P E E R
PR
R E V I E W E D

J
C
P

Haematological, Biochemical and Coagulation Changes in Mice, Guinea-pigs and Monkeys Infected with a Mouse-adapted Variant of Ebola Zaire Virus

M. Bray*, S. Hatfill*, L. Hensley† and J. W. Huggins*

*Virology and †Pathology Divisions, United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, Maryland 21702-5011, USA

Summary

Ebola Zaire virus from the 1976 outbreak (EBO-Z) was recently adapted to the stage of lethal virulence in BALB/c mice through serial passage. In the present study, various parameters were examined in groups of mice and guinea-pigs and in three rhesus monkeys after infection with mouse-adapted EBO-Z. The virus caused fatal disease not only in mice but also in guinea-pigs, in which the course of illness resembled that produced by guinea-pig-adapted EBO-Z. Mice, guinea-pigs and monkeys showed similar haematological and biochemical disturbances, but coagulopathy was less striking in mice than in the other two species. The virus caused severe illness in all three monkeys, one of which died. In the lethally infected monkey the degree of viraemia and the haematological, serum biochemical and coagulation changes were greater than in the other two animals, an observation that may prove to be of value in predicting fatal outcome. All three monkeys developed disseminated intravascular coagulation. The two survivors were completely resistant to challenge one year later with non-adapted EBO-Z. In general, the clinical and pathological changes produced in the three species resembled those previously described in guinea-pigs and non-human primates infected with non-mouse-adapted EBO-Z. It was noteworthy, however, that mouse-adaptation appeared to have resulted in a degree of attenuation for monkeys.

Introduction

Ebola Zaire virus (EBO-Z), a negative-sense non-segmented RNA virus belonging to the family *Filoviridae*, causes severe haemorrhagic fever in man (Peters *et al.*, 1996). Isolates from human patients cause fatal infections in non-human primates (NHPs) (Baskerville *et al.*, 1978; Bowen *et al.*, 1978, 1980; Fisher-Hoch *et al.*, 1983, 1985, 1992; Johnson *et al.*, 1995; Luchko *et al.*, 1995; Jaax *et al.*, 1996; Davis *et al.*, 1997) and non-fatal febrile illness in guinea-pigs (GPs), in which a small number of passages results in lethal virulence (Bowen *et al.*, 1978, 1980; Connolly *et al.*, 1999).

Bray *et al.* (1998) succeeded in producing a mouse-adapted EBO-Z, which gave rise to lethal infection in various mouse strains inoculated intraperitoneally. A detailed study of the pathogenesis of such infection in BALB/c mice was reported in the preceding paper (Gibb *et al.*, 2001). Within 2 days of inoculation, the virus infected mononuclear phagocytic cells, including those of the spleen and liver, and then spread to hepatocytes, fibroblasts and other cell types, in a pattern similar to that in EBO-Z-infected GPs and NHPs (Baskerville *et al.*, 1978; Jaax *et al.*, 1996; Peters *et al.*, 1996; Davis *et al.*, 1997; Connolly *et al.*, 1999).

Rapid viral replication results in extensive cytolysis, cytokine release, endothelial cell dysfunction, fluid shifts, coagulation abnormalities and

Correspondence to: M. Bray, Virology Division, USAMRIID, 1425 Porter Street, Ft Detrick, Maryland 21702-5011, USA.

physical interference with blood flow in the micro-circulation; these changes can be recognized by clinical laboratory tests (Fisher-Hoch *et al.*, 1983, 1985; Peters *et al.*, 1996). The purpose of the present study was two-fold: to investigate the clinical pathology of mice, GPs and NHPs infected with mouse-adapted EBO-Z; and to determine whether this virus would produce lethal infection in GPs and NHPs, as in mice.

Materials and Methods

Viruses and Cells

The viruses used were (1) mouse-adapted EBO-Z (Bray *et al.*, 1998; Gibb *et al.*, 2001), (2) "GP-adapted virus", EBO-Z '76 passaged twice in Vero cells and four times in GPs, and (3) EBO-Z '95, isolated from the 1995 Zaire outbreak and passaged three times in Vero cells. The latter two viruses were provided by Dr Peter Jahrling, USAMRIID.

Vero 76 monkey kidney cells (ATCC CRL 1587) and the E6 clone of Vero cells (Vero C1008, ATCC CRL 1586) were used for viral amplification and titration of samples. Cells were propagated in Eagle's minimal essential medium with Earle's salts (EMEM), non-essential amino acids, fetal bovine serum (FBS) 10%, glutamine, penicillin, and strep-tomycin at 37°C in a 5% CO_2 atmosphere. Viral titres were determined as described by Bray *et al.* (1998). The 50%-plaque-reduction neutralization titre ($PRNT_{50}$) of serum samples was determined by heating the samples at 56°C for 30 min, incubating serial two-fold dilutions with a fixed quantity of EBO-Z '95 virus for 30 min at 37°C, and identifying the dilution of serum that caused a 50% reduction in the number of plaques, as compared with normal serum.

EBO-Z Infection of Animals

Infectious material and animals were handled in maximum-containment biological safety level 4 (BSL-4) facilities at the USAMRIID. Laboratory personnel wore positive-pressure protective suits equipped with high-efficiency particulate air filters and supplied with "umbilical-fed" air. All experiments were conducted in accordance with animal welfare guidelines (Gibb *et al.*, 2001).

Mice. Adult female BALB/c mice were obtained from the National Cancer Institute (NCI), Frederick, MD, USA. In a typical experiment, a cohort of mice was infected intraperitoneally with from 1 to 1000 plaque-forming units (pfu) of mouse-adapted EBO-Z. One subgroup of 10 mice was then held for daily observation of weight loss, illness and death, while on each day over the next five days 3–6 mice from the remainder of the cohort were anaesthetized and exsanguinated, and serum or plasma was collected as described below. In two experiments, transponder chips were implanted subcutaneously several days before inoculation, enabling temperatures to be measured with a hand-held probe (Biomedic Data Systems, Maywood, NJ, USA). Temperatures were determined daily, beginning 2 days before infection with 1 or 100 pfu. In an initial experiment, in which mice were inoculated with 1 pfu, two animals were killed each day and serum viral titres were determined.

Guinea-pigs. Adult strain-13 GPs were obtained from the USAMRIID breeding colony and strain-2 GPs from the NCI. Experiments were performed as described above for mice, but with 3–6 animals in every subgroup. GPs were infected with mouse-adapted or GP-adapted EBO-Z by injecting 0·1 ml of viral suspension intraperitoneally or sub-cutaneously. The LD_{50} of each virus was estimated by infecting groups of four animals intra-peritoneally or subcutaneously with serial 10-fold dilutions of virus and observing them for illness and death. In one study, strain-2 guinea-pigs received implanted transponder chips and temperatures were monitored, beginning 2 days before infection. Blood was collected either by venipuncture under anaesthesia or by terminal exsanguination.

