ORIGINAL ARTICLE

Repeated infections of dengue (serotype DENV-2) in lung cells of BALB/c mice lead to severe histopathological consequences

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ABSTRACT

To determine the effect of DENV (serotype 2) repeated infections on lung cells is the main goal of this study. From the result, lung histology of control BALB/c mice showed normal alveolar morphology, while vehicle control BALB/c mice highlighted a slight thickening of the alveolar septum. Lung histopathology of BALB/c mice infected twice by DENV-2 showed the presence of hemorrhage, plasma leakage and presence of hemosiderin-laden macrophages (HLMs). Notably, in the lung of BALB/c mice infected four times by DENV-2, we observed thickening and disruption of the alveolar septum, inflammatory cell infiltration, plasma leakage and increased cellularity. Megakaryocyte releasing platelets were also found into the lung alveolus. Overall, our findings showed severe histopathological damage in lungs repeatedly infected by DENV-2, allowing us to argue that they can be linked to pulmonary complication. Result also showed that the number of infections with similar total DENV-2 titer led to different histopathological changes.

KEYWORDS

Arbovirus; dengue fever; hemosiderin-laden macrophages; mosquito-borne disease; recurrent infection; pulmonary complication

Introduction

The prevention and treatment of mosquito-borne diseases still represent a major challenge for modern parasitology and tropical medicine [1]. A dramatic expansion of dengue virus (DENV, hereafter) incidence has been reported in recent decades, representing dengue as the most momentous re-emerging arthropod-borne infectious disease [1]. DENV has become a major concern to public health worldwide, since it is affecting more than 100 countries, especially those in tropical and sub-tropical regions [1]. DENV endangers 40% world population's health. Each year it is anticipated that 50 million DENV infections occur with 5 million experiencing dengue hemorrhagic fever (DHF), a severe form of DENV infection, and death cases around 12,000 [2]. DHF causes potentially fatal consequences, leading to hemorrhage to different organs. Opposite to DHF, dengue fever (DF) is more self-limiting, however its complication can cause mortality in the 20% of cases [3].

A previous study on humans highlighted that DENV replicates in pulmonary macrophages, splenic macrophages and lymph nodes, and it has been isolated from the biopsy and necropsy of patients infected by DENV [4]. Another study reported that mesenteric lymph nodes, spleen and liver hold largely inactivated virus [5]. Lee et al., (2007) reported that DENV infects and proliferates in macrophages, vascular endothelium, developing in lung primary epithelium [6]. It has also been documented that DHF/DSS can cause acute respiratory distress syndrome (ARDS) and has become the 3rd foremost root of ARDS of in-depth pediatric care of DENV endemic zones [7]. In addition to these human cases, several study on animal species documented the involvement of lung in DENV infection with the performance of cytopathic effect. A previous lung morphological analysis in BALB/c mice by Barreto et al., 2007 showed increase number of mast cells at interalveolar septa and peribronchiolar spaces. Another study histopathological

study by Sakinah et al., 2017, showed extensive damage to the liver of BALB/c mice after dengue infections including loss of cell integrity, increase cells infiltrations, cytopathic effects, necrosis and apoptosis of liver cells. While study by Priya et al., 2017, exhibited histological changes in major blood vessels after DENV infection. These findings prove that the tissue morphological changes are like human DF and DHF, showing BALB/c are tolerant host for DENV-2 replication [8–15].

Overall, the above discussed studies have been scarcely considered and the potential impact of DENV on lung tissues still deserves major research attention [16]. Notably, detailed knowledge on the precise histopathological damage and the potential role of DENV repeated infections on lung tissues is still lacking. Therefore, the aim of this study is to determine the effect of DENV (serotype 2) repeated infections on lungs of BALB/c mice.

Materials and methods

Animals

In this study, BALB/c mice (25–30g) aged between 8–10 weeks were purchased. Twelve mice were used, they were divided into 4 groups consisting of 3 mice per group. The group 1 was the control, where mice were euthanized on day 0 of the experiment; group 2 was the vehicle control, where mice were euthanized on day 21 of the experiment as similar to group 3 and group 4. The DENV-2 in 500µl of Eagle's Minimum Essential Medium (EMEM, Biowest) was used to inoculate the mice in group 3 and 4 intraperitoneally. Group 3 was composed by mice infected with 2.5×10^5 FFU/day of DENV (serotype 2, DENV-2 hereafter) for 2 consecutive days, while in group 4 mice were infected with 1.25×10^5 FFU/day of DENV-2 for 4 consecutive days [17–19]. The mice were acclimatized for one week and the whole experiment was performed in the animal house, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. BALB/c mice were euthanized by cervical dislocation on day 21 (groups 2, 3 or 4) or 0 (group 1). This study was validated by the Animal Ethics Committee of IACUC, Universiti Putra Malaysia (UPM/IACUC/AUP-R017/2015).

