Selection of Neutralizing Human Monoclonal Antibodies against Dengue Virus

การคัดเลือกโมโนโคลนอล แอนติบอดีมนุษย์ที่จำเพาะต่อเชื้อไข้เลือดออกเพื่อป้องกันการติดเชื้อ

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ABSTRACT

In this study, the pool of CHO cells stably expressed the candidate NhuMAb numbers 54, that was prepared form previous study (Pitaksajjakul *et al.*, 2014) will be further selected for high producer subclone using cell sorter. Then, theirs activities of secreting NhuMAb against 4 serotypes of DENV, will be characterized using limiting dilution, and theirs specificity will be checked using immunofluorescence assay (IFA). To test the stability, high producer, stable CHO sub-clones will be cultured in 12 well plates until reached 20 passages, culture supernatants will be collected to determine IgG concentration using quantitative ELISA. Several stable subclones with highest productivity, and show the best stability and functionality will be selected for adaptation cultured from attached cell to suspension cell in serum free medium and further used for large scale production of NhuMAbs.

บทคัดย่อ

การวิจัยนี้ ได้ทำการศึกษา CHO cell ที่สามารถผลิต NhuMAbs โคลน 54 จากการศึกษาที่ผ่านมา โดยคัดเลือกโคลนที่สามารถให้ผลผลิตได้สูงสุด โดยใช้วิธี cell sorter หลังจากนั้น ทดสอบแอนติบอดีที่ได้ ว่าจำเพาะกับเชื้อไข้เลือดออก ทั้ง 4 สายพันธุ์ โดยใช้ทำการ limiting dilution เพื่อคัดเลือก โคลนเดี่ยว แล้วนำมาทดสอบด้วยวิธี immunofluorescence assay (IFA) จากนั้นทำการเลี้ยงเซลล์โคลนเดียวที่ได้ ทั้งหมด 20 passages เก็บ supernatants ไปทดสอบความเข้มข้นของ IgG ที่เก็บได้โดยวิธี quantitative ELISA พบว่ามี2-3โคลน ที่สามารถให้ความเข้มข้นของ IgG สูง หลังจากทำการเลี้ยงทั้งหมด 20 passage จากนั้นทำการปรับไปเลี้ยงในสภาวะ เซลล์ลอย โดยใช้อาหารเลี้ยงที่ปราศจากซีรัม และทำการขยายไปสู่การผลิตในระดับอุตสาหกรรม ต่อไป

Keywords: Dengue virus, Neutralizing human monoclonal antibodies

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Introduction

Dengue fever is the fastest-spreading tropical and vector-borne viral disease throughout the world (WHO Dengue guideline, 2011). The World Health Organisation (WHO) estimates 22,000 deaths per year. Until present, there has neither specific drug nor effective vaccine against all 4 serotypes of dengue virus (DENV) (Williams *et al*., 2013). Neutralizing monoclonal antibodies (MAbs) against all 4 serotypes of DENV has been considered as one alternative for DENV treatment (Fibriensah and Lok, 2016).

Twenty hybridoma cells producing neutralizing full human monoclonal antibody (NhuMAb) has been developed by a researcher team at the Center of Excellent for Antibody Research (CEAR), Faculty of Tropical Medicine (FTM), Mahidol University (MU) (Setthapramote *et al*., 2012; Sasaki *et al*., 2013). All 20 NhuMAbs has 90-100 % neutralizing activity against all 4 serotypes of DENV. Two out of 20 NhuMAbs (clones number 19 and 54) were selected as candidate for further neutralization tested with 20 clinical isolates DENV (5 isolates per each serotype

In this study, the pool of CHO cells stably expressed candidate NhuMAb numbers 54, that was prepared form our previous study (Pitaksajjakul *et al*., 2014) were further selected for high producer subclone using cell sorter. Then, theirs activities of secreting NhuMAb against 4 serotypes of DENV, were characterized using limiting dilution, and theirs specificities were checked using immunofluorescence assay (IFA). To test the stability, high producer, stable CHO sub clones will be cultured in 12 well plates until reached 20 passages, culture supernatants will be collected to determine IgG concentration using quantitative Enzyme-linked immunosorbent assay (ELISA). Several stable sub clones with highest productivity were selected for adaptation cultured from attached cells to suspension cells in the serum free medium and further used for large scale production of NhuMAbs.