Monkeys. Three 8-kg rhesus monkeys (*Macaca mulatta*), nos 8709, K439 and H873, were inoculated intramuscularly with 5000 pfu of mouse-adapted virus. The animals were then observed daily and examined and bled under anaesthesia from the femoral vein at intervals of 2–3 days. Two animals (K439 and H873) survived infection and remained in BSL-4 accommodation, where blood samples were collected every 2 months. Thirteen months after infection, both monkeys together with four *Macaca fascicularis* monkeys (three members of an experimental vaccine group and one untreated control) were challenged by intramuscular inoculation with 5000 pfu of EBO-Z '95. They were again observed daily and bled under anaesthesia at intervals of 2–3 days.

Clinical Pathology

Haematology. Total haemoglobin, haematocrit values, white blood cell counts and platelet counts

were determined from blood samples collected in tubes containing EDTA, by means of a Coulter T890 Haematologic Analyzer (Coulter Electronics, Hialeah, FL, USA). Cell differential counts on mice and monkeys were performed manually on Wright-stained blood smears.

Coagulation tests. On collection, blood was mixed with 1/10th of its volume of 0·1 M buffered sodium citrate and the plasma was separated by centrifugation. The prothrombin (PT), activated partial thromboplastin (aPTT), and thrombin (TT) times, and plasma fibrinogen concentration were determined on a Sysmex CA-1000 Automated Co-agulation Analyzer (Toa Medical Electronics, Kobe, Japan) with standard kits (Dade International, Miami, FL, USA). For mice, plasma samples from 2–3 animals at each time point were pooled to provide a sufficient volume for testing. Fibrin degradation products (FDPs) in monkey plasma were measured semiquantitatively by visual scoring of a latex agglutination test ("Spli-Prest"; Diagnostica Stago, Asnières, France). D-dimers were quantitated by an enzyme-linked immuno-sorbent assay ("Asserachrom D-Di"; Diagnostica Stago).

Serum biochemistry. Serum samples from individual mice and monkeys were frozen at $-70°C$, thawed and tested as a batch for sodium, potassium, chloride, bicarbonate, calcium, phosphorus, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), gamma-glutamyl transferase (GGT), total bilirubin, urea nitrogen (BUN) and creatinine (Cr) concentrations, by means of a Kodak 250 Chemistry Analyzer (Eastman Kodak, Rochester, NY, USA). GPs were not examined biochemically.

Results

Course of Illness and Viraemia

Mice. Signs of illness (ruffled fur, decreased activity, weight loss) were seen 3–4 days after inoculation. Increasing the inoculum from 1 to 1000 pfu only slightly shortened the mean time to onset of illness and death. Virus could not be detected in the serum on day 1 after any dose, but was present on day 2 and rose rapidly thereafter (Fig. 1A). By day 4, serum viral titres were in the range of 10^8–10^9 pfu/ml, regardless of the size of the inoculum. In two experiments in which body temperatures were monitored in mice infected with
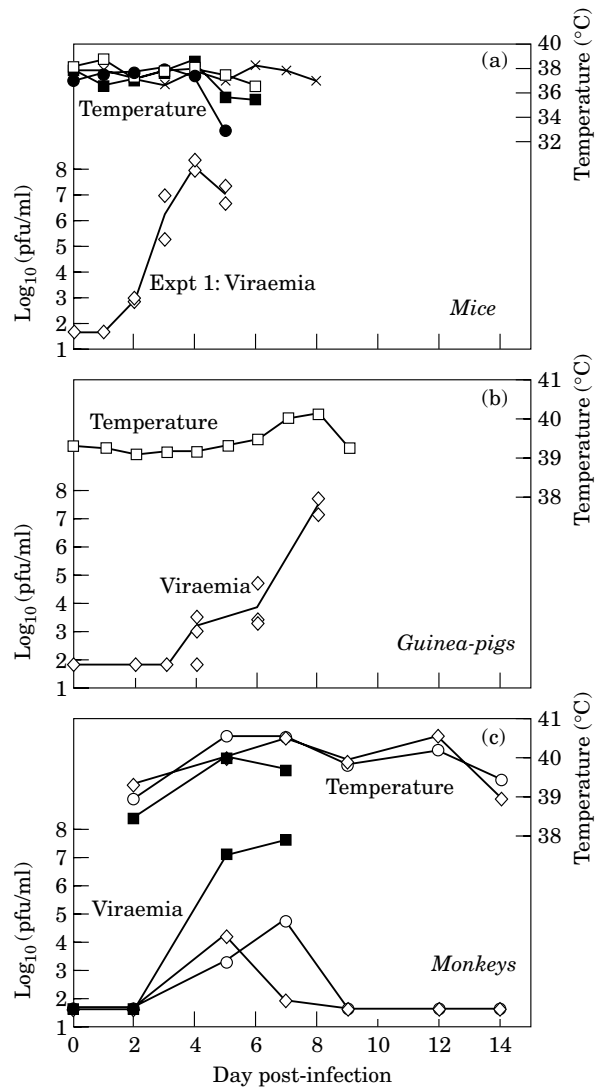

Fig. 1A–C. (A) Mean body temperature (upper lines, in °C) and viraemia (lower line, in pfu/ml; diamonds, individual values; line, geometric means) of BALB/c mice after intraperitoneal infection with mouse-adapted EBO-Z. Experiment 1: 1 pfu (30 LD_{50}; □); Experiment 2: 1 pfu (■), 100 pfu (●), or uninfected (×). In Experiment 1, viraemia was measured on samples from mice terminally exsanguinated after temperature determination. (B) Mean body temperature and viraemia of strain-2 GPs after intraperitoneal infection with 100 pfu of mouse-adapted EBO-Z. (C) Temperature and viraemia of three rhesus monkeys (8709, –■–; H873, –◇–; k439, –○–) infected with 5000 pfu of mouse-adapted EBO-Z.

