Journal of Virological Methods 159 (2009) 105–111

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Human single chain monoclonal antibody that recognizes matrix protein of heterologous influenza A virus subtypes

Ornnuthchar Poungpair[a], Wanpen Chaicumpa[b,*], Kasem Kulkeaw[b], Santi Maneewatch[b], Kanyarat Thueng-in[a], Potjanee Srimanote[a], Pongsri Tongtawe[a], Thaweesak Songserm[c], Porntippa Lekcharoensuk[c], Pramuan Tapchaisri[a]

[a] *Graduate Program, Faculty of Allied Health Science, Thammasat University, Pathumthani 12120, Thailand*
[b] *Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkok-noi, Bangkok 10700, Thailand*
[c] *Faculty of Veterinary Medicine, Kasetsart University, Thailand*

Article history:
Received 18 November 2008
Received in revised form 1 March 2009
Accepted 9 March 2009
Available online 20 March 2009

Keywords:
Influenza A
A/H5N1
Matrix protein (M1)
ScFv
Phage display

A B S T R A C T

Matrix protein (M1) is predominant and has pivotal role in the influenza A virus replication and assembly. It is therefore an attractive target for antiviral drugs, siRNA studies, and therapeutic antibodies. Nevertheless, therapeutic antibody that interferes with the M1 multiplex function has never been developed. In this study, human single monoclonal antibody fragments (HuScFvs) to M1 were generated. Full length recombinant M1 (rM1) was produced from cDNA prepared from genome of highly pathogenic avian influenza virus, A/H5N1. The rM1 was used as an antigen in phage biopanning to select phage clones displaying HuScFv from a human antibody phage display library. Several phage clones displaying HuScFv bound to the rM1 and harboring the respective *huscfv* gene inserts were isolated. RFLP experiments revealed multiple DNA banding patterns which indicated epitope/affinity diversity of the HuScFv. The HuScFv were tested for their binding to native M1 of homologous and heterologous influenza A viruses using ELISA as well as incorporating immunostaining and immunofluorescence studies with infected MDCK cells. One such protein produced from a selected phage clone blocked binding of M1 to viral RNA. The HuScFv in their *in vivo* functional format, e.g. cell-penetrating molecules, should be developed and tested as a broad spectrum anti-A/influenza.

1. Introduction

The genome of type A influenza viruses comprises 8 RNA segments encoding 11 functionally distinct proteins. The matrix protein-1 (M1), encoded by RNA segment 7, is predominant in the mature virion and relatively conserved among all influenza subtypes (McCauley and Mahy, 1983). It is juxtaposed at the inner surface of the viral lipid envelop which the virion acquired from the host plasma membrane during budding process. M1 is connected to the virus ribonucleoprotein (vRNPs) by binding to the viral RNA (Baudin et al., 2001). The protein has important role in virus replication. During the uncoating process, M1 releases vRNP into the host cytoplasm and permits rapid nuclear import of the vRNP (Martin and Helenius, 1991a). After viral replication, the M1 in conjunction with the viral nuclear export protein (NEP or NS2) encoded by RNA segment 8, transport the newly formed vRNPs from the nucleus to the cytosol (Martin and Helenius, 1991b; Bui et al., 2000). M1 is believed to be essential in releasing the newly formed vRNP from the nuclear matrix (Bui et al., 2000) and preventing nuclear re-import of the vRNP (Whittaker et al., 1996a,b). M1 has been shown also to have a critical role in the virus assembly and budding (Puertas et al., 2000; Nayak and Hui, 2002). Thus, both M1 and the M1-coding RNA segment are attractive targets for drug inhibitors and siRNA (Kawaoka et al., 1990; Hui et al., 2004).

