Neutralizing Potential of Fab IgG Hybrid Antibody Against Dengue Virus (DENV-1,2,3,4) Expressed on Mesenchymal Stem Cells

Mezenkimal Kök Hücreler Üzerinde Dengue Virüsüne Karşı (DENV-1,2,3,4) Fab IgG Hibrit Antikorunun Nötralize Edici Potansiyeli

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Cite this article as: Rantam, F.A., Ernawati, R., Rahmahani, J., Hendrianto, E., Susilowati, H., A'la, R., Mayasari, R.K., Aini, L.N., 2018. Neutralizing Potential of Fab IgG Hybrid Antibody Against Dengue Virus (DENV-1,2,3,4) Expressed on Mesenchymal Stem Cells. Acta Vet Eurasia 44: 12-19.

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Abstract

Dengue is an acute mosquito born viral infections in tropical and subtropical countries, that has been steadily increasing every year. Dengue haemorrhagic fever in Indonesia is caused by all 4 viral serotypes termed as DENV-1, DENV-2, DENV-3, and DENV-4. It is very difficult to treat the disease and there is no effective vaccine to eradicate Dengue virus infections .The aim of this research is to express the antibody fragment gene coding VL and VH of Fab IgG antibody in rat bone marrow mesenchymal stem cells (rat BM-MSCs). The genes were isolated from immunized mice using inactive virus of all dengue serotypes. Then genes were hybridized through ligation and insertion into plasmid pBR322, and transfected into rat BMMSCs. To analytically characterize Fab IgG hybrid binding ability, capacity and specificity, we used immune precipitation, western blotting, neutralization assay, and ELISA. All assays suggested that hybrid Fab IgG antibody have high reactivity and affinity, to efficiently neutralize all Dengue virus serotypes 1,2,3,4. Results of this study show that hybrid Fab IgG antibody can be used as neutralizing agent for the treatment of dengue infections in the future.

Keywords: Dengue virus, FabIgG hybrid antibody, generated expression ratBMMSCs, reactivity, neutralization

Öz

Dengue humması, tropik ve subtropikal ülkelerde sivrisineklerden kaynaklanan akut viral bir enfeksiyon olup, her yıl giderek artmaktadır. Endonezya'daki Dang kanamalı ateşine DENV-1, DENV-2, DENV-3 ve DENV-4 adı verilen 4 viral serotip neden olmaktadır. Hastalığın tedavisi çok zordur ve Dang virüsü enfeksiyonlarını yok etmek için etkili bir aşı yoktur. Bu araştırmanın amacı, sıçan kemik iliği mezenkimal kök hücrelerinde (sıçan BM-MSCs) bulunan Fab IgG antikorunun VL ve VH antikor fragmentini kodlayan geninin açığa çıkarılmasıdır. Genler, tüm dang serotiplerinin inaktif virüsü kullanılarak aşılanmış farelerden izole edilmiştir. Sonrasında genler, ligasyon ve plasmid pBR322 içine gömülmesi yoluyla hibridize edilmiş ve sıçan BMMSC'lerine nakledilmiştir. Analitik olarak Fab IgG'nin hibrid bağlama yeteneği, kapasitesi ve özgünlüğünü karakterize etmek için; bağışıklık çökeltmesi, western blotting, nötralizasyon testi ve ELISA kullanılmıştır. Bütün deneyler, hibrid Fab IgG antikorunun tüm Dang virüsü serotipleri 1,2,3,4'ü etkili bir şekilde nötrleştirmek için yüksek reaktiviteye ve afiniteye sahip olduğunu ortaya koymaktadır. Bu çalışmanın sonuçları, melez Fab IgG antikorunun gelecekte dang enfeksiyonlarının tedavisinde nötralize edici ajan olarak kullanılabileceğini göstermektedir.

