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Full Length Research Paper

Neutralizing human single-chain antibodies against Herpes Simplex Virus type 1 glycoprotein D from a phage display library

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Among the 12 glycoproteins of the Herpes simplex virus type 1 envelope (HSV-1), glycoprotein D (gD) plays a critical role in the entry of the virus into target cells and cell-to-cell spread. gD is an attractive target for molecular intervention and monoclonal antibodies to this glycoprotein has decreased the severity of experimental HSV-1 infection in animal studies. Single chain antibodies which are produced by antibody engineering are small high affinity recombinant antibodies with growing clinical importance especially when viral antigens have been used for targeted therapy. Here we report neutralizing single-chain fragment variable (scFv) antibodies against HSV-1 gD from a phage-display non-immune human scFv library. The phage antibody was panned against amino acid residues 12-21 derived from the N-terminal part of gD. Two scFvs, scFv-gD_1 and scFv-gD_2, with frequencies of 25% and 20% were isolated among scFv clones using PCR and MvaI fingerprinting. Phage ELISA analysis demonstrated high reactivity of scFv-gD_1 and scFv-gD_2 with the gD peptide. In neutralization assay, scFv-gD_2 exhibited neutralizing effect of 76%. Sequence analysis of scFv-gD_2 revealed the amino acid specific changes in FR1 region of heavy chain and FR1 and JL3 regions of light chain of antibody molecule. Also the sequence GADTAMAG in CDR3 region represented the specificity of the selected antibody. The results suggest that the specific neutralizing scFv-gD_2 can be considered as a new alternative in the prophylaxis and treatment of HSV-1 infections.

Key words: Herpes simplex virus type 1, glycoprotein D, single-chain antibody, neutralizing antibody, immunotherapy.

INTRODUCTION

Herpes Simplex Virus type 1 (HSV-1) belongs to the herpesviridae family and infects millions of people worldwide as a common viral pathogen causing wide-spreading disease (Whitley and Roizman, 2001). HSV-1 is a neurotropic virus that is able to establish a lifelong latent infection in the nervous system. Following the establishment of a latent infection in the trigeminal ganglia, periodic reactivation of the latent virus leads to recurrent infections in certain people (Lin et al., 2001; Wanger et al., 1997). HSV-1 is responsible for a wide range of human diseases from the localized infection such as orolabial or corneal lesions to life-threatening encephalitis and pneumonia in the immunocompromised individuals (Whitley and Roizman, 2001; Hwang and Spruance, 1999; Stanberry et al., 2000). Although antiviral drugs are used to reduce viral infection, there is an increase in resistance to these agents by the virus (Kleymann, 2003). Entry of HSV-1 into mammalian cells requires several glycoproteins on the surface of the enveloped virus. The HSV envelope has 12 glycoproteins that among those, glycoprotein B (gB), gD and the gH/gL

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heterodimer are functionally essential for both entry of extracellular virions and cell-to-cell spread. The gD binds to one of its different cellular receptors, herpes virus entry mediator (HVEM), nectin 1, or 3-O-sulfated heparansulphate (3-O HS). These receptors cause conformational changes in gD that starts the fusogenic signal of the core fusion machinery, constituted in gB and gH/gL (Krummenacher, 2005; Spear and Longnecker, 2003).

Neutralizing antibody-mediated immune mechanisms affect the result of HSV-1 infection *in vivo* which is shown in animal models (Balachandran et al., 1982; Kohl, 1990; Rector, 1984). Absence of neutralizing antibody has linked with severe HSV infection in human (Kohl et al., 1989). High levels of preexisting neutralizing antibodies may prevent HSV spread and viremia. Maternal HSV specific antibodies that reduce neonatal HSV transmission underline the protective effect of antibodies (Brown, 1991). Passive immunization with monoclonal antibodies to gD and immunization with purified gD decrease the severity of experimental HSV infection in mice (Long et al., 1984, Simmons and Nash, 1985). Antibodies to gD are divided into eight groups, of which four groups are type common to both gD of HSV-1 (gD-1) and HSV-2 (gD-2). There are only three groups against gD-1, and one group against gD-2 which are type specific (Eisenberg et al., 1982). The gD can induce neutralizing antibodies (Cohen et al., 1986; Fuller and Spear, 1985; Minson et al., 1986; Muggeridge et al., 1988; Para et al., 1985) that prevent entry of HSV-1 to host cell. Antigenic site VII is one immunodominant neutralizing region on gD (Minson et al., 1986; Bosch et al., 1987; Dix et al., 1981).

