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Neutralizing human recombinant antibodies against herpes simplex virus type 1 glycoproteins B from a phage-displayed scFv antibody library

Vahid Bagheri ^{a,b}, Foroogh Nejatollahi ^{a,c,*}, Seyed Alireza Esmaeili ^d, Amir Abbas Momtazi ^e,
Mohamad Motamedifar ^{f,c}, Amirhossein Sahebkar ^{g,h}

^a Recombinant Antibody Laboratory, Department of Immunology, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran

^b Student research Committee, Shiraz University of Medical Sciences, Shiraz, Iran

^c Shiraz HIV/AIDS Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

^d Student Research Committee, Immunology Research Center, Department of Immunology and Allergy, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

^e Student Research Committee, Nanotechnology Research Center, Department of Medical Biotechnology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

^f Department of Bacteriology and Virology, Shiraz University of Medical Sciences, Shiraz, Iran

^g Neurogenic Inflammation Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

^h Biotechnology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

The HSV-1 envelope glycoprotein B (gB) plays a critical role in virus entry into host cells. Neutralizing antibodies can therefore potentially prevent virus entry into target cells and cell-to-cell spread of infection. Our present study focused on the selection of neutralizing single-chain Fv (scFv) antibodies of a phage-displayed nonimmune human scFv antibody library against gB of HSV-1. To enrich specific scFvs, two phage antibodies were isolated against amino acid residues 31–43 derived from the N-terminal part of gB using panning technique. Two scFvs, scFv-gB₁ and scFv-gB₂, with frequencies of 45% and 20% were obtained from scFv clones after performing PCR and MvaI fingerprinting. In phage ELISA analysis, both gB₁ and gB₂ scFvs demonstrated high reactivity with the gB peptide. In the neutralization assay, scFv-gB₁ and scFv-gB₂ represented neutralizing effects of 55% and 59%, respectively. Upon further enhancement of the neutralizing effects of these antibodies, they can be considered as new potential alternatives in the treatment and prophylaxis of HSV-1 infections.

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1. Introduction

Herpes simplex virus (HSV) type 1 remains a significant pathogen and many people (60 to 80% of the adult population) around the world have been reported to be infected with this virus [1]. HSV-1 tends to the nerves and establishes a long-term latent infection in the nervous system [2]. In certain people, recurrent infections occur in the trigeminal ganglia following periodic reactivation of latent virus [3,4]. The virus causes a variety of diseases with a spectrum of severities, including cold sores (fever blisters), conjunctival and corneal lesions, pneumonia and meningoencephalitis in the immunocompromised individuals [1,5,6]. Antiviral drugs are able to reduce viral infection and are considered as efficacious treatments for HSV-1. However, virus resistance to these agents is increasing [7]. Several glycoproteins play a critical role in virus entry into mammalian cells and cell-to-cell spread, including gB, gD and gH/gL. The first stage to establish an infection is low-affinity and reversible attachment of the virus to the

cell surface that is mediated by gB binding to surface heparin sulphate proteoglycan (HSPGs) [8]. In the next stage, gD interacts with one of the multiple potential entry receptors, nectin 1, 3-O-sulfated heparan sulphate (3-O HS) or herpesvirus entry mediator (HVEM). Following gD attachment to one of its receptors, it undergoes conformational changes and a pH-independent fusion of an active multi-glycoprotein complex involving gB, gD, gH and gL will occur [9,10].

Formation of neutralizing antibodies can reduce the severity of HSV-1 infection. High levels of neutralizing antibodies may inhibit HSV-1 spread and viremia [11]. Among glycoproteins of HSV-1, gB has the most conserved entry glycoprotein that can act as fusion proteins. Four functional regions on gB have been identified by monoclonal antibodies. These regions include (i) initial 12 residues of the N-terminal part, (ii) residues 454 to 475, 391 to 410, and a less-defined zone within domain II, (iii) residues 697 to 725 of domain V and residues within domain I, and (iv) residues of domain IV nearby to domain III [12]. gB elicits a remarkable amount of neutralizing antibodies. Almost all neutralization activity is accounted for by epitopes in the N-terminal 298-amino acid portion of gB [13].

Isolation of specific antibodies by antibody phage display has become a popular method in recent years. In general, these libraries

* Corresponding author at: Department of Immunology, Shiraz University of Medical Sciences, P.O. Box 71348-45794, Shiraz, Iran.

