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ACTA VETERINARIA EURASIA
Formerly Journal of The Faculty of Veterinary Medicine İstanbul University

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Acta Veterinaria Eurasia (Acta Vet Eurasia) is an international, scientific, open access periodical published in accordance with independent, unbiased, and double-blinded peer-review principles. The journal is the official publication of İstanbul University Faculty of Veterinary Medicine and published biannually on January and July. The publication languages of the journal are English and Turkish.

Acta Veterinaria Eurasia (Acta Vet Eurasia) aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of veterinary medicine. The journal publishes original articles, reviews, case reports, short communications, and letters to the editor that are prepared in accordance with the ethical guidelines.

The scope of the journal covers all animal species including the topics related to basic and clinical veterinary sciences, raising livestock, veterinary genetics, animal nutrition and nutritional diseases, zooneses, veterinary medicinal products and public health, and food hygiene and technology.

The target audience of the journal includes specialists and professionals working and interested in all disciplines of veterinary medicine.

The editorial and publication processes of the journal are shaped in accordance with the guidelines of the International Committee of Medical Journal Editors (ICMJE), World Association of Medical Editors (WAME), Council of Science Editors (CSE), Committee on Publication Ethics (COPE), European Association of Science Editors (EASE), and National Information Standards Organization (NISO). The journal is in conformity with the Principles of Transparency and Best Practice in Scholarly Publishing (doaj.org/bestpractice).

Acta Veterinaria Eurasia is currently indexed in Web of Science- Zoological Records, Scopus, DOAJ, Embase, Gale, AgBiotechNet, Animal Breeding Abtracts, Animal Science Database, CAB Abstracts, Dairy Science Abstract, Helmintological Abstracts, Index Veterinarius, Nutrition Abstracts and Reviews Series B:Livestock Feeds, Nutrition and Food Database, Parasitology Database, Poultry Abstracts, Review of Medical and Veterinary Mycology, Tropical Diseases Bulletin, Veterinary Bulletin, VetMed Resource, TUBITAK ULAKBIM TR Index.

All expenses of the journal are covered by the of İstanbul University Faculty of Veterinary Medicine. Processing and publication are free of charge with the journal. No fees are requested from the authors at any point throughout the evaluation and publication process. All manuscripts must be submitted via the online submission system, which is available at http://dergipark.gov.tr/iuvfd. The journal guidelines, technical information, and the required forms are available on the journal's web page.

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Instructions to Authors

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Originality, high scientific quality, and citation potential are the most important criteria for a manuscript to be accepted for publication. Manuscripts submitted for evaluation should not have been previously presented or already published in an electronic or printed medium. The journal should be informed of manuscripts that have been submitted to another journal for evaluation and rejected for publication. The submission of previous reviewer reports will expedite the evaluation process. Manuscripts that have been presented in a meeting should be submitted with detailed information on the organization, including the name, date, and location of the organization.

Manuscripts submitted to Acta Veterinaria Eurasia will go through a double-blind peer-review process. Each submission will be reviewed by at least two external, independent peer reviewers who are experts in their fields in order to ensure an unbiased evaluation process. The editorial board will invite an external and independent editor to manage the evaluation processes of manuscripts submitted by editors or by the editorial board members of the journal. The Editor in Chief is the final authority in the decision-making process for all submissions.

An approval of research protocols by an Animal Ethics Committee in accordance with international principles is required for experimental, clinical and drug studies and for some case reports that are carried out on animals. If required, ethics committee reports or an equivalent official document will be requested from the authors. For studies carried out on animals, the measures taken to prevent pain and suffering of the animals should be stated clearly. The name of the ethics committee, and the ethics committee approval number should also be stated in the Materials and Methods section of the manuscript. For studies involving client-owned animals, author's must provide the information on informed consent from the client or the owner and adherence to a high standard (best practice) of veterinary care. The editor has the right to reject manuscripts on suspicion of animal welfare or research protocols that are not consistent with the international principles of animal research. For studies conducting questionnaire methodology should also require ethical approval and a statement should be included that shows that written informed consent of participants and volunteers was obtained following a detailed explanation of the study. The scientific and ethical responsibility of the research belongs to the authors.

