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Humanization and mammalian expression of a murine monoclonal antibody against Venezuelan equine encephalitis virus

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Abstract

The murine monoclonal antibody 1A4A1 can strongly neutralize Venezuelan equine encephalitis virus and is a good candidate for development of humanized antibody. Humanization of 1A4A1 variable domains was achieved by grafting 1A4A1 complementarity-determining regions (CDRs) onto the frameworks of human immunoglobulin germline variable and joining gene segments, whose CDRs have the highest similarities to 1A4A1 ones. The humanized 1A4A1 variable domains were further grafted onto human heavy and light chain constant domains to assemble the whole antibody gene, which was then synthesized and cloned to an adenoviral vector. After expression in HEK 293 cells and purification by protein L column, the humanized antibody was demonstrated to retain antigen-binding specificity and neutralizing activity.

Keywords: Humanization; Antibody; Venezuelan equine encephalitis virus

1. Introduction

Venezuelan equine encephalitis virus (VEEV), a member of the alphavirus genus of the family *Togaviridae*, is an important mosquito-borne pathogen in humans and equides (reviewed in ref. [1]). VEEV causes a spectrum of human diseases ranging from inapparent infection to acute encephalitis. Neurological disease appears in 4–14% of cases. The incidence of human infection during equine epizootics could be up to 30%. Mortality associated with the encephalitis in children is as high as 35%. Recent outbreaks in Venezuela and Colombia in 1995 resulted in around 100,000 human cases with more than 300 fatal encephalitis cases [2]. Furthermore, VEEV is highly infectious by aerosol. Therefore, VEEV is a potential biological warfare agent of concern. However, there are no antiviral drugs available that are effective against VEEV. Although currently there are two forms of VEEV vaccines available for human and veterinary use: TC-83, a live-attenuated Trinidad donkey strain and C-84, a formalin-inactivated TC-83 [3,4], these products are far from satisfactory. Approximately 20% of TC-83 vaccine recipients fail to develop neutralizing antibodies (Abs), while another 20% exhibit reactogenicity. In addition, TC-83 vaccine could reverse to wild-type form. C-84, is well tolerated, but requires multiple immunization, periodic boosts, and fails to provide protection against aerosol challenge in some rodent models.

Like the other alphaviruses, VEEV is an enveloped virus, consisting of three structural proteins: capsid encapsidating the viral RNA genome, and two envelope glycoproteins, E1 and E2. E1 and E2 form heterodimers, which project from the virus envelope as trimer spikes. Epitopes on the spikes are the targets of neutralizing Abs. Studies have shown that the viral neutralizing epitopes are mainly located on the E2 protein, and that the $E2^C$ epitope appears to be the hub of the neutralization epitopes [5,6]. Monoclonal antibody (mAb) 1A1A4 is specific for $E2^C$. This mAb has been shown to be efficient in protecting animals from a lethal peripheral challenge with virulent VEEV [7].

Murine mAbs, however, have serious disadvantages as therapeutic agents in humans [8]. They induce human anti-mouse Ab response. Repeat administration of murine mAbs may result in rapid clearance of the murine mAbs and anaphylaxis, which can sometimes be fatal. To overcome this hurdle, humanization of murine mAbs has been developed, by which murine Ab frameworks were replaced by human Ab

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ones in order to reduce immunogenicity of Abs in humans [9,10].

In this study, we grafted murine mAb 1A4A1 complementarity-determining regions (CDRs) of heavy and light chain variable domains (VH, VL) onto the frameworks of germline variable and joining (V, J) gene segments of human immunoglobulin (Ig) heavy and light chains, respectively, which were chosen based on the CDR similarities between human Igs and murine mAb 1A4A1. Furthermore, the humanized VH and VL were, respectively, grafted onto human gamma 1 heavy chain constant domains (CHs) and kappa 1 light chain constant domain (CL) to assemble the whole humanized Ab gene. The resultant whole humanized mAb gene was synthesized and cloned to an adenoviral vector. After the humanized Ab was expressed in HEK 293 cells and purified with protein L column, the Ab was demonstrated to retain antigen-binding specificity and neutralizing activity.