E-mail address: nejatolli@sums.ac.ir (F. Nejatollahi).

consist of either single chain fragment variable (scFv) or Fab fragments [14]. The smallest antibody fragments are scFv molecules (26–27 kDa) [15]. scFv antibodies have several advantages in terms of pharmacokinetic characteristics including lower retention in non-target tissues, higher penetration into target tissues, and lack of inducing human anti-mouse antibody responses [16,17]. Therefore, human scFv antibodies perform considerably better than conventional antibody molecules used for therapeutic applications.

Thus far, several neutralizing scFv antibodies have been produced against a number of viruses including scFv antibodies against human immunodeficiency virus type 1 (HIV-1) [18], scFvs to human cytomegalovirus [19] and scFv against influenza A virus H5N1 subtype [20] that can inhibit viral infections *in vitro*. Nejatollahi et al. described a neutralizing scFv-phage antibody against glycoprotein D of HSV-1 with neutralizing effect of 76%, which was capable of neutralizing HSV-1 and inhibiting virus entry to host cell [21]. Here, we selected two neutralizing anti-gB scFv antibodies to inhibit cytopathic effects in Vero cells infected with HSV-1.

2. Materials and methods

2.1. Rescue of scFv phages

A phage scFv display library in the form of frozen TG1 *E. coli* cells was previously provided as described [19]. Briefly, 10 µl of the expanded *E. coli* scFv library glycerol stock were grown overnight at 30 °C on 2TYG Agar/ampicillin plates (1.6% tryptone, 1% yeast extract, 0.5% Glucose, 0.5% NaCl, 1.5% agar, 0.005% ampicillin in deionized water). All grown cells were scraped in 50 ml 2TY broth and incubated at 37 °C for 1 h. After reaching an optical density of more than one, 10 µl of M13KO7 helper phage was added to medium and mixed. Then, it was incubated at 37 °C for 30 min without shaking followed by shaking at 37 °C for 30 min. Bacteria suspension was transferred to a 50 ml conical tube and centrifuged at 3500 RPM for 20 min. The bacterial pellet was transferred to 50 ml 2TY broth containing Ampicillin (100 µg/ml) and kanamycin (50 µg/ml) and cultured overnight with shaking at 30 °C. Afterwards, the culture was centrifuged at 5500 RPM for 20 min. The supernatant containing phage was filtered and stored at 4 °C.

2.2. Panning of phage scFv display library

The procedure of panning was performed using of the human phage display library against synthetic epitope (APSSPGTPGVAAA) of gB (Isogen, Netherland) as follows. In each round panning, the Peptide [10 µg/ml in phosphate buffered saline (PBS)] were coated on immunotube (Nunc, Roskilde, Denmark) overnight at 4 °C. The tubes were washed four times with 1 × PBS and blocked 2 h at 37 °C with 10% fetal bovine serum (FBS) and 2% skimmed milk in PBS. Then, the tubes were washed four times with PBS-Tween 20 (0.05%) and four times with PBS. For dilution of Phage library supernatant (10⁹ PFU per ml), an equal volume of blocking solution was added and incubated for 2 h at room temperature. Following washing, bound phages were eluted with log-phase *E. coli* TG1 and incubated at 37 °C for 1 h. After rescuing with phage M13KO7, other three rounds of panning were performed against the peptide.

2.3. Selection of scFv colonies among panned libraries

To analyze the diversity of the selected scFv antibodies, restriction fragment length polymorphism (RFLP) fingerprinting was performed on PCR-amplified scFv DNA as described. Briefly, DNA fingerprinting was carried out on 20 colonies after the fourth round panning. The scFvs inserts in Phagemids were amplified using forward (CCATGATTACGCCAAGCTTGGAGCC) and reverse (CGATCTAAGTTTTGCTGCTTTCC) primers. MvaI enzyme (Roche

Diagnostic GmbH, Mannheim, Germany) digested each product PCR at 37 °C for 2 h and the DNA product run on 2% agarose gel electrophoresis.

2.4. Measurement of scFv colonies concentration

For measuring of phage concentration of each phage rescued supernatant, 10 µl of phage antibody supernatant was added to 1 ml log-phase TG1 *E. coli* and incubated with shaking at 37 °C for 1 h. Serial dilution of bacteria was prepared and cultured onto 2TY/Ampicillin plates. After counting of Number of colonies per each dilution, scFv colonies concentration titer per milliliter was calculated.