All submissions are screened by a similarity detection software (iThenticate by CrossCheck).

In the event of alleged or suspected research misconduct, e.g., plagiarism, citation manipulation, and data falsification/fabrication, the Editorial Board will follow and act in accordance with COPE guidelines.

Each individual listed as an author should fulfill the authorship criteria recommended by the International Committee of Medical Journal Editors (ICMJE - www.icmje.org). The ICMJE recommends that authorship be based on the following 4 criteria:

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
2. Drafting the work or revising it critically for important intellectual content; AND
3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

In addition to being accountable for the parts of the work he/she has done, an author should be able to identify which co-authors are responsible for specific other parts of the work. In addition, authors should have confidence in the integrity of the contributions of their co-authors.

All those designated as authors should meet all four criteria for authorship, and all who meet the four criteria should be identified as authors. Those who do not meet all four criteria should be acknowledged in the title page of the manuscript.

Acta Veterinaria Eurasia requires corresponding authors to submit a signed and scanned version of the authorship contribution form (available for download through http://dergipark.gov.tr/iuvfd) during the initial submission process in order to act appropriately on authorship rights and to prevent ghost or honorary authorship. If the editorial board suspects a case of "gift authorship," the submission will be rejected without further review. As part of the submission of the manuscript, the corresponding author should also send a short statement declaring that he/she accepts to undertake all the responsibility for authorship during the submission and review stages of the manuscript.

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Manuscripts can only be submitted through the journal's online manuscript submission and evaluation system, available at http://dergipark.gov.tr/iuvfd. Manuscripts submitted via any other medium will not be evaluated.

Manuscripts submitted to the journal will first go through a technical evaluation process where the editorial office staff will ensure that the manuscript has been prepared and submitted in accordance with the journal's guidelines. Submissions that do not conform to the journal's guidelines will be returned to the submitting author with technical correction requests.

Authors are required to submit the following:

- Copyright Transfer Form,
- Author Contributions Form, and
- ICMJE Potential Conflict of Interest Disclosure Form (should be filled in by all contributing authors) during the initial submission. These forms are available for download at http://dergipark.gov.tr/iuvfd.

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Title page: A separate title page should be submitted with all submissions and this page should include:

- The full title of the manuscript as well as a short title (running head) of no more than 50 characters,
- Name(s), affiliations, highest academic degree(s), and ORCID ID(s) of the author(s),
- Grant information and detailed information on the other sources of support,
- Name, address, telephone (including the mobile phone number) and fax numbers, and email address of the corresponding author,
- Acknowledgment of the individuals who contributed to the preparation of the manuscript but who do not fulfill the authorship criteria.

Abstract: A Turkish and an English abstract should be submitted with all submissions except for Letters to the Editor. Submitting a Turkish abstract is not compulsory for international authors. The Abstract section of all types of articles should be unstructured. Please check Table 1 below for word count specifications.

Keywords: Each submission must be accompanied by a minimum of three to a maximum of six keywords for subject indexing at the end of the abstract. The keywords should be listed in full without abbreviations.

Manuscript Types

Original Articles: This is the most important type of article since it provides new information based on original research. The main text of original articles should be structured with Introduction, Materials and Methods, Results, and Discussion subheadings. Please check Table 1 for the limitations for Original Articles.

Statistical analysis to support conclusions is usually necessary. Statistical analyses must be conducted in accordance with international statistical reporting standards (Altman DG, Gore SM, Gardner MJ, Pocock SJ. Statistical guidelines for contributors to medical journals. Br Med J 1983: 7; 1489-93). Information on statistical analyses should be provided with a separate subheading under the Materials and Methods section and the statistical software that was used during the process must be specified.

Units should be prepared in accordance with the International System of Units (SI).