Anahtar kelimeler: Dengue virüsü, Fab IgG hibrid antikor, sıçan kemik iliği mezenkimal kök hücrelerinin ekspresyonu (ratBMMSC), reaktivite, nötrleştirme

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Received Date: 31 December 2016 • **Accepted Date:** 04 December 2017 • DOI: 10.5152/actavet.2018.004
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Introduction

Dengue virus, a single stranded RNA virus, has four serotypes with different properties and a homology of 60%-70%. In Indonesia, Dengue virus-infected-humans were found in 1968 in Jakarta and Surabaya, Java Island. Dengue then spread into all provinces. Dengue virus was primarily found in rural areas of Indonesia and in densely populated areas predominantly with serotypes 1, 2, and 3. In recent years, however, four serotypes were found in all provinces (Sumarmo, 1987; Putnak, 2003; Ong et al., 2008). Dengue virus has structural and non-structural proteins, like envelope protein (E), pre-membrane protein (PrM), non-structural proteins (NS1, NS3, and NS5) that can induce humoral immune response (Henchal and Putnak, 1990; Putnak et al., 2003). Dengue virus recognizes the target cell using E protein containing epitopes that can bind to the receptors of superficial membrane on the cell. Then, the RNA virus penetrates the cytoplasm of the target cell followed by the transcription, translation, and multiplication of the Dengue virus, a late stage of which releases from the cell. (Lai, et al., 2008). Although Dengue virus has properties with relative stability, in between serotypes has been found easy to displace. This caused the dominant serotype to replace the other serotype (Yamanaka, et al., 2011). Dengue virus has preferential affinity to dendritic cells and Langerhans cells and can produce permissive infection only by immature cells. Interferon types 1 and 2 play an important role during infection (Wu, et al., 2000; Sarathy et al., 2015).

Human infections by Dengue viruses have been increasing every year, especially in all provinces of Indonesia and in different countries with different serotypes (Ong, et al., 2008). Besides population density or environment, the other problem is that it is still difficult to find an effective antiviral agent for the treatment of Dengue virus infections, and vaccine development is still under investigation (Fried, et al., 2010). Dengue virus has four serotypes and each have different properties. Some of their proteins can induce protecting antibodies but do not constitute cross protection (Rothman, et al., 2010). On the other hand, during infections, immunoglobulins (Ig) play an important role in protecting people against infection. In this context, immunoglobulin G (IgG) has a special property as a neutralizing antibody in the Dengue virus infection. IgG antibodies are generated following class switching and maturation of the antibody response and thus participate predominantly in the secondary immune response (Meulenbroek and Zeijlmer, 1996; Rantam, et al., 2013). Based on these arguments, the IgG can be used for hyper immune therapy. This research was designed to produce IgG under an expression system using rat bone marrow mesenchymal stem cells (ratBMMSCs, Wistar rat strain). IgG can be secreted as a monomer that is small in size allowing it to easily perfuse tissues. IgG has a molecular weight of about 150 kDa and is made up of four peptide chains. It has two kinds of identical light chains which are 50 kDa and 25 kDa. Thus, a tetrameric quaternary structure makes it very easy to recognize Dengue virus epitopes with serotype specificity (Bielifeldt-Ohmann, et al., 2001). As such, extracted IgG hybrids can be used for the therapy of future Dengue virus infections. This research serves to express the fragment gene coded VL and VH of the FabIgG antibody in rat bone marrow mesenchymal stem cells (ratBM-MSCs).

Materials and Methods

This study was performed in the DHF Laboratory, Institute of Tropical Disease and Stem Cell Research and Development Center in 2015 and 2016 at Airlangga University, Surabaya, Indonesia. The Dengue virus was from DHF Laboratory, Institute of Tropical Disease Airlangga University, strain ITD UNAIR serotype 1,2,3 and 4.

Mesenchymal stem cells

The bone marrow mesenchymal stem cells (ratBMMSCs) were isolated from the short-term culture of the Wistar rat bone marrow. We performed stem cell isolation from the Wistar rat bone marrow in the stem cell laboratory of Airlangga University under the supervision of the animal ethics team of the veterinary faculty of Airlangga University. To maintain these cells, they were cultured using α-MEM (GIBCO, 12000-022) with 10% Fetal Bovine Serum (FBS) (GIBCO, 10270-106) (Rantam, et al., 2009). After seven passages, these cells were used to express a model of the FabIgG hybrid. F the reactivity assay of the hybrid product to DENV-1,2,3,4, vero cells (kindly supplied from Dr. Eric Van Gorp, Institute of Viroscience, Erasmus Medical Center, Rotterdam University) were used.

