Experimental Respiratory Infection of Marmosets (Callithrix jacchus) With Ebola Virus Kikwit

Sophie J. Smither,1 Michelle Nelson,1 Lin Eastaugh,1 Alejandro Nunez,2 Francisco J. Salguero,3 and Mark S. Lever1

1Microbiology Group, CBR Division, Defence Science and Technology Laboratory (Dstl), Salisbury, 2Pathology Department, Veterinary Laboratories Agency–Weybridge, Addlestone, and 3Department of Pathology and Infectious Diseases, School of Veterinary Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, United Kingdom

Ebola virus (EBOV) causes a highly infectious and lethal hemorrhagic fever in primates with high fatality rates during outbreaks and EBOV may be exploited as a potential biothreat pathogen. There is therefore a need to develop and license appropriate medical countermeasures against this virus. To determine whether the common marmoset (Callithrix jacchus) would be an appropriate model to assess vaccines or therapies against EBOV disease (EVD), initial susceptibility, lethality and pathogenesis studies were performed. Low doses of EBOV-Kikwit, between 4 and 27 times the 50% tissue culture infectious dose, were sufficient to cause a lethal, reproducible infection. Animals became febrile between days 5 and 6, maintaining a high fever before succumbing to EVD between 6 and 8 days after challenge. Typical signs of EVD were observed. Pathogenesis studies revealed that virus was isolated from the lungs of animals beginning on day 3 after challenge and from the liver, spleen and blood beginning on day 5. The most striking features were observed in animals that succumbed to infection, including high viral titers in all organs, increased levels of liver function enzymes and blood clotting times, decreased levels of platelets, multifocal moderate to severe hepatitis, and perivascular edema.

Keywords. Ebola virus; Ebola virus disease; filovirus; animal model; human disease correlates; marmoset; non-human primate; aerosol; pathology.

Marburg virus and Ebola virus (EBOV) cause highly lethal hemorrhagic fever [1, 2]. EBOV disease (EVD) initially presents with flulike symptoms, including fever, headaches, nausea, and muscle and joint aches; as the disease develops, a maculopapular rash or hemorrhaging may be observed. Death is due to multiorgan failure, shock, and/or disseminated intravascular coagulation [3]. During outbreaks, the virus is normally transmitted through direct contact with infected persons, animals, or their secretions.

The aerosol route of transmission is not generally associated with natural outbreaks; however, there is some evidence that droplet spread of Reston virus may have occurred [4], and animals can be experimentally infected with EBOV via the aerosol route [4, 5]. The role of aerosols and droplets in the transmission of EBOV during outbreaks is not fully understood [6]. The high infectivity and lethality of EBOV mean they are considered possible biowarfare/bioterrorist agents [5, 7].

Currently there are no licensed vaccines or medical countermeasures for disease caused by EBOV, but promising vaccine candidates and treatments are emerging at an accelerated rate owing to the continuing West African outbreak [8–10]. However, there is still a need for further understanding of the disease caused by aerosolized virus and the ability to test medical countermeasures against aerosolized virus [11, 12]. Licensure of a drug or vaccine to protect against EBOV infection is likely to rely on robust, reproducible, and appropriate animal models.

Several animal models have been developed to assess medical countermeasures for disease caused by filoviruses [13]. Rodents can be used for initial screening of compounds, but they have limitations because they are not naturally susceptible to infection by filoviruses [14–17]. Some nonhuman primate models of EVD have been developed. Primates are naturally susceptible to EBOV by multiple routes of infection and show clinical signs that disease progression similar to that observed in
human cases [12, 18–21]. Some of these models have been used for testing efficacy of vaccines and treatments [22–24].

The common marmoset (Callithrix jacchus) is a New World nonhuman primate and alternative option as a primate model [25]. C. jacchus has been reported elsewhere as a model for bacterial diseases [26–28] and for infection by viruses [29, 30], including the filoviruses [31, 32]. Studies were performed to determine whether the common marmoset was susceptible to EBOV infection by the aerosol route and could offer an alternative animal model for the development and testing of EVD vaccines and/or treatments [33].