1 pfu (30 LD_{50}) or 100 pfu, or were left uninfected, no temperature elevation was observed (Fig. 1A). Body temperatures fell as the mice became moribund.
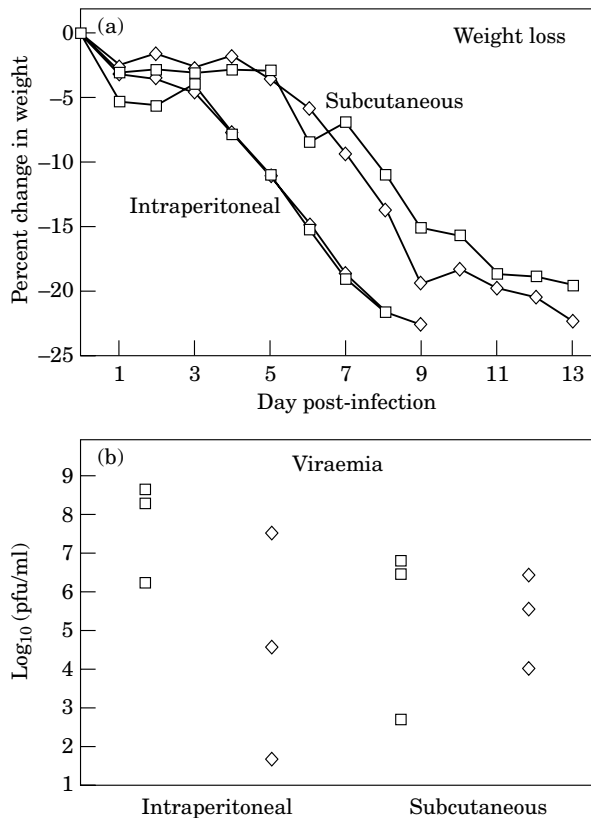
Fig. 2A,B. (A) Mean percentage change in body weight of groups of three strain-13 GPs inoculated subcutaneously or intraperitoneally with 1000 pfu of mouse-adapted (squares) or GP-adapted (diamonds) EBO-Z. (B) Serum viral titres of the same GPs on day 7 post-infection. Symbols as in Figure 2A.

Guinea-pigs. Mouse-adapted EBO-Z caused lethal illness with uniform signs in strain-2 and strain-13 GPs. By contrast, GP-adapted EBO-Z did not cause illness in adult mice (not shown). In both GP strains, the LD_{50} of mouse-adapted and GP-adapted EBO-Z was <1 pfu intraperitoneally and 10^3–10^4 pfu subcutaneously (in contrast to mice, in which subcutaneous inoculation of >10^6 pfu of mouse-adapted virus did not cause disease).

Mouse-adapted and GP-adapted EBO-Z caused almost identical illnesses after intraperitoneal inoculation, with onset of weight loss on day 4–5, and death at 8–9 days post-infection (Fig. 2A). Subcutaneous inoculation resulted in a later onset of illness. Viraemia appeared later in GPs than in mice (on day 5–6 after intraperitoneal inoculation with 100 pfu of mouse-adapted virus), and increased more slowly (Fig. 1B). Serum viral concentrations on day 7 post-infection were higher in intraperitoneally than subcutaneously inoculated

GPs. Unexpectedly, the highest titres occurred in GPs receiving mouse-adapted, rather than GP-adapted virus (Fig. 2B). Body temperatures began to rise above baseline on day 5–6, coinciding with an increase in serum viral titres to values of >10^3 pfu/ml (Fig. 1B). The animals remained febrile for 2–3 days, viral titres increasing to 10^7 pfu/ml, after which temperatures fell as death approached.

Monkeys. The mean body temperature of the three monkeys inoculated with mouse-adapted EBO-Z rose from 38·9°C on day 2 to 40·2°C on day 5 post-infection, and remained above 40°C up to day 12 (Fig. 1C). (Through an oversight, temperatures were not measured on day 0.) All three animals showed a reduction in activity and food intake on day 5. Monkey 8709 became markedly depressed over the next 2 days. By day 7 it had developed a fine reddish-purple maculopapular rash over the trunk and in the axillae. Conjunctival haemorrhages were present, but no frank bleeding was observed. Serum viral titres were $1·2 \times 10^7$ pfu/ml on day 5, and 4×10^7 pfu/ml on day 7 (Fig. 1C). The animal was found dead on the morning of day 8.

In the other two monkeys, decrease in activity after day 5 was milder, and neither developed a rash. Their peak serum viral titres were approximately 1000-fold lower than that of the fatally infected animal (H873, $1·7 \times 10^4$ on day 5; K439, 6×10^4 on day 7) (Fig. 1C). Both were free of viraemia on day 9, but remained febrile up to day 12. They became afebrile and were fully active by day 14. The $PRNT_{50}$ of both monkeys against EBO-Z '95 were 80 and 40 at 2 and 13 months after infection, respectively. Both remained healthy after challenge with EBO-Z '95 at 13 months, without detectable viraemia or changes in laboratory parameters (see below). Four *M. fascicularis* monkeys challenged at the same time became ill and died on day 6 or 7 post-infection.

Haematology

Haemoglobin and haematocrit (hg and hct). In mice, both hg and hct increased over the course of infection (Fig. 3). In the example shown (100 pfu inoculum) the hg increased from a mean of 15·5 g/dl on day 0 to values of 16·1–16·7 on days 4 and 5, and the hct rose from 43% on day 0 to 50% on day 4 and 51% on day 5. Similar increases in hg and hct were measured in GPs lethally infected with mouse-adapted EBO-Z (not shown).
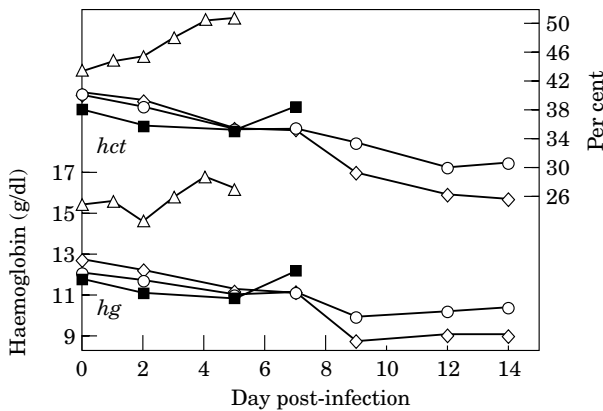
Fig. 3. Haemoglobin (hg) (in g/dl—left axis) and haematocrit values (hct) (per cent—right axis) of three rhesus monkeys (8709, –■–; H873, –◇–; k439, –○–) infected with mouse-adapted EBO-Z, and mean values of a cohort of mice (–△–) infected with 100 pfu of the same virus.

Fig 4A,B. (A) Counts (cells/mm^3) of WBCs (total), PMNs and lymphocytes in mice infected with mouse-adapted EBO-Z (100 pfu); mean values are indicated by lines. Diamonds indicate total WBC counts of individual mice. (B) Total WBC counts of three rhesus monkeys (8709, –■–; H873, –◇–; k439, –○–) infected with mouse-adapted EBO-Z.