2.5. Evaluation of the selected clones in phage ELISA

The reactivity of isolated scFv clones to the peptide was determined using phage ELISA. 150 µl of peptide (100 µg/ml in PBS) was coated on the 96 well ELISA plates overnight at 4 °C. After washing the wells with three times with PBS/Tween 20 (0.05%) and three times with PBS (three times), the wells blocked by 150 µl of blocking solution (5% w/v skimmed milk in PBS) and incubated at 37 °C for two h. Following washing, for dilution of phage rescue supernatant (10⁹ PFU ml⁻¹), blocking solution was added to it (1:1). Diluted phage supernatant incubated in wells at room temperature for 2 h. After washing wells, 150 µl of 1:100 dilution of anti-fd bacteriophage antibody (Sigma, UK) in PBS was added to their and incubated at room temperature for 1.5 h. The plate was washed and 150 µl of the HRP conjugated goat anti-rabbit IgG (1:4000 in PBS) added and incubated at room temperature for 1 h. The wells were washed and stained using 0.5 mg/ml ABTS (Sigma Chemical, Pool, USA) in citrate buffer (PH 8) containing 1 µl hydrogen peroxide in the dark at room temperature for 30 min. The absorbance of each well was read at 405 nm using an ELISA reader and calculated for each scFv antibody.

2.6. Virus stock

Patients that had lip lesions were selected and HSV-1 was isolated. Neutralization test confirmed virus genus using guinea-pig anti-HSV-1 serum (NIH, USA) and monoclonal (glycoprotein D and G) anti HSV-1 antibodies [22].

2.7. Cell culture

Vero cells (ATCC CCL-81) were cultured in DMEM medium and supplemented with 8% fetal calf serum (GIBCO, Australia), 0.14% (v/v) sodium bicarbonate, 100 units/ml penicillin, 100 µg/ml streptomycin sulphate, and 0.25 µg/ml amphotericin B (GIBCO, Germany). Cells were grown in the humidified air with 5% CO₂ at 37 °C about 24–48 h to be confluent.

2.8. Antiviral assessment of specific scFvs phages

The anti-HSV-1 activities of scFvs were evaluated using plaque reduction assay as following described. HSV-1 was diluted in DMEM (sigma-aldrich, Germany) to 50 pfu/ml. Equal amounts of virus and each scFv phage rescue supernatant were mixed and incubated at 37 °C for 1 h. A 1 ml of each mixture was added into each well of 24-well plates containing confluent Vero cells (performed in triplicate), which were then rocked gently 45 min at 37 °C. After removing mixture, DMEM containing 1 ml of 1% w/v Carboxymethyl Cellulose (CMC) supplemented with 2% fetal calf serum, 0.14% (v/v) sodium bicarbonate, 100 unit/ml penicillin, 100 µg/ml streptomycin sulphate, 0.25 µg/ml amphotericin B and 0.1 N NaOH added. The number of micro plaques was counted during five days and compared with the number of plaques seen in the virus control wells which were without scFv.

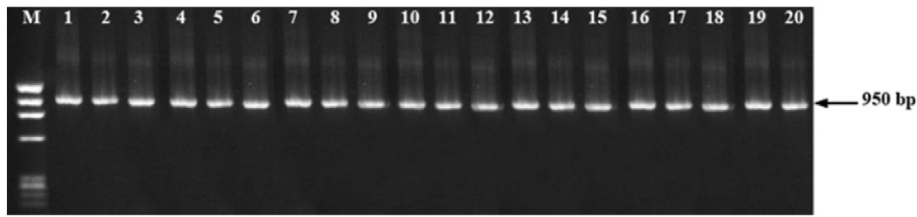


Fig. 1. PCR result of isolated clones against the gB peptide after panning. M, Marker- ϕ X174 DNA.

3. Results

3.1. Selection of scFv-phage antibodies by panning and fingerprinting

After four rounds of panning against peptides of gB, 20 clones were randomly analyzed by PCR to confirm the presence of the inserted segment. The predictable band (950 bp) was observed in all clones (Fig. 1). DNA fingerprinting of 20 scFv clones against epitope of gB after four rounds of panning is shown in Fig. 2. Two patterns, including pattern one (lanes 1, 3, 4, 5, 6, 7, 9, 10 and 18) and two (2, 8, 11 and 12) with the frequencies of 45% (scFv-gB₁) and 20% (scFv-gB₂) were obtained. The patterns of scFvs were used as predominant patterns for further investigation.

3.2. Assessment of scFv-phage antibodies in phage ELISA

The reactivity of scFvs-phage antibodies (scFv-gB₁ and scFv-gB₂) with the gB peptide produced positive ELISA readings and was significantly higher than those of controls (unrelated peptide, unrelated scFv-phage antibody and without peptide) at 405 nm (Fig. 3).

3.3. Neutralization of virus infectivity

The neutralization capacity of the anti-HSV-1 scFv antibodies was evaluated using percentages of plaque reduction, and was 55% for scFv-gB₁ and 59% for scFv-gB₂ (Table 1).