MATERIALS AND METHODS

Animals
Healthy, sexually mature common marmosets (Defence Science and Technology Laboratory [Dstl] breeding colony) were housed in pairs as a vasectomized male and female. Animals had free access to food, water, and environmental enrichment. All animals were surgically implanted with a telemetry device to record core body temperature (Remo Technologies). Before challenge animals were bled from the femoral vein to obtain baseline blood parameters. Animals were housed in purpose built cages held inside a rigid half-suit isolator maintained at negative pressure. Animal studies were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986, Codes of Practice for the Housing and Care of Animals Used in Scientific Procedures 1989, and US Animal Care and Use Review Office regulations.

Virus Growth, Sequencing, and Enumeration
EBOV Kikwit (EBOV/H.sapiens-tc/COD/1995/13625 Kikwit; hereafter EBOV-Kikwit) was provided by Public Health Agency of Canada. Passage 2 material was grown in Vero C1008 cells (ECCAC catalog No. 85 020 206) at 37°C/5% carbon dioxide hereafter EBOV-Kikwit). Passage 3 stock is an 8U variant [34]. Viral titers were determined using whole body real-time plethysmography with a Fleisch pneumotacograph (EMMS). The concentration of virus was quantified during each exposure, and the inhaled dose for each animal was calculated from the inhaled volume and the aerosol concentration.

Animal Studies
To determine whether the common marmoset was susceptible to EBOV-Kikwit by the aerosol route, a pair of animals were challenged with an initial 1:10 dilution of EBOV-Kikwit stock (10^6 TCID50) to give an inhaled dose of approximately 10 TCID50. Three more pairs were challenged with the same dose and doses a log higher (10^7 TCID50 stock, for an inhaled dose of approximately 100 TCID50) and a log lower (1:100 dilution of stock, for an inhaled dose of approximately 1 TCID50) to determine the effect of a range of aerosol doses. Then, to ensure reproducibility of aerosol infection, 2 more pairs of marmosets were challenged with a 1:10 dilution of EBOV-Kikwit. This reproducible dose was used in a natural history study with 16 animals, wherein 4 animals were culled at predetermined time points 24, 72, 120 or 144 hours after infection.

Postmortem Examinations
Postmortem examinations were performed on all animals after humane culling, as described elsewhere [32]. Complete blood counts were measured using a laser flow cytometry-based hematological analyzer (LaserCyte; IDEXX laboratories). Plasma concentrations of different chemicals were determined using a “dry-slide” technology biochemistry analyzer (VetTest; IDEXX laboratories). Citrated blood was used to obtain clotting times with a cassette-based coagulation analyzer (DX Coag; IDEXX laboratories).

Histology
Tissues were fixed in 10% buffered formalin solution (Sigma) and processed for paraffin wax embedding. Sections 4 μm ± 2 μm thick were prepared and stained with hematoxylin-eosin or phosphotungstic acid hematoxylin to allow visualization of fibrin or used for immunohistochemical detection of EBOV antigen, macrophages and neutrophils, or cleaved caspase 3 (Table 1).

Sections used for immunolabeling were dewaxed and dehydrated, endogenous peroxidase activity was quenched in hydrogen peroxide 3% in methanol for 15 minutes, and antigen was retrieved by various methods. (Table 1). The sections were then assembled into Sequenza coverplates (Shandon Scientific) and rinsed with Tris-buffered saline (TBS) (pH 7.6; 0.005 mol/L) (Sigma–Aldrich). Sections were blocked with 1.5% normal goat serum for 20 minutes, followed by incubation with the primary antibody diluted in TBS for 1 hour. Sections were washed in TBS and then incubated for 30 minutes with Dako REAL apparatus and AeroMP system (BiAera). The accumulated tidal volume for each animal during a 10-minute exposure was determined using whole body real-time plethysmography with a Fleisch pneumotacograph (EMMS). The concentration of virus was quantified during each exposure, and the inhaled dose for each animal was calculated from the inhaled volume and the aerosol concentration.

Aerosolized Ebola Virus in Marmosets • JID 2015:212 (Suppl 2) • S337
EnVision polymer (Dako UK). The immunohistochemical signal was visualized using 3,3-diaminobenzidine (Sigma-Aldrich), and sections were counterstained in Mayer hematoxylin (Surgipath UK), dehydrated in absolute alcohol, cleared in xylene, and mounted using Dibutyl Phthalate Xylene (DPX) and glass coverslips.