By contrast, two different patterns of change in hg and hct were observed in the monkeys (Fig. 3). The two animals whose infection was not fatal underwent a slowly progressive decline in both parameters over a period of 3 weeks after infection, consistent with the effect of repeated blood sampling. The fatally infected monkey, however, showed no decline in hg or hct from day 2 to day 5, and displayed a sharp increase from day 5 to 7, the day before death. These changes were consistent with haemoconcentration due to dehydration and fluid shifts.

White blood cells (WBCs). EBO-Z-infected mice showed a marked rise in total WBC count, with an increase in the percentage of polymorphonuclear leucocytes (PMNs). In the 100 pfu challenge experiment cited above, total WBC counts (cells/ mm^3) were 2000–5000 on days 0 and 1, 4000–12 000 on day 3 and 5000–20 000 on day 5 (Fig. 4A). This resulted from an increase in PMNs, accompanied by a decline in the total lymphocyte count (Fig. 4A).

All three monkeys developed a leucocytosis by day 5, with WBC counts (cells/mm^3) of from 6000– 8000 on day 0 and 10 000–16 000 on day 5 (Fig. 4B). This was accompanied by an increase in percentage of PMNs, from 23–47% on day 0 to 63– 79% on day 5. The rapid rise was followed by a precipitous fall in WBC count on day 7, with a shift to lymphocytosis, and a return to high circulating counts (Fig. 4B). The lethally infected monkey had the highest percentage of PMNs on day 5 (79%),
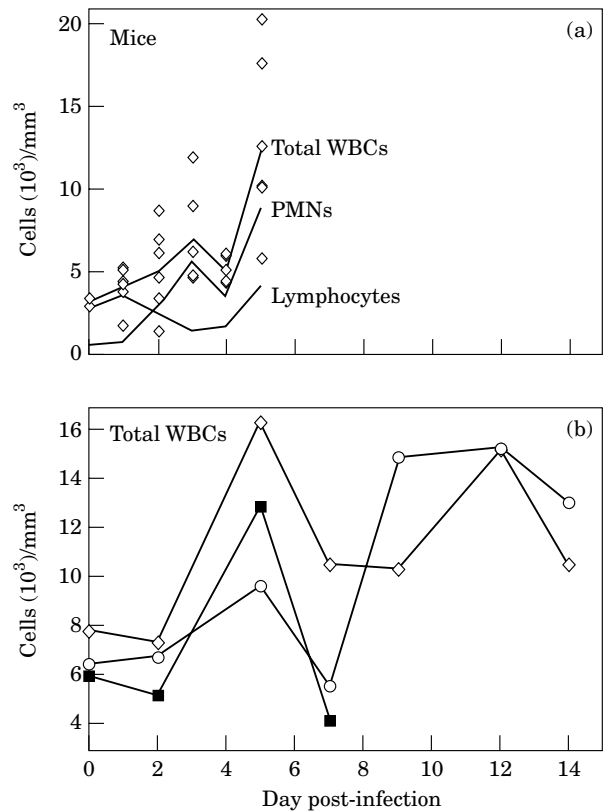
but its total WBC count on that day did not differ from those of the other two animals.

Platelets. Mice showed a decline in platelet counts over the course of illness (Fig. 5A). In the 100-pfu infection experiment, the platelet count had dropped 55% by day 4, while a decline of 67% from baseline was observed by day 5 in a second experiment. Strain-2 GPs infected with mouse-adapted EBO-Z also showed a profound drop in platelet count, similar to that previously observed in strain-13 GPs infected with GP-adapted EBO-Z (Connolly *et al.*, 1999). In monkeys, the lethally infected animal showed the most rapid and profound decline in platelet count, namely a 76% drop by day 7 (Fig. 5B). The platelet counts of the other two monkeys declined more slowly, to 50–69% by day 9, before starting to recover.
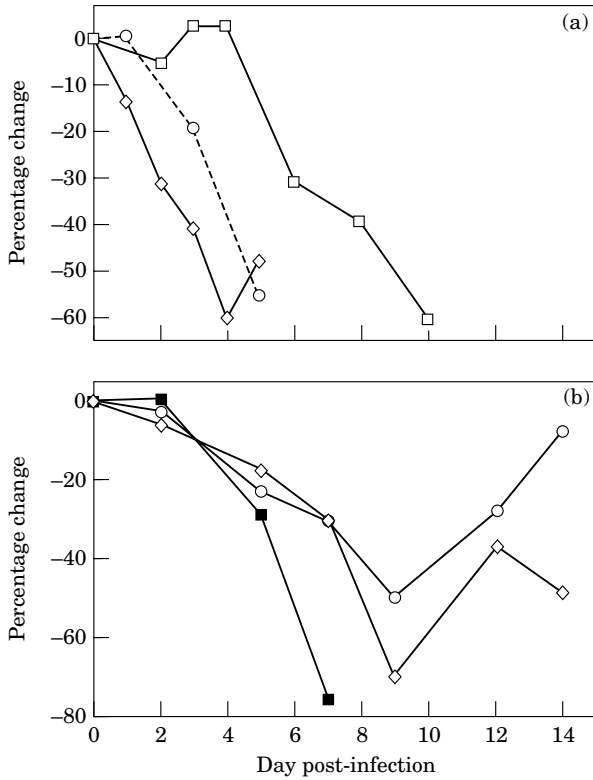
Fig. 5A,B. (A) Percentage change from baseline value of the mean platelet counts (in cells/mm^3) of groups of mice (diamonds: experiment 1; circles: experiment 2) or strain-2 GPs (squares) infected with mouse-adapted EBO-Z virus. (B) Percentage change in platelet counts of three rhesus monkeys (8709, –■–; H873, –◇–; k439, –○–) infected with mouse-adapted EBO-Z.

Coagulation

Mice. In a series of experiments, infection with mouse-adapted EBO-Z did not consistently cause a progressive coagulation defect over the course of illness. Figure 6A shows results obtained in the 100-pfu infection experiment and in a repeat experiment with the same dose. Although some plasma samples showed a modest prolongation of the aPTT, others from the same time point or later in the disease yielded values within the normal range. Plasma fibrinogen concentration also showed no change from baseline values.

