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The Zaire subtype of Ebola virus (EBO-Z) is lethal for newborn mice, but adult mice are resistant to the virus, which prevents their use as an animal model of lethal Ebola infection. We serially passed EBO-Z virus in progressively older suckling mice, eventually obtaining a plaque-purified virus that was lethal for mature, immunocompetent BALB/c and C57BL/6 inbred and ICR (CD-1) outbred mice. Pathologic changes in the liver and spleen of infected mice resembled those in EBO-Z–infected primates. Virus titers in these tissues reached 10^9 pfu/g. The LD_{50} of mouse-adapted EBO-Z virus inoculated into the peritoneal cavity was ∼1 virion. Mice were resistant to large doses of the same virus inoculated subcutaneously, intradermally, or intramuscularly. Mice injected peripherally with mouse-adapted or intraperitoneally with non-adapted EBO-Z virus resisted subsequent challenge with mouse-adapted virus.

Infection with Ebola (EBO) virus, subtype Zaire (EBO-Z), produces severe or fatal illness in nonhuman primates, guinea pigs, and suckling mice [1]. The virus causes lethal hemorrhagic fever in rhesus and cynomolgus macaques (*Macaca rhesus* and *Macaca fascicularis*), African green monkeys (*Cercopithecus aethiops*), and baboons (*Papio hamadryas*) [2–5]. It replicates to high titers in the liver, spleen, lymph nodes, and other organs, causing tissue necrosis, hemorrhage, and shock [6–8]. Cells of the mononuclear phagocytic system (MPS) are major targets of infection [8]. Hepatocytes, endothelial cells, and other cell types are also infected.

Guinea pigs inoculated with infectious material from human EBO-Z cases develop a nonlethal febrile illness [9, 10]. Sequential passage from animal to animal of virus in splenic homogenates results in a progressive increase in virulence, soon reaching uniform lethality [3]. Connolly et al. [11] (this supplement) recently provided quantitative data on the adaptation process: They compared the level of viremia induced by non-adapted EBO-Z in strain 13 guinea pigs with that produced by virus from each of 4 sequential passages. The mean serum virus titer 7 days after infection increased from $10^{1.2}$ to $10^{5.2}$ pfu/mL, paralleling an increase from 20% to 100% in the lethal-

ity of the virus. Another report of histologic changes associated with increasing virulence during sequential passage in outbred guinea pigs found that the virus was fatal for some animals after 3 passages, and its lethality increased through 4 additional passages [12]. In passages 1–3, viral replication was observed only in small numbers of MPS cells, including Kupffer's cells of the liver. Virus titers in the tissues were very low. Beginning with the fourth passage, there was increasing infection of MPS cells, hepatocytes, and endothelial cells.

Intraperitoneal (ip) or intracerebral (ic) inoculation of EBO-Z causes lethal infection in newborn mice [13, 14]. Smaller quantities of virus can be detected by titration in newborn mice than by plaquing in tissue culture: Ryabchikova et al. [10] reported that a 0.1-pfu ic dose of EBO-Z was lethal for 50% of mice. Only very young suckling mice are sensitive to EBO infection [14]. However, EBO-Z was also lethal for adult SCID BALB/c mice, in which the virus replicated to high titers in liver, spleen, and other tissues (Huggins J, unpublished data). EBO infection in SCID mice followed a very different course from that in the above-mentioned animals: While monkeys, guinea pigs, and suckling mice became acutely ill 4–6 days after inoculation of EBO-Z and died 6–11 days after infection, SCID mice developed gradual, progressive weight loss and slowing of activity and died 20–25 days after challenge.

There are no approved EBO vaccines and no therapeutic measures of proven efficacy. There is an urgent need for a model of EBO infection in immunocompetent adult mice in order to accelerate the pace of vaccine development and to make it possible to evaluate limited quantities of experimental antiviral drugs in animals smaller than guinea pigs. We therefore decided to attempt the adaptation of EBO-Z to adult mice of the common BALB/c inbred laboratory strain.

Materials and Methods

Biologic containment. Infectious material and animals were handled in maximum-containment biosafety level 4 facilities at

the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). Laboratory personnel wore positive-pressure protective suits (ILC Dover, Frederica, DE) equipped with high-efficiency particulate air filters and supplied with umbilical-fed air.

Virus and cells. The 1976 strain (Mayinga) of EBO-Z virus (passed either once in Vero cells or three times ic in suckling mice and then once in Vero cells [15]) and the 1995 strain of EBO-Z virus (passed twice in Vero cells) were provided by Peter Jahrling (USAMRIID). The viruses were amplified in Vero E6 cells, and the supernatant was collected to produce stocks of EBO-Z '76 Vp2, EBO-Z '76 Mp3 Vp2, and EBO-Z '95 Vp3. The E6 line of Vero African green monkey kidney cells (Vero C1008, ATCC CRL 1586) was propagated in modified Eagle medium (MEM) with Earle's balanced salts, nonessential amino acids, 10% fetal bovine serum (FBS), glutamine, penicillin, and streptomycin; the same medium with 2% FBS was used as replacement medium after cells were infected.

Titration by plaquing and by electron microscopy (EM). Virus stocks were serially diluted in MEM, adsorbed onto confluent Vero E6 cells in 12-well dishes, incubated for 1 h at 37°C, and covered with an agarose overlay [16]. A 1:5000 dilution of neutral red in buffered saline solution was added 6 days later, and plaques were counted the following day. EBO virions were also directly enumerated by EM [17]. Virus preparations were mixed with a suspension of 200-nm polystyrene spheres (Duke Scientific, Palo Alto, CA) at a final concentration of 2.15×10^8 spheres/mL. Aliquots (5 μL) of the virus-sphere mixture were applied to 300-mesh nickel grids, which were precoated with formvar and carbon, and glow-discharged. After drying, the grids were immersed sequentially in 1% glutaraldehyde in PBS, PBS alone, osmium tetroxide, and distilled water. They were then treated with uranyl acetate and examined at 80 kV with a transmission electron microscope (1200 EX; JEOL, Peabody, MA). All virions and spheres were counted in a total of 100 grid squares, and the virus titer was calculated.