**RESULTS**

**Susceptibility and Lethality Studies**

A summary of initial studies with aerosolized EBOV-Kikwit is shown in Table 2. After challenge with a Collison nebulizer concentration of 10^6 TCID_{50} EBOV-Kikwit, the mean impinger count was 43.5 TCID_{50}, yielding a mean aerosol concentration of 18 TCID_{50} for a 10-minute spray. Actual inhaled doses varied with the inhaled volume for each individual animal, which ranged from 0.26 to 1.5 L, resulting in an estimated inhaled dose range of 4–27 TCID_{50}.

All animals challenged with a starting concentration of 10^7 or 10^6 EBOV-Kikwit succumbed to infection (and were euthanized), however when a pair of animals was challenged with a 1:10 dilution of EBOV-Kikwit (10^5), 1 animal survived. The impinger count was below the detection limit of the assay, but based on the mean impinger count for a 1:10 dilution and the volume this animal inhaled during challenge, the inhaled dose for the survivor was estimated to be 1 TCID_{50}.

The temperature profile for all the animals that succumbed to infection was similar, with a normal diurnal rhythm for 4–5 days (Figure 1). From 100 hours after challenge animals became febrile (Table 2). Fever typically lasted 2–2.5 days and animals succumbed at 7 days after infection, although the time to death (euthanasia) was extended to 10 days for the single animal that succumbed to the lowest dose given (Table 2). Typically, there was a rapid decrease in temperature detected during the last 6–10 hours before euthanasia. This rapid temperature drop formed a key trigger for the humane end point. For the animal that survived, there were no clinical signs and no change in temperature from the typical diurnal rhythm (Figure 1).

Animals showed clinical signs between 24 and 48 hours before succumbing to disease. Overt signs of infection included a hunched posture, unkempt fur, altered respiration, subdued nature, and reluctance to move, eat or drink. External hemorrhaging from the genitals was observed in 4 animals, but no rash was observed in any of them.

---

**Table 1. Summary of Primary Antibodies and Antigen Retrieval Methods Used for Immunohistochemistry**

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Source</th>
<th>Antigen Retrieval Method</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse vs EBOV VP40</td>
<td>Dstl, UK</td>
<td>Microwave (pH 6.0)</td>
<td>1:7500</td>
</tr>
<tr>
<td>Mouse vs human macrophages, clone MAC387</td>
<td>AbD Serotec, Kidlington, UK</td>
<td>0.5% Trypsin-chemotrypsin</td>
<td>1:400</td>
</tr>
<tr>
<td>Rabbit vs human active caspase 3</td>
<td>Abcam, Cambridge, UK</td>
<td>Microwave (pH 9.0)</td>
<td>1:900</td>
</tr>
</tbody>
</table>