Review Articles: Reviews prepared by authors who have extensive knowledge on a particular field and whose scientific background has been translated into a high volume of publications with a high citation potential are welcomed. These authors may even be invited by the journal. Reviews should describe, discuss, and evaluate the current level of knowledge of a topic in the field and should guide future studies. The main text of review articles should be begun with an Introduction section and finalized with a Conclusion section. The remaining parts can be named relevantly to the essence of the research. Please check Table 1 for the limitations for Review Articles.

Case Reports: There is limited space for case reports in the journal and reports on rare cases or conditions that constitute challenges in diagnosis and treatment, those offering new therapies or revealing knowledge not included in the literature, and interesting and educative case reports are accepted for publication. The text should include Introduction, Case Presentation, and Discussion subheadings. Please check Table 1 for the limitations for Case Reports.

Short Communications: Short communications are the narrow-scoped research articles that provides new scientific information. These types of articles should be prepared in the original article format and contain Introduction, Materials and Methods, Results, and Discussion subheadings.

Letters to the Editor: This type of manuscript discusses important parts, overlooked aspects, or lacking parts of a previously published article. Articles on subjects within the scope of the journal that might attract the readers' attention, particularly educative cases, may also be submitted in the form of a "Letter to the Editor." Readers can also present their comments on the published manuscripts in the form of a "Letter to the Editor." Abstract, Keywords, and Tables, Figures, Images, and other media should not be included. The text should be unstructured. The manuscript that is being commented on must be properly cited within this manuscript.

Table 1. Limitations for each manuscript type

Type of manuscript	Word limit	Abstract word limit	Reference limit	Table limit	Figure limit
Original Article	3500	250 (Structured)	No limit	6	7 or tatal of 15 images
Review Article	5000	250	50	6	10 or total of 20 images
Case Report	1000	200	15	3	10 or total of 20 images
Short Communication	1500	200	15	5	5 or total of 10 images
Letter to the Editor	500	No abstract	5	No tables	No media

Tables

Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

Figures and Figure Legends

Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

All acronyms and abbreviations used in the manuscript should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

The Latin scientific names of a species should be written in italics. Apart from the names of species, italicization should be avoided as much as possible.

When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Chelex-100 (BioRad, California, USA)"

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text.

Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

References
While citing publications, preference should be given to the latest, most up-to-date publications. In the main text of the manuscript, references should be cited by author's name and the publication year in parenthesis. In the case of direct citations in the main text, only publication year should be stated in parenthesis after the name of the author. Please see below for examples:

For single authored reference: (Bell, 2005)

For double authored reference: (Nielsen and Engberg, 2006)

For direct citation in a sentence: "According to Bell (2005)……." or "According to Nielsen and Engberg (2006)……."

Reference with multiple authors: (Doyle et al., 2007)

For multiple references, in order of year: (Bell, 2005; Bell, 2008; Doyle et al., 2007; Nielsen and Engberg, 2006; Willis and Murray, 1997)

For references with the same author and year: (Bell, 2005a; Bell, 2005b; Bell, 2005c)

Reference List
The list of references should only include works that are cited in the text and that have been published or accepted for publication. The references must be listed alphabetically according to the last name of the author. The author names and the publication year should be written in bold. Journal titles should not be abbreviated. If an ahead-of-print publication is cited, the DOI number should be provided. Authors are responsible for the accuracy of references.

The reference styles for different types of publications are presented in the following examples:

Journal Article: **Cohen, N.D., Vontur, C.A., Rakestraw, P.C., 2000.** Risk factors for enterolithiasis among horses in Texas. Journal of the American Veterinary Medical Association 216, 1787-1794.

Book Section: **Kramer, J.M., Gilbert, R.J., 1989.** Bacillus cereus. In: Doyle, M.P. (Ed.), Foodborne Bacterial Pathogens. Marcel Dekker, New York, pp. 22-70.

Books with a Single Author: Combs, G.F., 1992. The Vitamins: Fundamental Aspects in Nutrition and Health. Academic Press, San Diego.