Serial passage of EBO virus in mice. Newborn litters of BALB/c mice and adult female BALB/c, C57BL/6, and ICR (CD-1) mice were obtained from the National Cancer Institute, Frederick, MD. Serial passage was initiated by inoculating litters of 2-day-old suckling BALB/c mice ip with EBO-Z '76 Mp3 Vp2. Eight days later, the litters were killed, and livers and spleens were removed aseptically and pooled. They were then ground in a mortar with sterile sand and growth medium, and the suspension was clarified by low-speed centrifugation and titrated. In passages two through eight, the pooled liver suspension was inoculated subcutaneously (sc) into new litters, and the process was repeated. Animals were killed 8–10 days after inoculation. Passage 2 was carried out in 2-day-old, passages 3–6 were carried out in 4-day-old, passages 7 and 8 were carried out in 8-day-old, and passage 9 was carried out in 15-day-old mice. Mice at each passage level were inoculated with .02 mL of undiluted liver suspension, which ranged in titer from 200 to 5×10^6 pfu/mL. Some ninth-passage mice were inoculated sc and some ip.

Plaque purification. Vero E6 cells were infected with diluted liver suspension from the ninth mouse passage and covered with an agarose overlay. After staining with neutral red, 11 single plaques were removed by pipette, suspended by freeze-thawing and vortexing, and used to infect flasks of Vero E6 cells. Once generalized cytopathic effect was observed, the supernatants were collected, and a 100-fold dilution of each was inoculated ip into groups of 3-week-old mice. The supernatant that caused 100% mortality in the shortest time period was diluted for another round of plaque purification, and a 100-pfu dose of each of 8 stocks derived from second-round plaques was tested in mice. The most virulent isolate (EBO-Z '76 Mp3 Vp2 Mp9 GH) was amplified, stored as aliquots, and used in all subsequent experiments.

Evaluation of lethality and viral replication in mice. Lethality was determined by diluting EBO-Z virus in growth medium and inoculating it ip, sc, or ic in suckling mice or ip, sc, intradermally (id), or intramuscularly (im) in adult mice. The LD_{50} dose of mouse-adapted EBO-Z '76 Mp3 Vp2 Mp9 GH inoculated ip into adult BALB/c mice was determined by inoculating groups of 6–10 mice with serial 10-fold dilutions of virus in 3 separate experiments and analyzing the combined results by use of a linear regression program (SPSS, Chicago). In one experiment, groups of 16-week-old mice were anesthetized and exsanguinated at daily intervals after ip infection with 100 pfu of mouse-adapted virus, the serum was collected, and the livers and spleens were removed aseptically. The virus titers of the serum and of a weighed portion of each organ were determined as described above, and the remainder was preserved for microscopy study. In another experiment, the same procedure was performed 4 days after ip inoculation of 100 pfu of EBO-Z '76 Vp2, EBO-Z '76 Mp3 Vp2, or mouse-adapted virus EBO-Z '76 Mp3 Vp2 Mp9 GH.

Histology, immunohistochemistry, and EM. Portions of liver and spleen were immersion-fixed for 30 days in 10% neutral-buffered formalin and then embedded in paraffin. Sections were mounted on glass slides and stained with hematoxylin and eosin; replicate sections were stained immunohistochemically. In brief, sections were treated with a 0.05% solution of Protease VIII (Sigma, St Louis) for 3 min at 37°C, blocked at room temperature with normal goat serum for 20 min, and incubated for 1 h with rabbit anti-EBO serum diluted 1:500 with 1% powdered milk in PBS. Sections were incubated for 30 min each with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and alkaline phosphatase-labeled streptavidin (Life Technologies Gibco BRL, Gaithersburg, MD). Slides were placed in naphthol AS-BI phosphate/hexazotized new fuchsin (Kirkegaard & Perry, Gaithersburg, MD) for color development and counterstained with Mayer's hematoxylin.

Necropsy tissues from EBO-infected monkeys served as positive controls. Liver and spleen from uninfected mice and the substitution of normal rabbit serum for rabbit anti-EBO serum served as negative controls. Small fragments of tissue for EM were immersion fixed for 1 h in 2% glutaraldehyde in 0.1 *M* Millonig's phosphate buffer (pH 7.4), rinsed in buffer, and postfixed for 1–2 h in 1% osmium tetroxide in buffer. The material was then rinsed, stained with 0.5% uranyl acetate in ethanol, dehydrated in ethanol and propylene oxide, and embedded in POLY/BED 812 resin (Polysciences, Warrington, PA) for sectioning [15].

Neutralization of virus with antiserum. Aliquots of EBO virus in growth medium were mixed with serial dilutions of a commercially acquired purified preparation of IgG from horses hyperimmunized with EBO-Z (equine IgG; Epidbiomed, Novosibirsk, Russia; provided by Peter Jahrling [5, 18, 19]) and incubated at 34°C for 1 h. Controls consisted of virus mixed with immune serum from guinea pigs that had survived infection with Marburg (MBG) Musoke virus (provided by Alan Schmaljohn, USAMRIID) or with

normal serum or medium alone. For in vitro plaque-reduction tests, the mixture was used to infect Vero E6 cells, and plaques were counted 1 week later. For in vivo assessment of lethality for mice, the mixture was inoculated ip into adult BALB/c mice, which were monitored for survival.

Immunization and challenge.   Adult mice were inoculated ip with 1–1000 pfu of EBO-Z '76 Vp2, EBO-Z '76 Mp3 Vp2, or EBO-Z '95 Vp3, or with plaque-purified MBG Musoke virus or MBG Ravn virus (provided by Alan Schmaljohn) or medium alone. In one experiment, aliquots of EBO-Z '95 Vp3 equivalent to 100 pfu/dose were inactivated by heating at 75°C for 30 min or by gamma-irradiation with 6 MR from a ^{60}Co source prior to ip inoculation. These samples showed no residual infectivity for Vero E6 cells. In other experiments, mice were inoculated sc, id, or im with mouse-adapted EBO-Z virus and then challenged 3 weeks later by ip injection of 100 pfu of the same virus.