Dengue virus propagation and stock preparation

A clinical isolate of dengue virus serotype 2 (DENV-2) (Reference ID: 3878) was obtained from the Virology Laboratory, FPSK, UPM (Malaysia). The DENV-2 was propagated into confluent *Aedes albopictus* mosquito lineage cells (C6/36 cell) (ATCC CRL-1660) by adding 1ml of DENV-2 stock solution with unknown titre (provided for this study at passage 3) and agitating

for 1 h to allow the viral adsorption at maximum level. After 1 hour, 7ml of fresh Eagle's Minimum Essential Medium (EMEM, Biowest) was added with low fetal bovine serum (FBS, Biowest) concentration (2%), and the cultures were incubated for 8 days in a 75 cm² sized flask at 28°C in 5% CO₂ incubator [19]. Therefore, the supernatant was aspirated and stored at –80°C. To obtain high concentration, the DENV-2 was passaged up to passage 10. After 10 passages the virus stock was quantified through foci forming assay with the titer of 5×10^5 FFU/ml.

Histology studies

Lungs from each mouse were harvested and immediately fixed in 10% formalin. Then, the tissues were processed for histology studies. The lung tissues from BALB/c mice were embedded with paraffin wax. The tissue was manually placed into the appropriate mould size which was pre-poured with molten paraffin with diagonal orientation across the block. Immediately, the cassette was placed on top of the mould, added with additional molten paraffin and allowed to cool. The paraffin block containing lung tissue was then sectioned in 4µm thickness, and stained with hematoxylin and eosin (H&E) using Leica ST5010 autostainer XL [20]. Three sections were prepared in total, with 10µm of intervals between each section. Histopathological changes in the lungs of BALB/c mice were analyzed using an OLYMPUS DP72 light microscopy. Each mouse slide, having three sections, was examined on 6 different areas with a surface of $18 \times 10^4 \mu\text{m}^2$, and each group was examined for $54 \times 10^4 \mu\text{m}^2$ using Olympus Soft Imaging Solution image analysis software Cell[^]F (Tokyo, Japan). The hemorrhagic and plasma leakage areas were observed in 20× magnification, which includes a wider area ($216 \times 10^4 \mu\text{m}^2$ per group). The histopathological changes were scored using a 0–4 point scale. A score of 0 indicates normal lungs, 1 indicates mild lung injury (< 25%), 2 indicates moderate lung injury (25% – 50%), 3 indicates severe lung injury (50% – 75%) and 4 indicates very severe lung injury (> 75%). The parameters observed for this scoring were degree of edema, hemorrhage, thickening of alveolar septum, reduction of alveolar elasticity, alveolar space collapse and inflammatory cells infiltration. The presence of hemosiderin-laden macrophages (HLMs) and megakaryocytes were also counted.

Statistical analysis

Data on the degree of edema and hemorrhage, thickening of alveolar septum, reduction of alveolar elasticity, alveolar space collapse, inflammatory cells

infiltration, HLMs and megakaryocytes data were analyzed by parametric test (One-Way ANOVA followed by post hoc multiple comparison) at the 5% significance level. All tests were performed using SPSS (IBM SPSS Statistic 23). $P = 0.05$ was used as threshold to assess significant differences among treatments.

Results

Figure 1 summarized the DENV-2-triggered changes in the lung histopathology. Lung histology of control BALB/c mice showed normal alveolar morphology (Figure 1(a)), while lung histopathology of vehicle control BALB/c mice highlighted a slight thickening of the alveolar septum (Figure 1(b)). The lung histopathology of BALB/c mice infected twice by DENV-2 showed the presence of severe hemorrhage (mean lung injury score 3.67 ± 0.11), plasma leakage and presence of HLMs (Figures 1(c), and 2(e)). Notably, in the lungs of BALB/c mice infected four times by DENV-2, we observed severe thickening and disruption of the alveolar septum (lung injury score 3.44 ± 0.12), inflammatory cell infiltration, plasma leakage and increased cellularity (Figure 1(d)).

Micro-anatomical details about DENV-2-triggered changes in lung histopathology were described in Figure 2. Severe edema (lung injury score 3.39 ± 0.14), high presence of macrophages and HLMs (45.5 ± 4.05) characterized BALB/c mice infected twice by DENV-2 (Figures 2(e) and 3(c)). In addition, inflammatory cells infiltration and increased cellularity were observed in both DENV-2 infected groups, with more abundance in group 4 (lung injury score 3.44 ± 0.17) (Figure 1(d,e), 2(d), and 3).