Primers

Fusion fragment genes of each Fab-VH and LH of IgG to E protein of DENV-1, 2, 3, and 4 were prepared by PCR methods using various specific primers. Our primer design with reference from NCBI with access number ab073322 for heavy chain and bc094049 for light chain, using BLAST primer at NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). For Fab-IgG, amino acids 200 residue was amplified from the lymphocytes of mice, which were immunized by mixing (70% adjuvant, 20% antigen, 10% water) the inactive virus strain ITD UNAIR serotype 1, 2, 3 and 4 with Montanide™ ISA 70 VG adjuvant (SEPPIC).

Construction of transfer vector for fusion of heavy and light chains' antibody genes

The template encoding the chimeric construct was selectively amplified using external primers (T3 Promoter, Forward primer GCAATTAACCCTCACTAAAGG, T7 promoter, forward primer TAATACGACTCACTATAGGG, T7 terminator, reverse primer GCTAGTTATTGCTCAGCGG). Each primer contains appropriated restriction sites (Amp and tet) for sub cloning into the GC5 vector as competent cells. The resulting PCR fragment genes were ligated into the pBR322 (Geneon, Germany, G30-325), and transferred into ratBMMSCs, which were selected. Next, the hinge, CH2 and CH3 domains (amino acid 200 residue) of rat

Table 1. Primers used to amplify the gene-coded immunoglobuline G

Code	Sequence nucleotide	Function
Forward (F)	5'-TGGGGGTTCTCTGAGTCTCTC-3'	Primers for synthesis of cDNA
Reverse (R)	3'- CAGCTCTCAGGGCATTCATT-5'-	
FGH1	5'-AGGTTCAGCTTCAGCAGTCT-3'	Primers specific for heavy chain
RGH1	3'-CGTAGTAAAAATCCTTTGAACAGTA-5'	
FGH2	5'TGAAGAACTCTGCCGTCTATT-3'	
RGH2	3'-GCACAATTTTCTTGTCCACT-5'	
FGL1	5'-GTACCATCTTCCCACCATC-3'	Primers specific for light chain
RGL1	3'-CTCATTCCTGTTGAAGCTCT-5	
FGL2	5'- GTCCAGTCAGAGCCTTTTAT-3'	
RGL2	3'- TGGGGTAGAAGTTGTTCAAG-5'	
FGL3	5'- GCCCAGGTTCTTATGTTACT-3'	
RGL3	3'- GAGAGTGAAATCTGTCCCAG-5'.	
FGH1	5'-AGGTTCAGCTTCAGCAGTCT-3'	Primers for second round PCR
RGH1	3' CGTAGTAAAAATCCTTTGAACAGTA-5'	

FabIgG were amplified by RT-PCR from cells ratBMMSCs, using the following primers FGH1 and RGH1, as shown in Tabel 1. This PCR product was cloned and inserted into ratBMMSc by using the method modified from Liang and others (1997).

Purification of hybrid FabIgG

A modified method (Kihira and Aiba 1992; Leickt, et al., 1998; Huse, et al., 2002) was used to purify the FabIgG hybrid. Briefly, after the centrifugation of the supernatant of lysed cells at 20,000 × g (13,000 rpm in SS-34 rotor) at 4°C, the supernatant was filtered using a 0.45-µm filter. Then, the pH of the MAb supernatant was adjusted to 8.0 by dialyzing against PBS using dialysis tubing (Serva, 44144.01). In the meantime, the protein A–Sepharose column was prepared and attached to the fraction collector, and then the column was equilibrated with PBS, pH 8.0, at room temperature. A layer antibody solution was washed into the resin bed column with several volumes of PBS, pH 8.0. It was then eluted with 0.1 M citric acid at pH 6.5. The eluent was collected in vials and dialyzed against PBS, pH 7.3 and was then used for analysis.