2. Materials and methods

2.1. Humanization of murine mAb 1A4A1

Murine mAb 1A4A1 was provided by Dr. J.T. Roehrig (Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Colins, CO, USA). The VH and VL of mAb 1A4A1 were cloned in a single chain fragment variable format, mA116 previously, which showed to retain the same binding specificity as mAb 1A4A1 [11]. The humanization of VH and VL of murine mAb 1A4A1 was done by Absalus Inc. (Mountain View, CA, USA). Briefly, in order to select human VH and VL frameworks 1–3, the VH and VL amino acid sequences of murine 1A4A1 were separately subjected to IgBlast and IMGT searches against the entire human Ig germline V gene segments and then human heavy and light chain germline V gene segments were selected based on their highest CDR 1 and 2 similarities with those of murine 1A4A1 VH and VL without consideration of framework similarity. Both human VH and VL framework 4 were selected, respectively, from human heavy and light chain J gene segments based on the highest similarities between human J gene segments and murine 1A4A1 VH and VL CDR3. Finally, CDRs of murine 1A4A1 VH and VL were, respectively, grafted onto the frameworks of selected germline V and J gene segments of human Ab heavy and light chains, resulting in humanized 1A4A1 (hu1A4A1). Furthermore, the hu1A4A1 VH and VL were, respectively, grafted onto human gamma 1 heavy chain CHs and kappa 1 light chain CL to assemble the whole humanized Ab gene, resulting in humanized 1A4A1IgG1 (hu1A4A1IgG1).

2.2. Construction, expression and purification of hu1A4A1IgG1

The hu1A4A1IgG1 DNA sequence (2 kb) was synthesized as follows. A light chain leader sequence was upstream from the light chain, followed by a furin cleavage linker before the heavy chain. The whole DNA sequence flanked by *Kpn* I and *Hind* III was synthesized by GenScript Corporation (Scotch Plaines, NJ, USA) and cloned into pUC57 vector, resulting in pUC57-hu1A4A1IgG1. A recombinant adenovirus vector expressing hu1A4A1IgG1 was constructed using AdEasy system (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, *Kpn* I–*Hind* III fragment of pUC57-hu1A4A1IgG1 was ligated to a *Kpn* I–*Hind* III-digested pShuttle-CMV vector. The resulting pShuttle construct was co-transformed with the pAdEasy-1 vector into *Escherichia coli* BJ5183 cells to produce recombinant adenoviral genomic construct for hu1A4A1IgG1 protein. The recombinant adenoviral construct, pAd-hu1A4A1IgG1 was linearized with *Pac* I and transfected into HEK 293 cells (American Type Culture Collection, Manassas, VA, USA) cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum (FBS) for amplification and then the amplified adenovirus was purified by a chromatographic method.

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

2.3. Sodium dodcyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Abs were separated by 10% SDS-PAGE gels using a Mini-PROTEAN II apparatus (Bio-Rad Laboratories, Mississauga, Ont., Canada). The bands were visualized by SimplyBlue safestain staining (Invitrogen, Burlington, Ont., Canada). The molecular weights of the samples were estimated by comparison to the relative mobility values of standards of known molecular weights.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The reactivity of purified hu1A4A1IgG1 to VEEV E2 antigen was determined by ELISA. Nunc Maxisorp™ flat bottomed 96-well plates (Canadian Life Technologies, Burlington, Ont., Canada) were coated overnight at 4 °C with recombinant VEEV E2 antigen at a concentration of

10 μg/ml in carbonate bicarbonate buffer, pH 9.6. The plates were washed five times with PBS containing 0.1% Tween-20 (PBST) and then blocked in 2% bovine serum albumin for 2 h at room temperature. After five washes with PBST, the plates were incubated for 2 h at room temperature with various concentrations of Abs diluted in PBST. Following five washes with PBST, the plates were incubated for 2 h at room temperature with horseradish peroxidase-conjugated rabbit anti-human IgG fragment crystallizable portion (Fc) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:5000 in PBST. Finally, the plates were washed five times with PBST and developed for 10 min at room temperature with a 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories). The reactions were read at an absorbance of 650 nm by a microplate autoreader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Neutralization assay in vitro

Neutralizing activity of hu1A4A1IgG1 against VEEV (strain TC-83) was analyzed by plaque reduction assay. Briefly, Ab was serially two-fold diluted and mixed with an equal volume containing 50 plaque-forming units of virus per 100 μl. After mixtures were incubated for 1 h at room temperature, 200 μl of the mixture was inoculated in duplicate into wells of six-well plates containing confluent Vero cell monolayers and incubated at 37 °C for 1 h. At the end of the incubation, the virus/Ab mixtures were removed from the wells before the wells were overlaid by tragacanth gum and then incubated for 2 days. The wells were stained with 0.3% crystal violet and plaques were counted. Neutralization titer was expressed as the highest Ab dilution that inhibited 50% of virus plaques.