Isolation of specific antibodies by antibody phage display has become a popular method in recent years. Antibody-phage display libraries generally consist of either single chain fragment variable (scFv) or Fab fragments (Lillo et al., 2011). ScFv molecules (26–27 kDa) are small antibody fragments (Nuttall et al., 2000). Pharmacokinetic properties of human scFv antibodies have improved because of deep and fast penetration to target tissues, low retention times in non-target tissues and low immunogenicity (Batra et al., 2002; Curigliano et al., 2010). ScFv antibodies perform significantly better than conventional antibodies in therapeutic applications. Many neutralizing scFv antibodies are produced against viruses, including scFv against influenza A virus H5N1 subtype (Maneewatch et al., 2009), single chain antibodies to human immunodeficiency virus type 1 (HIV-1) (Wang et al., 2005) and scFvs to human cytomegalovirus (Nejatollahi et al., 2002) that can inhibit viral infections *in vitro*. Chen et al. (2004) reported a non human neutralizing scFv (DL l l) against gD of HSV produced from a IgG secreting hybridoma. In this study, we selected specific anti-gD human scFv antibodies from a phage antibody display library and assessed their neutralizing effects against HSV-1 in cell culture.

MATERIALS AND METHODS

Selection of anti-gD scFv

The peptide (ADPNRFRGKD) was synthesized (Isogen, Netherland) and used as gD epitope to select specific anti-gD scFvs by panning process. The diluted peptide (10 $\mu g ml^{-1}$ in PBS) was coated overnight in Immunotubes (Nunc, Roskilde, Denmark) at 4°C. The tubes were washed with PBS and blocked with 2% skimmed milk at 37°C for 2 h. A phage antibody display library of scFv was produced as described previously (Nejatollahi et al., 2008). The library was phage rescued using M13KO7 helper phage. The phage supernatant (10^{10} PFU/ml) was added to an equal volume of blocking solution and incubated in the tubes for 2 h at room temperature. Following washing, bound phages were eluted with log-phase TG1 *E. coli* and incubated at 37°C for one hour. Three further rounds of panning were repeated to select specific scFv against the peptide. PCR was performed on the clones obtained after panning and each product was digested by MvaI restriction enzyme (Roche, Mannheim, Germany) at 37°C for 2 h in DNA fingerprinting assay to determine whether panning had been successful in selecting for specific scFv clones. Two specific scFvs were selected which were used for further investigations.

Measurement of scFv concentration

The scFv concentration in phage rescue supernatant of specific clones, 10 µl of phage antibody supernatant was added to one ml log-phase TG1 *E. coli* and incubated with shaking at 37°C for 1 h. Serial dilutions of bacteria were prepared and cultured onto 2TY/Ampicillin plates. After counting the number of colonies per dilution, phage concentration titer per milliliter was calculated.

Phage ELISA

The reactivity of isolated scFv clones to the peptide was determined using phage ELISA. Peptide (100 $\mu g ml^{-1}$ in PBS) was coated on the 96 well ELISA plates and placed overnight at 4°C. The wells were washed three times with PBS/Tween 20 and three times with PBS. Blocking solution (5% w/v skimmed milk in PBS) was added to the wells and incubated at 37°C for 2 h. Following washing, phage rescue supernatant (10^{10} pfu ml^{-1}) was diluted 1:1 with blocking solution, added to the wells and incubated at room temperature for 2 h. The plate was washed and anti-fd bacteriophage (Sigma, Chemical co, UK) was added to each well and incubated at room temperature for 1.5 h. HRP conjugated goat anti-rabbit IgG (Sigma, Chemical co, UK) was added following washing and incubated at room temperature for 1 h. The wells were stained using 0.5 mg/ml ABTS (Sigma Chemical Co, UK) in citrate buffer (PH 8) containing 1 µl hydrogen peroxide. The optical density (OD) of each well was read at 405 nm after 30 min using an ELISA reader, and the average absorbance for each scFv antibody was calculated. Wells

containing unrelated peptide, unrelated scFv and M13 KO7 helper phage instead of specific scFv, and no peptide were also considered as controls.