Guinea-pigs. In contrast to mice, lethally infected GPs showed increasing prolongation of PT and aPTT over the course of illness. The former value had increased from *c.* 14 to >25 s, and the latter from 30 to 70 s, by day 8 (Fig. 6B). Plasma fibrinogen concentration showed no change from baseline values.
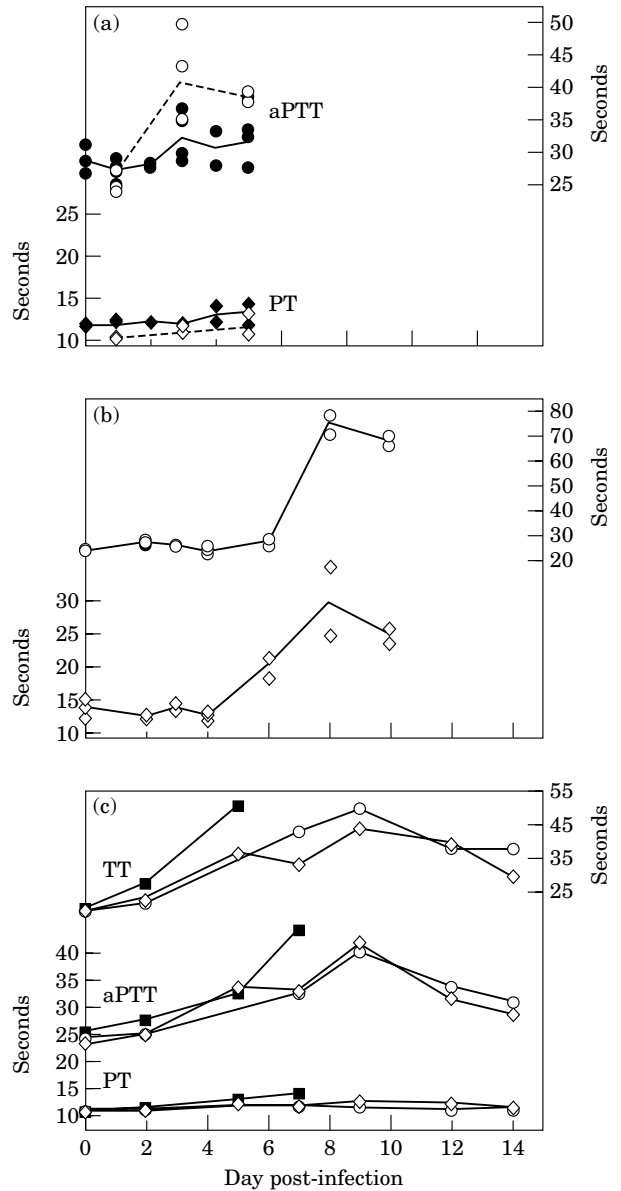

Fig. 6A–C. (A) Individual and mean plasma PTs and aPTTs (in seconds, left and right axes, respectively) of groups of BALB/c mice, at daily intervals after intraperitoneal infection with 100 pfu of mouse-adapted EBO-Z, in two experiments (first: solid symbols, solid lines; second: open symbols, dashed lines). Each test was performed with pooled plasma from two or three mice terminally exsanguinated on the indicated day. (B) Individual and mean plasma PTs (diamonds) and aPTTs (circles) of a cohort of strain-2 GPs after intraperitoneal infection with 100 pfu of mouse-adapted EBO-Z. (C) Plasma PTs, aPTTs (seconds, scale left axis) and TTs (seconds, scale right axis) of three rhesus monkeys (8709, –■–; H873, –◇–; k439, –○–) infected with mouse-adapted EBO-Z.

Monkeys. All three animals developed significant coagulation abnormalities, which were most striking in the lethally infected monkey. This animal showed a marked prolongation of aPTT and TT and a lesser change in PT, which increased up to day 7 (Fig. 6C). The two survivors displayed similar prolongation of the aPTT, but it was slower in onset, peaking at day 9 and returning gradually to normal. The plasma fibrinogen concentration in the lethally infected monkey decreased from the baseline value, but in the other two animals it increased and remained elevated up to day 14 (Fig. 7A).

FDPs and D-dimers, detected in the plasma of all three monkeys, were consistent with disseminated intravascular coagulation (DIC) (Fig. 7B,C). The lethally infected monkey was distinguished by a rapid rise in both parameters; it was the only one positive for FDPs on day 5. FDPs and D-dimers rose more slowly in the other two monkeys but remained positive in both up to day 14. (Blood samples were not collected beyond that point.)

Serum Biochemistry

Mice. EBO-Z-infected mice resembled NHPs in having marked elevations of the liver enzymes AST and ALT in the serum. In the 100-pfu infection experiment, the AST became elevated on day 3 and was 20-fold above the baseline value on day 5 (Fig. 8A). The ALT increased approximately 10-fold (not shown). The AP and GGT were also mildly elevated by day 5, and there was a slight increase in total bilirubin (Fig. 8A). The BUN decreased over the course of illness, and the Cr remained stable.

Monkeys. The lethally infected monkey showed increases in AST and ALT similar to those in mice, reaching concentrations on day 7 more than 20 times the baseline value (Fig. 8B). Smaller increases in AP, LDH and total bilirubin were also noted. By contrast, the AST and ALT of the two survivors were only 2–3 times the baseline values on day 7. In the lethally infected monkey, the BUN values were 14 and 58 U/ml on days 0 and 7, respectively. The corresponding Cr values were 0·9 on day 0 and 3·5 on day 7 (Fig. 8C). These parameters barely changed in the other two animals.

Discussion

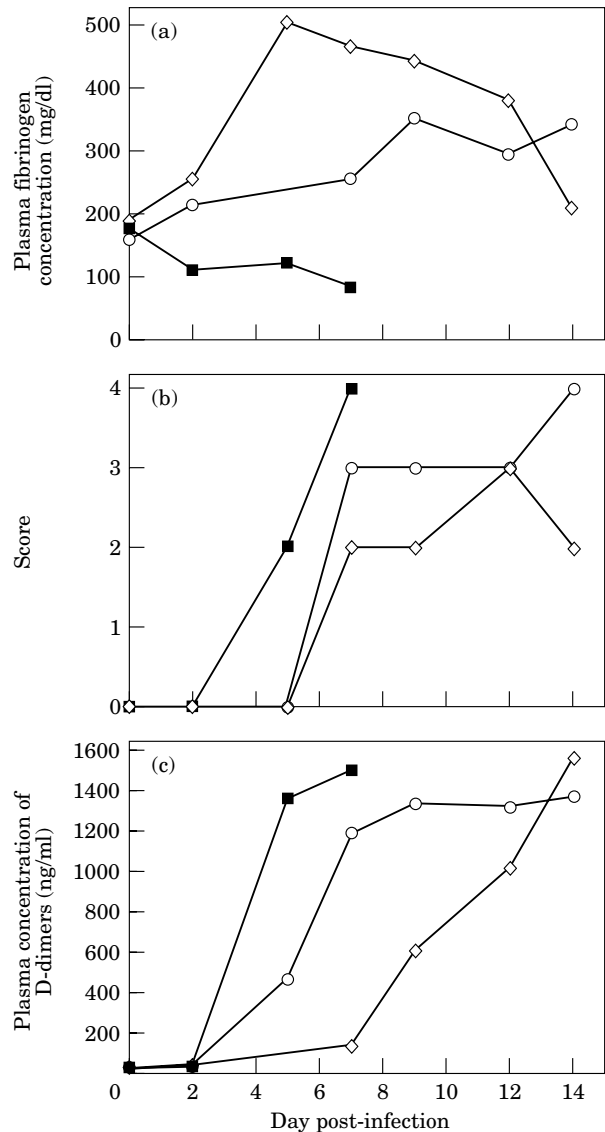The course of illness in EBO-Z-infected mice differed slightly from that in GPs and the lethally