3. Results and discussion

Different approaches have been developed to humanize murine Abs in order to reduce the antigenicity of murine Abs in humans [9,10]. The most widely used one is CDR-grafting, to graft all murine CDRs onto the human Ab frameworks, which are chosen based on their highest similarities to the frameworks of murine Ab to be humanized. This CDR-grafting approach has proven itself to be successful in some cases. However, in many more instances, this humanization process could result in CDR conformation changes, which affected the antigen-binding affinity. To restore the affinity, additional work for back-mutation of several murine framework amino acids, which are deemed to be critical for CDR loop conformation, had to be done. Recently, Hwang et al. employed an approach to graft murine CDRs onto human germline Ab frameworks based on the CDR sequence similarities between the murine and human Abs while basically ignoring the frameworks [12]. Because the selection of the human frameworks is driven by the sequence of the CDRs, this strategy minimizes the differences between the murine and human CDRs. This approach has the potential to generate humanized Abs that retain good binding to their cognate antigen. Besides, since all residues in frameworks are from human Ab germline sequences, the potential immunogenicity of non-human Abs is highly reduced. Using this approach, an anti-VEEV murine mAb 1A4A1 was humanized in this study. The amino acid sequences of the VH and VL from murine 1A4A1 were first aligned with human Ig germline V and J genes. Human heavy chain V gene segment H5-51 and J gene segment JH4 were selected to provide the frameworks for the murine 1A4A1 VH. For the murine 1A4A1 VL, human light chain V gene segment L15 and J gene segment Jk3 were selected. The identities of the CDR1 and CDR2 amino acid sequences between murine 1A4A1 VH and human H5-51 gene segment were 20 and 47%, while the identity of CDR3 between murine 1A4A1 VH and JH4 gene segment was 33%. For the light chain, the identities of CDR1 and CDR2 between murine 1A4A1 VL and human L15 gene segment were 27 and 14%, while the identity of CDR3 between murine 1A4A1 VL and human Jk3 gene segment was 22%. The CDRs of murine 1A4A1 VH were then grafted onto the frameworks of selected human Ig germline H5-51 and JH4 gene segments, while the CDRs of murine 1A4A1 VL were grafted onto L15 and Jk3 gene segments. The hu1A4A1 VH was further grafted onto the human gamma 1 heavy chain CHs to form a complete heavy chain, while the VL was grafted onto the human kappa 1 light chain CL to form a whole humanized light chain (Fig. 1).

In order to express heavy and light chains in a monocistronic construct, a six-residues linker, RGRKRR containing the recognition site for protease furin was incorporated between the two chains. Furin is a ubiquitous subtilisin-like proprotein convertase, which is the major processing enzyme of the secretory pathway [13]. The furin minimal cleavage site is R–X–X–R. However, the enzyme prefers the site R–X–(K/R)–R. An additional R at the P6 position appears to enhance cleavage. To get the expressed Ab to be secreted to culture media, a leader sequence was added upstream to the Ab gene. The whole DNA sequence including the human Ab kappa light chain L15 leader sequence, the humanized light chain (VL + CL), furin cleavage linker, and the humanized heavy chain (VH + CH1 + CH2 + CH3), around 2 kb was synthesized as shown in Fig. 1 and then cloned into an adenoviral vector. The unique restriction sites flanking the V domains, which allow for efficient V domain replacement and at the heavy chain CH1-CH2 domain junction for generation of fragment antigen-binding portion of Ab (Fab) were also designed (Fig. 1).

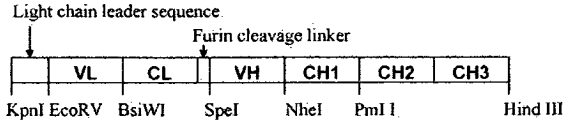

Fig. 1. Schematic diagram of the hu1A4A1IgG1 DNA sequence.