Objective of the study

The aim of this study was to select sub clone from candidate NhuMAb numbers 54 and characterize their specificities.

Materials and methods

Stable Chinese hamster ovary (CHO) –K1 cell pool

Stable cell pool of CHO-K1 cells secreting NHuMAb 23-1B3B9(clone 54) was previously established by transfection of heavy chain (HC) and light chain (LC) gene of NHuMAb 23-1B3B9(clone 54), and selection by 2 antibiotics of 800 µg/ml Hygromycin and 8 µg/ml Puromycin. These stable cell lines were cultivated in Minimum Essential Medium Eagles with Earle's Balanced Salts (MEM/EBSS) medium (Hyclone®, USA) plus 10% heat-inactivated FBS (Hyclone®, USA) and MEM Non Essential Amino Acid (MEM/NEAA) (Gibco, Invitrogen, USA), at 37 °C in a humidified 5% CO_2 incubator.

Screening of high-producer stable cells by Fluorescence Activated Cell Sorter (FACS)

Transfected pool of stable CHO-K1 cells secreting NHuMAb 23-1B3B9 from 10 cm dish were trypsinized, and resuspended with 10 ml culture medium. The cell suspension was centrifuged at 1,000 rpm, for 5 mins to pellet the cells. The medium was then aspirated. The cells were resuspended with PBS solution supplemented with 2 mM EDTA

and 10% FBS. The number of cells was counted and 2-4 × 10^6 viable cells/ml each of stained samples (with anti-human Alexa Flour 488 at 1:1000) and unstained samples were washed with PBS/EDTA/FBS for 3 times. Non-overlapping region of stained and unstained samples were gated and sorted based on fluorescence signal detected by the S3e cell sorter (Bio-Rad, USA.). The cells that showed the highest signal in the top 5% were sorted for further culturing

Cell cloning by limiting dilution method

From the sorted stable cell population, a single cell will be cloned by limiting dilution. Limiting dilution was simply performed by counting the cell using hemocytometer (Gibco, USA.). The cell solution was prepared to contain 1 cell/well, and 2 cells/well in 200 µl/well of culture medium containing antibiotics seeded in 96-well cell culture plate. The plates were incubated at 37 ºC, 5% CO_2 for 14 days and observed the wells under light microscope to select wells that shown single original clone. The cultured medium were harvested for IFA to detect anti-DENV antibody secreting positive clones. The positive clones were further scaled up and stocked. The culture supernatant was collected for anti-DENV detection by IFA and IgG quantitation by ELISA. All single stable clones were screened to obtain approximately 5-10 individual clones to proceed for stability test.

Immunofluorescence assay (IFA)

Fifty microliter of culture supernatant of stable CHO cells was added to the well of IFA plate, and incubated at 37 °C for 1 hr. The wells were washed with 150 µl of PBS for 3 times. Goat anti human IgG conjugated with Alexa Fluor® 488 (Invitrogen, USA.) at 1:1000 dilution in PBS was then used as secondary antibody and added to the well, then incubated for 1 hr. at room temperature, and washed again. The positive interaction of anti-DENV with clones showed green fluorescence under Fluorescence microscope (Olympus DP 72, Japan).

ELISA

IgG Quantitative ELISA was used to determine the IgG concentration containing in the culture supernatant obtained from CHO stable cells. The ELISA assay was performed for screen high producers after limiting dilution, and during stability test. Goat anti-Human IgG-Fc fragmented affinity purified coating Antibody (Bethyl, USA) was coated on 96-well plate and incubated at room temperature for 1 hour. The wells were washed 5 times with 300 µl of washing solution followed by of blocking solution at 200 µl/well. Then, the plate was incubated at room temperature for 30 minutes and washed again. Then, 100 µl of culture supernatant and human reference serum (Bethyl, USA) (human reference serum was used as standards, concentration varied form 15.6, 31.25, 62.5, 125, 250, 500) was added to the well, and incubated at room temperature for 1 hr. Next, Goat anti-human IgG-Fc Fragmented HRP-conjugated (Bethyl, USA) (1:150,000), was added and incubated at room temperature for 1 hour, and washed again. To determine interaction signal, 100 µl of Tetramethyl benzidine (TMB) solution (KPL, USA.) was added as a substrate, and put at room temperature for 5-10 mins. For stopping the reaction, 25 µl of H_2SO_4 was added into each well. IgG concentration was meassured by using an ELISA reader (Tecan®, Switzerland).