**Table 2. Outcome After Aerosol Exposure of Marmosets to Ebola Virus-Kikwit**

<table>
<thead>
<tr>
<th>Animal Identifier (Sex)</th>
<th>Step</th>
<th>TCID_{50} of EBOV-Kikwit in Collison (Dilution)</th>
<th>Volume Inhaled, mL</th>
<th>Time to Death (Euthanasia), h/d</th>
<th>Time to Fever, h</th>
<th>Length of Fever, h</th>
<th>Mean TTD per Dose, h/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>93M (M)</td>
<td>Susceptibility</td>
<td>10^6 (1:10)</td>
<td>1507</td>
<td>156/6.5</td>
<td>102</td>
<td>54</td>
<td>160.5/6.7</td>
</tr>
<tr>
<td>26N (F)</td>
<td>Dose range: high</td>
<td>10^7 (undiluted)</td>
<td>789</td>
<td>165/6.9</td>
<td>101</td>
<td>64</td>
<td>169.0/7.0</td>
</tr>
<tr>
<td>90P (F)</td>
<td>Dose range: medium</td>
<td>10^6 (1:10)</td>
<td>685</td>
<td>161/6.7</td>
<td>102</td>
<td>59</td>
<td>202.5/8.4</td>
</tr>
<tr>
<td>205N (M)</td>
<td>Dose range: low</td>
<td>10^6 (1:100)</td>
<td>737</td>
<td>177/7.4</td>
<td>117</td>
<td>60</td>
<td>169.0/7.0</td>
</tr>
<tr>
<td>61N (F)</td>
<td>Dose range: medium</td>
<td>10^7 (1:10)</td>
<td>526</td>
<td>193/7.6</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>116N (M)</td>
<td>Dose range: low</td>
<td>10^7 (1:100)</td>
<td>705</td>
<td>222/9.2</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>42N (F)</td>
<td>Dose range: low</td>
<td>10^7 (1:100)</td>
<td>705</td>
<td>222/9.2</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>49N (M)</td>
<td>Dose range: medium repeat 1</td>
<td>10^6 (1:10)</td>
<td>750</td>
<td>185/7.7</td>
<td>124</td>
<td>61</td>
<td>176.0/7.3</td>
</tr>
<tr>
<td>86P (M)</td>
<td>Dose range: medium repeat 2</td>
<td>10^6 (1:10)</td>
<td>1124</td>
<td>185/7.7</td>
<td>124</td>
<td>61</td>
<td>176.0/7.3</td>
</tr>
<tr>
<td>64P (F)</td>
<td>Dose range: medium repeat 2</td>
<td>10^6 (1:10)</td>
<td>575</td>
<td>159/6.6</td>
<td>123</td>
<td>36</td>
<td>176.5/7.3</td>
</tr>
<tr>
<td>232N (M)</td>
<td>Dose range: medium repeat 2</td>
<td>10^6 (1:10)</td>
<td>227</td>
<td>194/8.1</td>
<td>135</td>
<td>59</td>
<td>NA</td>
</tr>
<tr>
<td>111P (F)</td>
<td>Dose range: medium repeat 2</td>
<td>10^6 (1:10)</td>
<td>227</td>
<td>194/8.1</td>
<td>135</td>
<td>59</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Abbreviations**: EBOV, Ebola virus; F, female; M, male; NA, not applicable (animal survived); ND, not determined (equipment failure; no data available); TCID_{50}, 50% tissue culture infectious dose; TTD, time to death.
Viral Load, Blood Analysis, and Pathology in Susceptibility and Median Lethal Dose Studies

High organ titers (>10⁷ TCID₅₀ EBOV-Kikwit) were observed in the livers, lungs, kidneys, spleens and blood of all animals that succumbed to infection (data not shown). No virus was detected in the organs of the animal that survived aerosol EBOV-Kikwit challenge.

A wide range of blood parameters was measured before challenge and at the point of culling. A number of cell types and chemistry values showed significant changes from baseline to after infection (Figure 2). Platelet counts, reticulocyte percentage, and plasma amylase levels were decreased at postmortem examination compared with baseline, and alkaline phosphatase, alanine aminotransferase, and globulin levels increased at postmortem examination. By linear model analysis, 4 factors were found to be significantly different from baseline to terminal: amylase, urea, platelets, and clotting time as measured by activated partial thromboplastin time. Blood data from the single animal that survived EBOV-Kikwit challenge showed a varied response, with most values similar to the baseline values, but some of the complete blood count data were similar to the values seen in animals that succumbed (eg, monocyte, basophil, and platelet counts) (Figure 2). Because the challenge delivered to this animal was very low, there was no evidence that it actually received an infectious dose.

Gross examination of organs at postmortem examination showed signs of hemorrhage in livers (Figure 3) or lungs (data not shown). Livers typically appeared mottled, and spleens and lymph nodes were enlarged. Histopathological features were observed in organs after hematoxylin-eosin staining (Figure 3). Livers typically showed random multifocal areas of hepatocellular degeneration and necrosis with occasional neutrophil infiltration, intracytoplasmic inclusion bodies, and hemorrhaging. Lungs showed interstitial pneumonia with occasional thrombosis or microthrombosis, hyperemia, focal alveolar hemorrhages, and infrequent presence of fibrin. Spleens showed more widespread fibrin deposition in the red pulp, severe lymphoid depletion in most samples, and frequent hemorrhages in the marginal zone. Kidneys typically showed a variety of pathologic changes, including thrombosis in the glomerular capillaries, hyperemia, varying levels of glomerulonephritis, tubular necrosis, fibrin, and multifocal hemorrhages.

