Histopathology of Natural Ebola Virus Subtype Reston Infection in Cynomolgus Macaques during the Philippine Outbreak in 1996

Tetsuro IKEGAMI, Mary Elizabeth G MIRANDA, Alan B CALAOR, Daria L MANALO, Noel J MIRANDA, Masahiro NIKURA, Masayuki SAIJO, Yumi UNE, Yasuo NOMURA, Ichiro KURANE, Thomas G KSIAZEK, Yasuhiro YOSHIDA, and Shigeru MORIKAWA

1Special Pathogens Laboratory, Department of Virology 1, National Institute of Infectious Diseases, 4–7–1 Gakuen, Musashimurayama, Tokyo 208-0011, 2Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan, 3Veterinary Research Department, Research Institute for Tropical Medicine, Department of Health, Muntinlupa City 1770, 4INA Research Philippines, Inc., Laguna, Philippines, 5Laboratory of Veterinary Pathology, Azabu University, Kanagawa, Japan, 6Special Pathogens Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Abstract: We investigated the livers, spleens, kidneys, and lungs collected from 24 cynomolgus macaques (Macaca fascicularis) naturally infected with Ebola virus subtype Reston (EBO-R) during the Philippine outbreak in 1996, in order to reveal the histopathologic findings. These macaques showed necrotic hepatocytes with inclusions, slight to massive fibrin deposition in splenic cords, depletion of lymphoid cells in the white pulp of the spleen, and fibrin thrombi in some organs. Immunohistochemical analysis using anti-leukocyte antigen L1 antibody revealed an increase in blood-derived macrophages/monocytes in the livers, kidneys, and lungs of EBO-R infected macaques. EBO-R NP antigens were detected in the macrophages/monocytes, endothelial cells, and fibroblasts in the liver, spleen, kidney, and lung. These results indicate that EBO-R infection is characterized by systemic coagulopathy and an increase in blood-derived macrophages/monocytes in accordance with the EBO-R propagation in macrophages/monocytes.

Key words: cynomolgus macaque, Ebola virus, Histopathology, subtype Reston

Introduction

Ebola virus belongs to the family Filoviridae and is divided into 4 subtypes: Zaire (EBO-Z), Sudan (EBO-S), Ivory Coast (EBO-IC), and Reston (EBO-R) [12]. EBO-R outbreaks have occurred in 1989 (Virginia, USA), 1990 (Texas, USA), 1992 (Siena, Italy), and 1996 (Texas, USA) among macaques imported from the Phi-

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Address corresponding: S. Morikawa, Special Pathogens Laboratory, Department of Virology 1, National Institute of Infectious Diseases, Gakuen 4–7–1, Musashimurayama, Tokyo 208-0011, Japan
ippines [7–9, 16, 19, 20, 23, 26, 32]. A high mortality rate among EBO-R-infected cynomolgus macaques was documented in each epidemic; however, there was no illness or fatalities among the serologically confirmed, infected humans [8, 9, 23, 32]. Histopathological findings of cynomolgus macaques naturally infected with EBO-R during the 1989 American outbreak have been reported [11, 15, 19]. EBO-R virions were demonstrated in circulating macrophages/monocytes, interstitial fibroblasts, interstitial macrophages, adipose cells, vascular endothelial cells, hepatocytes and adrenal cortical cells, but rarely in the renal tubules and type-II alveolar epithelial cells in the monkeys infected in the 1989 outbreak [15]. Simian hemorrhagic fever virus (SHFV), belonging to the family Arteriviridae was also isolated from some of the macaques during the EBO-R outbreaks [11, 16, 19]. However, the involvement of SHFV in the pathogenesis of EBO-R has not yet been clarified.

Experimental infection of non-human primates [4, 5, 13, 14, 18, 21, 28] and guinea pigs [10, 29] with EBO-Z demonstrated that EBO-Z propagates initially in mononuclear phagocytic system (MPS) cells, such as monocytes and macrophages, and subsequently in hepatocytes, adrenal cortical cells, endothelial cells, and fibroblasts in EBO-Z infected monkeys [28] and guinea pigs [10].