Cell culture

Vero cells were cultured in 1× Dulbecco's modified Eagle's medium (DMEM) in 24-well plates in the humidified air with 5% CO2 at 37°C for 24-48 h to be confluent. Medium supplemented with 8% fetal calf serum (FCS; Gibco-BRL), 0.14% (v/v) sodium bicarbonate, 100 U penicillin ml^{-1}, 100 µg streptomycin sulphate ml^{-1}, and 0.25 µg amphotericin B ml^{-1} (Gibco-BRL).

Neutralization assay

The anti-HSV-1 activities of scFvs were evaluated using plaque reduction assay. 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 for 1 h. 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. The mixture was removed and one ml of 1% w/v Carboxymethyl Cellulose (CMC) in 1× DMEM supplemented with 2% fetal calf serum, 0.14% v/v sodium bicarbonate, 100 U penicillin ml^{-1}, 100 µg streptomycin sulphate ml^{-1}, 0.25 µg amphotericin B ml^{-1} and 0.1 M sodium hydroxide was added. The number of micro plaques were counted after five days and compared with the number of plaques in the virus control wells which contained no scFv.

Sequencing of scFv-gD_2 gene

To determine the nucleic acid sequence of the scFv with better neutralizing effect, the amplified scFv-gD_2 was sequenced using the BigDye Terminator 3.1 Sequencing reaction (Applied Biosystems). The amino acid sequence of VH and VL regions of the antibody were determined using ExPASy-Translate tool (www.expacy.org translate) and the amino acid sequence alignments were obtained from VBASE2 (http://www2.mrc-lmb.cam.ac.uk/vbase/alignments2.php).

RESULTS

Selection of scFv-phage antibodies by panning

Figure 1 shows DNA fingerprinting of 20 clones against gD epitope after four rounds of panning. MvaI fingerprinting of PCR-amplified scFv inserts revealed two predominant patterns: pattern one (lanes 1, 3, 10, 12 and 14) with frequency of 25% and pattern two (lanes 4, 5, 16 and 20) with frequency of 20%, the scFv-gD_1 and scFv-gD_2, respectively. These scFvs were used for further investigations.

Phage ELISA

The reactivity of scFv-phage antibodies to the gD peptide was determined in phage ELISA. Figure 2 shows that optical density (OD) of scFv-gD_1 and scFv-gD_2 were significantly higher than those of controls (unrelated peptide, unrelated scFv, M13 KO7 helper phage and no peptide) at 405 nm.

Neutralization of HSV

The neutralization capacity of the anti-HSV-1 scFv antibodies was evaluated by percentages of plaque reduction which were 45% for scFv-gD1 and 76% for gD2 (Table 1).

Sequencing

Sequence analysis revealed that the VH and VL regions of neutralizing scFv-gD_2 belonged to VH1 and VL1 gene families, respectively. In Figure 3, the amino acid changes are shown in comparison with the amino acids of the VH and VL gene families. The sequences were submitted to Gen Bank. The assigned Accession Numbers for nucleotide sequences of VH and VL regions are KC788508 and KC788509, respectively. The amino acids specificity changes in comparison with the VH and VL gene families are shown.

DISCUSSION

Following the steps viruses take to enter target cells, the first stage of infection associated with the specific interaction of viral surface proteins with cellular proteins, lipids, or carbohydrates, can be blocked by neutralizing monoclonal antibodies (Krawczyk et al., 2011). Three groups of anti-gD monoclonal antibodies against HSV-1 (groups II, V and VII) react with continuous antigenic sites. Monoclonal antibodies which reacted with oligo-peptides 9-21, 10-24, 7-23, 7-21 and 11-32 of gD, were classified as group VII antibodies and neutralized HSV-1 *in vitro* (Minson et al., 1986; Eisenberg et al., 1982; Geerligs et al., 1990; Cohen et al., 1988). The greatest virus neutralization capacity was associated with antisera raised to the peptide conjugate of the N-terminal amino acid residues 2 to 21 of gD (Geerligs et al., 1990; Strynadka et al., 1988). The sequence 12 to 21 located in residues 2 to 21 elicited antibodies that neutralized HSV-1 in the absence of complement (Strynadka et al., 1988). Interaction of these antibodies with synthetic peptides (peptides 1 to 23, peptides 8 to 23 and 11 to 23) (Cohen et al., 1984; Dietzschold et al., 1984) suggested that one of the neutralizing epitopes in the N-terminal 1 to 23 sequence is located between residues 11 and 19. This suggestion is confirmed since antiserum to peptide