Reverse transcriptase–polymerase chain reaction (PCR) and nucleotide sequence analysis.   Total cellular RNA was isolated from one 25-cm^2 flask each of Vero E6 cells infected with EBO-Z '76 Mp3 Vp2 or EBO-Z '76 Mp3 Vp2 Mp9 GH virus 4 days after infection, by using Trizol reagent (Life Technologies Gibco BRL). The RNA pellet was resuspended in 10 μL of RNase-free water and heated at 60°C for 10 min; a 2-μL aliquot was then used for first-strand cDNA synthesis using a Superscript kit (Life Technologies Gibco BRL) and random hexamer primers. PCR amplification with the Expand High Fidelity kit (Boehringer Mannheim, Indianapolis) used the forward primer 5′-GATCAG-ATCTACAATGGGCGTTACAGG-3′ and the reverse primer 5′-GATCAGATCTCTAAAAGACAAATTTG-3′, which amplify nucleotides corresponding to positions 6037–8070 with respect to the genome of the 1976 Mayinga strain of EBO-Z (GenBank accession number L11365) [20, 21]. PCR amplification was done in 100-μL volumes, using a PCR 9600 thermocycler (Perkin-Elmer, Foster City, CA). Forty cycles (each consisting of 94°C for 40 s, 38°C for 45 s, and 72°C for 1 min) were followed by 1 cycle of 72°C for 5 min. PCR products were cloned into the pCRII vector (Stratagene, La Jolla, CA), and large-scale preparations were made by using purification columns (Qiagen, Chatsworth, CA). Nucleotide sequences were determined by the dideoxy chain-termination method, with a Sequenase 2.0 kit (United States Biochemicals, Gaithersburg, MD).

Results

Serial mouse passage.   Non–mouse-adapted viruses EBO-Z '76 Vp2 and EBO-Z '76 Mp3 Vp2 were lethal for many or all 4-day-old suckling BALB/c mice when inoculated ip or ic, but they did not cause illness in 8- or 15-day-old mice (data not shown). Serial passage was therefore begun in young suckling mice. In the first passage, EBO-Z '76 Mp3 Vp2, which had previously been passed 3 times in suckling mice inoculated ic, was injected ip in 2-day-old mice. About 10^3 pfu of virus in pooled liver suspension recovered from these animals was then injected sc into 2-day-old mice, producing illness (ruffled fur and diminished activity) or death in several mice before exsanguination of the remaining animals at day 9 after infection. The livers and spleens of these second-passage mice each

yielded a total of 10^4–10^5 pfu of virus, demonstrating that sc inoculation resulted in systemic infection. Passage was continued in 4-day-old mice, with similar results.

After 6 passages, the virus caused illness in 8-day-old mice, 1 of which died before the remainder were killed at day 10. In the eighth passage, the virus was lethal for 5 of 16 8-day-old mice before the remainder were killed on day 9. Routine titration of liver suspension recovered from eighth-passage mice revealed a mixture of plaque phenotypes of the virus on Vero E6 cells: In addition to pale, moderately well-defined ''bull's eye'' plaques, which had been seen up to that point, a new population of similarly sized but sharply outlined, transparent plaques was also present. Vero E6 cells infected with this material showed typical replicating EBO virus by EM. No other infectious agent was observed. This virus and those from all previous passages were neutralized in vitro by equine IgG raised against EBO-Z [5, 18, 19], confirming that all isolates consisted of EBO virus (data not shown). Passage 8 liver suspension did not produce visible illness when tested in sc-inoculated 15-day-old mice, but ip inoculation was lethal for 14 of 18 mice.

Liver suspension from a moribund ninth-passage mouse yielded a pure population of clear plaque virus. Virus derived from a single clear plaque (plaque G) caused 100% mortality in 6 days when inoculated ip in 3-week-old mice, as did virus isolated in a second round of plaque purification (plaque GH). This doubly plaque-purified ninth-passage virus, designated EBO-Z '76 Mp3 Vp2 Mp9 GH or mouse-adapted virus, was used in all subsequent experiments.

Lethality for suckling and adult mice.   Mouse-adapted virus inoculated ip or ic was uniformly lethal for 8- and 15-day-old mice. The same virus inoculated sc was lethal for 8-day-old animals, but killed less than half of the 15-day-old sucklings and did not cause visible illness in 3-week-old mice. An ip dose of 100 pfu of mouse-adapted virus was lethal for 5-week-old BALB/c, C57BL/6, and ICR (CD-1) mice (figure 1). Additional studies with smaller quantities of virus showed that BALB/c and CD-1 mice did not differ in their susceptibility to the virus. Mouse-adapted virus was lethal for BALB/c mice ranging in age from 5 to 16 weeks. The LD$_{50}$ fell within the range of 0.025–.04 Vero E6 pfu (i.e., a dose of 1 pfu was equivalent to ~30 LD$_{50}$). Direct counting by EM of virions mixed with a known concentration of microspheres revealed that 1 pfu was equivalent to 25–30 virions (figure 2). Therefore, 1 LD$_{50}$ equaled roughly 1 virion. Titration of a stock of the 1995 strain of EBO-Z virus by EM gave a similar ratio of virions to plaque-forming units.

EBO disease in adult mice.   In a series of experiments, mice developed ruffled fur, showed progressive slowing of activity, and began to lose weight 3 days after ip injection of 1 or 100 pfu (30 or 3000 LD$_{50}$) of mouse-adapted virus (figure 3A). The smaller dose of virus caused a slightly less precipitous onset of disease and weight loss, but the rate of weight loss, once it commenced, was about the same for the 2 doses. A
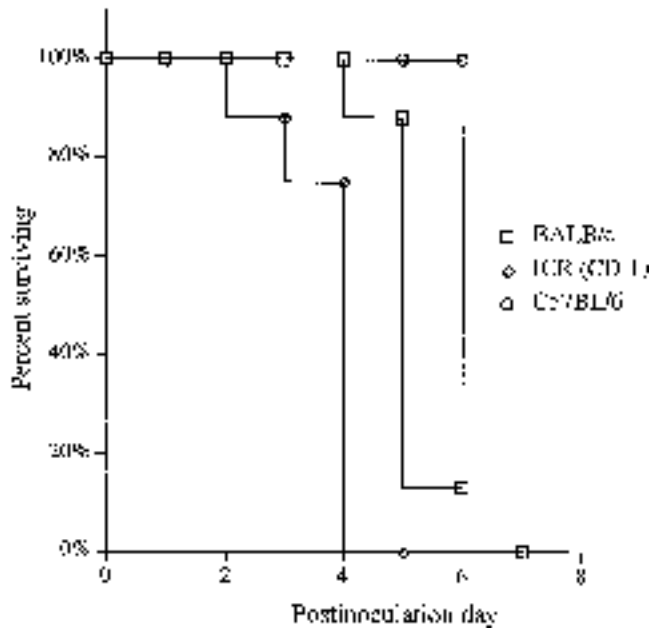
Figure 1. Mouse-adapted Ebola virus, subtype Zaire (EBO-Z), was lethal for 3 strains of mice. Groups of 8 5-week-old ICR (CD-1) outbred and BALB/c and C57BL/6 inbred female mice were inoculated intraperitoneally with 100 pfu (3000 LD$_{50}$) of EBO-Z '76 Mp3 Vp2 Mp9 GH virus and monitored for survival. All mice became ill within 3–4 days after injection. Further studies with smaller doses of virus showed no difference in susceptibility between CD-1 and BALB/c mice.

