Reports of Biochemistry & Molecular Biology
Vol.8, No.3, Oct 2019

Original article

www.RBMB.net

Isolation of Neutralizing Human Single Chain Antibodies Against Conserved Hemagglutinin Epitopes of Influenza A Virus H3N2 Strain

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Abstract

Background: Immunotherapies using monoclonal antibodies against influenza A hemagglutinin (HA) has been an effective means for controlling Influenza spread. An alternative method for viral prophylaxis and treatment is the development of human single-chain variable fragment (scFv) antibodies with no human anti-mouse antibody (HAMA) response and high specificity. In the present study, two highly conserved sequences of HA were used to select specific neutralizing scFvs against H3N2 strain of influenza A virus.

Methods: Biopanning process was performed to isolate specific scFv antibodies against highly conserved HA sequences, aa173-181 and 227-239, of the influenza A H3N2 strain from a scFv library. The peptide-binding specificity of the selected clones was examined via phage ELISA. The soluble forms of the clones were prepared and assessed using western blot analysis and neutralization efficiency of the selected clones were examined by TCID50 neutralizing assay and real-time PCR.

Results: scFv 1 and scFv 2 were selected against HA of H3N2 influenza A virus with frequencies of 95% and 30% in the panning process, respectively. Western blot analysis confirmed the scFv band size. Significant neutralization in the presence of scFv 1 and scFv 2 were obtained. Real time PCR revealed significant decrease in viral copy number.

Conclusions: Two specific neutralizing scFvs against two highly conserved neutralizing epitopes of the influenza A virus HA glycoprotein were selected. A strong neutralization effect of scFv1, showed the potential of this antibody for H3N2 influenza A controlling in the viral spread.

Keywords: Hemagglutinin, H3N2 Influenza A, Neutralization, scFv.

Introduction

Influenza virus infects roughly 5–10% of the world's population each year (1).The virus is classified into three distinct types, A, B, and C, all of which infect different host species including humans, birds, wild mammals, pigs, and horses (2). Influenza A viruses are the main etiological agents responsible for the occasional pandemics and seasonal flu (3). Influenza A contains two antigenic surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which further sub-classifies the influenza A strain (4).

Hemagglutinin protein facilitates viral entry into the target cell through recognizing and binding to host proteins bearing sialic acid on their surface. This engages receptor-mediated endocytosis which leads to a decrease in pH, triggering conformational changes in HA. This change in the structure of HA leads to the fusion of the viral and host membranes and the release of the viral genome into the host cell cytoplasm. This HA glycoprotein is critical for viral infection into the host cell and is a major vaccine target while NA

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Received: 23 Dec, 2019; Accepted: 6 Jul, 2019

enables the virus to be released from the host cell and is required for influenza virus replication (5). The influenza A genome contains eight gene segments of single-stranded, negative-sense RNA that encodes for 13 proteins (3). The segmented structure of the influenza A genome enables the exchange of gene segments among different influenza strains during a coinfection of a single host cell, a process called "genetic reassortment". This is a key mechanism contributing to the emergence and spread of pandemic viral strains throughout immunologically naive populations (6). The two pandemic strains of influenza that emerged in 1957 and 1968 were the result of genetic reassortment between human and avian viruses (7). H3N2 subtypes are reported in some pandemic and epidemic episodes. The H3N2 influenza A subtype appeared due to reassortment between human and avian influenza viruses; the HA and PB1 segments of the H2N2 virus were replaced with those from an avian H3 virus (8). The H3N2 virus that emerged in the 1968 pandemic and accounted for 48 percent mortality rate (9). Two pandemic waves of A/H3N2 occurred during the winters of 1968-1969 and 1969-1970 in both Europe and Hong Kong. Two severe H3N2 epidemics were also reported in 1975–1976 and 1980–1981 (10). Vaccination is one of the most effective strategies for preventing the spread of disease and mortality caused by influenza infection (11). However, the effectiveness of the seasonal influenza vaccines is reduced due to antigenic drift. Antiviral drugs are effective in reducing the symptoms and complications that can occur due to influenza virus infections but drug- resistant isolates rendering the treatments insufficient (12, 13).

