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Alanine scanning of the rabies virus glycoprotein antigenic site III using recombinant rabies virus: Implication for post-exposure treatment

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ABSTRACT

The safety and availability of the human polyclonal sera that is currently utilized for post-exposure treatment (PET) of rabies virus (RABV) infection remain a concern. Recombinant monoclonal antibodies have been postulated as suitable alternatives by WHO. To this extent, CL184, the RABV human antibody combination comprising monoclonal antibodies (mAbs) CR57 and CR4098, has been developed and has delivered promising clinical data to support its use for RABV PET. For this fully human IgG1 cocktail, mAbs CR57 and CR4098 are produced in the PER.C6 human cell line and combined in equal amounts in the final product. During preclinical evaluation, CR57 was shown to bind to antigenic site I whereas CR4098 neutralization was influenced by a mutation of position 336 (N336) located within antigenic site III. Here, alanine scanning was used to analyze the influence of mutations within the potential binding site for CR4098, antigenic site III, in order to evaluate the possibility of mutated rabies viruses escaping neutralization. For this approach, twenty flanking amino acids (10 upstream and 10 downstream) of the RABV glycoprotein (G) asparagine (N336) were exchanged to alanine (or serine, if already alanine) by site-directed mutagenesis. Analysis of G expression revealed four of the twenty mutant Gs to be non-functional, as shown by their lack of cell surface expression, which is a requirement for the production of infectious RABV. Therefore, these mutants were excluded from further study. The remaining sixteen mutants were introduced in an infectious clone of RABV, and recombinant RABVs (rRABVs) were recovered and utilized for in vitro neutralization assays. All of the viruses were effectively neutralized by CR4098 as well as by CR57, indicating that single amino acid exchanges in this region does not affect the broad neutralizing capability of the CL184 mAb combination.

1. Introduction

Rabies is a major zoonotic disease that causes 55,000 annual deaths worldwide with an estimated 10 million people receiving post-exposure treatment (PET) each year [1]. PET for category III bite exposures consists of rabies vaccine and anti-rabies antibodies in order to provide adequate protection against rabies. Unfortunately, only limited quantities of human and equine polyclonal anti-rabies immune globulin (HRIG and ERIG) are available due to cost and manufacturing limitations, resulting in treatment under-usage in areas where rabies is endemic. There are also potential health risks associated with the use of HRIG and ERIG: (i) allergic reactions, (ii) contamination with various known or unknown pathogens. These points illustrate an obvious need for the replacement of HRIG and ERIG with a safer and more easily acquired product [2,3].

RABV belongs to the genus *Lyssavirus* in the *Rhabdoviridae* family. It is a negative-stranded RNA virus with a genome size of about 12 kb encoding five proteins: the nucleoprotein, which encapsidates the RNA genome (N); a phosphoprotein (P), which serves as a non-catalytic polymerase subunit; the matrix protein M, which bridges the viral core and the host cell derived viral membrane; a glycoprotein (G), which mediates infection of the host cell; and the viral polymerase (L) (for review see ([4]). Virus transmission is typically via animal bite during the time when the host's saliva contains infectious virions. Upon infection of its new host, RABV travels in a retrograde manner from the peripheral inoculation site, through neuromuscular junctions, up axons and neuronal cell bodies and into the central nervous system (CNS) where it continues to

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replicate. In so doing, the virus can evade the host's immune response for days to weeks. By this time, damage to the CNS is irrecoverable, and the host's delayed humoral response is futile [5,6].

As RABV G is primarily responsible for both attachment to a cellular receptor as well as pH-dependent fusion of the endosomal vesicle to release the capsid into the host cell [4], antibodies directed against RABV G neutralize the virus by preventing receptor binding or its cell-to-cell spread [7]. More specifically, as antibodies bind to specific antigenic sites on the RABV G, they prevent its attachment to a new host cell. In doing so, RABV G antibodies prevent the spread of infection and, in theory, allow antibody-mediated clearance of virions [6]. More recently Gomme et al. [8] showed that clearance of RABV from the brain is not cytolytic. Although the crystallized structure of RABV G is not yet fully determined, antigenic sites for RABV G have been mapped onto the known similar trimeric structure of vesicular stomatitis virus G [9]. Currently, the five antigenic sites are termed I, II, III, IV and minor site a [10].

Previous research has led to the development of human monoclonal antibodies (mAbs) CR57 and CR4098, which thus far have proven to effectively neutralize many RABV strains [10,11]. By combining these two mAbs, a new RABV mAb combination named CL184 has been developed and has delivered promising clinical data supporting its use for RABV PET [12]. This human IgG1 mAb combination consists of equal amounts of CR57 and CR4098 produced in the PER.C6 human cell line. The CL184 combination was developed using the following criteria to ensure its safety and effectiveness: (i) mAb binding epitopes must not overlap, (ii) mAbs must effectively neutralize their counterpart's escape viruses [10], (iii) CL184 must ensure adequate coverage of natural RABV isolates, and (iv) in vivo efficacy of CL184 should be comparable to HRIG when administered with rabies vaccine.