The histopathological findings of EBO-R infected cynomolgus macaques in the Philippine outbreaks have not yet been reported. In this study, we investigated the characteristic light microscopic findings of the livers, spleens, kidneys and lungs from 24 dead or sacrificed cynomolgus macaques during the EBO-R outbreak of 1996 in the Philippines, and demonstrated an increase of blood-derived macrophages/monocytes in EBO-R infected macaques.

**Materials and Methods**

**Animals**

The EBO-R epidemic occurred in a monkey breeding and export facility in the Philippines from March to September in 1996 [23, 26]. During the outbreak at this facility, some monkeys died of Ebola virus infection, and some monkeys held in pens with antigen-positive animals were sacrificed to control the spread of the virus.

Tissues from 29 cynomolgus macaques (*Macaca fascicularis*) were histopathologically examined. Twenty-four of the 29 monkeys were infected with EBO-R, while the remaining 5 were uninfected. The EBO-R infection was previously confirmed by detection of the antigen in liver homogenates by antigen-capture enzyme-linked immunosorbent assay (ELISA) at the Research Institute for Tropical Medicine, Philippines [23]. Of the 24 EBO-R-infected macaques, 12 died of the virus infection, and 12 were sacrificed to prevent the viral spread. The available clinical signs of these macaques are shown in Table 1.

**Light Microscopy**

Tissue samples from the livers, spleens, lungs, and kidneys of the macaques were fixed in 10% formalin. The tissues were embedded in paraffin, cut into 3-μm sections and stained with either hematoxylin and eosin (HE) or phosphotungstic acid hematoxylin (PTAH).

**Immunohistochemistry**

The paraffin sections were examined by immunohistochemistry using a commercial kit (VECTASTAIN elite ABC kit: Vector Laboratories, Inc. Burlingame, USA). They were pre-treated in 10 mM citrate buffer at 95°C for 15 min in a microwave (H2500 Microwave Processor, Energy Beam Sciences, Inc. Massachusetts, USA) prior to immunostaining. We used a rabbit polyclonal antibody to EBO-Z recombinant nucleoprotein (NP) [30] to detect EBO-R NP, since the rabbit serum showed considerable cross-reactivity with the EBO-R antigen in the indirect immunofluorescent assay, immunoglobulin G-ELISA and Western blotting (data not shown). We also used a mouse monoclonal antibody to a leukocyte antigen L1 protein (Novoceastra Lab, Inc, Newcastle, UK). Biotinylated horse antibody to mouse IgG was used as secondary antibody for the primary antibodies. Then, peroxidase-conjugated streptavidin was reacted. Diaminobenzidine and hydrogen peroxide were used as a substrate for visualization. We counted the number of L1 antigen-positive cells in the liver, medulla of kidney, and lung in 5 fields at 50X magnification, using computer software (the public domain NIH Image program, National Institutes of Health, Bethesda, USA). The number of positive cells was compared with those of 5 non-infected monkeys by Student’s t-test.
### Table 1. Case history, viral antigen localization, and light microscopic findings in EBO-R infected macaques

<table>
<thead>
<tr>
<th>macaque No.</th>
<th>Recorded health statusa</th>
<th>Viral antigen locationb</th>
<th>Inclusions in hepatocytes in red pulp</th>
<th>Fibrin deposition in spleen</th>
<th>Lymphoid cell depletion in renal tubules</th>
<th>Hemorrhage in alveoli</th>
<th>Thrombi formationc</th>
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<td>(spleen)²</td>
<td>(kidney)²</td>
<td>(lung)²</td>
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<tr>
<td>2671</td>
<td>H</td>
<td>L(0), S(1), K(0), P(1)</td>
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<tr>
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<td>H, W</td>
<td>L(1), S(1), K(0), P(1)</td>
<td>-</td>
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<td>2386 W</td>
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<tr>
<td>2377 NR</td>
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<tr>
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<th>III. Negative macaques (Uninfected)</th>
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<td>2784 H</td>
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<td>2757 H</td>
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<td>2758 H</td>
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### Results

**Histopathologic findings in macaques that died of EBO-R infection**

All the macaques that had died of Ebola virus infection during the outbreak showed similar pathological findings (Table 1). Moderate to severe infiltration of mononuclear cells was present in the portal areas in the livers. Hepatocytes contained irregular shaped intracytoplasmic acidophilic inclusion bodies, which were widely scattered throughout the lobules (Fig. 1a). Necrosis of hepatocytes was occasionally observed. A prominently increased number of mononuclear cells and fewer neutrophils were present in the hepatic sinuses and veins. Some of the mononuclear cells were enlarged and contained acidophilic inclusions in the cytoplasm. PTAH staining demonstrated fibrin thrombi in the sinuses.