Results

The CHO stable cells from pool clone number 54 were sorted by Flow cytometry and used limiting dilution to select a single clone, eight sub clones were found as a single clone(Fig.1). Sub clone number 54s-18, 54s- 45, 54s-55, 54s-64, 54s-82, 54s-124, 54s-136, 54s-165 were cultured to the 20th passage, culture supernatant were collected for each passage to checked the IgG yield. Four clones were contaminated in the passage 15th (54s-45, 54s-64, 54s-124, 54s-136) and 4 clones were completed to the passage 20th (54s-18, 54s-55, 54s-82, 54s-165), 8 sub clones (54s-18, 54s-45, 54s-55, 54s-64, 54s-82, 54s-124, 54s-136 and 54s-165) were selected to test with IFA and ELISA.

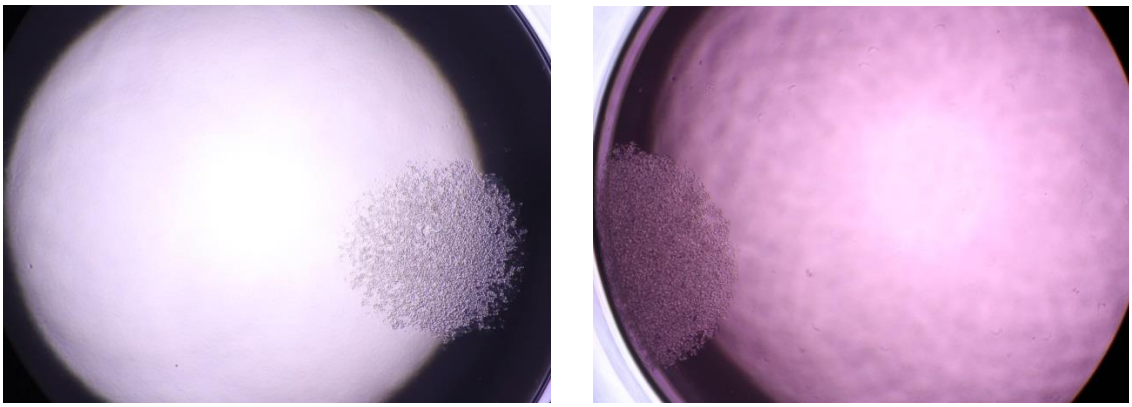

Figure 1 Single clone form limiting dilution. The picture show the single clone in 96 well/plate(A and B)

Culture supernatant form each sub clone were checked for reactivity by using IFA. Eight sub clones were cultured and collected culture supernatants form every passage. IFA test by using Alexa Fluor were shown positive results from all sub clones (Fig. 2)