Several preclinical *in vivo* studies using animal models have shown that anti-influenza virus HA monoclonal antibodies offer protection when administered during the late phase of infection (5, 14). Monoclonal antibodies with neutralizing activity have been used to prevent the development severe influenza infection including: CR9114 against influenza A and B viruses (15), and FI6 against group 1 and 2 viruses (14). Although monoclonal antibodies have been demonstrated to be useful in the treatment and diagnosis of influenza infection, they have several drawbacks that limit their clinical application. Since the monoclonal antibodies are murine derived, will induce a human anti-mouse antibody (HAMA) response, even in humanized forms of monoclonal antibodies (16, 17). In addition, the technology used to produce monoclonal antibodies is very difficult and time consuming (18). The use of phage display technology in producing specific human single-chain variable fragment (scFv) antibodies provided specific and effective alternatives with no HAMA response (19-21). ScFv fragments consist of the variable heavy (V_H) and light (V_L) chains of an immunoglobulin that are bound together via a flexible linker and can maintain their specificity and affinity for their antigens (22, 23). Moreover, small size and high penetration into tissues have made these antibodies very attractive agents for immunotherapy approaches (24, 25). In this study, we selected specific scFvs against two conserved sequences of HA of H3N2 influenza virus, aa 173-181 and aa 227-239. Soluble forms of the antibodies were produced and the neutralizing effects of both the phage displayed and soluble forms of the selected scFvs in MDCK cell culture were evaluated by Reed and Muench method and real time PCR.

Materials and methods

Selection of anti-HA scFv

The peptides KFDKLYIWG (aa 173-181) and SSRISIYWTIVKP (aa 227-239) were used as the HA epitopes to select for specific anti-HA scFvs via biopanning. Immunotubes were coated overnight with peptides (Nunc, Roskilde, Denmark). The page antibody display scFv library was constructed as previously described (20). The library was phage rescued using M13KO7 helper phage. Phage supernatant was diluted in blocking solution (10^{10} pfu /ml) and added to the coated immunotubes then incubated at room temperature for 1 hour. Following washing, bound phages were eluted with log-phase TG1 *E. coli*. The bacterial pellet was grown on 2TY-ampicillin agar plates. Four rounds of panning was performed to select for the specific scFvs against each peptide. PCR was performed on the clones obtained after panning and its product was digested with Mva I restriction enzyme using a DNA fingerprinting assay. The clone with the most frequent pattern was selected against each peptide and phage-rescued for further evaluations.

Measurement of scFv concentration

To determine the scFv concentration of each rescued phage, 10 µl of phage antibody supernatant was added to 1ml of log phase TG1 *E. coli*. Serial dilutions of bacteria were performed and plated onto a 2TY Agar/Ampicillin medium. The number of clones per dilution was calculated and the appropriate scFv concentrations were used for the following experiments.

Phage ELISA

ELISA plates were coated with 100 µg/ml of each peptide per well at 4 °C overnight. Wells were blocked 2 hours at 37 °C with blocking solution. After washing, phage supernatant (concentration $2x10^{13}$) was diluted with blocking solution at a 1:1 ratio then added to each well and incubated at room temperature for 2h. The wells were washed, then the anti-fd antibody was added and incubated for 2 h. Any non-binding antibodies were removed via washing. HRP conjugated anti-fd antibody was added to each well. Wells were washed and TMB solution was added. Following 10 minutes of incubation, 40 µl of stop solution was added to each well and the optical density of each well was measured at 450 nm using ELISA reader (BP-800, Biohit, USA).

Production of soluble antibody fragments

Phage supernatant of the selected clones was added to HB2151 *E. coli* in a logarithmic growth phase and incubated for 1h. The pellet was cultured onto 2TYG Agar/Ampicillin plates and incubated overnight. The transfected HB2151 bacteria was supplemented with 1mM IPTG (isopropyl β-D-thiogalactoside) then incubated overnight at 30 °C. The supernatant was separated from the bacterial pellets. Lysis buffer (10 mM Tris HCL + 50 mM NaCl + 100 mM Na2HPO4 + 8 M urea, PH: 8) was added to the pellet and incubated on ice for 2 hours, then incubated at 37 °C for 2 hours with shaking (100 rpm). The bacteria was sonicated for 30 seconds for a total of 10 rounds. The sonicated bacteria was then centrifuged and the supernatant containing the soluble scFv, was collected and stored at -20 °C.