In the white pulp of the spleen, the number of lymphoid cells was severely decreased, and hemorrhage was occasionally present in the follicle (Fig. 1b). The number of MPS cells in the red pulp cords was dramatically decreased. The splenic cords were prominently engorged with acidophilic materials (Fig. 1c). The acidophilic...
Fig. 1. Light microscopic lesions observed in macaques that died of EBO-R infection. (a) Liver, No. 2386 monkey. Some hepatocytes have irregular acidophilic inclusion bodies in the cytoplasm (arrow). HE. Bar = 50 µm. (b) White pulp in spleen, No. 2386. The number of lymphoid cells is prominently reduced in the follicle and hemorrhage is also observed. HE. Bar = 200 µm. (c) Red pulp in spleen, No. 2386. The number of constituent cells of the red pulp cord is prominently decreased. The red pulp cord is engorged, and acidophilic material (fibrin) is deposited (arrow head). Enlarged macrophages are seen in the splenic sinus (arrow). HE. Bar = 50 µm. (d) Red pulp in spleen, No. 2386. Fibrin deposition is demonstrated in the red pulp cord. PTAH. Bar = 50 µm. (e) Liver, No. 2377. EBO-R NP antigens are detected in the cytoplasm of macrophages / monocytes (arrow) in the sinus and endothelial cells (arrow head). Immunohistochemistry, Mayer’s hematoxylin counterstain. Bar = 50 µm. (f) Red pulp in spleen, No. 2377. Macrophages (arrow) in the splenic sinus and endothelial cells (arrow head) have EBO-R NP antigens. Immunohistochemistry, Mayer’s hematoxylin counterstain. Bar = 50 µm.
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materials were demonstrated to be massive cross-linked fibrin deposits by PTAH staining (Fig. 1d). Fibrin deposition was located along the basement membrane of the endothelial cells of the splenic sinuses and the interstitial space in the red pulp cord. Fibrin thrombi were also observed in the sinuses. In the splenic sinuses, enlarged mononuclear cells containing acidophilic inclusions in the cytoplasm were present (Fig. 1c).

In the kidneys, mild to moderate mononuclear cell infiltration was present around the arcuate veins. An increased number of circulating mononuclear cells was present in the renal vessel lumens. Many thrombi composed of acidophilic materials and small amounts of fibrin were present in the medulla of renal capillaries in some macaques. Six macaques (Nos. 2377, 2338, 2334, 2872, 2882 and 2878) showed hemorrhage in the renal tubules.

Mild to moderate perivascular infiltration of mononuclear cells was observed in the pulmonary parenchyma. Some macaque lungs showed evidence of fibrinous exudate and hemorrhage in the intra-alveolar area. Similar thrombi to those observed in the renal medulla were also present in the pulmonary venular.

Histopathologic findings in sacrificed macaques

The twelve sacrificed macaques showed more variable pathological changes than those that died of EBO-R infection (Table 1). Among them, 4 macaques (Nos. 2671, 2182, 2612 and 2669) showed no clinical signs, while 3 macaques (Nos. 956, 2939 and 2615) were ill at euthanasia (Table 1). Data on clinical manifestations were not available for the remaining 5 macaques.

Two macaques (Nos. 2671 and 2182) showed minimal fibrin deposition along the basement membrane of the splenic sinus. In addition, enlarged mononuclear cells in the sinuses of the liver and spleen occasionally contained acidophilic intracytoplasmic inclusions. However, inclusions in hepatocytes were not present in these 2 macaques.