Antibody-induced escape variant testing was previously performed for CR57 and CR4098 [10,13–15]. The linear binding site for CR57 has been defined as RABV G K226 to L231, which corresponds to antigenic site I [10,14]. The binding site for CR4098 has been determined to be conformational and mapped to antigenic site III (aa 330–338) based on the observed N336 mutation in CR4098 escape viruses [10,13]. Here, alanine scanning was performed to further characterize the importance of amino acid residues adjacent to N336 (including antigenic site III) for viral neutralization by CR4098.

2. Materials and methods

2.1. Plasmids and site-directed mutagenesis

Alanine scanning of RABV G antigenic site III (strain SAD B19, GenBank accession no. M31046) was performed in the parental vector pCAGGS [16] containing RABV G (pCAGGS-G) [17] (pCAGGS-1A, pCAGGS-2S and pCAGGS-3A) or in pTITG [18] (pTITG4A to pTITG20A) due to difficulties in obtaining mutations 4A through 20A with the pCAGGS vector (QuikChange II Site-Directed Mutagenesis Kit, Stratagene). All inserted G genes were entirely sequenced using primers RP24 (5′-GAGACTTGCGGCTTTGTAGATG-3′) and RP651 (5′-CGAGACCCATGTTCCATCCATAAG-3′) to confirm the mutation of interest and the integrity of the RABV G sequence.

2.2. G expression test

BSR cells seeded 24 h prior on glass coverslips in 6 well plates in DMEM with 5% FBS were transfected with plasmids at a 2 μg:4 μl DNA to Fugene ratio (Roche Diagnostics). Plates were incubated at 37 °C for 24 h. For pTITG plasmids, transfected cells were then infected with a recombinant vaccinia virus (VACV) expressing T7 RNA polymerase [19,20] at a multiplicity of infection (MOI) of 5, and incubation was continued for another 24 h at 37 °C. Coverslips were fixed with 4% paraformaldehyde and stained with primary rabbit anti-RABV G antibody (Pacific Immunology) and secondary antibody Cy-2 conjugated donkey anti-rabbit (Jackson Immuno). Coverslips were mounted onto glass slides for analysis via fluorescent microscopy. For internally stained specimens, fixed cells were permeabilized with 1% Triton X-100 in PBS/10 mM Glycine for 5 min prior to the addition of primary antibody.

Table 1
Site-directed mutagenesis of RABV G D326 thru R346 and initial characterization in a transient mammalian expression system. Amino acids in bold indicate position of RABV G antigenic site III.

Constructs	AA position	WT	Mutant	Surface expression	Fusogenicity
pCAGGS-1A	326	D	A	Yes	Low
pCAGGS-2S	327	A	S	Yes	Low
pCAGGS-3A	328	H	A	Yes	Low
pTITG-4A	329	Y	A	No	None
pTITG-5A	330	K	A	Yes	WT
pTITG-6A	331	S	A	Yes	Moderate
pTITG-7A	332	V	A	Yes	Low
pTITG-8A	333	R	A	Yes	WT
pTITG-9A	334	T	A	Yes	WT
pTITG-10A	335	W	A	No	None
	336	N			
pTITG-11A	337	E	A	Yes	Low
pTITG-12A	338	I	A	Yes	Low
pTITG-13A	339	L	A	Yes	Low
pTITG-14A	340	P	A	Yes	Moderate
pTITG-15A	341	S	A	Yes	WT
pTITG-16A	342	K	A	Yes	WT
pTITG-17A	343	G	A	Yes	WT
pTITG-18A	344	C	A	No	None
pTITG-19A	345	L	A	Yes	None
pTITG-20A	346	R	A	Yes	WT

2.3. G functionality test

Medium on VACV-T7-infected, plasmid-transfected BSR cells (as above) was replaced with PBS/10 mM MES/10 mM HEPES buffered to pH 5.0 for 5 min as described previously by others [21]. Wells were washed with PBS, and DMEM/5%FBS was added back. Plates were then incubated for at least 4 h or up to 24 h at 37 °C. Coverslips were fixed and stained as described above.

2.4. Cloning

To introduce the G mutants into our infectious RABV clone, cSPBN [22], pTITG-5A thru -20A (Table 1) were digested with PspXI and PpuMI, and the resulting fragment was used to replace the corresponding fragment in cSPBN. To introduce pCAGGS-1A, -2S and -3A into cSPBN, the entire G gene in cSPBN was replaced by utilizing the XmaI and NheI restriction sites. cDNA was sequenced with primer RP24 to confirm the introduced mutation.

2.5. Recovery of rRABVs from cDNA

Recombinant RABVs were recovered by transfection of BSR cells as described previously [23] and modified recently [17]. Briefly, FuGENE 6 Transfection Reagent (Roche Diagnostics) was used to co-transfect 6 plasmids into BSR cells (a BHK-21 cell clone) in 6-well plates. The concentrations of plasmids (per 6-well plate) were as follows: 2.5 μg recombinant RABV cDNA, 1.25 μg RABV-N, 0.75 μg T7 polymerase, 0.63 μg RABV-P and RABV-L, and 0.5 μg RABV-G. The plasmids encoding the recombinant RABV cDNA and RABV-N, P, G, and L were under the control of the T7 promoter; the

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