Western blot analysis

Both the 5% stacking and 10% running gel were prepared. Periplasmic extract was mixed with the sample buffer then loaded into each well of the gel. Following electrophoresis, gel was soaked into the transfer buffer. A sandwich of paper/gel/membrane/paper was placed directly between positive and negative electrodes. Protein-transfer was carried out for 20 minutes. The unoccupied spaces of the PVDF membrane were blocked with 5% skimmed milk in PBS at 4 °C overnight. The PVDF membrane was washed then incubated with HRP conjugated goat anti-c-myc antibody for 1.5 hours. The PVDF was washed with PBS/Tween. Following washing, for visualization, a Fermentase ECL chemiluminescence system was used and a radiology film was developed.

Cell culture

Madin–Darby canine kidney (MDCK) cells were grown in DMEM medium. The media was supplemented with 10% fetal bovine serum (GIBCO), 100 u/ml penicillin and 100 µg/ml streptomycin. Cells were stored in an incubator with 5% CO2 at 37 °C for 24-48 h.

Neutralization assay

Influenza neutralization assay was performed using MDCK cells and 100 TCID50 of influenza virus. Separately, the soluble scFv and phage displayed scFv antibodies were mixed with 100 TCID50 of virus and incubated for 1 hour at room temperature. The mixture was added to a monolayer of MDCK cells. The plate was incubated at 37 ºC for 3–4 days. Infectivity was identified by the presence of CPE and the titer was calculated by the Reed-Muench method.

Real-Time Polymerase Chain Reaction

In order to measure the copy number of the virus in cell culture, quantitative real-time PCR was used. Using a viral RNA extraction kit (Roch, Mannheim, Germany), 200 µl of the sample was extracted. The master mix was prepared and the amplification conditions were set according to the manufacturer's instruction**.** The neutralizing effect of the antibodies was evaluated according to the copy number of the virus following antibody treatment.

Results

Selection of scFv-phage antibodies by panning

Figure 1 shows the DNA fingerprinting of the selected clones against peptide 1 and peptide 2. The frequencies of the two scFvs against peptide 1 and peptide 2 were 95% and 30%, respectively. One colony from each pattern was used for further investigations (Fig. 1).

