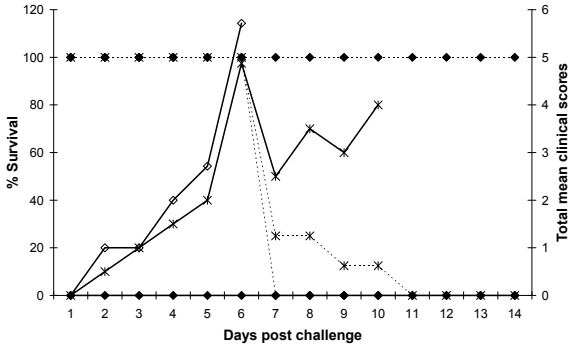

Fig. 6a. Protection (dashed lines) of mice against s. c. VEEV TrD challenge by prior treatment with hu1A4A1IgG1-2A (n=8). Mean total clinical scores are also indicated (solid lines). Filled diamonds = hu1A4A1IgG1-2A, empty diamonds = untreated, asterisks = human IgG control.

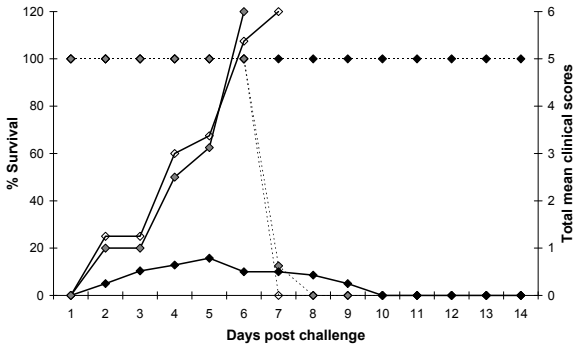

Fig. 6b. Protection (dashed lines) of mice against s. c. VEEV TrD challenge by post treatment with hu1A4A1IgG1-2A (n=8). Mean total clinical scores are also indicated (solid lines). Black filled diamonds = treated 24 hr post challenge, grey filled diamonds = treated 72 hr post challenge, empty diamonds = untreated.