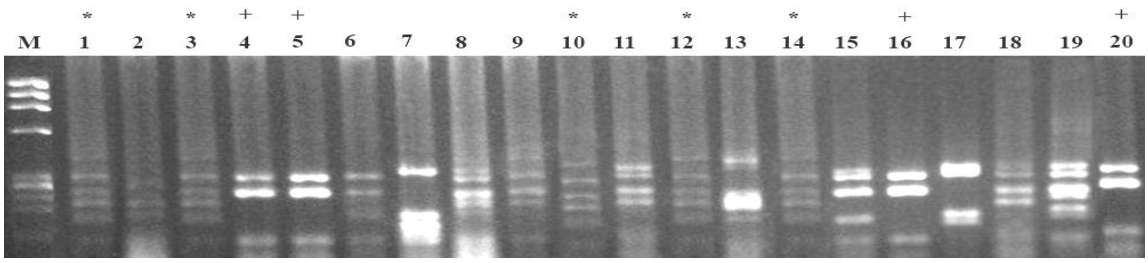

Figure 1. DNA fingerprinting pattern of 20 panned clones against the gD peptide.
MvaI fingerprinting of scFv genes revealed two patterns: pattern 1 (lanes 1, 3, 10, 12 and 14) and pattern 2 (4, 5, 16 and 20) with the frequencies of 25% (scFv-gD_1) and 20% (scFv-gD_2) respectively.