The alveolar septum was disrupted, the elasticity of the alveolar wall was severely reduced (lung injury score 3.33 ± 0.11) and the repeated infections caused the collapse of alveolus in both DENV-2 infected groups, severely in group 4 (lung injury score 3.67 ± 0.11) (Figures 1(c-e), 2(c,d) and 3(a,b)). Lastly, megakaryocyte releasing platelets into the alveolus (0.66 ± 0.18) was observed in lung tissues from group 4 (Figure 3(b,c)).

Discussion

Dengue hemorrhagic fever (DHF) and DSS increase the rate of mortality, if compared to DF cases. Both of them are characterized by hemorrhages and shock syndrome [21]. DENV-2 infection raises the virus production within the body and affects most of the internal organs, as recently pointed out by researches conducted on liver tissues and blood cells from experimentally infected mice populations [9,10]. With its well known characteristics, DENV increases the permeability of endothelium bed of the vessel and leads to the fluid extravasations from the endothelium bed

into the body cavity. This eventually reduces the blood pressure in the circulation, due to less circulating blood, resulting in low blood supply to all over the body, with highly detrimental consequences on vital organs. Moreover, the consumption of host mechanism by the DENV during its replication and host immunological storm results in organ malfunctioning. One of the organ malfunctions during DENV is bone marrow, results in low platelet production, increases the risk of bleeding and led to major complication to other vital organs [22].

Several studies have been carried out using mice as a model for DENV infection. The mice susceptibility to various serotypes of DENV was demonstrated, including DENV-2 [8–15]. In this study, BALB/c mice did not show physical alteration or mortality after repeated infections with DENV-2, except for ruffled fur. The findings are varying from previous studies due to several factors, these include the condition of the mice immune system, the virulence of the DENV itself, the DENV load being injected into the mice and also the route of infusion [23].

Clinical studies conducted on DENV infected patients have pointed out that DENV infection causes organ injuries. It has been reported that DENV replicates in lymph nodes, pulmonary and splenic macrophages of DENV-infected patients [4]. Furthermore, DENV also infects and replicates in the primary epithelium of human lungs [21], causes breathing complications [24] and histopathological changes to the lung tissues, which eventually cause organ failure. In the present research, the BALB/c mice infected by DENV-2, both from group 3 and 4, showed marked alterations of the lung histology. BALB/c mice in both group 3 and 4 were infected with a total of 5×10^5 FFU; 21 days after, BALB/c mice showed severe thickening of alveolar septum with the increased inflammatory cells infiltration, cellularity and macrophages. The increased cellularity surrounding the inter-alveolar septa may indicate increased permeability in the lungs, as the principal characteristic of DENV infection is vascular permeability [25]. The presence of inflammatory cells' infiltration indicates the host immune reaction at the local area due to DENV infection. Moreover, the disruption of the alveolar septum was observed, resulting in reduced elasticity and collapse of the alveolus, in agreement with earlier researches [8,26,27]. The congestion of the alveolar septa causes the rupture of alveolar walls [27,28]. In addition to that, the presence of inflammatory cells infiltration in the alveolar septa results in septa thickening [29]. This thickening may result in blockage during partial diffusion of gas exchange [30]. The loss of elasticity and collapse of alveoli were found in emphysema, which suggests that DENV infection probably causes emphysema during extensive lung involvement. Apart from this, the infection of type II pneumocytes by DENV

Figure 1. DENV-2-triggered changes in the lung histopathology (40 $\times$ magnification). (a). Lung histopathology of control BALB/c mice showing normal alveolar morphology; (b). Lung histopathology of vehicle control BALB/c mice showing slight thickening of alveolar septum pointed with blue arrow ($\nearrow$); (c). Lung histopathology of BALB/c mice infected twice by DENV-2 showing hemorrhage with green arrow ($\blacktriangledown$), plasma leakage represented by yellow arrowhead ($\blacktriangledown$) and presence of hemosiderin-laden macrophages (HLM) showed with red arrow ($\nearrow$); (d). Lung of BALB/c mice infected four times by DENV-2 showing thickening and disruption of alveolar septum, inflammatory cell infiltration, plasma leakage and increased cellularity; (e). Lung injury scores by the analysis of H&E-stained sections. The DENV-2 infected groups (3 & 4) showed significant changes compared with the control (group 1) (* $P < 0.05$) and vehicle control (group 2) (# $P < 0.05$).