similar pattern of acute onset of weight loss has been observed in EBO-infected guinea pigs [11]. Averaged over 8 experiments each, the mean time to death of 98 mice inoculated with 30 LD$_{50}$ was 6.9 days, and that of 78 mice inoculated with 3000 LD$_{50}$ was 5.8 days. In various experiments, a small number of animals bled spontaneously from the orbit, bladder, gastrointestinal tract, or within the abdominal cavity before death. Two mice showed thrombosis of the hepatic portal veins and infarcts in the liver at necropsy. Blood collected in the last 1–2 days of illness failed to clot, indicating the presence of a coagulation defect, the nature of which has not yet been determined.

To study in vivo viral replication and organ pathology, we inoculated a group of 16-week-old mice ip with 100 pfu (3000 LD$_{50}$) of mouse-adapted virus; 1–3 animals were killed each day. Virus titers in the liver and spleen exceeded 10^7 pfu/g on the second day and then increased more slowly, reaching a maximum of $>10^9$ pfu/g in both organs of the only surviving mouse on the fifth postinoculation day (figure 3B). The mean serum virus titer peaked on the third day at 7.5×10^7 pfu/mL.

We compared the replication of non–mouse-adapted and mouse-adapted EBO viruses in adult mice by inoculating animals ip with 100 pfu of EBO-Z '76 Vp2, EBO-Z '76 Mp3 Vp2, or EBO-Z '76 Mp3 Vp2 Mp9 GH and killing them 4 days later. In the 2 groups inoculated with non-adapted virus, the virus titers in the spleen ranged from 1.6×10^4 to $1.2 \times$

10^5 pfu/g (figure 4), roughly 3×10^3 to 2×10^4 pfu per spleen. Similarly, virus titers in the liver indicated a total of $\sim4 \times 10^3$ to 1.5×10^4 pfu/organ (not shown). The combined quantity of virus in each animal's liver and spleen ranged from $\sim8 \times 10^3$ to 3×10^4 pfu, a larger quantity than had been inoculated, giving evidence of viral replication. By contrast, the spleens of mice inoculated with mouse-adapted EBO-Z yielded a mean of 6.2×10^7 pfu/g, >1000 times greater than the mean titer of EBO-Z '76 Vp2 or EBO-Z '76 Mp3 Vp2 at the same time point. A comparable disparity in titers was observed in the liver.

Histology, immunohistochemistry, and ultrastructure in liver and spleen. Two days after ip inoculation of 100 pfu (3000 LD$_{50}$) of mouse-adapted EBO-Z, the liver appeared essentially normal upon routine light microscopy, but EBO virus antigen was detected in sinusoidal lining cells, consistent with Kupffer's cells, by immunohistochemistry (not shown). By day 4, the liver had developed disseminated, often coalescing, foci of hepatocellular vacuolar change, degeneration, and necrosis (figure 5A). Hepatocytes and, less frequently, Kupffer's cells contained large (3–15 μm), pleomorphic, acidophilic, cytoplasmic viral inclusions. The sinusoids contained necrotic cellular debris and small
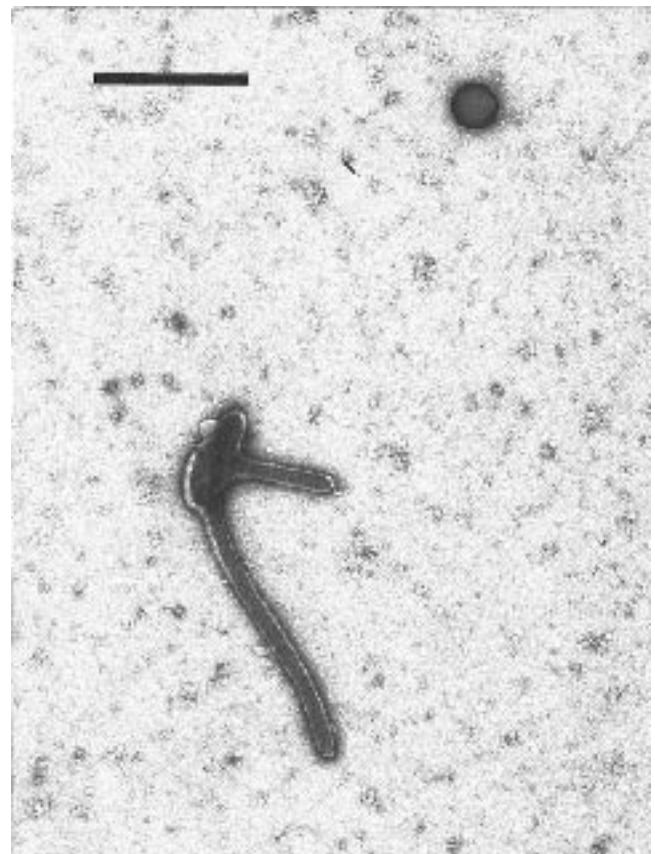