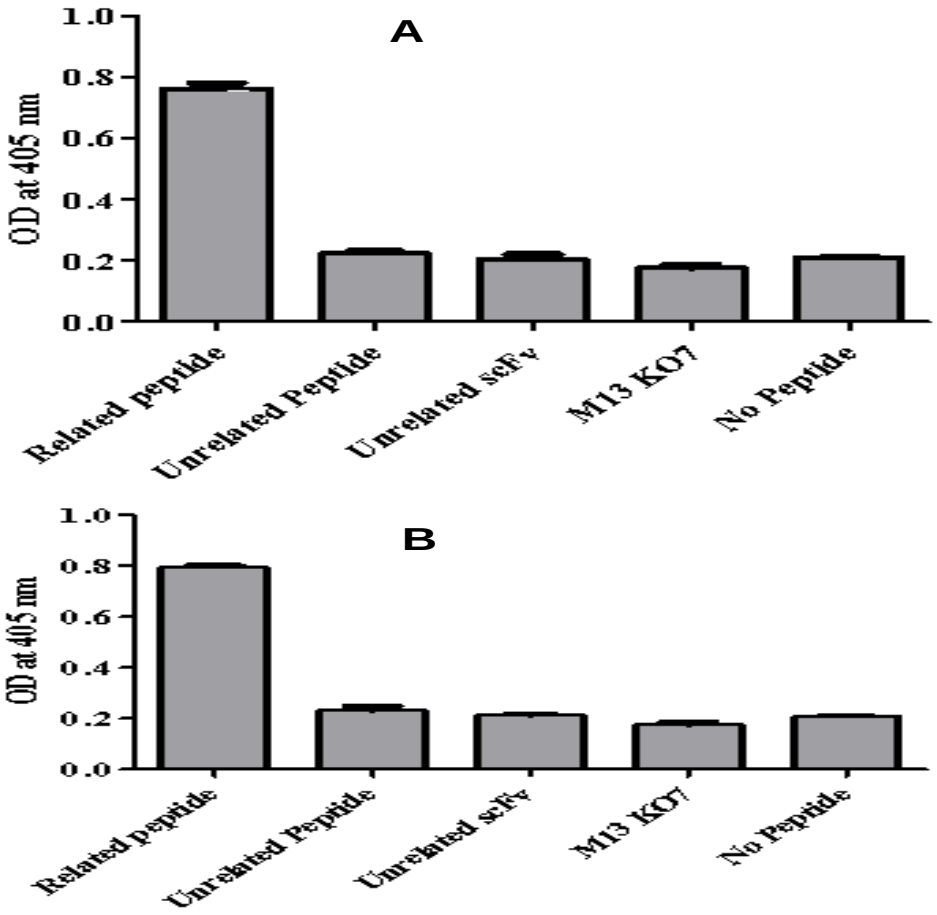

Figure 2. Phage ELISA of positive scFv clones against the gD peptide. ScFv-gD_1 (A) and scFv-gD_2 (B).

Table 1. Percentage of plaque reduction in three plaque reduction assay experiments.

Well	Number of plaques									Mean	% Reduction
	Experiment 1			Experiment 2			Experiment 3				
scFv-gD_1	27	18	29	28	25	22	20	23	19	23.4	45
scFv-gD_2	10	12	8	12	10	11	10	9	12	10.4	76
Control	43	41	40	42	43	42	39	43	45	42	-

Well treated with scFv-gD_2 showed the highest plaque reduction, 76%. Control well contained no antibody.

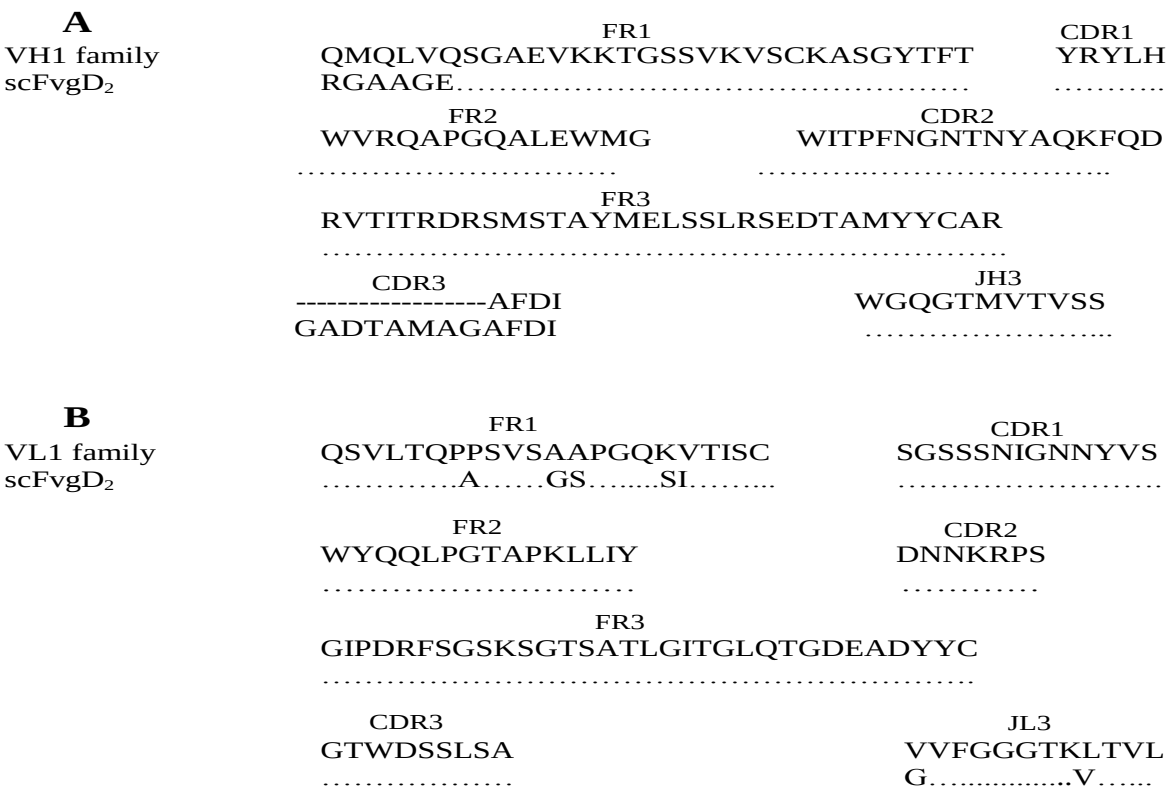

Figure 3. Amino acid sequences of scFv-gD_2. Heavy chain (A) and light chain (B). The amino acids specificity changes in comparison with the VH and VL gene families are shown.