In the present study, human single chain antibody fragments (HuScFv) specific to recombinant M1 of the influenza A virus were produced by using a human antibody phage display library. The HuScFv not only bound to recombinant and native M1 of various influenza A virus subtypes both *in vitro* and in viral infected Mardin–Darby Canine kidney (MDCK) cells *ex vivo*, but also blocked viral RNA binding of the native M1. Data indicate that it might be worth developing these HuScFv further into a cell-penetrating vari-

* Corresponding author. Tel.: +66 2 4196491; fax: +66 2 4196491.
E-mail address: tmwcc@mahidol.ac.th (W. Chaicumpa).

0166-0934/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2009.03.010

ant and testing the possible potential of the preparation as a broad spectrum anti-influenza agent.

2. Materials and methods

2.1. Viruses

The influenza A viruses used in the study were: A/duck/Thailand/144/05 (H5N1) (Songserm et al., 2006a), A/dog/Thailand-Suphanburi/KU-08/04 (H5N1) (Songserm et al., 2006b), H8N4 (isolated in 2006 from asymptomatic duck of Nakhon Pathom province, Thailand), A/swine/Iowa/15/30 (H1N1)-ATCC333, and A/swine/TH/KU-21/04 (H3N2). The H5N1 virus was propagated in embryonated eggs and the other strains were from MDCK cell cultures.

2.2. Preparation of recombinant M1

Total RNA was extracted from A/duck/Thailand/144/05 (H5N1) isolated freshly from allantoic fluids of infected embryonated eggs by using Trizol® reagent (Invitrogen, California, USA). Complementary DNA (cDNA) was synthesized using the Uni-12 primer and RevertAid™ H MinusM-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania). The cDNA was used as a template for PCR amplification of a full length M1 coding sequence (759 bp). The PCR nucleotide primers were designed according to the M1 gene sequence deposited in the GenBank database (accession no. AY626114). BamHI and HindIII endonuclease restriction sites (underlined) were incorporated into the primers, i.e. forward: 5′-AGGATCCGATGAGTCTTCTAAC-3′ and reverse: 5′-TAAGCTTCTTGAATCGCT GCATC-3′, respectively. The PCR program was set sequentially: 94 °C for 10 min, 30 cycles of 94 °C for 90 s, 50 °C for 90 s, and 72 °C for 90 s, and 72 °C for 10 min. The PCR product was ligated into the pGEM®-T Easy vector (Promega, Wisconsin, USA) and transformed into JM109 *Escherichia coli*. The verified M1 gene sequence was subcloned into the pET20b$^+$ expression vector (Novagen, Wisconsin, USA) and the recombinant plasmid was introduced into BL21(DE3) *E. coli*. Full length recombinant M1 was produced from the selected transformed *E. coli* clone grown under IPTG induction and the protein was purified from the bacterial lysate by Ni-NTA agarose (Invitrogen).

2.3. Preparation of mouse and rabbit polyclonal antibodies to recombinant M1

For production of mouse anti-recombinant M1, the protein was subjected to 12% SDS–PAGE using mini-PROTEAN-III cell (Bio-Rad, California, USA). Gel strips containing the Coomassie Brilliant Blue G-250 stained M1 band were excised and minced into tiny gel pieces with a tissue grinder and 500 μl PBS were added. This preparation was mixed (2:1, v/v) with alum (Pierce, Illinois, USA) and injected intramuscularly into 5-week-old BALB/c mice (50 μl per animal). Two boosters were then given at 14-day intervals. Blood samples were collected from individual mice 1 week following the second booster and the sera were pooled (mouse polyclonal anti-M1; mPAb).

A New Zealand white rabbit, weighed ∼2 kg, was injected intramuscularly with purified recombinant M1 (1 mg in 400 μl PBS) mixed with 200 μl alum into both thighs. Two boosters were given at 14-day intervals. Blood was collected 7 days following the second booster and the serum was extracted (rabbit polyclonal anti-M1; rPAb).

Animal experiments were approved by Ethical Committee of Thammasat University, Thailand.