Fig. 7A–C. (A) Plasma fibrinogen concentrations (in mg/dl) of three rhesus monkeys infected with mouse-adapted EBO-Z. (B) Plasma concentrations of fibrin degradation products (FDPs), judged by visual scoring (0–4) with a latex agglutination assay. (C) Plasma concentrations of D-dimers (in ng/ml). 8709, –■–; H873, –◇–; k439, –○–.

infected rhesus monkey. In mice, the onset of visible illness and weight loss was associated with a rapid rise in viraemia on day 3 post-infection, but no temperature elevation was observed. It is known that a number of infectious agents do not induce a febrile response in mice. In fact, several investigators have shown that artificial elevation of body temperature increases the resistance of mice to severe infection, at least in part by "up-regulating" the release of cytokines (Arif *et al.*, 1999; Jiang *et al.*, 1999, 2000). It is thus possible that the
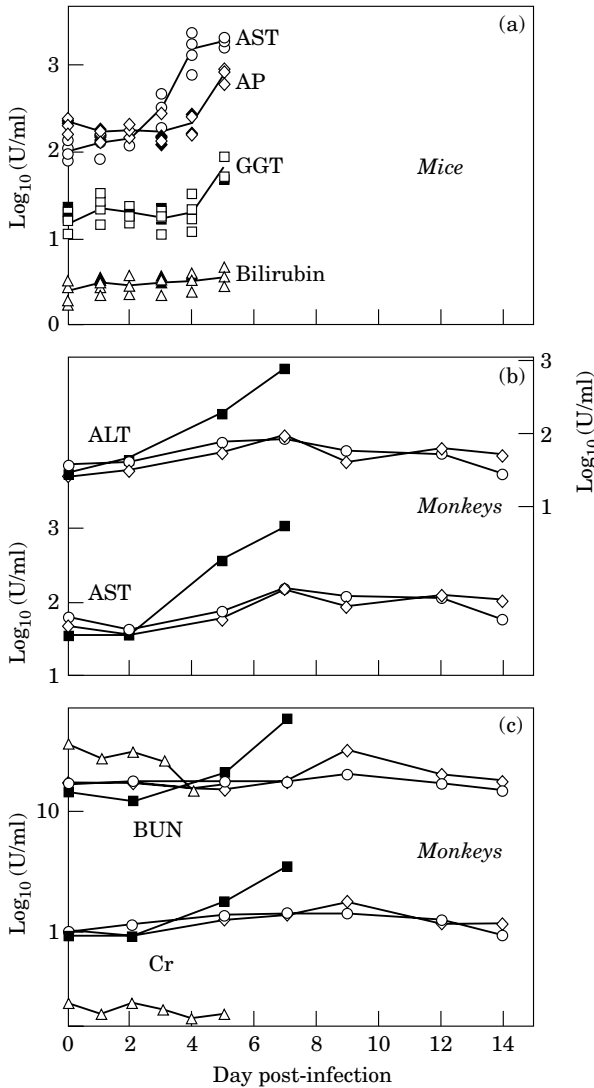
Fig. 8A–C. (A) Individual and geometric mean concentrations (in U/ml) of liver-associated enzymes and total bilirubin in sera of mice infected intraperitoneally with 100 pfu of mouse-adapted EBO-Z and terminally exsanguinated on the indicated day. For abbreviations, see text. (B) Serum ALT and AST concentrations of three rhesus monkeys infected with mouse-adapted EBO-Z. (C) Serum BUN and Cr concentrations of monkeys and mice (–△–) in the same experiments. Monkeys: 8709, –■–; H873, –◇–; k439, –○–.

absence of a febrile response in the presence of lethal infection results from a suppressive effect of the invading pathogen on the innate immune response. The progression of illness was slower in GPs than in mice or monkeys. Thus, after infection with 100 pfu, viraemia was not detected until day 4, weight loss began on day 4–5, body temperatures did not begin to rise until day 6, and the peak serum viral titre was not reached until day 8.

Mouse-adapted EBO-Z caused similar changes in hg, hct, WBC count and platelet count in all three animal species. The hg and hct increased over the course of illness in all lethally infected animals, consistent with haemoconcentration due to dehydration or fluid shifts. Such changes were previously observed in GPs infected with GP-adapted EBO-Z (Connolly *et al.*, 1999). Neither hg nor hct increased in the two monkeys that survived infection, indicating that the increase in these parameters was associated with the development of shock. Mice showed a rise in WBC count and in percentage of PMNs, similar to that previously observed in GPs (Connolly *et al.*, 1999). All three monkeys, on the other hand, showed a rise and fall in total WBCs, with an early increase in percentage of PMNs. The same pattern was previously observed in NHPs and in a human case of Ebola Côte d'Ivoire (EBO-CI) infection (Fisher-Hoch *et al.*, 1983, 1985; Formenty *et al.*, 1999). The lethally infected monkey could not be distinguished from the other two on the basis of the total WBC count, but it had the highest percentage of PMNs. Mouse-adapted virus caused a marked decline in platelet counts in all three animal species. This began earlier in the fatally infected monkey than in the two surviving animals.

Initial observations of lethally infected mice revealed the occasional occurrence of gross haemorrhage and the failure of blood samples to clot, suggesting the presence of a coagulation defect (Bray *et al.*, 1998). However, in contrast to findings in guinea-pigs and monkeys, pathological studies failed to reveal significant amounts of fibrin in the tissues of EBO-Z-infected mice, suggesting a lesser degree of consumptive coagulopathy (Jaax *et al.*, 1996; Davis *et al.*, 1997; Connolly *et al.*, 1999; Gibb *et al.*, 2001). In the present study, coagulation testing failed to demonstrate a consistent prolongation of PT or aPTT in mice over the course of illness. GPs, by contrast, showed a progressive prolongation of PT and aPTT. Lack of diagnostic reagents prevented us from testing for FDPs and D-dimers in the plasma of GPs and mice.