Protein G and A columns are widely used for a quick purification for Abs because of protein G and A binding to the Fc of Ig. However, protein G and A cannot only bind to human Ig, but also bind to bovine Ig, therefore they cannot be used for purification of hu1A4A1IgG1 in our study since pAd-hu1A4A1IgG1-infected HEK 293 cells were cultured in the medium with 5% FBS containing high percentage of bovine Ig. Unlike protein G and A, protein L binds Ig through interactions with the light chains. Protein L only binds to Ig containing light chains of type kappa 1, 3 and 4 in human and kappa 1 in mouse. Most importantly, protein L does not bind to bovine Ig. Since our humanized Ab has human kappa 1 chain, we chose protein L column to purify hu1A4A1IgG1 to eliminate co-purification of bovine Ig. In this way, the purity of hu1A4A1IgG1 was pretty high in SDS-PAGE as shown in Fig. 2.

When the purified product was subjected to 10% SDS-PAGE, one obvious band of ~150 kDa in non-reducing condition (intact disulfide bridges) and one clear band of ~75 kDa in reducing condition (cleavage of disulfide bridges) were observed (Fig. 2). Since the Ab molecule is composed of two identical heavy chains (50 kDa each) and light chains (25 kDa each) connected by disulfide bridges, human IgG should show one band of ~150 kDa band in non-reducing condition, and two bands of ~50 and ~25 kDa in reducing condition by SDS-PAGE. In our study, only 75 kDa showed up in reducing condition by SDS-PAGE, indicating furin cleavage linker between heavy chain and light chain was not cleaved. However, the same furin cleavage linker sequence was cleaved in our another Fab construct expressed in a

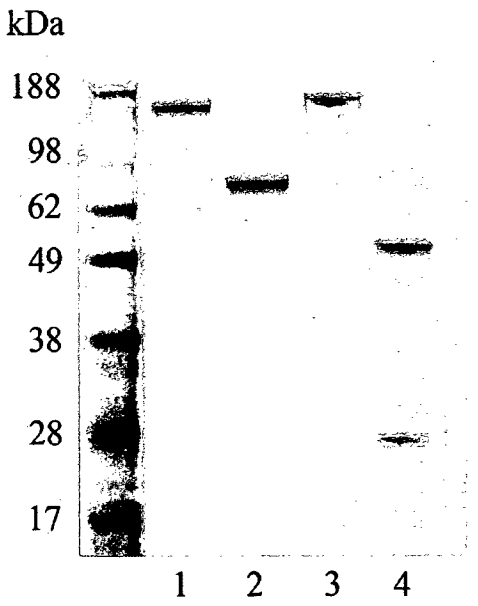

Fig. 2. SDS-PAGE analysis of the purified hu1A4A1IgG1. Samples were resolved by SDS-PAGE. Lanes (1 and 3) purified hu1A4A1IgG1 and control human IgG1 in non-reducing condition and lanes (2 and 4) purified hu1A4A1IgG1 and control human IgG1 in reducing condition.

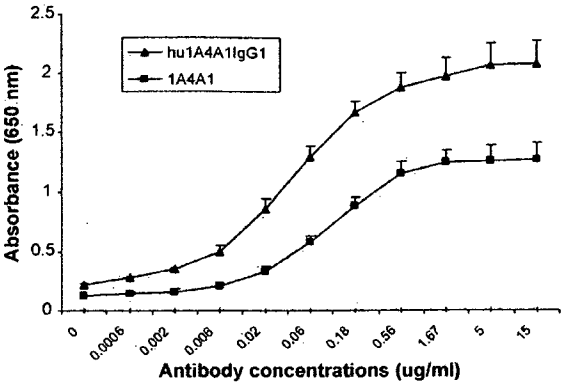

Fig. 3. Hu1A4A1IgG1–antigen-binding assay by ELISA.

mammalian system (data not shown), which indicated the conformation of expressed hu1A4A1IgG1 probably made the furin cleavage linker be inaccessible by furin or the sequence surrounding furin linker could influence furin cleavage.

The specific binding reactivity of purified hu1A4A1IgG1 to VEEV E2 antigen was examined by ELISA. The hu1A4A1IgG1 bound to VEEV E2 in a dose-dependent manner, similar to the binding to VEEV E2 of its parental murine 1A4A1 (Fig. 3), indicating this non-cleaved Ab was still reactive to VEEV E2 antigen in ELISA. Furthermore, this humanized Ab was evaluated for its ability to block VEEV infection in Vero cells using a standard plaque-reduction assay. The hu1A4A1IgG1 showed a strong neutralizing activity with 50% plaque reduction neutralization titers at 0.78 μg/ml.

This humanized Ab, hu1A4A1IgG1 deserves further evaluation as an immunotherapeutic agent and should enhance its potential therapeutic value by reducing its overall immunogenicity.

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