2.4. Preparation of human single chain variable antibody fragments (HuScFv) to recombinant M1

Phage library used in this study consists of a multitude of M13 phages displaying human single chain variable fragments (VH-peptide linker-VL; HuScFv) constructed in our laboratory from immunoglobulin genes of 60 Thai blood donors using pCANTAB5E phagemids and M13KO7 helper phages. This large repertoire library possesses $\sim 2.6 \times 10^8$ antibody diversity. Prior to bio-panning, the library was propagated in TG1 *E. coli* and the bacteria were co-infected with the M13KO7 helper phages to produce complete phages with a titer of $\sim 6 \times 10^{12}$ cfu/ml.

For phage bio-panning, the recombinant M1 was diluted to 1 μg in 100 μl carbonate–bicarbonate buffer, pH 9.6 and used to coat each well of microtiter ELISA plate (Costar, NY, USA) followed by incubation at 37 °C overnight. The wells were then washed with PBS, pH 7.4 containing 0.05% Tween-20 (PBST), and the unoccupied sites were blocked with 200 μl blocking buffer (3% skim milk in PBS) at 25 °C for 1 h. After a further washing step, the phage library ($\sim 3 \times 10^{11}$ particles) was dispensed into each antigen-coated well and plate was incubated for 1 h. Unbound phages were removed by washing with the PBST and the bound particles were eluted from individual wells with 50 μl of 0.1 M glycine–HCl, pH 2.2. The eluted phages were mixed immediately with 3 μl of 2 M Tris, which was followed by adding log phase culture of TG1 *E. coli.* These phage-bacteria mixtures were kept at 25 °C for 1 h to allow the phage transduction. Aliquots were plated onto 2× YT agar containing 100 μg/ml ampicillin and 2% glucose (2× YT-AG). Transformed TG1 *E. coli* with *huscfv*-phagemids were screened by PCR using primers specific to the *huscfv*, i.e. *R1* (forward): 5′-CCA TGA TTA CGC CAA GCT TT-3′ and *R2* (reverse): 5′-GCT AGA TTT CAA AAC AGC AGA AAG G-3′. The expected size of the *huscfv* amplicon was ∼1000 base pairs. The *huscfv*-phagemids were extracted from transformed TG1 *E. coli* colonies and subcloned into non-suppressor HB2151 *E. coli*. HB2151 *E. coli* clones harboring the *huscfv*-phagemids were selected randomly from 2× YT-AG agar plates and appropriate clones were used as factories for the production of soluble HuScFv.

For preparing soluble HuScFv, individually selected HB2151 *E. coli* colonies were grown under 1 mM IPTG induction for 5 h. The bacterial cells were harvested, subjected to sonication and centrifuged. The HuScFv were purified from individual bacterial lysates using DEAE anion exchange column chromatography.

2.5. Restriction fragment length polymorphism (RFLP) of the huscfv

The RFLP patterns of the *huscfv* sequences in the selected HB2151 *E. coli* clones were studied. The *huscfv* sequences were amplified from recombinant phagemids in the transformed HB2151 *E. coli* clones and were digested with MvaI. The DNA-enzyme mixture (25 μl) consisted of 5 units MvaI (Fermentas; 10 units/μl), 2.5 μl 10× buffer red, 0.1 μg of DNA (in 2.5 μl), and 19.5 μl of ultrapure distilled water. Mixtures were incubated at 37 °C for 5 h. The digested DNA was separated in 12 % polyacrylamide gel and stained with ethidium bromide. The DNA banding patterns were visualized under UV transillumination.

2.6. Western blot analysis (WB)

WB was used for detecting recombinant M1 and HuScFv in *E. coli* lysates. Antigenic preparations were separated by 12% SDS–PAGE and the separated components were transblotted onto a nitrocellulose membrane (NCM). Unoccupied areas on the NCM were blocked with blocking solution and probed with HisProbe™-HRP, DCIP + H_2O_2 substrate (for recombinant M1 detection) or mouse monoclonal anti-E-Tag (for HuScFv detection). Anti-mouse