Enzyme linked immunosorbent assay (ELISA)

This method was used to analyze the antibody titer of the secreting FabIgG hybrid. After coating the ELISA plate with the Dengue virus antigen 10^7, with each volume well at 100µl, the plate was then incubated for 24 hour at 4°C. It was then washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS-T containing 3% of skimmed milk for 1 hour at room temperature. After washing it three times with PBS-T, 100 µL of the two fold dilutions of ratBMMSc culture supernatants were added to the wells. The plates were then incubated for 1 hour at 37°C and washed four times with PBS-T. In the next step, 100 µL of 1:1600 dilution of HRP conjugated antimouse FC specific antibody (Invitrogen, 31430). The plate was then incubated for 1 hour at 37°C. After washing, 100 µL of substrate (Invitrogen, 31430) was added and incubated at room temperature for 15 min. Finally, the absorbance was measured using an ELISA reader at 450 nm (Immuno Mini NJ-2300) (Chu, et al., 1994).

Immunoprecipitation of the FabIgG hybrid binding to E protein of Dengue virus

The immunoprecipitation was used to analyze reactivity between the epitop and the FabIgG hybrid expressed on ratBMMSCs. Briefly, about $2x10^7$ cells in 0.5 ml DMEM were on ice for 1 hour to maintain cells so as not to fragment. Then, cells were washed with PBS and lysed by 0.5 ml of ice using an NP-40 lysis buffer (50 mM Tris-buffer pH 7.4, 250 mM NaCl, 5mM EDTA, 0.1% NP-40, plus protease inhibitors) (Merck). After removing the insoluble cell debris by microcentrifugation (11,500 rpm for 1 hour at 4°C), the FabIgG hybrids were precipitated by protein G conjugated sheparose beads (Amersham Bioscience, 212-006-168) overnight. The beads were then washed with PBS and re-suspended with a loading buffer containing 0.1M DTT, and heated for 10 min at 75°C. They were then resolved in 10% NuPAGE (Invitrogen, NP0001) with a Morpholinepropanesulfonic acid (MOPS) running buffer. Finally, the FabIgG hybrid was mixed with a whole antigen of Dengue virus (strain ITD UNAIR serotype 1, 2, 3 and 4) and after that was immunoprecipitated with an anti-FabIgG hybrid (Yoshida, et al., 2010).

Neutralizing of FabIgG hybrid to E protein in cell culture

Neutralization assay was performed to determine the neutralizing capacity of purified the FabIgG hybrid. Briefly, the FabIgG hybrid was incubated with 10^3 PFU of the whole Dengue virus (strain ITD UNAIR, DENV-1, 2, 3 and 4). A mixture of the FabIgG hybrid and the virus were diluted equally with a ratio of 300µl After a 1-hour incubation at room temperature, they were then

Figure 1. Prototype of the FabIgG hybrid specific envelope protein of Dengue virus (DENV-1, 2, 3, and 4) infected ratBMMSCs. By using pBR322-plasmid

Table 2. Neutralizing activity of the FabIgG hybrid to all Dengue virus serotypes

Reagent	Dilution	Focus Forming Unit (FFU) (mean of reduction)
Vaccinated positive control serum	1:1	0 (100)
Negative control serum	1:1	100 (0)
The FabIgG hybrid	1:1	0 (100)
	1:2	10
	1:4	21
	1:8	41
	1:16	81

Data Focus Forming Unit assay using Dengue virus serotypes 1, 2, 3, and 4 have shown the average of duplicate assays. The FabIgG hybrid was diluted as shown in Table 2.

inoculated to the cell target by ratBMMSc derived from bone marrow stem cells, followed by another incubation at 37°C in a 5% CO_2 incubator for one hour. After the removal of the medium, a growth medium was added and incubated for 6 days at 37°C in a 5% CO_2 incubator. Finally, Dengue viruses released from infected cells were detected by indirect ELISA using conjugated FabIgG labeled with HRP (Invitrogen, SA5-10226). These activities were analyzed using a binding index of virus