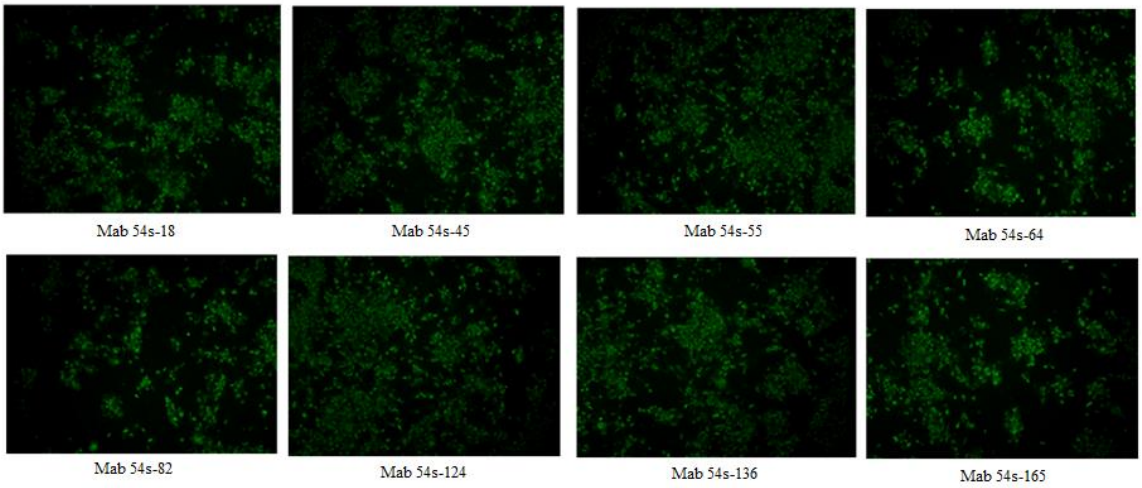

Figure 2 Immunofluorescence assay from single clone

Culture supernatants form the passage 2nd, 5th, 10th, 15th and 20th were measured by quantitation using ELISA. Goat anti-Human IgG-Fc fragmented affinity purified antibody was coated on 96 well plates as the primary Antibody. Human reference serum was used as standards varied to 500, 250, 125, 62.5, 31.25 and 15.625 ng/ml . Goat anti-human IgG-Fc Fragmented HRP-conjugated was used as the secondary antibody. Tetramethyl benzidine (TMB) solution and H2SO4 were added as a substrate and stop solution.IgG concentrations were measured by ELISA reader and calculated to pg/cell (Fig.3), clone 18 and 124 were good candidates with high and more stable concentration (Fig.4).

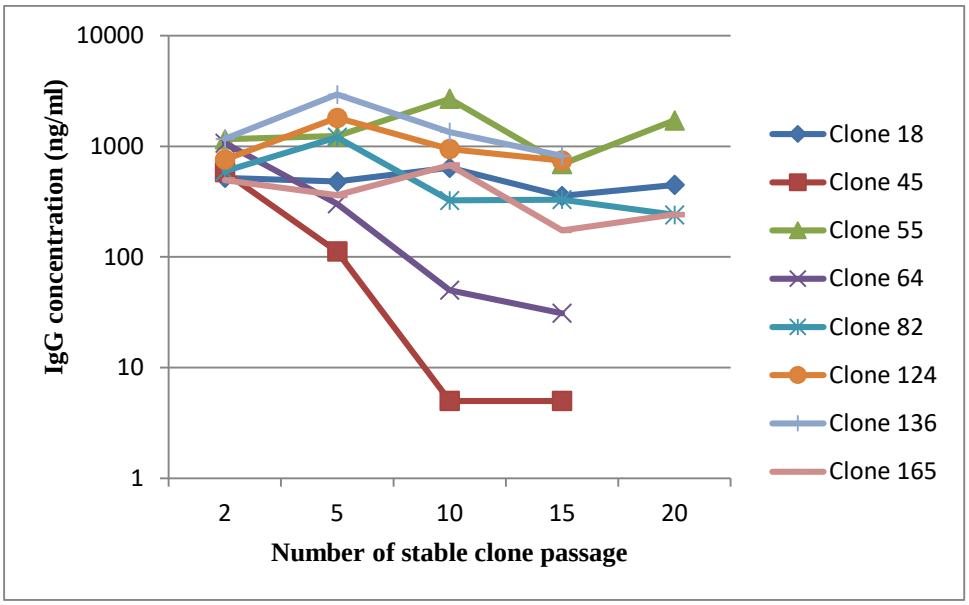

Figure 3 IgG concentrations (ng/ml) of sub clone No.54 sorted (54s-18, 54s- 45, 54s-55, 54s-64, 54s-82, 54s-124, 54s-136, 54s-165) in enzyme-link immunosorbent assay. clone 18, 55 and 124 are the high level concentration, clone 45 and 64 are low concentration.