Figure 2. Ebola virus particle and polystyrene sphere used for counting virions. Preparation negatively contrasted with 1% uranyl acetate. Bar = 615 nm.
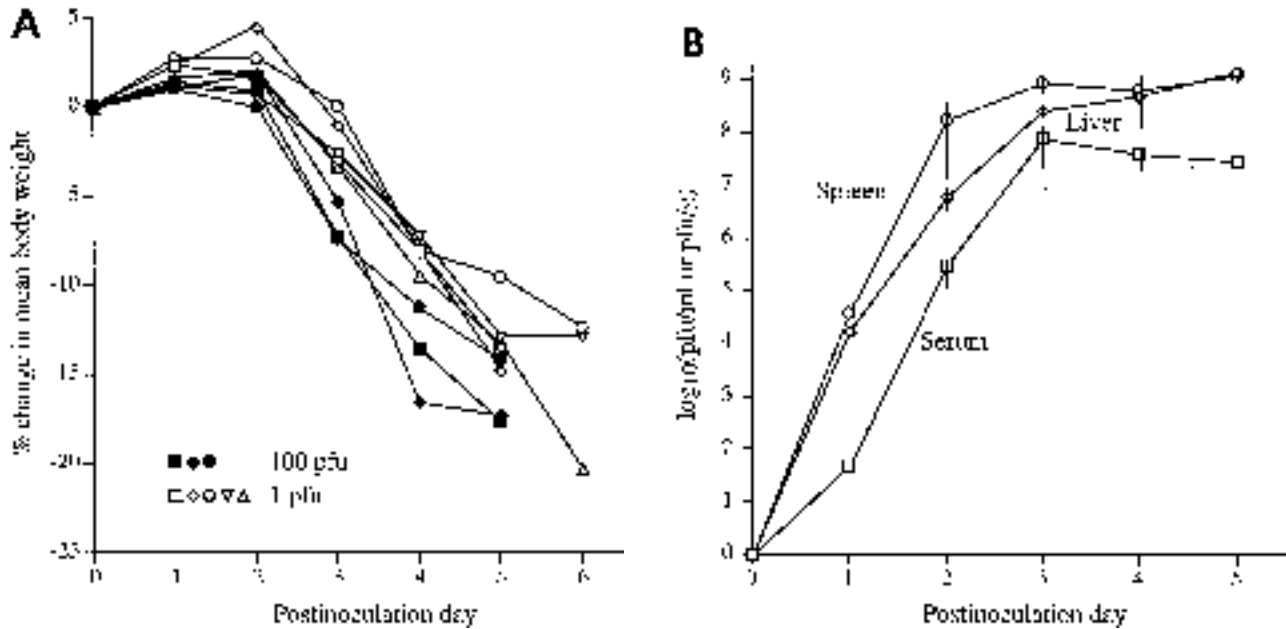
Figure 3. **A**, BALB/c mice infected with mouse-adapted Ebola virus, subtype Zaire, begin to lose weight 3 days after inoculation. Eight groups of 6–15 mice (5–16 weeks old) were injected intraperitoneally (ip) with 1 pfu (30 LD_{50}; 5 groups, open symbols) or 100 pfu (3000 LD_{50}; 3 groups, solid symbols) of mouse-adapted Ebola virus, and their mean weight was determined daily. All mice were dead by day 7 after infection. **B**, Virus titers in spleen, liver, and serum increased rapidly after ip infection. A group of 16-week-old female BALB/c mice was inoculated ip with 100 pfu (3000 LD_{50}) of mouse-adapted virus. One animal was killed on day 1, 2 were killed on day 2, 3 each were killed on days 3 and 4, and only survivor was killed on day 5. Virus titers in serum (pfu/mL) and liver and spleen (pfu/g) were determined. Mean and range values (bars) are shown.
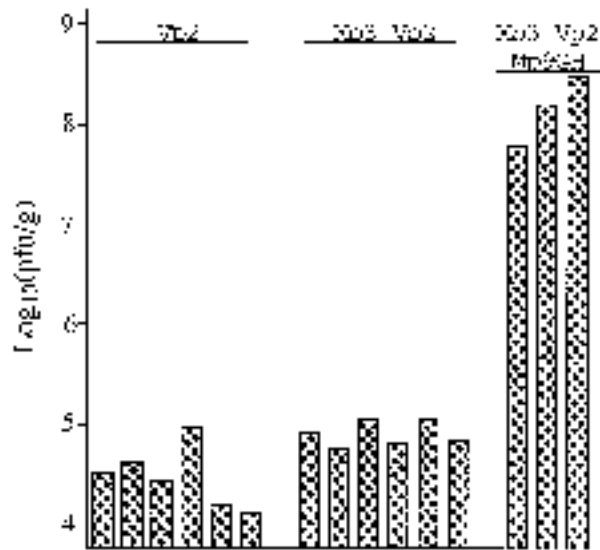

Figure 4. Mouse-adapted Ebola virus, subtype Zaire (EBO-Z), replicated to higher titers than non-adapted EBO-Z viruses in spleens of adult mice. Five-week-old female BALB/c mice were inoculated intraperitoneally with 100 pfu of EBO-Z '76 Vp2 (6 mice), EBO-Z '76 Mp3 Vp2 (6 mice), or EBO-Z '76 Mp3 Vp2 Mp9 GH (3 mice). Four days later, animals were killed, blood and tissue were collected, and virus titers in serum, spleen, and liver were determined. Titers in spleen are shown.

numbers of neutrophils and monocytes. Kupffer's cells, sinusoidal endothelial cells, and hepatocytes within and adjacent to areas of hepatocellular degeneration and necrosis were immunoreactive for EBO virus antigen (figure 5B).

Antigen was also present in circulating monocytes (not shown). Cell-free and cellular debris–associated immunoreactive material was present in the sinusoids. Characteristic EBO virus inclusions and budding virions were observed in hepatocytes, Kupffer's cells, and sinusoidal endothelial cells by EM (figure 6). Large numbers of free virions were present in the sinusoids and Disse's spaces. In the spleen, EBO virus antigen was detected by immunohistochemistry in numerous large mononuclear cells in marginal zones 2 days after infection (figure 5C). Ultrastructural examination showed replicating EBO virus in marginal zone macrophages and in macrophages of the splenic white pulp but not in lymphocytes. Infrequent small deposits of fibrin were observed in the red pulp late in the course of infection.