On the other hand, two macaques (Nos. 2921 and 2728) showed minimal acidophilic intracytoplasmic inclusions in a few hepatocytes. Three macaques (Nos. 2739, 2644 and 2612) showed hepatic inclusions in many hepatocytes. No. 2644 showed massive fibrin deposition in the red pulp cords like most of the macaques that died of EBO-R infection while the other 4 macaques showed minimal deposition. None of these 5 macaques showed any lymphoid cell depletion in the white pulp of spleens. Hemorrhage and enlarged macrophages were present in the renal proximal tubules of No. 2644.

The other 5 macaques (Nos. 956, 2400, 2939, 2669 and 2615) showed pathological changes similar to those in the macaques that died of EBO-R infection in the liver and the spleen. Nos. 956 and 2939 showed hemorrhage in the renal proximal tubules, and Nos. 2669 and 2615 showed hemorrhage in the intra-alveolar area.

All twelve sacrificed macaques showed fibrin thrombi in the livers and the spleens. Five macaques (Nos. 2612, 956, 2400, 2939, and 2669) showed thrombi composed of acidophilic materials and small amounts of fibrin in the kidneys and/or the lungs.

The five uninfected macaques did not show any pathological changes in any of tissues examined.

EBO-R NP antigen distribution

The localization of EBO-R NP antigen was examined by immunohistochemistry, and the result is summarized in Table 1.

In all macaques that died of EBO-R infection, EBO-R NP antigens were detected in macrophages/monocytes, endothelial cells and fibroblasts in the livers (Fig. 1c), spleens (Fig. 1f), kidneys and lungs. Although the acidophilic inclusions were frequently observed in the hepatocytes by HE staining, EBO-R NP antigens were not frequently detected in these cells (Fig. 1e).

On the other hand, EBO-R NP antigen distribution among sacrificed macaques was variable compared to that of macaques that died of EBO-R infection. One sacrificed macaque (No. 2671) had the viral antigens only in the MPS cells of the spleen. Five sacrificed macaques (Nos. 2671, 2182, 2921, 2728, and 2739) had the viral antigens in circulating macrophages/monocytes in the livers, spleens, kidneys and lungs but not in the endothelium in these organs.

In addition, two macaques (Nos. 2644 and 2878) that had hemorrhage in the renal proximal tubules had EBO-R NP antigens in the epithelium of renal proximal tubules.

Increase in leukocyte antigen L1 Positive Cells

As shown above, an increased number of mononuclear cells was observed in the vessel lumens of livers and kidneys. These mononuclear cells were morphologically indistinguishable from macrophages and monocytes. Thus, we analyzed the number of blood-derived macrophages/monocytes in the livers, kidneys and lungs by counting the numbers of leukocyte antigen L1-positive
cells. Since the fibrin was deposited in the red pulp cord of the spleen and the number of cells in the spleen were prominently decreased in severely affected macaques, we did not analyze the number of L1-positive cells in the spleen. The numbers of L1 antigen-positive cells in the livers, kidneys and lungs counted in 5 fields at 50X magnification are shown in Fig. 3. All sacrificed macaques and macaques that died through infection with EBO-R had increased numbers of L1-positive cells in the hepatic vasculature in comparison with the average of the 5 uninfected ones (P<0.01) (Figs. 2a, 2b, 3). Furthermore, 6 of 11 (55%) sacrificed and 10 of 11 (91%) dead macaques showed increased numbers of L1-positive cells in renal vessels, and 8 of 10 (80%) sacrificed macaques and all of the macaques that died of EBO-R infection showed increased numbers of L1-positive cells in the pulmonary venular (P<0.01) (Figs. 2c, 2d, 3).

**Discussion**

In this study, we histologically examined formalin-fixed tissues from 24 EBO-R infected cynomolgus macaques, and compared the outstanding findings in sacrificed EBO-R infected macaques with those of macaques that died during the 1996 outbreak in the Philippines. Regrettably, the gross findings and sufficient clinical data were not available in this study. The light microscopic findings such as acidophilic inclusion bodies in the hepatocytes, fibrin deposition in the red pulp of the spleen, lymphoid cell necrosis in the white pulp of the spleen and fibrin thrombi formation were consistent with those reported in the 1989 American outbreak [11, 15, 19]. However, the histological changes in 7 (Nos. 2671, 2182, 2921, 2728, 2739, 2644 and 2612) of 12 sacrificed macaques were minimal or moderate as compared with
Fig. 3. The increase in leukocyte antigen L1 positive cells in the livers, kidneys and lungs of EBO-R infected macaques. Each column represents the total number of leukocyte antigen L1 positive cells in the fields of the livers, kidneys and lungs from the EBO-R-infected sacrificed macaques, EBO-R-infected dead macaques and non-infected macaques (5 fields at ×50, mean ± SD). *P<0.05, **P<0.01 versus the average of 5 negative controls.