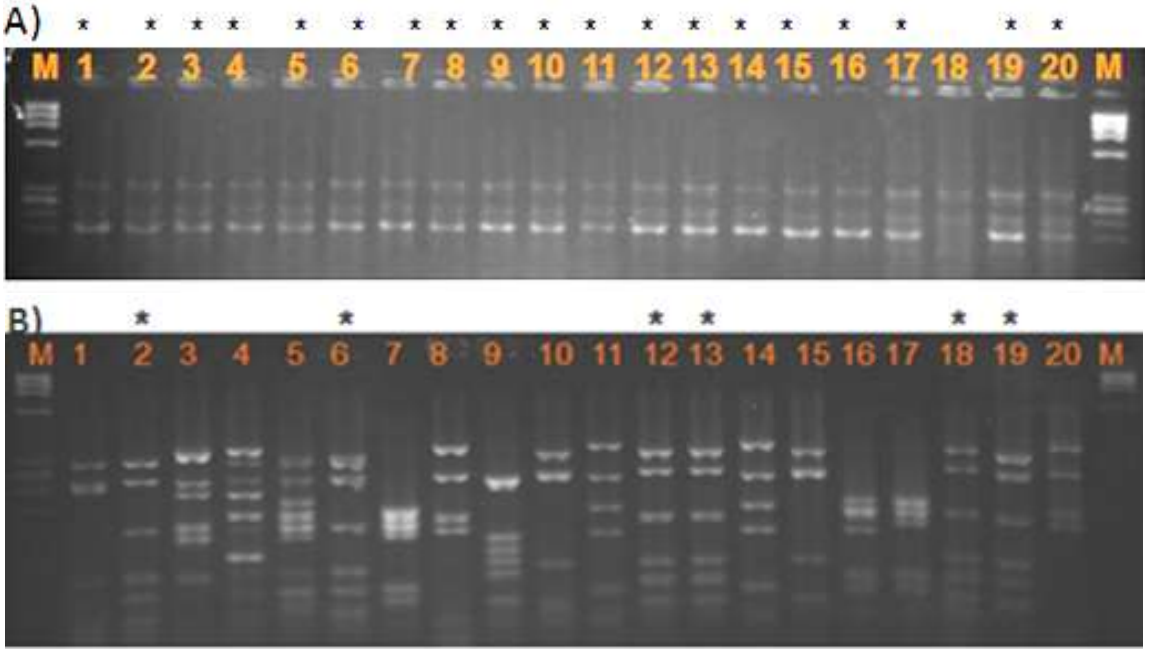

Fig. 1. DNA fingerprinting of selected clones against peptide 1 (A) and peptide2 (B). M: Marker.

Positive phage ELISA

Specific binding of the selected scFv antibodies to peptides 1 and 2 of HA was evaluated by phage ELISA assay. The wells containing unrelated peptide, no peptide and M13KO7 were used as controls. The absorbance at 450 nm are shown in Figure 2. OD values obtained from the reactions of each of the selected scFvs with the corresponding peptide two-fold higher than those of the negative controls (Fig. 2).

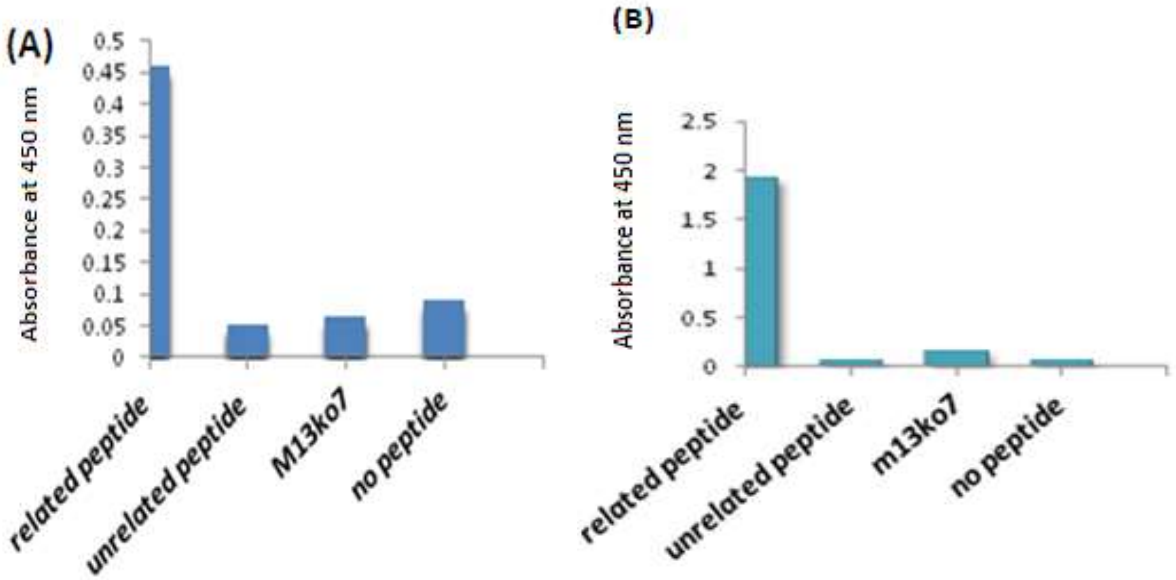

Fig. 2. Phage ELISA results of the scFv against peptide 1(A) and scFv against peptide 2(B).

Western blot

Figure 3 shows the western blot results of scFv1, which was expressed production in HB2151. Production of the soluble scFv1 in culture medium induced by IPTG was detectable, a 25KD band was observed. No band was observed in the culture medium without IPTG induction (Fig. 3).