Neutralization of virus by antiserum. The 50% plaque-reduction neutralization titer of equine IgG against mouse-adapted EBO-Z was 1:6400, which was equal to its titer against EBO-Z '76 Vp2 and differing <2-fold from titers against EBO-Z Mp3 Vp2 and EBO-Z '95 Vp3. Preincubation of mouse-adapted virus with equine IgG blocked its lethality for mice. A challenge dose of 30 LD_{50} was neutralized by a 1:50 or 1:500 dilution of IgG, while a 1:5000 dilution of IgG or various
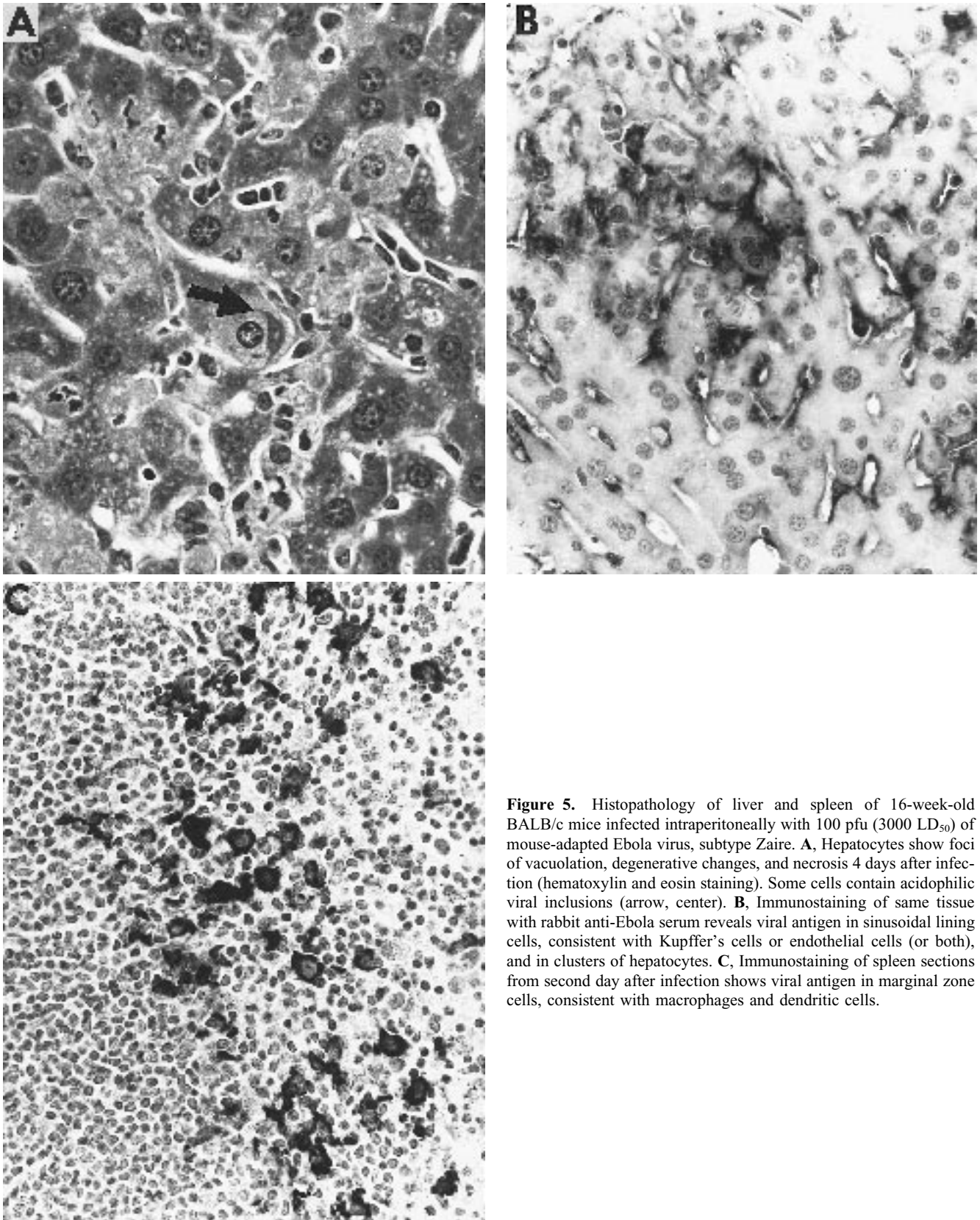
Figure 5. Histopathology of liver and spleen of 16-week-old BALB/c mice infected intraperitoneally with 100 pfu (3000 LD_{50}) of mouse-adapted Ebola virus, subtype Zaire. **A**, Hepatocytes show foci of vacuolation, degenerative changes, and necrosis 4 days after infection (hematoxylin and eosin staining). Some cells contain acidophilic viral inclusions (arrow, center). **B**, Immunostaining of same tissue with rabbit anti-Ebola serum reveals viral antigen in sinusoidal lining cells, consistent with Kupffer's cells or endothelial cells (or both), and in clusters of hepatocytes. **C**, Immunostaining of spleen sections from second day after infection shows viral antigen in marginal zone cells, consistent with macrophages and dendritic cells.

Figure 6. Thin section through hepatic sinusoid of 3-week-old mouse 5 days after infection with mouse-adapted Ebola (EBO) virus, subtype Zaire. Characteristic EBO virus inclusions (*) are present in hepatocytes (H), Kupffer's cells (K), and sinusoidal endothelial lining cell (E). EBO virions are seen budding from Kupffer's cells into lumen of sinusoid (S). Space of Disse's (D) is congested with EBO virus particles. Bar = 2.5 μm.
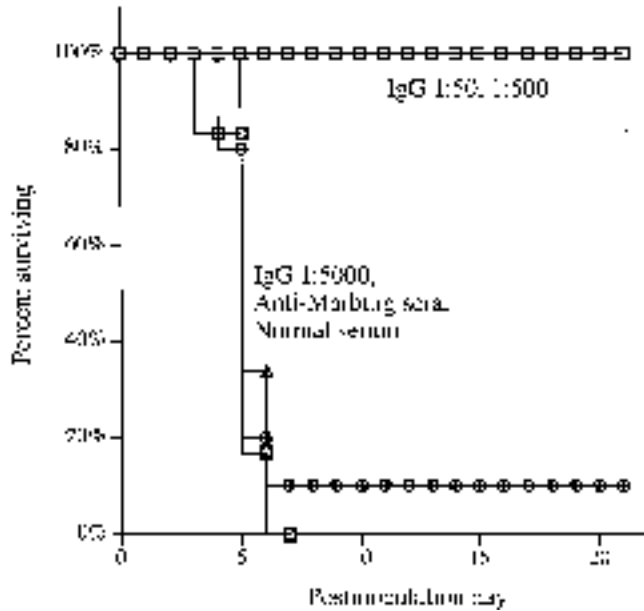
Figure 7. IgG from horses immunized with Ebola virus, subtype Zaire (EBO-Z) neutralizes mouse-adapted EBO-Z virus. Mice were inoculated intraperitoneally with 30 LD_{50} of mouse-adapted virus preincubated with various dilutions of equine IgG or with control sera. A 1:50 or 1:500 dilution of IgG neutralized virus, so mice neither became ill nor died. 1:5000 dilution of IgG, 1:20 or 1:200 dilution of guinea pig anti-Marburg serum, 1:20 dilution of normal mouse serum, and growth medium alone failed to protect mice (results for groups partially overlap).

dilutions of anti-MBG serum or normal serum failed to protect against lethal infection (figure 7).

