Molecular Immunology 68 (2015) 168–175

Contents lists available at ScienceDirect

Molecular Immunology

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

A novel variable antibody fragment dimerized by leucine zippers with enhanced neutralizing potency against rabies virus G protein compared to its corresponding single-chain variable antibody fragment

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ARTICLE INFO

Article history:
Received 13 March 2015
Received in revised form 18 June 2015
Accepted 26 June 2015
Available online 29 August 2015

Keywords:
Rabies virus
Leucine zipper
Single chain variable fragment
Zip variable fragment
Glycoprotein
Virus-neutralization antibody

ABSTRACT

Fatal rabies can be prevented effectively by post-exposure prophylactic (PEP) with rabies immunoglobulin (RIG). Single-chain variable fragments (scFv), which are composed of a variable heavy chain (V_H) and a variable light chain (V_L) connected by a peptide linker, can potentially be used to replace RIG. However, in our previous study, a scFv (scFV57S) specific for the rabies virus (RV) G protein showed a lower neutralizing potency than that of its parent IgG due to lower stability and altered peptide assembly pattern. In monoclonal antibodies, the V_H and V_L interact non-covalently, while in scFvs the V_H is connected covalently with the V_L by the artificial linker. In this study, we constructed and expressed two peptides 57V_L-JUN-HIS and 57V_H-FOS-HA in *Escherichia coli*. The well-known Fos and Jun leucine zippers were utilized to dimerize V_H and V_L similarly to the IgG counterpart. The two peptides assembled to form zipFv57S *in vitro*. Due to the greater similarity in structure with IgG, the zipFv57S protein showed a higher binding ability and affinity resulting in notable improvement of *in vitro* neutralizing activity over its corresponding scFv. The zipFv57S protein was also found to be more stable and showed similar protective rate as RIG in mice challenged with a lethal dose of RV. Our results not only indicated zipFv57S as an ideal alternative for RIG in PEP but also offered a novel and efficient hetero-dimerization pattern of V_H and V_L leading to enhanced neutralizing potency.

1. Introduction

Rabies virus (RV) is a member of the genus *Lyssavirus*, family Rhabdoviridae (Rupprecht et al., 2002; Warrell and Warrell, 2004). It has a single-stranded, negative-sense RNA genome encoding the glycoprotein (G), matrix protein (M), nucleocapsid protein (N), phosphoprotein (P), and RNA-dependent RNA polymerase protein (L) (Albertini et al., 2011). The G protein is one of the components which assemble the surface of RV. Therefore, it is highly immunogenic and can trigger the production of neutralizing antibodies (Dietzschold et al., 1978; Perrin et al., 1985).

RV infection can cause lethal rabies disease in all mammalians including humans (Hemachudha et al., 2002). Human exposures are mainly attributed to the bites of rabid dogs without vaccination, particularly in rural areas of developing countries (Dodet and Africa Rabies, 2009; Hampson et al., 2008). Other rare pathways of infection such as aerosol exposures in caves with high numbers of bats and in laboratories have also been reported (Constantine et al., 1972; Winkler et al., 1973). When RV infects surrounding neurons and invades the central nervous system, leading to the initial symptoms of acute encephalitis, no remedy or therapy is effective against the disease which has the highest known fatality rate of all infectious diseases. The World Health Organization (WHO) estimates that >55,000 deaths per annum are associated with rabies and recommends swift administration of post-exposure prophylaxis (PEP), which can prevent the emergence of symptoms effectively (Both et al., 2012; Knobel et al., 2005). PEP consists of three steps: prompt wound cleaning, injection of a rabies vaccine,

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http://dx.doi.org/10.1016/j.molimm.2015.06.027
0161-5890/

and infiltration of rabies immunoglobulin (RIG) such as human immunoglobulin (HRIG) or equine immunoglobulin (ERIG).

However, RIG has various drawbacks, such as batch-to-batch variations, limited supply in endemic areas and potential contamination related to blood-derived products. In order to address these problems, monoclonal antibodies (mAbs) have been investigated as a replacement for RIG (Both et al., 2013; Lee et al., 2013; van Dolleweerd et al., 2014). However, they also have some shortcomings, including instability of human hybridoma cell lines as well as the high cost of production, which may render treatment cost-prohibitive to the poor in developing countries. Recombinant antibodies like Fab and Fv (Cheung et al., 1992; Horne et al., 1982), which can be expressed in *Escherichia coli*, would decrease production costs and may be a viable alternative. The Fab fragment is composed of two polypeptide chains. Due to requiring assembly in the periplasm of *E. coli*, the yield of functional Fab fragment is usually significantly low (Hust et al., 2007). The variable fragment (Fv), a heterodimer of the light chain variable domain (V_L) and the heavy chain variable domain (V_H), is the smallest recombinant antibody with antigen-binding ability. However, no interchain disulfide interactions are present, and the dissociation constant between the V_H and V_L domains of an Fv ranges only from 10^{-5} to 10^{-8} M, which is not sufficient to keep the two domains associated with each other (Jager and Pluckthun, 1999a,b).