Conference Proceedings: **Cardinali, R., Rebollar P.G., Mugnai, C., Dal Bosco, A., Cuadrado, M., Castellini, C., 2008.** Pasture availability and genotype effects in rabbits: 2. development of gastro-intestinal tract and immune function of the vermiphorm appendix. In: Proc. 9th World Rabbit Congress, Verona, Italy, 1159-1164.

Thesis: **Bacınoğlu, S., 2002.** Boğa spermasında farklı eritme süreleri ve eritme sonrasında oluşturulan soğuk şoklarının spermatolojik özelliklere etkisi. Doktora Tezi, İstanbul Üniversitesi Sağlık Bilimleri Enstitüsü, İstanbul.

Manuscripts Published in Electronic Format: **Thierry, F., 2006.** Contagious equine metritis: a review. Equine Reproductive Infections: http://www.equinereproinfections.com (Accessed on 07.07.2006].

REVISIONS
When submitting a revised version of a paper, the author must submit a detailed "Response to the reviewers" that states point by point how each issue raised by the reviewers has been covered and where it can be found (each reviewer's comment, followed by the author's reply and line numbers where the changes have been made) as well as an annotated copy of the main document. Revised manuscripts must be submitted within 30 days from the date of the decision letter. If the revised version of the manuscript is not submitted within the allocated time, the revision option may be canceled. If the submitting author(s) believe that additional time is required, they should request this extension before the initial 30-day period is over.

Accepted manuscripts are copy-edited for grammar, punctuation, and format. Once the publication process of a manuscript is completed, it is published online on the journal's webpage as an ahead-of-print publication before it is included in its scheduled issue. A PDF proof of the accepted manuscript is sent to the corresponding author and their publication approval is requested within 2 days of their receipt of the proof.

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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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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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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 FabIgG, 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

VH	LH
GGSMKLSCVASGFTFSNYWMNWVRQSPEKGLEWVAEIRLKS NNYATHYAESVKGRFTISRDDSKSSVYLQMNNLRAEDTGIYYC TGTVRSLYYEPIHKPCCRFSEAH*GEHSG*VNQGTGA	WRSSQSIVHSNGNTYLEWYLQIPGQSSKLLIYKVSNRFS GVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQASHV PWTFGGGTKLDIKRADAAPTVSIFAPX

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

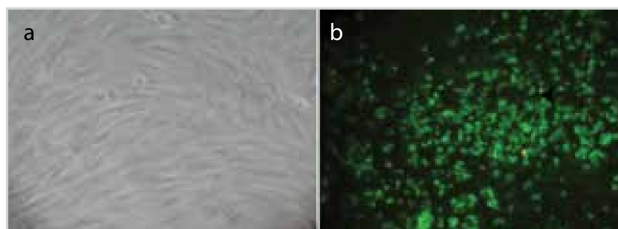

Figure 2. a, b. The characterization and culturing of bone marrow mesenchymal stem cells (ratBMMSCs). (a) Cells are already growing 90%. (b) characterization of cells using immunocytochemistry stained with CD105 labeled with FITC