All three monkeys showed typical features of DIC, which have previously been detected in EBO-Z-infected NHPs and in human patients infected with EBO-CI and Marburg virus. These features included decreased platelet counts, prolongation of PT and aPTT, circulating FDPs, decreased plasma fibrinogen and extensive fibrin deposition in tissues (Fisher-Hoch *et al.*, 1983, 1985; Gear, 1989; Fisher-Hoch and McCormick, 1998; Ryabchikova *et al.*, 1998, 1999; Formenty *et al.*, 1999). The consumptive coagulopathy is generally assumed to be

caused by extensive virus-induced tissue injury and the dysfunction or damage of platelets and endothelial cells (Fisher-Hoch *et al.*, 1983, 1985) . As noted by other investigators, the increase in the aPTT was more marked than that of the PT, consistent with a greater disturbance of the intrinsic than the extrinsic coagulation pathway (Fisher-Hoch *et al.*, 1983). Interestingly, maximal levels of FDPs and D-dimers were similar in the lethally and non-lethally infected animals, although they developed earlier in the former. The monkey that died was the only one to show a decrease in fibrinogen, consistent with a more severe degree of consumptive coagulopathy. This may reflect severe liver involvement, as indicated by the high serum AST concentration, rendering the animal unable to synthesize fibrinogen at a rate sufficient to maintain a normal serum level. The increased fibrinogen levels in the other two monkeys probably represented part of an acute phase reaction.

Elevations in serum concentrations of the liver-associated enzymes AST and ALT were similar in mice and in the lethally infected monkey. They resembled those previously reported for lethally infected GPs and NHPs and for human cases of EBO CI and Marburg infection (Gear *et al.*, 1975; Fisher-Hoch *et al.*, 1985; Connolly *et al.*, 1999; Formenty *et al.*, 1999; Ryabchikova *et al.*, 1999). This finding was not unexpected, since the hepatic lesions in EBO-Z-infected mice, guinea-pigs, monkeys and human patients are virtually identical (Bray *et al.*, 1998; Ryabchikova *et al.*, 1998; Zaki and Goldsmith, 1998; Connolly *et al.*, 1999; Gibb *et al.*, 2001). Interestingly, mice differed from GPs and the lethally infected monkey in displaying a decrease in BUN, rather than an increase, over the course of illness, perhaps reflecting a severe degree of hepatic injury and a consequent decline in urea synthesis.

As shown in this study and the accompanying paper by Gibb *et al.* (2001), lethal infection with mouse-adapted EBO-Z in normal adult BALB/c mice has many points in common with the lethal disease caused by non-adapted EBO-Z in primates. Of greatest importance are the virus's predilection for cells of monocyte/macrophage lineage, its rapid rate of replication together with the lytic nature of infection, and the ability of the virus to infect other cell types. This combination of factors leads, within days of initial exposure, to infection of mobile and fixed tissue macrophages throughout the body, and to spread of the virus to hepatocytes, fibroblasts, adrenal cortical and other cells, with massive cytolysis, coagulopathy, endothelial cell dysfunction,

fluid shifts, shock and death. These severe disruptions explain many of the abnormalities seen in clinical laboratory tests: haemoconcentration, with a rise in hg and hct; mobilization of granulocytes; consumption of platelets; disturbance of coagulation pathways; and release of enzymes from infected hepatocytes. The principal difference between the terminal stages of illness observed in mice and monkeys, as discussed above, relates to the apparent paucity of features of coagulopathy in the former, in contrast to the classic manifestations of DIC observed in the latter. We cannot currently determine whether DIC does or does not occur in mice, because of the lack of reagents for measuring FDPs and D-dimers.

Mouse-adapted EBO-Z is the only EBO virus that causes lethal illness in mice and GPs and severe disease in non-human primates. Human EBO-Z isolates do not cause disease on injection into adult immunocompetent mice and cause only mild illness in GPs (Van der Groen *et al.*, 1979; Bowen *et al.*, 1980). GP-adapted EBO-Z also does not cause disease in mice, but is fully lethal for monkeys (Bowen *et al.*, 1978; Fisher-Hoch *et al.*, 1985; Bray, unpublished data). Various pieces of evidence suggest that mouse-adapted EBO-Z was selected over the course of serial passage in mice for its ability to subvert or evade innate antiviral resistance mediated by the type I interferon (IFN-α/β) response, enabling it to overwhelm its host (Bray, 2001). A single mutation appears to permit the virus to suppress the type I IFN response in GPs, but makes it more susceptible to innate antiviral mechanisms of monkeys, causing it to be partially attenuated. A back-mutation at this site may have been responsible for the lethal illness in monkey 8709.

This conclusion, although based on the survival of only two of three animals, may be significant if "historical" controls are included in the considerations. Studies cited above, performed over more than 20 years, have demonstrated that EBO-Z is lethal for rhesus and cynomolgus macaques, African green monkeys and baboons. Of more than 60 animals described in published reports, only one survived infection (Fisher-Hoch *et al.*, 1983). (By contrast, EBO Sudan and EBO Reston do not cause uniform mortality in NHPs [Fisher-Hoch *et al.*, 1992].) If infection of monkeys with mouse-adapted EBO-Z consistently results in the survival of a significant percentage of animals, such a model may prove useful for studying the role of host responses in determining the outcome of EBO-Z infection. It is worth noting that the two surviving monkeys represent the first NHPs in which solid

resistance to a large (5000 pfu) EBO-Z challenge has been demonstrated. Immune animals generated through "vaccination" with mouse-adapted virus would be of great interest for defining the humoral and cell-mediated basis of immunity to EBO-Z infection. In particular, their repertoire of B cells might serve as a library for the recovery of mRNA for use in creating monoclonal antibodies.

The uniform lethality of non-adapted or Guinea-pig-adapted EBO-Z for NHPs has made it impossible to study the relationship between abnormal values in clinical laboratory tests and a fatal outcome of infection. Such an analysis has been performed, for example, in human cases of Crimean-Congo haemorrhagic fever; in such cases, threshold values of platelet count, fibrinogen level and other parameters may identify a patient as being at high risk of death (Swanepoel *et al.*, 1989). In the present study it was possible to examine data from day 5 to identify factors associated with a fatal outcome. Thus, the lethally infected monkey was the only animal on day 5 with a rash, a decrease in serum fibrinogen below the baseline value, detectable FDPs, an increase in AST, and no decline in hg or hct. The same monkey differed from the other two on the same day in showing a 1000-fold higher titre of circulating virus, a greater decline in platelets, a higher percentage of circulating PMNs, a greater prolongation of the TT, a higher level of D-dimers, and a greater increase in BUN and Cr. This constellation of changes identified the animal as being at the greatest risk of death. Such predictors, if found to be reliable in further studies of NHPs infected with mouse-adapted EBO-Z, will aid in guiding the development of new therapies for filovirus infection.

References

Arif, A. A., Gao, L., Davis, C. D. and Helm, D. S. (1999). Antibody response to heat shock proteins and histopathology in mice infected with *Trypanosoma cruzi* and maintained at elevated temperatures. *Journal of Parasitology*, **85**, 1089–1099.