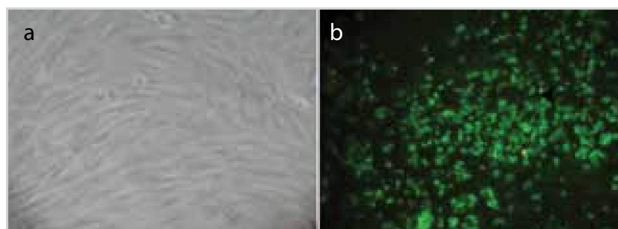

Figure 2. a, b. The characterization and culturing of bone marrow mesenchymal stem cells (ratBMMSCs). (a) Cells are already growing 90%. (b) characterization of cells using immunocytochemistry stained with CD105 labeled with FITC

to ratBMSCs, and used Immunoassay. Abstractly cells were infected by strain ITD UNAIR Dengue virus; all serotype then reactivated using hybrid immunoglobulin and then staining using HRP (Pauli, et al., 1984). The neutralizing ability was used and compared with vero cells as positive control.

Results

Characterization of Bone Marrow - Mesenchymal Stem Cells (ratBMMSCs)

The ratBMMSCs cells were expanded in the Petri dish until they grow confluents about 90% as seen in Figure 1 and were char-

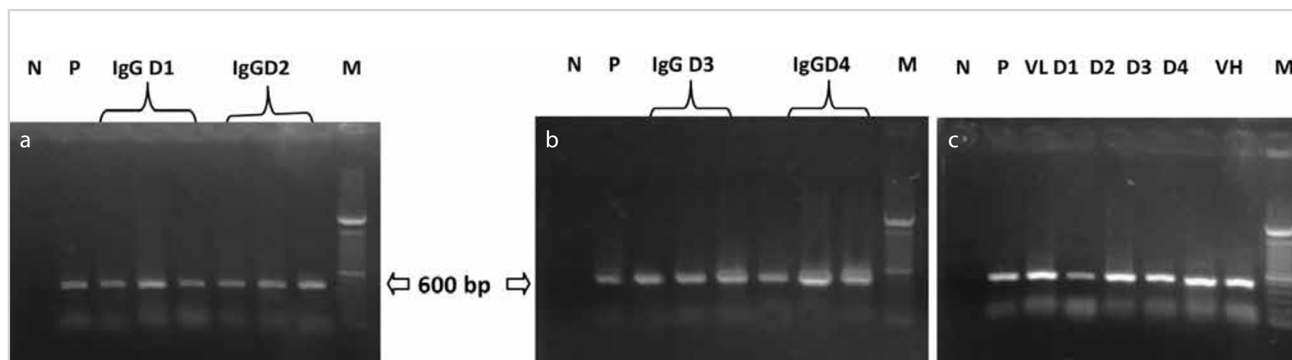

Figure 3. a-c. PCR product of a fragment gene coded FabIgG -D1, 2, 3, 4 VH and VL. (a) PCR product of a fragment gene coded FabIgG D1, D2; (b) PCR product of a fragment gene has coded FabIgG D3, D4; (c) PCR product of a fragment gene has coded FabIgG D1, D2, D3, and D4 of VL

