Viral Replication and Host Gene Expression in Alveolar Macrophages Infected with Ebola Virus (Zaire Strain)

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In order to characterize the cellular response to and identify potential diagnostic markers for the early detection of Ebola virus, an in vitro culture system involving nonhuman primate alveolar macrophages was developed. Ebola virus replication in the alveolar macrophages was characterized by plaque assay, immunohistochemical analysis, and in situ hybridization. Fluorogenic 5′-nuclease assays specific for nonhuman primate proinflammatory cytokines and chemokines were designed and used to evaluate mRNA transcription in macrophages infected with Ebola virus. Transient increases in cytokine and chemokine mRNA levels were observed immediately following exposure to Ebola virus. At 2 h postexposure, levels of cytokine and chemokine mRNAs were markedly reduced. Although Ebola virus infection of alveolar macrophages failed to induce a sustained increase in proinflammatory cytokine and chemokine mRNA transcription (potentially reducing the use of these markers as diagnostic tools), the fluorogenic 5′-nuclease assays developed may have prognostic value for individuals infected with Ebola virus. Recently published data have indicated that persons who remain asymptomatic after exposure to Ebola virus are capable of mounting an early proinflammatory cytokine response and that those who become clinically ill are not. If implemented immediately after exposure, these assays could be used to predict which individuals will be more likely to remain asymptomatic as opposed to those who will be more likely to develop clinical signs and eventually succumb to the virus.

Ebola virus is one of two members of the family Filoviridae. Members of this family are responsible for sporadic epidemics of hemorrhagic fever in human and nonhuman primates, with mortality rates ranging from 22 to 88% (21, 25). The disease course associated with Ebola hemorrhagic fever is acute and progresses rapidly. The incubation period after exposure to Ebola virus can range from 2 to 10 days. Patients often present with flu-like symptoms, such as fever, myalgia, headache, diarrhea, and vomiting. Clinical signs may progress rapidly and include severe nausea, pharyngitis, diarrhea, hematemesis, and melena. The primary routes of transmission are improper needle hygiene, direct contact with infected tissue or fluid samples, and close contact with infected patients (3, 4, 6, 18). Other modes of filovirus transmission, including oral, conjunctival, and aerosol routes, have been identified through experimental infection of laboratory animals (15, 16, 18).

To study the host response to and identify potential diagnostic markers for Ebola virus infections, an in vitro culture system involving nonhuman primate alveolar macrophages was developed and characterized. Macrophages are known to be primary targets for Ebola virus replication, and there is both experimental and epidemiological evidence suggesting that aerosol spread of this virus can occur (2, 8, 15, 18). Aerosol spread has been validated experimentally with nonhuman primates exposed to Ebola virus (Zaire subtype) and Marburg virus (2, 18). In addition, there is evidence suggesting that aerosol spread of the virus occurred among monkeys housed in distant cages in a quarantine facility during the Ebola virus (Reston subtype) outbreak (15). During this same outbreak, several of the animal caretakers in contact with infected monkeys were found seropositive for Ebola virus antibodies by an indirect immunofluorescent-antibody assay (5). Three out of four of the animal caretakers who seroconverted had no known history of parenteral or trauma-induced exposure to Ebola virus; therefore, the possibility of aerosol transmission in these cases has been suggested (18).

Normal host defenses in the lungs include both innate (non-specific) and acquired (specific) immune responses. Alveolar macrophages are the major immune effector cells resident in the airways and thus are key players in both arms of the immune response (24). They constitute the first line of phagocytic defense against infectious agents (innate response) and orchestrate the acquired (specific) response by the ordered expression and secretion of cytokines and chemokines (27). During a viral infection, they participate in the innate response by producing alpha/beta interferons (IFN-α/β) and the acquired response through antigen presentation, lymphocyte activation (through the secretion of soluble mediators), and IFN-α/β-induced major histocompatibility complex (MHC) class I gene expression (32).

Based on previously published studies, we hypothesized that infection of alveolar macrophages with Ebola virus would alter the expression of proinflammatory cytokines, chemokines, and IFN-α/β (1, 10, 29). Ebola virus infection of monocytes/macrophages can induce tumor necrosis factor alpha (TNF-α) production and, when compared to patients who recovered from Ebola virus infection, those with fatal outcomes had markedly elevated levels of gamma interferon (IFN-γ), IFN-α, interleukin 10 (IL-10), IL-2, and TNF-α (29). In addition, a more recent study showed that the VP35 protein of Ebola virus altered IFN-α/β production (1).