---

**Figure 1.** Temperature profiles of marmosets challenged with Ebola virus (EBOV) Kikwit by the aerosol route. Representative temperatures profiles showing a febrile response are shown for animals challenged by the aerosol route with EBOV-Kikwit. Each color represents a single animal. Four animals (solid lines) were challenged with a Collison concentration of 10⁶ times the 50% tissue culture infectious dose (TCID₅₀) of EBOV-Kikwit (for a mean inhaled dose of approximately 10 TCID₅₀), and 2 animals (dotted lines) were challenged with a log higher dose (for a mean inhaled dose of approximately 100 TCID₅₀). One animal challenged with an estimated inhaled dose of 1 TCID₅₀ (dashed black line) survived the challenge.
Natural History Study

To investigate the natural history of the disease, 16 animals were exposed to a target dose of 10 TCID\textsubscript{50} EBOV-Kikwit. Four animals were culled at each of 4 time points; (days 1, 3, 5, and 6 after infection). Aside from an elevated temperature in animals culled at day 5 or 6 after infection, no other clinical signs or gross pathology was observed.

No virus was detected in tissues on day 1 after infection, and low levels (<10\textsuperscript{3} TCID\textsubscript{50}/g EBOV-Kikwit) were detected in some animals on day 3 after infection (Figure 4). At day 5 after infection, as fever began, systemic spread of EBOV-Kikwit had occurred, with a range of virus titers seen (<10\textsuperscript{2} to >10\textsuperscript{6} TCID\textsubscript{50}/g organ EBOV-Kikwit). At day 6 after infection, viral titers were consistently high (>10\textsuperscript{5} TCID\textsubscript{50}/g EBOV-Kikwit) in all organs (Figure 4).

A number of blood chemistry values changed over time after infection with EBOV-Kikwit (Figure 5). Alkaline phosphatase levels increased (Figure 5); as did globulin, creatinine kinase, and lactate dehydrogenase levels (results not shown). Generally, amylase levels decreased over time (Figure 5). From the complete blood cell count, reticulocyte counts decreased over time and the white blood cell count increased (Figure 5). There was minimal change in clotting times after challenge until day 6 after infection, when an increased clotting time was observed (Figure 5).

Minimal histopathological features were observed in animals during the natural history study. Spleen tissue at day 5 and 6 after infection showed acute splenitis, moderate hyperemia, and abundant neutrophils in the red pulp but no evidence of necrosis or fibrin deposition. At day 6 after infection, there was also mild lymphoid depletion. No histopathological features were observed in the livers until day 6 after infection, when multifocal areas of hepatocellular degeneration and necrosis and limited neutrophilic infiltration were observed.

In the lungs at days 1 and 3 after infection mild interstitial pneumonia, alveolar microhemorrhage and hyperemia were evident. On day 5 the lung of 1 animal showed pathological findings similar to those seen earlier, and on day 6 the lung of another animal showed focal interstitial pneumonia. There were minimal histopathological features in kidney tissue, with no remarkable changes seen on day 1, 3, or 5 after infection; on day 6, mild glomerulonephritis was evident in 1 animal.

Immunohistochemical, MAC387, and Caspase 3 Staining

Additional staining was used to assess the level of viral antigen, macrophages, monocytes or polymorphonuclear cell or apoptosis and phosphotungstic acid hematoxylin was used to detect fibrin (Figure 6). EBOV antigen was detected in the livers, spleens, and lungs of all animals at day 6 after infection and in the kidneys of...
3 of the 4 animals at day 6 after infection. The presence of viral antigen was observed in the livers (hepatocytes, Kupffer cells, or endothelial cells), spleens (macrophages in the red pulp), kidneys (glomerular cells), and lungs (alveolar macrophages) of all animals that succumbed to infection (Figure 6, set 1).

MAC387 staining suggested a high abundance of macrophages, monocytes, or granulocytes in all organs of all animals that succumbed to infection and the distribution was similar to that of the viral antigen (Figure 6, set 2). Caspase 3 staining was used to investigate the presence of apoptosis. In spleen tissue, there was minimal staining at day 6 after infection, but more evidence of apoptosis was observed in the spleens of animals that succumbed to infection. Lymph nodes also showed evidence of apoptosis (Figure 6, set 3).