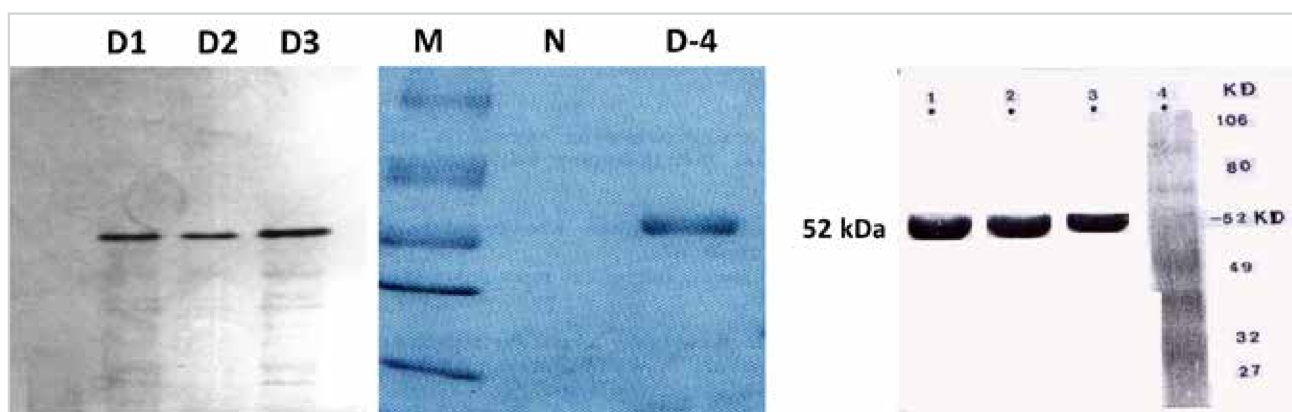

Figure 4. Electrophoresis analysis product of the FabIgG hybrid D1, 2, 3, and 4. A. Product electrophoresis was stained by using Silver stain (AgNO3). B. Product electrophoresis was stained by using Commasie blue. C. Immunoprecipitation of protein E.

acterized using CD105. The results were seen as in Figure 2: a characterization using CD105 was done to make sure the cell was a stem cell because CD105 are cell surface markers for Mesenchymal Stem Cells. The PCR product of the gene coded FabIgG as well, as in Figure 3, and showed 600 bp that were used to design the FabIgG hybrid expressed in ratBMMSCs.

The cloning product of the gene fragment of FabIgG DENV 1, 2, 3 and 4 was inserted into the plasmid pBR322, and was then transfected into ratBMMSc cells by using electrophorator. The results of FabIgG expression are shown in Figure 4.

The results of the purification of the FabIgG hybrid, those expressed in ratBMMSCs and analyzed using a western blot are shown in Figures 4A, B and C. The FabIgG hybrid showed a nearly identical pattern of protein bands under reduced conditions. The protein was found to have a molecular weight of 52 kDa, and seemed like a dominant band, which likely represents the monomer of the FabIgG hybrid in Figure 5.

Based on the reactivity of the FabIgG hybrid of DENV-1, 2, 3 and 4 to E protein, we designed an analysis on the neutralization effect of the FabIgG hybrid expressed using the ratBMMSCs model. The results suggested that the FabIgG hybrid can neutralize the Dengue virus serotypes 1, 2, 3 and 4 infected with ratBMMSCs. The whole of the reduction plaque neutralization test (PNRT) potential of IgG hybrid in cells is shown in Figure 6 and Figure 7.

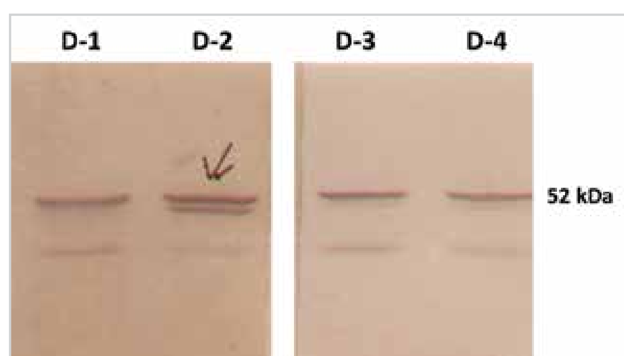

Figure 5. A western blotting analysis of E protein of Dengue virus with different serotypes of DENV-1, 2, 3, and 4 then reacted with the FabIgG hybrid, and was stained by using Pierce