The purpose of these studies was to determine the effects of
Ebola virus (Zaire subtype) on alveolar macrophage cytokine and chemokine and IFN-α/β production. In this report, we describe the development and evaluation of a one-step fluorogenic reverse transcription (RT)-PCR assay for evaluating RNA transcription of nonhuman primate cytokine and chemokine genes and discuss the potential of these markers for detecting filovirus infections. We report on the replication of Ebola virus in nonhuman primate alveolar macrophages and on the effects of viral infection on the expression of cytokine and chemokine and IFN-α/β mRNAs. We specifically focused on TNF-α, IL-1β, IL-8, IL-6, macrophage inflammatory protein 1α (MIP-1α), and IFN-β.

MATERIALS AND METHODS

Virus preparation. Ebola virus (Zaire subtype, 1995 strain) was propagated in Vero E6 cells. Upon visualization of 60% to 70% cytopathic effects, supernatants were harvested, clarified by centrifugation, and stored at −70°C. Viral titers were determined by performing plaque assays on Vero E6 cells as described previously [22]. Briefly, viral stock was diluted serially in minimal essential medium with Earle’s salts without phenol red, and 0.1 ml was added to each well of a 12-well dish, incubated for 1 h at 37°C, and then washed once in sterile PBS−EDTA, centrifuged at 240 × g for 5 min, and stored at 4°C until Wright staining was performed or until used in immunohistochemical (IHC) and in situ hybridization (ISH) assays.

IHC assays. IHC assays were performed on formalin-fixed cytospin preparations and positive control tissue sections with an ENVISION kit (Dako Corp., Carpinteria, Calif.). A cocktail of two mouse monoclonal antibodies (anti-Ebola virus GP and anti-Ebola virus VP40) was used as the primary antibody (16). Positive control tissue sections were deparaffinized, rehydrated, and pretreated with proteinase K solution (Dako) for 6 min at room temperature before being immunostained. Cytospin preparations of alveolar macrophages were washed in PBS and pretreated with proteinase K solution for 6 min at room temperature before being immunostained. Before adding the primary antibody, we blocked endogenous peroxidase activity by adding peroxidase (0.33% hydrogen peroxide) for 25 min. The primary antibody was diluted 1:5,000 in antibody diluent. After applying the primary antibody to tissue sections, we collected the remainder of the protocol using a streptavidin-peroxidase conjugate, followed by development with diaminobenzidine containing hydrogen peroxide. All steps were completed according to the manufacturer’s instructions. Tissues from a monkey experimentally infected with Ebola virus in a previous study were included as positive controls. For negative controls, duplicate cytospin preparations and tissue sections were incubated with normal mouse serum pretreated as described for the primary antisera. After immunostaining was done, all sections were counterstained with hematoxylin, and coverslips were applied.

ISH assays. Replicate 5-μm tissue sections to be used as positive controls were floated on RNase-free water (Quality Biologicals), collected on glass slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, Pa.), and air dried. The sections were deparaffinized and rehydrated through graded alcohols to RNase-free water. Tissue sections and alveolar macrophage cytospin preparations were treated with 2× standard saline citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 10 min at 70°C and then placed in Tris-buffered saline (TBS; 50 mM Tris HCl, 0.15 M NaCl, 2 mM MgCl2) at room temperature before being immunostained. All steps were completed according to appropriate safety protocols, washed in nuclease-free water, and allowed to air dry before we added hybridization solution.

The probe used for hybridization was comprised of two plasmids, one with the pCRII backbone (Invitrogen, San Diego, Calif.) containing a 2.03-kb Ebola virus glycoprotein cDNA insert and one with the pCR2I backbone containing a 2.4-kb Ebola virus nucleoprotein cDNA insert. Plasmid DNA was purified using a plasmid midi kit (Qiagen, Valencia, Calif.) according to the manufacturer’s instructions. The probe was labeled with digoxigenin-11-dUTP by nick translation according to the manufacturer’s instructions (Boehringer). The probe was denatured at 95°C for 5 min and placed in an ethanol ice bath. Fifty microliters of hybridization solution (45% [vol/vol] deionized formamide (Ambion Inc., Austin, Tex.), 4× SSC, 4 μg of bovine serum albumin (BSA)/ml, 5 μg of poly(A), 25 ng of digoxigenin-labeled probe, nuclease-free H2O to 50 ml) was applied to each tissue section. Coverslips, which were placed on the tissue sections, and the sections were incubated in a humidified chamber for 16 h at 37°C. Coverslips were floated off the slides with 1× SSC. Sections were washed in 1× SSC and incubated for 15 min at room temperature in TBS containing 0.1% BSA and 0.1% Triton X-100. Sections were then covered with 500 μl of alkaline phosphatase-conjugated antidigoxigenin antibody (Fab fragment; 1:600 in TBS−0.1% BSA; Boehringer) and incubated for 60 min at 37°C. Sections were washed twice in TBS−0.1% BSA, rinsed in RNase-free water, and placed in chromogen buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2, pH 9.5). Bound antibody was visualized by incubating slides in a 1:11 molar ratio of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate containing levamisole (Gibco-BRL) for 1 h at 37°C. Slides were rinsed in water and counterstained with nuclear fast red (Vector Laboratories). A positive signal was identified by blue-purple cytoplasmatic staining. Controls included uninfected tissues.