Figure 3. Gross pathological appearance (A–C) and hematoxylin-eosin staining (D–G) of marmoset organs after aerosol challenge with Ebola virus (EBOV) Kikwit. A, B, An enlarged spleen (A) and the normal-size spleen (B) of the animal that survived EBOV-Kikwit challenge for comparison. C, Mottled liver with apparent blood clots. Scale bars in A-C represent approximately 10 mm. D–G, Staining showing perivascular (open arrow) and peribronchiolar (line arrow) edema in the lung (D), necrotic hepatitis and signs of hemorrhage in the liver (arrows) (E); necrosis and hemorrhage in the red pulp and lymphoid depletion in the spleen (arrow) (F), and glomerulonephritis and hemorrhaging in the kidney (arrow) (G).
There was no evidence of fibrin deposition in animals at day 6 after infection. However, fibrin deposition was apparent in the kidney and spleen of animals that succumbed to an aerosol dose of approximately 10 TCID50 of EBOV-Kikwit. The fibrin was associated with the capillaries in the kidney and spleen, and in some networks in the red pulp of the spleen. Fibrin was also detected in the lung and liver (Figure 6, set 4).

For the single animal that survived low-dose aerosol challenge, no viral antigen was detected in any of the organs analyzed. Staining revealed the presence of macrophages in all organs assessed. There was no evidence of apoptosis or fibrin deposition in this animal (data not shown).

**DISCUSSION**

EVD in the marmoset was characterized, after aerosol challenge with EBOV-Kikwit, by assessing viral load, hematological, physiological, and pathological features over time. The results were consistent with other nonhuman primate model data [12, 20, 31, 32, 38, 39], other animal models [40, 41] and with the previously limited data on human infections [42].

This study demonstrated that the common marmoset is susceptible to aerosolized EBOV-Kikwit, with doses of 4–27 TCID50 sufficient to cause lethal infection. A median lethal dose by the aerosol route could not be definitively calculated, but 1 of 2 animals challenged with the lowest dose of EBOV-Kikwit, estimated to be approximately 1 TCID50, survived with no clinical signs or evidence of viral replication. This suggests that the median lethal dose of EBOV-Kikwit in marmosets, by the aerosol route, may be approximately 1 TCID50. Aerosol challenges were performed based on a standard exposure time (10 minutes) and measurement of the volume of air each individual animal inhaled during challenge. The breathing rates of the animals differed, which ultimately affected the viral challenge dose delivered, and a broad correlation between delivered dose and time to death was observed. Such variable dosing is a common phenomenon and a recognized limitation of the aerosol route of delivery.

In particular, reproducible disease patterns and clinical signs were demonstrated with a target aerosolized virus dose of 10 TCID50. Low infectious doses are typical for infection by filoviruses in other animal models [12, 14, 17, 31, 38].

Fever is a key feature of human infection with EBOV [42–44] and is observed in other animal models [31, 39], and elevated temperatures were seen in all marmosets that succumbed to EBOV-Kikwit infection. For humans, the date of infection is not typically known, but the length of time between onset of fever/symptoms and death (or recovery) in the current West African outbreak is >8 days [43, 44], whereas marmosets succumbed within 3 days after the onset of fever.

No rash was observed in any marmosets in these studies. This is consistent with findings in other marmosets and African green monkeys infected with EBOV [13, 39, 45]. A rash is a common feature of EVD in rhesus macaques [20] but is seen in a low proportion of EBOV-infected humans [42, 44].

Onset of clinical signs coincided with the isolation of virus within blood and organs. This was consistent with results of EBOV infection in macaques [20] and marmosets [31]. Humans that succumb to EBOV infection also have high viral loads in multiple organs [42–44]. High viral titers were matched by abnormalities in blood parameters; liver and kidney enzyme levels, white blood cell counts, and blood clotting times were increased compared with baseline in animals that succumbed, and platelet levels were decreased. These findings are consistent with early findings in nonhuman primates [46, 47]. In humans, patients present with low lymphocyte counts, which increase as disease progresses [42]; a similar pattern was observed in our marmosets and in previous studies [31]. Thrombocytopenia is a well-documented feature of EVD in humans, as are elevated levels of alanine and aspartate aminotransferase, blood urea nitrogen, and creatinine and prolonged blood clotting times [42, 44];
we observed similar trends in marmosets infected with EBOV-Kikwit.