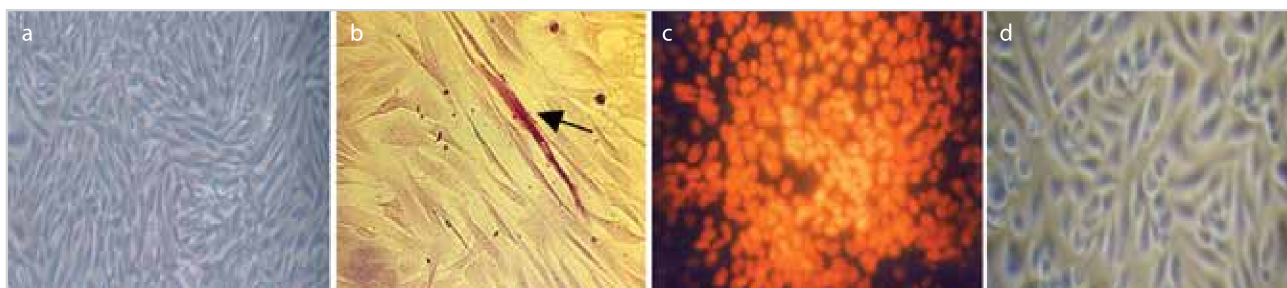

Figure 6. a-d. Neutralization assay using ratBMMSc. The purification of the FabIgG hybrid was performed using IgG-Sepharose affinity chromatography and then reacted with Dengue virus. After 1 hour it was incubated at room temperature and then inoculated into ratBMMSc. Three days after incubation in a 5% CO_2 incubator, prepared cells were analyzed by ELISA. (a) Normal ratBMMSCs. (b) ratBMMSCs inoculated with DENV-1, 2 and DENV-3, 4 with the FabIgG hybrid. (c) Vero cells inoculated with DENV-1, 2 and DENV-3, 4, and with the FabIgG hybrid. (d) Normal vero cells.

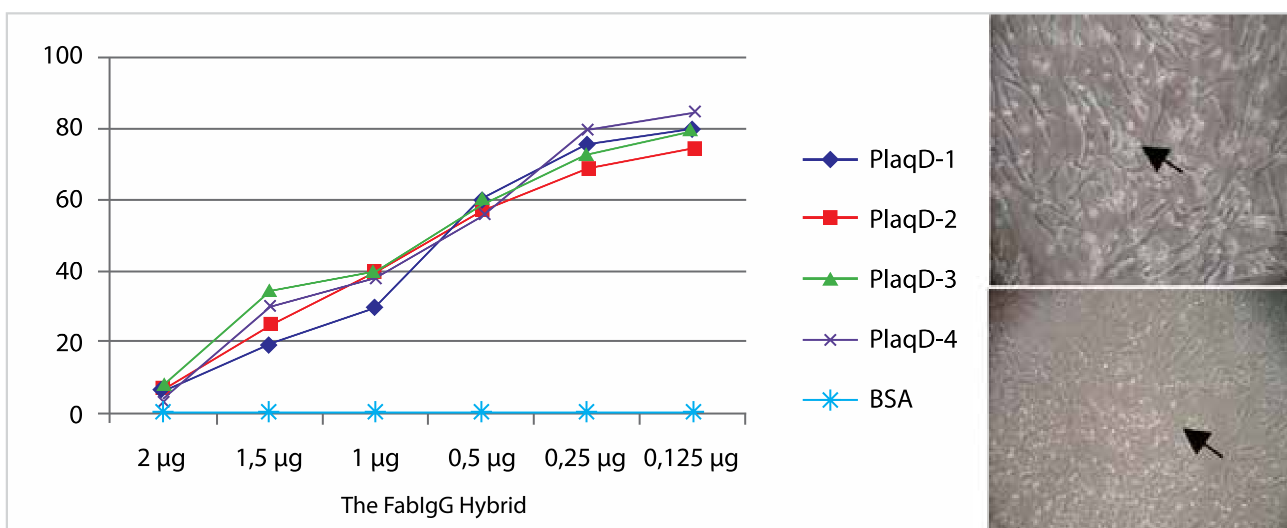

Figure 7. Comparison of the neutralizing properties of the FabIgG by plaque neutralization tests using ratBMMSCs to DENV-1, 2, 3, and 4 serotypes. The neutralization results using vero cells not shown in this diagram. Two-fold dilutions of the affinity of purified FabIgG collected from the supernatant of culture ratBMMSCs assayed by plaque reduction neutralization tests. BSA is a negative control. A. DENV-1, 2, 3, and 4 inoculated vero cells. B. DENV-1, 2, 3, and 4 inoculated ratBMMSCs

Discussion

In this study, we characterize the ratBMSCs as shown in Figure 2, and the generated transformed ratBMMSCs that expressed the immunoglobulin (FabIgG) hybrid specific serotype of DEN-1, 2, 3, 4 as shown in Figure 1. The prototype of the hybrid showed that the coding gene of VH and LH immunoglobulin were cloned and ligated with others, that we called the FabIgG hybrid, but the mechanism of integration of genes into the ratBMMSc cells genome was not characterized. Although, the genome of ratBMMSCs may enhance transcription, thereby improving the yield of foreign proteins obtained from transformed cell cultures.