Figure 2. Micro-anatomical details about DENV-2-triggered changes in lung histopathology (100 × magnification). (a). Lung histopathology of control BALB/c mice showing normal alveolar morphology; (b). Vehicle control mice showing slight thickening of alveolar septum pointed with blue arrow (↗); (c). Lung histopathology of BALB/c mice infected twice by DENV-2 showing edema and presence of macrophages. The green arrowhead represents (▼) the edematous and hemorrhagic area while the red arrow (↗) shows the hemosiderin-laden macrophages (HLM); (d). Lung of BALB/c mice infected four times by DENV-2 showing thickening and disrupted alveolar septum, increased inflammatory cells infiltration, cellularity and plasma leakage showed in yellow arrowhead (▼); (e). The DENV-2 infected groups (3 & 4) showed significantly increased hemorrhage and edema compared with the control (group 1) (*P < 0.05) and vehicle control (group 2) (#P < 0.05).

may result in alveolar collapse since its functioning to release surfactant into alveolus to reduce collapse. Rodrigues et al., (2014) found hyperplasia of type II pneumonia cell in histological analysis from fatal DENV cases, suggesting that there is involvement of type II pneumonia cell in dengue infection [31].

Besides, fluid extravasations and hemorrhage were observed in DENV-2 infected BALB/c mice, markedly in group 3. It has been proven that DHF

may lead to ARDS [22,32,33]. Since lung endothelial cells are involved DENV infection, several inflammatory mediators such as RANTES and IL-8 can be released and activate the involvement of neutrophils, eventually increasing the vascular permeability [6]. Thus, the increase of alveolar vascular permeability can result in fluid extravasations and hemorrhage in the alveolar spaces (Figure 4) [2,12,33–35].

Figure 3. Lung morphology in BALB/c mice from the group infected twice with DENV-2. (a). BALB/c mouse lung showing high presence of hemosiderin-laden macrophages (HLM), hemorrhage and edema; (b). Lung of BALB/c mouse showing the presence of megakaryocytes and platelets in the alveolus. Images were captured under 100 × magnification; (c). The DENV-2 infected groups (3 & 4) showed significantly increased HLMs, while group 4 showed significant increased megakaryocytes compared with the control (group 1) (* $P < 0.05$) and vehicle control (group 2) (# $P < 0.05$).

HLMs were observed in DENV-2 infected BALB/c mice, predominantly in group 3. Previous studies have demonstrated the presence of HLMs as biomarkers for alveolar hemorrhage [36,37]. DENV infection increases the vascular permeability and causes plasma leakage into the alveolar spaces, thus increases the deposition of iron into the interstitium, and can increase the presence of HLMs. This condition was similarly found in idiopathic pulmonary fibrosis (IPF) occurring due to pulmonary hemodynamics imbalance associated with increased alveolar septal capillary density and microvessel density [38,39]. It has been diagnosed that the presence of HLMs causes alveolar hemorrhage in the BAL or lung tissue [40]. It is suggested that the mechanisms of hemorrhage include the deformity of the alveolar endothelium layer and immunological failing that obstruct the alveolar endothelium integrity.

Interestingly, we observed the presences of megakaryocytes in the lung of BALB/c mice from group 4. Megakaryocytes were usually found in bone marrow, which produces platelets into the blood vessels. Previous study stated that megakaryocytes were found in the mammalian lungs [41]. In the forensic necropsies of human lungs, megakaryocytes were identified routinely with increased number in ARDS/

ALI samples [42,43]. Hansen et al. (1979) reports that megakaryocytes count was higher in the blood vessel of patients with pulmonary inflammation [44]. An investigation by Alexander et al., (1996) proved that during stem cells inadequacy and thrombocytopenia, the progenitor cells can drift out from the lung, supporting the bone marrow cells repopulation and platelet counts reconstitution [45]. It has been indicated that platelets found in the lungs are the key for pulmonary defense, however could also lead to injuries in lung tissues [41]. Thus, the presence of megakaryocytes in lung tissues highlighted by our findings can be suggested as the host response to aid in recovery of lung injury due DENV-2 infection.

Conclusions

Overall, our findings showed severe histopathological damage in lungs infected repeatedly with DENV-2, and this may lead to pulmonary complications if immediate action for the treatment are not taken. Our findings also parallel to the reports in human DENV infection studies that results in morphological changes. Furthermore, findings exhibited that the numbers of infections with DENV led to different histopathological changes, even

Figure 4. Flow diagram explaining the consequences and mechanisms potentially involved in repeated DENV-2 infections on lung tissues of BALB/c mice.

the total DENV-2 titer in BALB/c mice infected two and four times were comparable. Finding from this study will be a useful key to resolve unresolved problems in relation to DENV infection of lungs.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

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