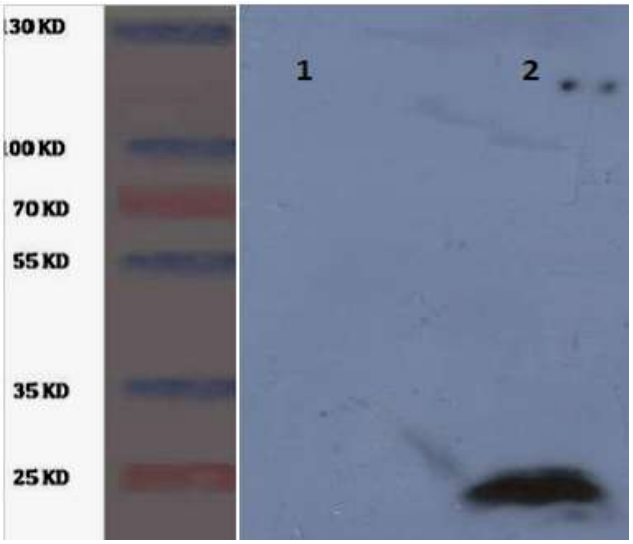

Fig. 3. Western blot analysis of soluble scFv1. Lane 1: culture medium from HB2151 without induction by IPTG. Lane 2: scFv production of preplasmic extract upon induction by 1 mM IPTG.

Neutralization assay

The neutralization capacity of the anti-HA scFv antibodies were evaluated via CPE in cell culture. CPE was reduced when the viruses were treated with scFvs antibodies. The number of the virus particles in the virus control (without antibody treatment) was 10^5/ml and the number of virus particles in the presence of phage displayed and soluble scFv against peptide 1 were $10^{1.33}$ and $10^{2.3}$. The phage displayed scFv against peptide 2 was $10^{2.33}$, per milliliter. The M13KO7 and HB2151 were used as negative controls (Table1).

Table 1. Results of the TCID50 assay. In the presence of the phage displayed scFv1, the highest neutralization was observed ($\mathbf{10^{1.33}}$). M13KO7 and HB2151 were used as negative controls for the phage displayed and soluble forms of the antibodies, respectively.

		Virus	Cell	Mean log_{10} TCID50/ml
Phage displayed antibody	1-scFv against peptide 1	H3N2	MDCK	1.33
	2-scFv against peptide 2	H3N2	MDCK	2.33
Soluble antibody	1-scFv against peptide 1	H3N2	MDCK	2.3
Control negative	1-M13KO7	H3N2	MDCK	4.66
	2-HB2151 lysate	H3N2	MDCK	4.55
	3-no Ab (virus control)	H3N2	MDCK	5

Quantitative Real-Time PCR assay

Real time PCR was used to determine the viral copy number (CN) following antibody treatment in the MDCK cell culture. Our findings show the lowest viral copy number in the phage display antibody to be associated with the scFv against peptide 1 (811 CN/5 µl). The copy numbers of the virus following treatment with soluble scFv against peptide 1 was 1141 CN/5 µl. Copy number of virus in samples containing anti-peptide 1 phage displayed scFv was over 70 times lower than the viral copy number in the samples containing M13KO7 (negative control). This antibody showed the best neutralization effect on the influenza A virus. The decrease of viral copy number after treating with scFv 2 was more than 57 times.

Table 2. Results of the real time PCR. Viral copy number in samples containing phage displayed scFv1 showed the lowest copy number. HB2151 and M13KO7 were used as negative controls.

Antibody/control sample	Copy number
Phage display scFv against peptide 1	811
Phage display scFv against peptide 2	999
M13KO7	57190
Soluble scFv against peptide 1	1141
HB2151	5752
Virus control	423300

Discussion

The use of antiviral drugs has been a critical means of treatment for combating viral infections, however, the development of drug resistant strains significantly diminishes the efficacy of these therapeutic strategies. Anti-influenza virus HA monoclonal antibodies, which showed protection when administered during the late phase of infection, conducted for antibody uses in the treatment and prophylaxis against influenza (5, 14). The development of monoclonal antibodies is an alternative tool for mitigating viral infection. However, the clinical success of monoclonal antibodies is constrained due to the high cost of production, storage requirements, and the limited routes suitable for administration (26). The effectiveness of monoclonal antibodies against infectious disease has been explored in several studies (27-30).