4. Discussion

The first stage of HIV-1 infection is associated with the specific interaction of viral surface proteins with cellular proteins, lipids, or carbohydrates, and can be blocked by neutralizing monoclonal antibodies [23]. In this study, monoclonal antibodies (MAB) were mapped to an identified domain (domain I to VI) in the crystal structure of gB and classified as groups 1 to 6. Monoclonal antibody H1817 that belonged to 6A subgroup bound functional region 4 (FR4) in domain VI of gB. FR4 containing the first 12 residues of the N terminus formed the epitope of MAB H1817. MAB H1817 had high neutralizing effect and reduced the plaque number on Vero cells by >50% [24]. The antibody recognized a single peptide spanning residues 31 to 50 as previously reported. Pereira et

al. mapped neutralizing epitopes in continuous regions of the amino-terminal half of gB [25]. Neutralizing antibodies H1396 [26] and H1397 [25] recognized residues 31–50. Precise peptide mapping of MAb revealed that the epitope for MAb H1817 was residues 31 to 43 and constituted FR4 [24]. Therefore, we chose peptide fragment APSSPGTPGVAAA (amino acids residues 31–43) as an immunodominant epitope that was recognized by neutralizing monoclonal antibodies.

Antibody display on phages is a powerful tool that provides ligand specificity for targets of interest through binding to pure antigens or synthetic peptides coated on a solid phase in repetitive panning rounds [27–29]. In this study, phages expressing the scFv were selected on gB epitope after the fourth round of panning. scFv-gB₁ and scFv-gB₂ were chosen because of more frequency compared with other scFv-phage antibodies (Fig. 2).

Antibody library with antigen can be screened in several ways, such as direct phage ELISA and indirect or capture phage ELISA [30]. The resultant phage clones can be tested using phage ELISA to determine whether scFvs displayed on phages are specific to the peptides. Several studies have previously used this method to evaluate the specificity of antibodies for the targeted epitopes or antigens. For instance, specificity of scFvs against *Helicobacter pylori* [31], hepatitis B virus surface antigen [32], staphylococcal enterotoxin B (SEB) [33], and *Mycobacterium avium* subsp. *paratuberculosis* surface proteins [34] have been certified using phage ELISA. If optical density (OD) of specific scFvs against interest targets at 405 nm are two folds higher than negative controls, a positive phage ELISA is confirmed [35]. Our results revealed that scFv-gB₁ and scFv-gB₂ are specific for the epitope of gB and the mean OD of both antibodies at 405 nm were two folds higher than control ODs (Fig. 3).

Plaque reduction assay was used to test the neutralizing activity of scFv antibodies. Hitherto, neutralizing scFv antibodies have been produced against many viral proteins, but surface glycoproteins have been the most appealing target owing to their important role in virus attachment to receptors of host cells; examples of such antibodies include scFvs against HA (Haemagglutinin) of influenza A virus H5N1 subtype [20], gB and gH cytomegalovirus [19], respiratory syncytial virus [36], rabies virus [37], and Enteric coronavirus [38]. In a previous study, one recombinant Fab-phage antibody (Fab54–7) was selected against the gB of HSV-1 and HSV-2 using panning. This Fab isolate was not capable of neutralizing either strain when used alone [39]. Nejatollahi et al.

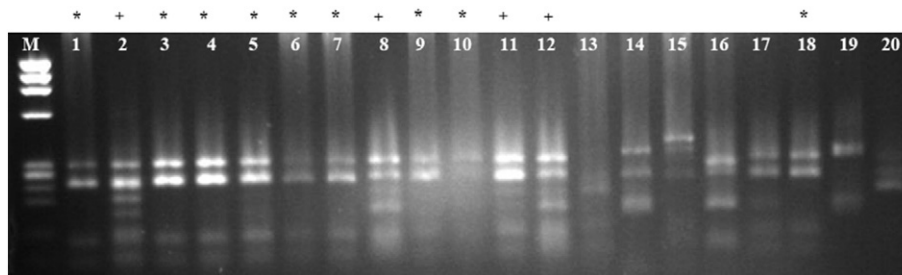


Fig. 2. DNA Fingerprinting pattern of 20 panned clones against the peptide of gB. Mval fingerprinting of scFv genes revealed two patterns, including pattern 1 (lanes 1, 3, 4, 5, 6, 7, 9, 10 and 18) and 2 (2, 8, 11 and 12) with the frequency of 40% (scFv-gB₁) and 20% (scFv-gB₂), respectively. Common patterns were represented with asterisk and plus sign. M, Marker- ϕ X174 DNA.