Immunization and challenge. Adult mice inoculated ip with 100 pfu of EBO-Z '76 Vp2 or with doses of 1–1000 pfu of EBO-Z '95 Vp3 showed no visible illness. When challenged ip 3 weeks later with 100 pfu (3000 LD_{50}) of mouse-adapted virus, all mice survived without any sign of disease (data not shown). However, mice that were inoculated ip with a quantity of EBO-Z '95 Vp3 equivalent to 100 pfu, which had been inactivated by heat or by gamma irradiation before injection, were not protected against subsequent challenge with the same dose of virus. ip inoculation of MBG virus did not cause visible illness and did not cross-protect against subsequent challenge with mouse-adapted EBO-Z.

Mouse-adapted EBO-Z virus caused illness in adult mice only when injected into the peritoneal cavity. sc inoculation of as much as 10^6 pfu of mouse-adapted virus did not cause visible illness or death in adult mice (not shown). In one experiment, sc, im, or id inoculation of 100 pfu of mouse-adapted virus did not cause illness in mice, with the exception of 1 animal that died 6 days after id inoculation (figure 8). The mice in that experiment were shown to be immune when challenged ip 3 weeks later with 100 pfu (3000 LD_{50}) of the same virus.

Sequence of the glycoprotein (GP) gene. We determined the sequence of the GP gene of our starting virus for serial passage, EZ '76 Mp3 Vp2, and of the mouse-adapted virus, EZ '76 Mp3 Vp2 Mp9 GH, and compared them to the GenBank sequence of the GP gene of the Mayinga strain of EBO-Z virus. The starting stock and the mouse-adapted virus differed identically from the GenBank sequence at the following 3 positions, listed with the GenBank nucleotide first: 6232 A>G, 6775 T>C, and 7670 T>C. These nucleotide differences would result in the following amino acid changes, with the amino acid predicted by GenBank sequence listed first: S>P, S>P, and I>T, respectively. The nucleotide sequence of EZ '76 Mp3 Vp2 also differed from the GenBank sequence at the following 3 positions, with the GenBank nucleotide listed first: 6165 A>G, 6909 C>T, and 7212 A>C. The sequence of the mouse-adapted virus was the same as the GenBank sequence at these sites. These 3 nucleotides form the third bases of codons, and the differences are not predicted to result in amino acid changes.

Discussion

Serial passage of the 1976 strain of EBO-Z virus in suckling mice resulted in the emergence of an agent with a novel dual phenotype: clear plaque morphology on Vero E6 cells and
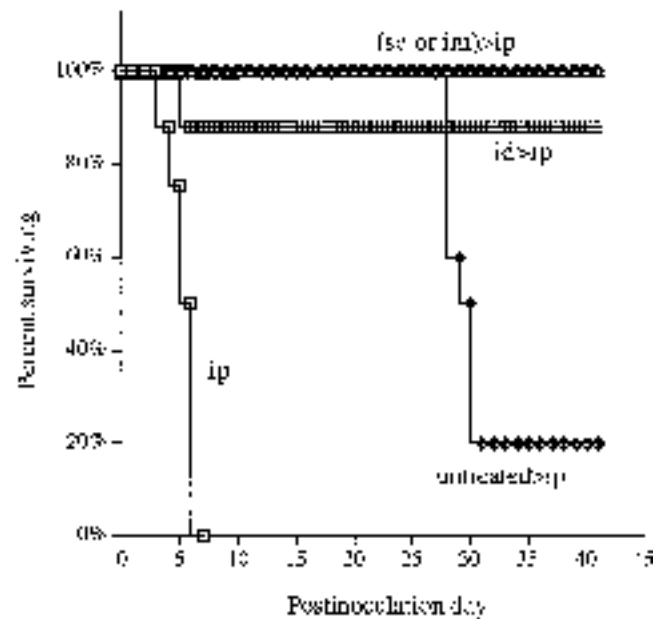

Figure 8. Peripheral inoculation of mouse-adapted Ebola virus, subtype Zaire, protects against subsequent lethal intraperitoneal (ip) challenge with same virus. Six-week-old female BALB/c mice were either inoculated with 100 pfu of mouse-adapted virus subcutaneously (sc) in upper back or at base of tail, intramuscularly (im) in quadriceps muscle, intradermally (id) in mid-back, or ip, or they were left untreated. All mice inoculated ip and 1 mouse injected id died, while rest remained healthy. Three weeks later, all surviving mice were challenged ip with 100 pfu (3000 LD_{50}) of same virus. All previously inoculated mice remained healthy.

lethality for adult mice. A series of experiments confirmed that the clear plaque agent was EBO virus rather than a contaminant and that EBO virus caused the illness and death of adult mice. Examination of a suspension of purified mouse-adapted virus by EM showed typical EBO virions. Assay of these particles demonstrated that lethality depended predictably on inoculation of virus: a dose of ~3 virions (3 LD_{50}) killed 18 of 20 mice, while one-tenth of that dose caused 2 deaths in 20 mice. Inoculating this suspension into mice resulted in the appearance of characteristic replicating EBO virus in the liver and spleen. The virus titer in these organs reached 10^8 pfu/g by the third day after inoculation, coinciding with the onset of illness, and continued to increase until death.

As further evidence that lethal infection of mice was caused by EBO-Z, we found that the in vitro and in vivo infectivity of the virus suspension was neutralized by IgG from horses hyperimmunized against EBO-Z and that mice previously inoculated with the 1976 or 1995 strains of EBO-Z virus were protected against challenge with the agent.

The EBO mouse model has highly desirable features for an animal model of an exceptionally virulent human disease. The entire course of illness is played out in a week or less: All mice become acutely ill 3 days after infection, similar to the rapid onset of disease in primates, and die 5–8 days after challenge. The mouse-adapted agent is fatal for BALB/c mice over a broad age range. The use of purified, aliquotted virus and genetically identical mice of the same sex and similar age yields virtually uniform results. Such reproducibility will facilitate statistical evaluation of the effect of prophylactic or therapeutic interventions.