A study by Kubto-Koketsu et al. (31) prepared human monoclonal antibodies from the peripheral blood of patients infected with or vaccinated against different strains of influenza. They were able to obtain 10 hybridoma clones stably producing anti-influenza virus antibodies: one for influenza A H1N1, four for influenza A H3N2 and five for influenza B. Epitope mapping showed that the two broadly neutralizing antibodies to H3N2 that were derived from different patients both recognized the same epitopes. Specifically, the HA globular region located underneath the receptor binding site, aa 173-181 and 227-239. These two regions of HA are highly conserved among the H3N2 strains. In the present study, we used these highly conserved HA regions aa 173–181 and aa 227-239 to select for and isolate specific neutralizing scFv antibodies against these peptides. Two conserved epitopes, peptides 1 and 2, (corresponding to aa171-181 and aa227-239, respectively) of the influenza A H3N2 HA protein were used as the panning antigen. Following four rounds of panning, PCR was used to confirm the presence of the VH Linker-VL inserts in the clones. DNA fingerprinting was used to determine the homogeneity of the isolated clones and isolate the specific clones against each peptide. Two clones were selected against peptide 1 and peptide 2 with frequencies of 95%, and 30%, respectively. To determine the peptide binding specificity of the isolated scFvs against their respective peptides a phage ELISA was performed (32, 33). A positive phage ELISA is reported when the average OD value is at least two-fold greater than that of the well with no peptide (34). The absorbance against peptide 1 and 2 were 0.46 nm and 1.92 nm, which was greater than 2 times that of the no peptide absorbance, 0.091 nm and 0.074 nm. These findings demonstrate that the panning process was successful and selected for scFvs specific to peptides 1 and 2 of HA. SDS-PAGE and western blot detected soluble scFv1. A distinct 25KD band of the soluble scFv1 was observed. Production of the soluble scFv in culture medium by induction with IPTG was detectable, however the culture medium without IPTG induction showed no detectable band (Fig. 3). This finding is in accordance with Wang et al., in which a recombinant scFv was constructed against two important virulence factors of *Staphylococcus aureus*. Western blot analysis of the scFv showed a 30kDa band visible in the sample of the IPTG-induced culture, no band was observed in the sample of the non-induced culture (35). No distinct band was observed for scFv against peptide 2, which may be a result of less soluble scFv expression. The neutralizing effects of both the phage displayed and soluble forms of scFv1 and the phage displayed scFv2 were evaluated via TCID50 and real time PCR. The neutralization assay is based on the microscopic observation of viral induced cytopathic effects (CPE) (36). To determine the infectious titer of the virus in tissue culture, 50% cell culture infectious dose (TCID50) assay was used. The TCID50 values were calculated by the Reed and Muench method or Spearman and Karber (37). The TCID50 assay was used to determine the infectious titer of the H3N2 virus, which can cause CPE in cell culture. As shown in Table 1, the

number of the virus particles in the virus control group without scFv antibody treatment was 10^5/ml, while the number of virus particles reduced in the presence of the isolated scFv antibodies. In the presence of scFv1 and scFv 2, $10^{3.67}$ and $10^{2.67}$ times of CPE reduction were observed. Of the four scFvs, the phage display scFv antibody against peptide 1 showed a better effect than the other scFvs. In the presence of M13KO7 and HB2151, no reduction in influenza viral titer was observed. Real-time PCR measured the viral copy number (CN) in each specimen following antibody treatment in MDCK cell culture. The sample containing the phage display scFv antibody against peptide 1 had the lowest viral copy number per 5 µl (811 CN/5 µl). The maximum neutralization effect against H3N2 Influenza virus was attributed to the scFv1 antibody, over 70-fold decrease in viral copy number. The scFv 2 showed over 57-fold decrease in the viral copy number.

The unique characteristics of scFvs, such as their small size, high binding affinity, human origin, and low immunogenicity (38-41) has led to their use as immunotherapies targeting several infectious diseases including, HCMV, HBV, H5N1 influenza, rabies, and HIV (44, 46). In the present study, we isolated two scFv antibodies with significant neutralization efficiency against highly conserved epitopes of the H3N2 HA envelope protein. The ability of the scFv against peptide 1 to significantly reduce viral copy number over 70-fold suggests a potential role for scFvs as an effective neutralizing antibody for the prophylaxis and treatment of high risk patients during an influenza epidemic. Since the antibody is selected against conserved sequences of the influenza A virus, it also has the potential to be effective against different influenza strains. Further investigation is required to examined the effects of the isolated scFvs *in vivo*.

Acknowledgment

The authors would like to express their appreciation to Shiraz University of Medical Sciences for financial support. The present article was extracted from a thesis written by Mahboubeh Poursiyami, grant no.7243.

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