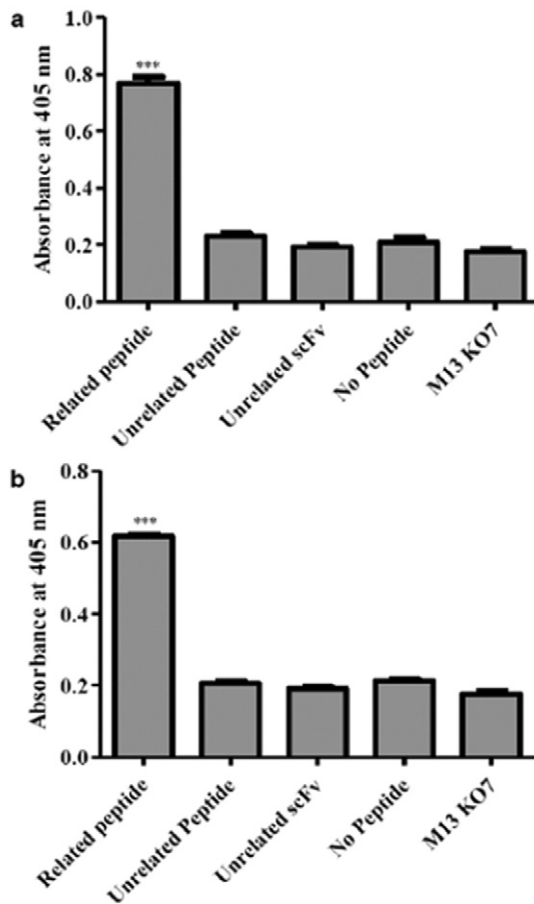


Fig. 3. Phage ELISA of positive scFv phage clones against the gB peptide. scFv-gB₁ (a) and scFv-gB₂ (b). OD (405 nm) of both scFvs were significantly higher than controls (unrelated peptide, unrelated scFv, M13 KO7 helper phage and without peptide and reacted specifically with the gB peptide. The data are presented as the mean \pm standard error. $P < 0.05$.

isolated one scFv-phage antibody (scFv-gD₂) against glycoprotein D with a neutralizing effect of 76% [21]. In this study, two scFv-phage antibodies (scFv-gB₁ and scFv-gB₂) could neutralize virus with neutralizing effects of 55% and 59%, respectively, and significantly decreased cytopathic effects in Vero cells. Moreover, both scFvs could inhibit the virus *in vitro* when used alone (Table 1), and did not require dimerization to exert neutralizing effects.

Owing to their several advantages, single chain antibodies could serve as useful tools for antibody-based therapies. Better penetration to target tissues because of the small size [16,40] has made scFvs more effective than antibodies in therapeutic applications especially when viral antigens are the targets [41,42].

Development of the selected neutralizing antibodies with the human origin is essential for HSV-1 because these antibodies play a major role as therapeutic agents for the central nervous system infection and pneumonia induced by HSV-1 in immunocompromised individuals. In addition, about 30% of neonatal infections are caused by

Table 1

Percentage of plaque reduction in three plaque reduction assay experiments. The scFvs-gB₁ and scFvs-gB₂ antibodies showed reduction of 55% and 59% in plaque numbers, respectively. Control well contained no antibody.

Well	Number of plaques								Mean	% Reduction	
	Experiment 1		Experiment 2		Experiment 3						
scFv-gB ₁	18	13	18	17	16	19	20	17	19	17.4	59
scFv-gB ₂	23	20	17	20	18	20	19	18	17	19.1	55
Control	45	43	42	40	39	45	44	41	42	42.3	-

this virus [43]. Risk of acquisition of neonatal infection is increased with low maternal HSV-1 antibody avidity. Thus, HSV-1 neutralizing antibodies can find potential application in preventing neonatal Herpes infection [44].

In conclusion, the present study aimed at selecting scFv antibodies of a phage-displayed nonimmune human scFv antibody library against gB of HSV-1 and two scFvs, scFv-gB₁ and scFv-gB₂, with frequencies of 45% and 20% were obtained from scFv clones, and showed neutralizing effects of 55% and 59%, respectively. These results encourage further attempts (mainly through genetic engineering) to promote the development of scFv antibodies with preserved binding capacity but enhanced neutralizing effects. Also, future studies are warranted to test the neutralizing effect of the identified scFv antibodies in a purified and phage-free form to obtain a better assessment on their neutralizing effect and also test the dose-response association for the neutralizing effects of ScFvs. Optimized scFv antibodies with enhanced neutralizing effects could also be relevant for testing in clinical trials.

Conflict of interest

The authors declare that they have no conflict of interest.

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