The other tests performed in this research demonstrated a molecule of FabIgG using SDS-PAGE 12% with a molecular weight of 52 kDa as seen in Figure 4. They then analyzed the reactivity of the hybrid using Western blotting as shown in Figure 5. This reactivity showed that the FabIgG hybrid has a good function since the immunoglobulin hybrid can capture antigens of Dengue virus. These properties of the FabIgG hybrid can recognize antigen especially envelope (E) of all Dengue virus serotypes, because they were designed four hands powerful to capture of different serotypes like in Figure 6. That means these hybrids have potential for the development of passive vaccines for all serotypes of Dengue virus. However, the envelope (E) protein is a major antigen for inducing protective antibody (Ab) responses (Halstead, 1998; 2003) and can avoid the risk of Antibody-Dependent Enhancement (ADE). The strength of both molecule binding sites is in the Fab of variable heavy (VH) and light (VL). This way, they have the possibility of a very easy way to build an interaction with an antigen-antibody complex, which will stimulate immediate reactivity of macrophage to opsonisation. Then, peptide

expression will follow on the superficial membrane cell through the major histocompatability complex I (MHC I), and then induce cell-mediated immune response through an endogenous process (Kehry and Castle, 1994). This mechanism may play an important role as a basic how-to design for a preventive model like a blocking agent (de Carvalho Nicacio, et al., 2000; Gigler et al., 1999; Gould et al., 2005).

If we compare the FabIgG hybrid and the active vaccine tetravalent, the hybrid cannot induce polyclonal antibodies, nor generate an immune system. The hybrid FabIgG, however, have to neutralize viral infections by binding to epitops of E protein of Dengue virus. This model has two kinds of functions. The first is a signaling of complex proteins to activation of cellular and humoral immune response through expression of tool-like receptors (TLR) on the surface membrane of macrophage. This pathway can usually provoke the activation of the $CD4^{+}$ T cell through MHC-II of antigen presenting cells (APC). Secondly, it can be used as neutralizing agent to prevent viral infection. Although, the effectiveness of the hybrid FabIgG is still in trial for certain pathogens (like HIV and Salmonella) (Abaitua, et al., 2006; Tagliabue and Rappuoli, 2008).

The results of the ability of the FabIgG hybrid in neutralizing have shown that the hybrid is good at working with this cell, although the neutralizing level still less then using polyclonal antibodies (data not shown). Based on this data, there is still a need to establish a hybrid model expression using a ratB-MMSCs cell system, although we have seen that they can work very well. On the other hand, the goal in this study here would be to provide an antibody that can protect immediately, although all theFabIgG hybrids show similar protective effects at local infection sites or in DENV-infected cell culture.

In conclusion, we have shown in this study that the neutralizing effect provided by the FabIgG hybrid against Dengue virus-infected rat bone marrow mesenchymal stem cell (ratBMMSCs) model do not cross-react with uninfected mesenchymal stem cells. Our findings suggest that the FabIgG hybrid may be an attractive strategy for developing a vaccine against Dengue virus as well as passive vaccine.

Acknowledgements: We are grateful to Dr. Eric Van Gorp, for helpful discussions and for providing Vero cells. Also to our team at Stem Cell Research and Development Center, Laboratory of DHF, Institute of Tropical Disease (ITD). This work was supported by grants from the RUPT Airlangga University (DIPA Ditlitabmas at 2015 No. 519/UN3/2015, March, 26, 2015 and No.018/SP2H/LT /DRPM/II/2016, February, 17, 2016.

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