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-

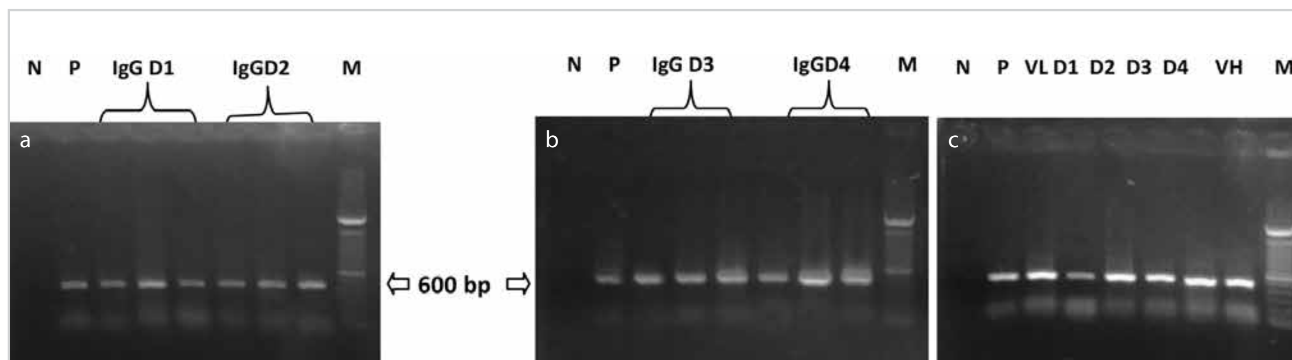

Figure 3. a-c. PCR product of a fragment gene coded FabIgG -D1, 2, 3, 4 VH and VL. (a) PCR product of a fragment gene coded FabIgG D1, D2; (b) PCR product of a fragment gene has coded FabIgG D3, D4; (c) PCR product of a fragment gene has coded FabIgG D1, D2, D3, and D4 of VL

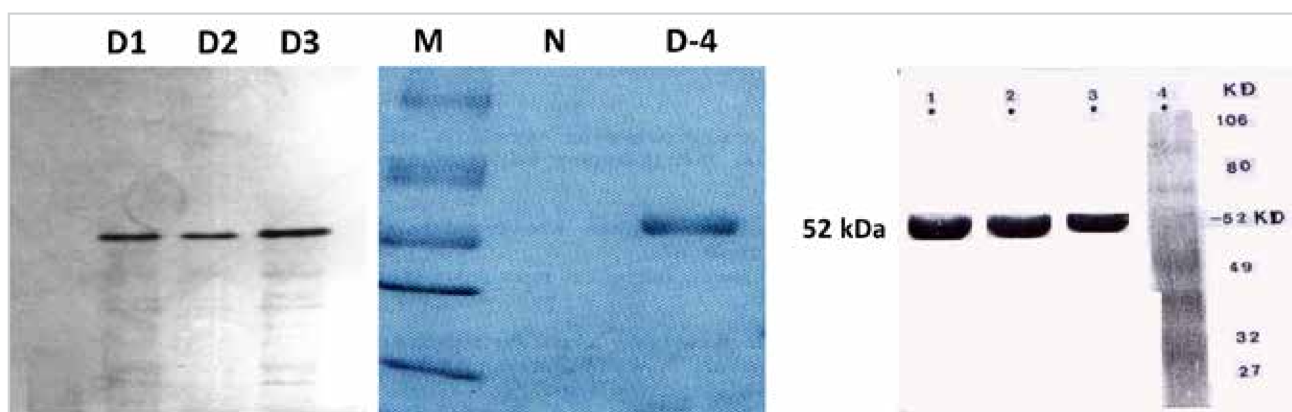

Figure 4. Electrophoresis analysis product of the FabIgG hybrid D1, 2, 3, and 4. A. Product electrophoresis was stained by using Silver stain (AgNO3). B. Product electrophoresis was stained by using Commasie blue. C. Immunoprecipitation of protein E.

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.

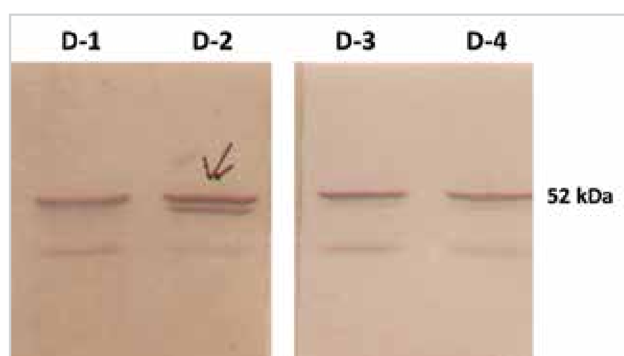

Figure 5. A western blotting analysis of E protein of Dengue virus with different serotypes of DENV-1, 2, 3, and 4 then reacted with the FabIgG hybrid, and was stained by using Pierce