those which died. These 7 sacrificed macaques showed no dramatic lymphoid cell depletion in the white pulp of the spleens which was evident in macaques that died. In addition, EBO-R NP antigens were not detected in the sera from 2 sacrificed macaques (Nos. 2182 and 2921) by antigen-capture ELISA, while EBO-R antigens were detected in the sera from 6 sacrificed macaques (Nos. 2728, 2739, 2644, 2612, 2669 and 2615) (data not shown) [24]. Therefore, the sacrificed macaques examined in this study were thought to be at various stages of the EBO-R infection.

The fibrin deposition in the red pulp of spleen was an
outstanding finding in EBO-R infected macaques in this study. Experimental EBO-R infection in cynomolgus macaques also revealed similar findings [13, 20]. It was reported that monkeys infected with SHFV showed similar fibrin deposition in the red pulp of the spleen [1]. SHFV was also isolated from cynomolgus macaques from the first outbreak of EBO-R in the Philippines [16]. We performed reverse transcription polymerase chain reaction (RT-PCR) to detect SHFV genomic RNA (p15 coding region) from frozen tissues (liver or spleen) of 7 EBO-R-infected monkeys during the same outbreak using primers p15F (5’-GTC CAG AGG GAA TAG GCT-3’) and p15R (5’-GCA GCA AAA TTG ATT CTC TGT CCG T-3’). We could not detect any evidence of SHFV infection (data not shown). However, the possibility of SHFV infection could not be excluded in all the EBO-R infected macaques in this study, because we could not examine all the monkey specimens with the RT-PCR. Experimentally induced endotoxemia also resulted in the fibrin deposition in the red pulp cord of the spleens of rhesus monkeys [3, 22]. Thus, fibrin deposition in the spleen may be a common change in macaques that suffer diseases with vessel disorders.

Considering the fibrin thrombi formation in the livers and spleens and fibrin deposition in the spleen in most of the EBO-R infected macaques, systemic coagulopathy seemed to occur in the EBO-R infected macaques. Increased levels of TNF-α, interleukin (IL)-2, IL-10, and interferon (IFN-α) were reported in fatally EBO-Z-infected human patients [2, 31]. Recently, EBO-R infected cynomolgus monkeys were also reported to have increased levels of TNF-α, IFN-γ, IL-2, IL-1β, and IL-6 in the blood [17]. Procoagulant activity in the endothelium was most likely enhanced by these cytokines produced in the process of EBO-R propagation, following tissue destruction and macrophage activation.

Thrombi composed of acidophilic materials and small amounts of fibrin in the kidneys and lungs were only observed in the macaques in which viral antigens were detected in the endothelium. Since these thrombi were immunohistochemically stained with a rabbit polyclonal antibody to CD62P (PharMingen, Co., Ltd. San Diego, USA) specific to platelet and endothelium (data not shown), the thrombi may contain platelets and/or the debris of endothelium as their ingredient.

We confirmed an increase in the number of leukocyte antigen L1 positive macrophages/monocytes in the liver, spleen, kidney and lung of the EBO-R infected macaques. A monoclonal antibody, MAC387, recognizes leukocyte antigen L1 (calprotectin) which is expressed in neutrophils and monocytes [6], and is a useful marker for newly blood-derived macrophages because monocytes gradually lose calprotectin after migration from blood into tissues [25, 27]. It was reported that MPS cells were the primary targets of Ebola virus [10, 28]. Granulomatous inflammation was demonstrated in the liver of guinea pigs experimentally inoculated with EBO-Z [29]. It is of interest to know whether the infected macrophages/monocytes work to gather other macrophages/monocytes by producing cytokine. The increase in the number of macrophages/monocytes in the vessels may also be advantageous to the rapid dissemination of EBO-R.

Acknowledgments

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