As a more stable alternative to Fv, single chain variable fragment (scFv) are composed of V_L and V_H connected by a flexible polypeptide linker, which allows them to be produced as a single polypeptide. Several scFvs directed against the RV G protein with neutralizing potency *in vivo* have been reported (Ray et al., 2001; Yuan et al., 2014). However, in some cases, scFvs have demonstrated a much lower neutralizing potency than their parent IgG due to insufficient stabilization (Duan et al., 2012). It may also be attributed to differences in assembly patterns of V_H and V_L in the scFv compared to its intact IgG counterpart or Fab. In an intact IgG or Fab, the V_H and V_L are connected non-covalently via hydrophobic interactions and disulfide bonding between the constant domain of the light chain (C_L) and the constant domain one of the heavy chain (C_HI), so the aminos at both the terminus of V_H and V_L are free. In a scFv, one of the aminos at the terminus of V_H and V_L is not free. This non-free amino is connected covalently with the carboxy at the terminus of the polypeptide linker to form a peptide bond, then it can block the antigen-binding domain of scFv in some extent. Moreover, the design of scFv's linker is not easy because the length and sequence of the linker control so many characteristics such as charge, conformation, flexibility, interaction with the Fv and antigen, resistance to proteolysis, extent of protein aggregation (Bird and Walker, 1991).

In this study, in order to overcome the drawbacks of scFv and simulate the natural structure of Fv in an intact Y-shaped IgG or Fab, we constructed two peptides $57V_H$-FOS-HA and $57V_L$-JUN-HIS from the RV neutralizing antibody scFv57S (Duan et al., 2014). Fos and Jun are well-known leucine zipper peptides which can form heterodimers efficiently and preferentially (de Kruif and Logtenberg, 1996; Kostelny et al., 1992). Therefore, in this study $57V_H$-FOS-HA and $57V_L$-JUN-HIS containing the Fos and Jun leucine zippers, respectively, were assembled *in vitro* to form a novel anti-RV G protein Fv fragment designated zipFv57S. As disulfide bonds linking V_H and V_L have been demonstrated to improve the stability of Fv (Kuan and Pastan, 1996; Reiter et al., 1994), we also added cysteine residues via Gly-Gly spacers at the N- and C-terminal ends of the Fos and Jun sequences. The affinity, stability and the neutralizing potency of zipFv57S were assessed and compared with scFv57S both *in vitro* and *in vivo*. These data provide evidences that the protective efficacy of zipFv as a potential treatment for RV infection may be enhanced by dimerizing V_H and V_L with the aid of the Fos and Jun leucine zippers.

2. Materials and methods

2.1. Cells and virus

BHK-21 (baby hamster kidney-21) and 293T (SV40-transfected human embryonic kidney 293) cells for the pseudotype virus neutralizing assay (PNA) and BSR cells for the rapid fluorescent focus inhibition test (RFFIT) were propagated in DMEM supplemented with 10% fetal bovine serum (FBS). The challenge virus strain CVS-11 for the RFFIT and CVS-24 for RV challenge *in vivo* were preserved at −80 °C in our laboratory.

2.2. Construction of expression plasmids

The heavy variable chain ($57V_H$) and the light variable chain ($57V_L$) genes utilized in this study were based on the previously published sequence of $FV57^{VL85Ser}$ in which the cysteine of V_L at position 85 was mutated to serine to prevent the false formation of disulfide bonds (Duan et al., 2014). For clarity, $FV57^{VL85Ser}$ is named scFv57S in this study.

The $57V_H$-FOS-HA gene was synthesized using gene splicing by overlap extension PCR (SOE PCR). The $57V_H$ gene was cloned as the template pBV220-scFv57S (kept in our laboratory). The modified FOS-HA gene template (PGH-FOS-HA) was synthesized by Shanghai Generay Biotech Co., Ltd., containing CGG (cysteine, glycine, glycine), FOS, GGC and HA nucleotide sequences in that order from the N- to C-terminal ends. The modified FOS-HA contained a sequence overlapping with $57V_H$. Therefore, the target gene $57V_H$-FOS-HA was gained by using both the modified FOS-HA and $57V_H$ fragments as templates for PCR amplification. The $57V_H$-FOS-HA sequence was then inserted into the pGEM-T-EASY vector and transformed into *E. coli* Top 10 for sequencing. The correct plasmid was digested with *Nde*I and *Xho*I and cloned into the pET20b (+) vector. The method for obtaining the $57V_L$-JUN-HIS gene was the same as that for $57V_H$-FOS-HA.

2.3. Expression, assembly, purification, and identification of zipFv57S and scFv57S

The methods of bacterial expression and dissolving the inclusion bodies of the $57V_H$-FOS-HA, $57V_L$-JUN-HIS and scFv57S proteins were previously described (Duan et al., 2012). Solutions containing $57V_H$-FOS-HA and $57V_L$-JUN-HIS at the same molar quantities were mixed and then diluted with 8 M urea to the final total concentration of 0.08 mg/ml. The assembly and renaturation were performed by dialysis as in our former study (Gu et al., 2011). After assembly, zipFv57S was obtained by two steps of purification at 4 °C. First, the proteins after concentration were loaded on a HisTrap immobilized metal ion affinity chromatography (IMAC) column. Second, size exclusion chromatography (SEC) was performed to remove the aggregates. Samples eluted by imidazole were injected in a HiLoad 16/60 Superdex™ 200 prep grade column (GE Healthcare) on an AKTA system (GE Healthcare). In order to identify the zipFV57S and scFv57 proteins, both reducing and non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot were performed. Detection of $57V_H$-FOS-HA was performed with a mouse anti-HA antibody. A mouse anti-His monoclonal antibody was used to detect $57V_L$-JUN-HIS and scFv57S. The concentrations of zipFv57S and scFv57S were determined by the bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology).

2.4. RFFIT

In order to compare the neutralizing potency of zipFv57S and scFv57S, the RFFIT was performed. The procedure was the same