We initially suspected that the changes in plaque phenotype and virulence that occurred during serial passage resulted from mutations in the GP gene, which is involved in virus binding to cellular receptors and entry into cells [22]. However, we found that the predicted peptide sequences of the GP of the pre- and postpassage viruses were identical: Although there were 3 nucleotide differences between the GP sequence of the starting stock and that of the mouse-adapted virus, none of the differences was predicted to result in an amino acid change. It thus appears that accelerated replication of the adapted virus in mice resulted from an alteration in events subsequent to virus entry into cells, which was brought about by a change in a viral protein other than GP.

A prominent feature of lethal EBO infection in mice is the involvement of multiple cell types, similar to the pattern of infection in guinea pigs and primates [6–8, 10, 11, 14]. In the livers, we observed viral replication in Kupffer's cells, hepatocytes, and endothelial cells, leading to disseminated, multifocal necrosis, which resembled pathology in other animal models. The spleens of mice, guinea pigs, and primates showed a similar immunohistochemical localization of EBO virus antigen in cells of the MPS system. However, we noted few fibrin deposits in the spleens of moribund mice, while fibrin was more prominent in the spleens of guinea pigs, and copious deposits were observed throughout the red pulp of the spleens of nonhuman primates dying of EBO infection [8]. Despite the paucity of fibrin deposition, EBO-infected mice showed other signs of coagulation abnormalities, including occasional spontaneous bleeding from external and internal sites. More detailed findings from a larger time-course study of EBO-Z in mice will be presented in a later report.

Another notable characteristic of EBO disease in mice is the rapidity of virus proliferation. Mouse-adapted EBO-Z replicated in the liver and spleen to titers $>10^8$ pfu/g within 3 days after infection, eventually reaching 10^9 pfu/g. These titers exceed reported peak concentrations in the liver and spleen of monkeys ($>10^7$ pfu/g) and guinea pigs ($>10^6$ pfu/g) [11]. Peak viremia in mice ($>10^7$ pfu/mL) was comparable to maximum titers observed in monkeys (10^6–10^7 pfu/mL) but exceeded those in guinea pigs ($>10^5$ pfu/mL) [2, 11, 23]. Virus titers were higher in the spleen than in the liver or serum, as has been observed in guinea pigs [11].

An interesting feature of the EBO mouse model is the disparity between the results of ip and peripheral inoculation. The agent was highly infectious when injected ip: the LD_{50} equaled a single EBO virion. In contrast, mice injected sc with doses as large as 10^6 pfu of the same virus did not become ill. This phenomenon is not observed in guinea pigs or nonhuman primates, which are susceptible to EBO or MBG virus inoculated sc, im, or ip. On the basis of these other animal models, there is no reason to believe that peripherally inoculated EBO virus simply remains sequestered at the injection site in mice rather than entering lymphatic vessels and reaching regional lymph nodes. In fact, we found evidence that virus inoculated sc, id, or im was taken up by or infected (or both) antigen-presenting cells of the MPS system before or after entry into regional lymph nodes, as mice developed a protective immune response and were protected against subsequent ip challenge.

Viral replication in lymph nodes draining an injection site has been observed after sc inoculation of EBO-Z in guinea pigs [11]. However, it appears that either the corresponding cells in mice were refractory to virus replication or that an infection that began in regional lymph nodes was rapidly eliminated through immune mechanisms. We have not determined whether systemic dissemination of peripherally inoculated virus takes place.

Virions delivered into the peritoneal cavity are distributed very differently from those injected into the soft tissues. Material inoculated ip passes rapidly through peritoneal lymphatic vessels and enters the bloodstream; even red blood cells make this transit within minutes after injection [24]. Virions inoculated ip may thus reach the bloodstream and initially infect MPS cells in the liver and spleen. Mouse-adapted virus clearly infected these organs soon after ip inoculation, since titers in the liver and spleen already exceed 10^7 pfu/g 2 days after infection. In marked contrast to the failure of peripherally inoculated virus to cause systemic infection, it appears that the arrival of even a few

virions of mouse-adapted EBO-Z in the liver or spleen leads to rapid viral proliferation. This may result either from the presence of a permissive host cell or from a weakness in protective immune mechanisms. Alternatively, some virus inoculated ip may infect peritoneal macrophages, many of which would pass through diaphragmatic lymphatics and enter mediastinal lymph nodes [25], leading to viral proliferation.

We were able to detect replication of non–mouse-adapted EBO-Z '76 Vp2 and EBO-Z Mp3 Vp2 viruses in the liver and spleen of adult mice, but the yield was exceeded >1000-fold by that of mouse-adapted virus under the same conditions. Thus, non-adapted and adapted EBO viruses may share the same initial target cells, but the former viruses replicate slowly after infection, apparently giving the animal sufficient time to eliminate virus-infected cells through non-specific or specific (or both) immune mechanisms. Mouse-adapted virus, on the other hand, may simply proliferate too rapidly for the infection to be suppressed.

ip inoculation of as little as 1 pfu of the 1995 strain of non–mouse-adapted EBO-Z was sufficient to elicit a protective immune response in mice, enabling them to survive a subsequent 3000 LD_{50} challenge. However, the same dose of heat- or radiation-inactivated virus did not induce immunity. These findings indicate that viral replication was required for inoculation of a very small quantity of virus to result in protective immunity. We have not yet determined whether such protection is based on a humoral or a cell-mediated immune response, nor have we attempted to use larger amounts of inactivated EBO-Z to immunize mice, as has been used in guinea pigs [26].

The use of wild-type EBO-Z to vaccinate mice has no equivalent in primate models of EBO infection, but the results at least demonstrate that it is possible to obtain solid protection against an otherwise lethal virus. Our findings suggest that the mouse model could be used for the initial evaluation of EBO virus vaccines. We do not know whether results obtained with the mouse model will accurately predict the efficacy of the same measures in primates. The predictive value of vaccination experiments will be affected by differences in the pathogenesis of EBO infection in mice and primates and by variations in their immune responses to the same antigens. Similarly, differences in drug metabolism between mice and primates might limit the model's accuracy in predicting the efficacy of antiviral agents in treating human EBO cases. However, we are optimistic that this new animal model of EBO infection will contribute significantly to the effort to control this deadly emerging pathogen. The mouse model may also prove valuable as a tool of basic research, as the availability of immunologically characterized inbred strains and of transgenic and knockout mice may make it possible to design experiments that will help to elucidate the pathophysiology of EBO hemorrhagic fever.

Acknowledgments

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