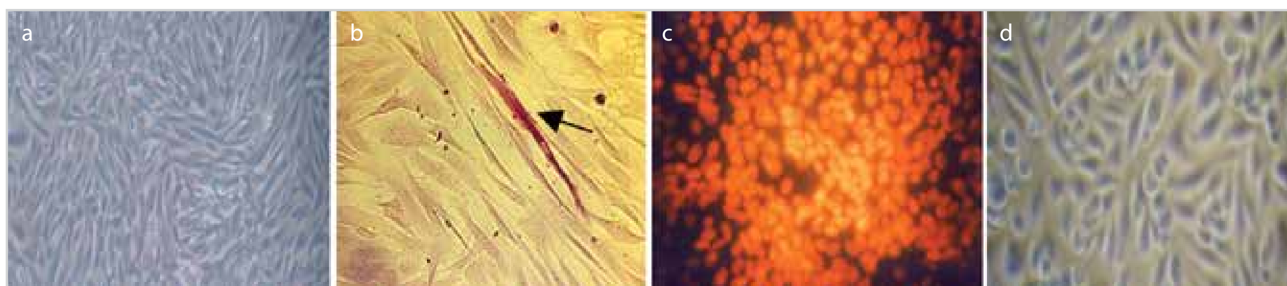

Figure 6. a-d. Neutralization assay using ratBMMSc. The purification of the FabIgG hybrid was performed using IgG-Sepharose affinity chromatography and then reacted with Dengue virus. After 1 hour it was incubated at room temperature and then inoculated into ratBMMSc. Three days after incubation in a 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.

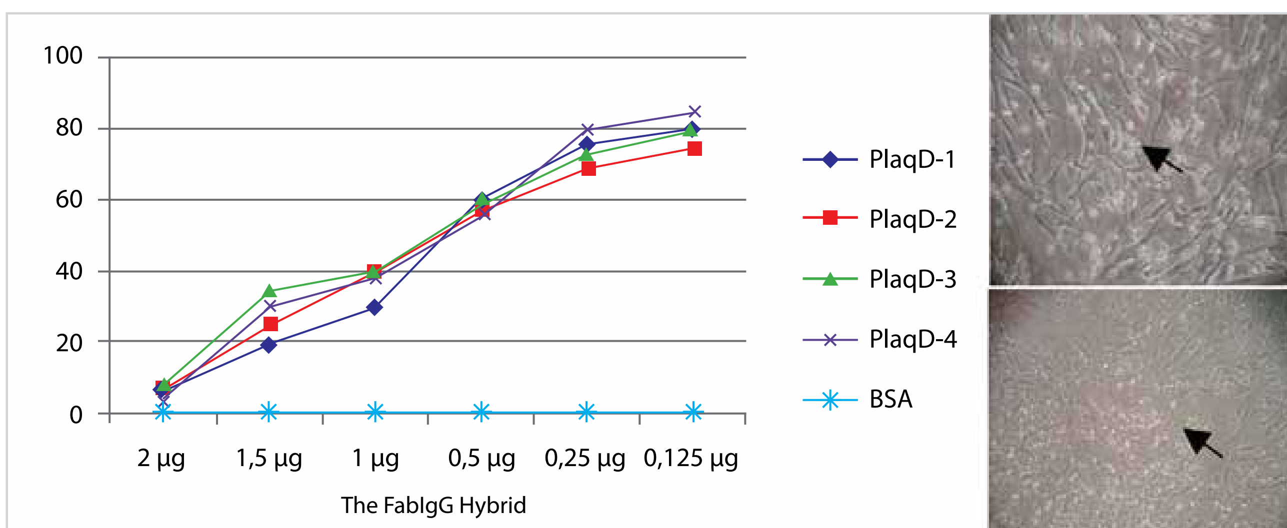

Figure 7. Comparison of the neutralizing properties of the FabIgG by plaque neutralization tests using ratBMMSCs to DENV-1, 2, 3, and 4 serotypes. The neutralization results using vero cells not shown in this diagram. Two-fold dilutions of the affinity of purified FabIgG collected from the supernatant 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.

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