Baskerville, A., Bowen, E. T., Platt, G. S., McArdell, L. B. and Simpson, D. I. H. (1978). The pathology of experimental Ebola virus infection in monkeys. *Journal of Pathology*, **125**, 131–138.

Bowen, E. T., Platt, G. S., Lloyd, G., Raymond, R. T. and Simpson, D. I. (1980). A comparative study of strains of Ebola virus isolated from southern Sudan and northern Zaire in 1976. *Journal of Medical Virology*, **6**, 129–138.

Bowen, E. T. W., Platt, G. S., Simpson, D. I. H., McArdell, L. B. and Raymond, R. T. (1978). Ebola haemorrhagic fever: experimental infection of monkeys. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **72**, 188–191.

Bray, M. (2001). The role of the Type I IFN response in the resistance of mice to filovirus infection. *Journal of General Virology*, **82**, 1365–1373.

Bray, M., Davis, K., Geisbert, T., Schmaljohn, C. and Huggins, J. (1998). A mouse model for evaluation of Ebola prophylaxis and therapy. *Journal of Infectious Diseases*, **178**, 651–661.

Connolly, B., Steele, K., Davis, K., Geisbert, T., Kell, W., Jaax, N. and Jahrling, P. (1999). Pathogenesis of experimental Ebola virus infection in guinea pigs. *Journal of Infectious Diseases*, **179**, Suppl. 1, S203–S217.

Davis, K. J., Anderson, A., Geisbert, T., Steele, K., Geisbert, J., Vogel, P., Connolly, B., Huggins, J., Jahrling, P. and Jaax, N. (1997). Pathology of experimental Ebola virus infection in African green monkeys. *Archives of Pathology and Laboratory Medicine*, **121**, 805–819.

Fisher-Hoch, S. P., Brammer, T. L., Trappier, S. G., Hutwagner, L. C., Farrar, B. B., Ruo, S. L., Brown, B. G., Hermann, L. M., Perez-Oronoz, G. I. and Goldsmith, C. S. (1992). Pathogenic potential of filoviruses: role of geographic origin of primate host and virus strain. *Journal of Infectious Diseases*, **166**, 753–763.

Fisher-Hoch, S. P. and McCormick, J. B. (1998). Experimental filovirus infections. *Current Topics in Microbiology and Immunology*, **235**, 117–143.

Fisher-Hoch, S. P., Platt, G. S., Lloyd, G., Simpson, D. I., Neild, G. H. and Barrett, A. J. (1983). Haematological and biochemical monitoring of Ebola infection in rhesus monkeys: implications for patient management. *Lancet*, **ii**, 1055–1058.

Fisher-Hoch, S. P., Platt, G. S. and Neild, G. H. (1985). Pathophysiology of shock and hemorrhage in a fulminating viral infection (Ebola). *Journal of Infectious Diseases*, **152**, 887–894.

Formenty, P., Hatz, C., Le Guenno, B., Stoll, A., Rogenmoser, P. and Widmer, A. (1999). Human infection due to Ebola virus, subtype Côte d'Ivoire: clinical and biological presentation. *Journal of Infectious Diseases*, **179**, Suppl. 1, S48–S53.

Gear, J. S. (1989). Clinical aspects of African viral hemorrhagic fevers. *Review of Infectious Disease*, **2**, Suppl. 4, S777–S782.

Gear, J. S., Cassel, G. A. and Gear, A. J. (1975). Outbreak of Marburg virus disease in Johannesburg. *British Medical Journal*, **4**, 489–494.

Gibb, T., Bray, M., Geisbert, T., Steele, K., Kell, W., Davis, K. and Jaax, N. (2001). Pathogenesis of experimental Ebola Zaire virus infection in BALB/c mice. *Journal of Comparative Pathology*, **125**, 233–242.

Jaax, N., Geisbert, T. W., Vogel, A., Topper, M. and Jahrling, P. (1996). Lethal experimental infection of rhesus monkeys with Ebola Zaire (Mayinga) virus by

the oral and conjunctival route of exposure. *Archives of Pathology and Laboratory Medicine*, **120,** 140–155.

Jiang, Q., Cross, A., Singh, I., Chen, T., Viscardi, R. and Hasday, J. D. (2000). Febrile core temperature is essential for optimal host defense in bacterial peritonitis. *Infection and Immunity*, **68,** 1265–1270.

Jiang, Q., Detolla, L., Singh, I. S., Gatdula, L., Fitzgerald, B., van Rooijen, N., Cross, A. S. and Hasday, J. D. (1999). Exposure to febrile temperature upregulates expression of pyrogenic cytokines in endotoxin-challenged mice. *American Journal of Physiology*, **276,** 1653–1660.

Johnson, E., Jaax, N., White, J. and Jahrling, P. (1995). Lethal experimental infections of rhesus monkeys by aerosolized Ebola virus. *International Journal of Experimental Pathology*, **76,** 227–236.

Luchko, S. V., Dadaeva, A., Ustinova, E. N., Sizikova, L. P., Ryabchikova, E. and Sandakhchiev, L. S. (1995). Experimental study of Ebola hemorrhagic fever in baboon models. *Bulletin of Experimental Biology and Medicine*, **9,** 302–304.

Peters, C. J., Sanchez, A., Rollin, P., Ksiazek, T. G. and Murphy, F. A. (1996). Filoviridae: Marburg and Ebola viruses. In: *Fields Virology*, B. N. Fields, D. M.

Knipe, P. M. Howley, Eds, Lippincott-Raven, New York, pp. 1161–1176.

Ryabchikova, E., Kolesnikova, L. and Luchko, S. (1999). An analysis of features of pathogenesis in two animal models of Ebola virus infection. *Journal of Infectious Diseases*, **179,** Suppl. 1, S199–S202.

Ryabchikova, E., Kolesnikova, L. and Netesov, S. (1998). Animal pathology of filovirus infections. *Current Topics in Microbiology and Immunity*, **235,** 147–173.

Swanepoel, R., Gill, D. E., Shepherd, A. J., Leman, P. A., Mynhardt, J. H. and Harvey, S. (1989). The clinical pathology of Crimean-Congo hemorrhagic fever. *Review of Infectious Disease*, **2,** Suppl. 4, S794–S800.

Van der Groen, G., Jacob, W. and Pattyn, S. R. (1979). Ebola virus virulence for newborn mice. *Journal of Medical Virology*, **4,** 239–240.

Zaki, S. R. and Goldsmith, C. (1998). Pathologic features of filovirus infections in humans. *Current Topics in Microbiology and Immunity*, **235,** 97–116.