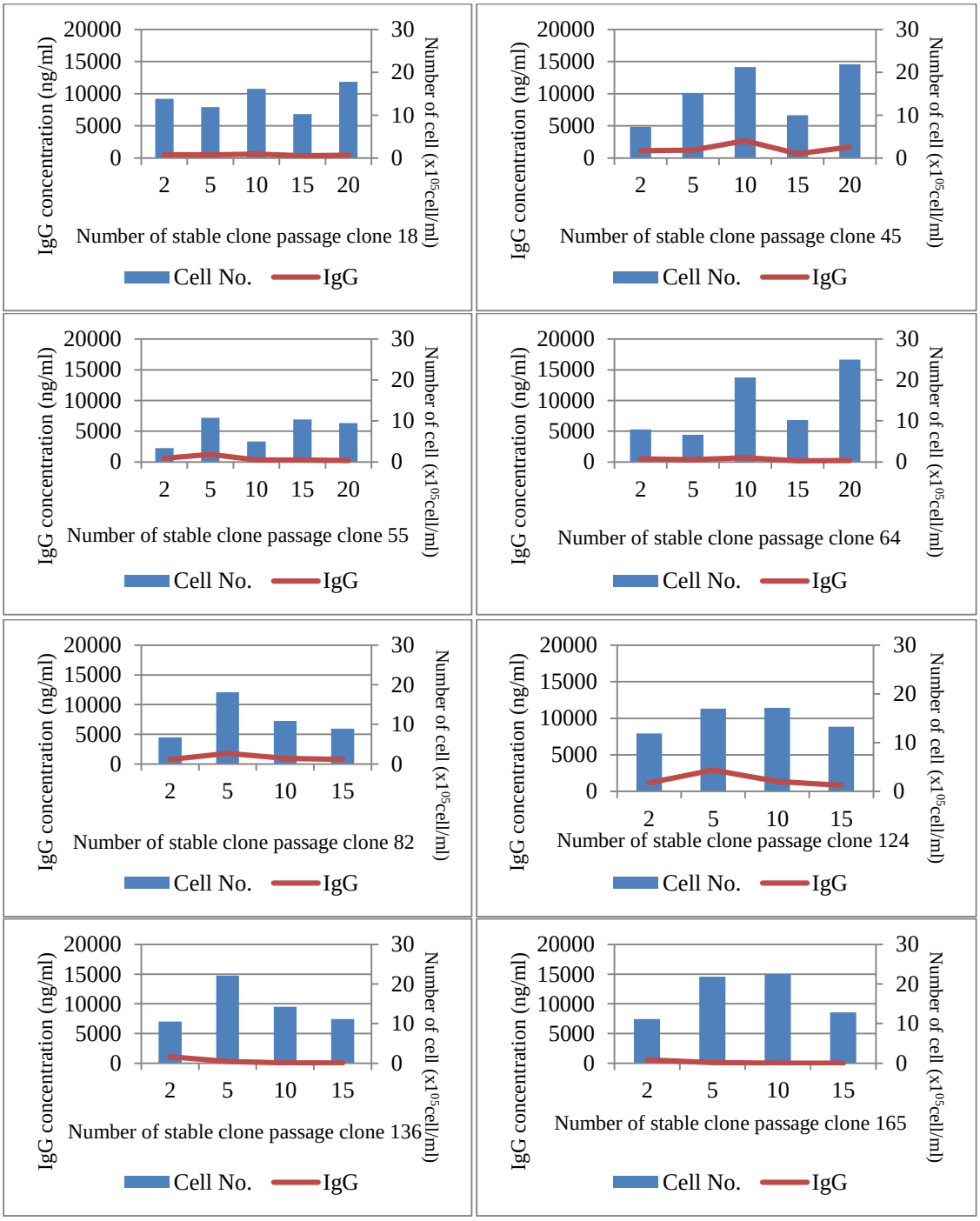

Figure 4 IgG concentration (ng/ml) of stable clone No.54 sorted (54s-18, 54s- 45, 54s-55, 54s-64, 54s-82, 54s-124, 54s-136, 54s-165) compared with number of cell (cell/ml).The picture show clone 18 and 124 are more stable than the other clone

Discussion

In this study we found 2 clones were candidate with high and more stable concentration (clone 18 and 124) when compare with number of cell (cell/ml). The concentrations are low at passage 2, increase at passage 5, 10, 15 and decrease at passage 20. We focus clone number 18 and 124 that have high concentration and more stable when compare with number of cell (cell/ml).

Further studies we will check inserted gene by using real-time PCR and culture 2 clones and adapt to suspension cell.

Acknowledgements

This study was granted by Thailand Research Fund-Master Research Grants: Grants no. MSD57I0140

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