12 to 21 reacted with isolated glycoprotein D and neutralized HSV-1 virions (Strynadka et al., 1988).

The identification of ligand specificity to targets of interest through antibodies displayed on phages is a powerful tool which is provided by pure antigens or synthetic peptides coated on a solid phase using repetitive panning rounds (Nejatollahi et al., 2011; Duan and Siegumfeldt., 2010; Griffiths et al., 1994; Hoogenboom., 2002). In this study, phages expressing scFv were selected on HSV-1 gD epitope through panning process. The epitope used for selection of specific scFv was sequence 12-21 of gD (ADPNRFRGKD) which was introduced as a neutralizing epitope of HSV-1 capable of inducing antibodies with greatest virus neutralization (Strynadka et al., 1988). The scFv-gD_1 and scFv-gD_2 were selected due to their patterns which were dominant in comparison to the patterns of other clones (Figure 1).

The phage-scFv clones were examined using phage ELISA to determine their specificity to the target epitopes. Specificity of scFvs against *Burkholderia mallei* (Zou et al., 2007), *Helicobacter pylori* (Pedroza-Roldan et al., 2011) and hepatitis B virus (Zhang et al., 2006) were confirmed by phage ELISA. Thathaisong et al. (2008) showed that optical density (OD) of specific scFvs against influenza-A virus H5N1 subtype at 405 nm were two fold higher than negative controls in a positive phage ELISA. We assessed the selected clones after four rounds of panning. The results of the phage ELISA revealed that panning method had been successfully performed and also selected clones were specific to the gD peptide. The mean optical densities (OD) of the both scFv-gD_1 and scFv-gD_2 at 405 nm were two folds higher than ODs of the controls (Figure 2).

In order to test neutralizing activity of scFv antibodies, plaque reduction assay was used. The scFv-gD_2 was able to neutralize HSV-1 with neutralizing effect of 76%. Although the neutralizing effect was observed in all selected clones, scFv-gD_1 showed neutralizing effect less than 50% (Table 1). Sanna et al. (1995) reported that none of the three recombinant Fab antibodies specific for the HSV-1 gD and gB selected in their study, were capable to neutralize either strain if used alone. Dimerization of Fabs was able to neutralize HSV-1. In our study, scFv-gD_2 alone was able to inhibit the virus significantly. Neutralizing non-human single chain antibody against gD of HSV-1 was also reported(Chen et al., 2004); the antibody reacted with amino acids 222 to 252 of gD and prevented genital herpes in guinea pig model whereas in the current study

a human scFv antibody originated from antibody engineering was used which was specific against amino acids 12-21 of gD.

Sequence analysis of scFv-gD_2 revealed that the VH region was derived from VH1 which was assembled with VL1 gene family. The amino acid alignment of heavy and light chains of scFv-gD_2 showed some amino acids differences (Figure 3) in comparison with VH and VL gene families. In FR1 of VH and VL regions of scFv-gD_2, there were amino acids residues of RGAAGE instead of QMQLVQ and A, GS and SI instead of P, AA and KV, respectively. The sequence GADTAMAG in CDR3 region represented the specificity of VH. There were also amino acids of G and V instead of V and L in JL3 of VL region, respectively. Remarkable specificity of antibody molecules is due to these changes (Kabat and Wu, 1991) and shows selection of specific antibody against the peptide of interest.

In conclusion due to several advantages of single chain antibodies, these recombinant molecules are useful tools for antibody-based therapies. The small size of scFvs which results in better penetration to targets (Batra et al., 2002; Shen et al., 2005) has made them more effective in therapeutic applications especially when viral antigens are the targets (Duan et al., 2012; Xun et al., 2013). The human origin of the selected neutralizing antibody also plays a major role in the effectiveness of therapies and offers its potential for clinical applications including neonatal herpes, central nervous system (CNS) infection and pneumonia induced by HSV in immune-compromised individuals.

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