The absence of significant histopathological features at later time points was perhaps surprising but consistent with an absence of any gross disease for all organs at any time point and low viral titers at earlier time points. It is possible that histopathological lesions and changes became apparent only very late in the disease course, after day 6, resulting in limited and discrete pathological changes confined to smaller areas. Immunohistochemical staining of organs indicated that levels of viral antigen increased during the course of disease. This finding is consistent with findings in rhesus macaques challenged with EBOV via the aerosol route [39].

Macrophages are early targets for EBOV, and infected macrophages result in an increase in proinflammatory cytokines, chemokines, and tissue factors that induce more cells to be infected and can lead to an overwhelming innate immune response that is a major part of EBOV pathogenesis [48, 49]. All animals that succumbed to disease in our study showed high levels of MAC387-stained cells in the liver, splenic red pulp, and lung. The presence of apoptotic phenomena is associated with lymphocyte depletion, which was also observed in the spleens and lymph nodes of the animals that succumbed to infection. In other nonhuman primates infected with EBOV, lymphocyte depletion/apoptosis was common in the lymph nodes and spleen [39, 50]. Fibrin deposits were detected in organs of marmosets during the latter stages of the disease. Similar responses have been reported in rhesus macaques challenged via the aerosol route, with fibrin detected in livers, spleens, and lungs at days 7 and 8 after challenge [39].

The common marmoset (C. jacchus) is a New World nonhuman primate species whose small size (300–500 g) offers some advantage over more commonly used Old World primates as an animal model [25]. As with other animal models, there are practical limitations to the use of marmosets within high containment laboratories, such as continuous blood sampling, but marmosets may offer an alternative small primate model for the development and testing of vaccines and/or treatments for EVD. The current study has shown that the common marmoset

Figure 5. Changes in selected blood parameters at different times after challenge with Ebola virus (EBOV) Kikwit by the aerosol route. After challenge with EBOV-Kikwit marmosets show altered blood parameters over time. A, Amylase levels. B, Alkaline phosphatase (ALKP) (dotted line; open symbols) and alanine aminotransferase (ALT) (solid line; closed symbols). C, Platelet counts. D, White blood cell (WBC) count. E, Reticulocyte counts. F, Blood clotting time measured by the prothrombin time (PT) (solid line; closed symbols) and activated partial thromboplastin time (aPTT) (dotted line; open symbols). Data from animals that succumbed after challenge with the same starting concentration (Terminal) are also shown for comparison, and prechallenge (Pre) data from all animals are included.
Figure 6. Staining for viral antigen, macrophages, apoptosis or fibrin in marmoset organs after aerosol challenge with Ebola virus (EBOV) Kikwit. Fixed liver (2 panels), spleen, kidneys, lungs (3 panels each), and lymph nodes (1 panel) showed reactivity after staining. Set 1, Stained with an anti-EBOV VP40 antibody; viral antigen stains brown. Set 2, Samples stained (brown) with MAC387 for monocytes, macrophages and granulocytes. Set 3, Caspase 3 staining for apoptotic cells, labeled brown (arrows), visible in spleen and lymph nodes only. Set 4, Phosphotungstic acid hematoxylin staining to visualize fibrin deposition, in kidney and lungs (arrows). (Magnification, ×10 [sets 1–3] and ×20 [set 4]).

is susceptible to EBOV-Kikwit and shows disease patterns, clinical signs, and blood parameters similar to those seen in humans and other nonhuman primates infected with EBOV. The marmoset may therefore provide a small representative primate model of EVD for testing medical countermeasures.

Notes

Acknowledgments. We thank our many colleagues in the Microbiology and Facilities group at the Defence Science and Technology Laboratory (Dstl) for assistance with animal care and monitoring; A. Phelps and P. Pearce for helping with implant surgery; T. Piggot, P. Rachwal, C. Lonsdale, and D. Cleary for sequence analysis; and T. Atkins and G. Donovan for project support.

Financial support. This work was supported by Medical Countermeasure Systems Joint Project Management Office (contract HDTRA1-11-C-0039).

Potential conflicts of interest. All authors: No potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References