Cytokine and chemokine assays (ELISAs). Cytokine and chemokine levels were determined by enzyme-linked immunosorbent assays (ELISAs). TNF-α (monkey), IL-1β (human), and IL-8 (human) ELISA kits were purchased from BioSource International (Camarillo, Calif.). IL-6 (human) and MIP-1α (human) kits were purchased from Pierce Endogen (Rockford, Ill.) and R&D Systems (Minneapolis, Minn.), respectively. ELISAs were performed according to the manufacturer’s protocols in a BSL-4 containment suite.

RNA extraction. Total RNA was extracted from alveolar macrophages using Trizol (Gibco-BRL). Cells were lysed by adding Trizol (Gibco-BRL) to individual wells of a six-well plate. After allowing sufficient time for cell lysis to occur,
by incubating samples for 15 min at 95°C with RNase-free DNase I (Ambion). The DNase I was subsequently inactivated containing the RNA was harvested. The RNA was precipitated by adding isopropanol, and the pellet was washed in 75% ethanol. Total RNA was resuspended in 200 μl of RNase-free water (Ambion) and treated for 30 min at 37°C after the DNase I was subsequently inactivated by incubating samples for 15 min at 95°C. RNA was quantitated by spectrometry, and A260/A280 ratios were determined.

Cytokine and chemokine primer and probe design. Primers and probes specific for macaque TNF-α, IL-1β, IL-8, IL-6, and MIP-1α genes were designed by evaluation of both published sequences (GenBank) and an unpublished sequence (the cDNA sequence for MIP-1α was kindly provided by Francois Villinger, Emory University, Atlanta, Ga.) using Primer Express Software (Applied Biosystems, Foster City, Calif.) (Table 1). The primers and probes were designed to meet the guidelines recommended by Applied Biosystems. The primers were designed with melting temperatures (Tm) of 58 to 60°C. The probes were designed to have a Tm of at least 7°C higher than the primer Tm. The presence of a guanine at the 5’ end of the probes was avoided, and the amplicons were designed to be less than 200 bp long. Primer and probe dimers, hairpins, stem-loop structures, and false priming sites were minimized. Primers specific for IFN-β were designed by evaluation of published sequences (GenBank) using Primer Express Software as described above (Table 1).

Fluorogenic 5’-nuclease assays. RT-PCR was performed with an ABI PRISM 7700 sequence detection system and an Applied Biosystems TaqMan EZ RT-PCR kit according to the manufacturer’s instructions. Thermocycling conditions for newly designed cytokine and chemokine primer sets were identical and were as follows: 55°C for 45 min; 94°C for 1 min, and 40 cycles at 94°C for 15 s and 60°C for 30 s. Final concentrations used in the 50-μl reaction mixture were as follows: 0.5 μM each primer, 0.2 mM probe, 5 U or rTth enzyme, 300 μM each deoxynucleotide triphosphate, 1× TaqMan EZ RT-PCR buffer containing the passive reference dye ROX, and 2.5 mM manganese acetate. To account for variations in the amount of input RNA, RNA encoded by an endogenous housekeeping gene, that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified and quantitated, and all results were normalized to this value. All assays were performed in triplicate, and averages from the three threshold cycle (Ct) values were used for quantitating cytokine and chemokine mRNA levels. Twenty-five nanograms of total RNA was used in all RT-PCRs.

Quantitation of cytokine and chemokine RNA transcription. Three methods exist to quantitate target mRNA levels in a sample using fluorogenic 5’-nuclease assays. They are the relative and absolute standard curve methods and the comparative Ct method (user bulletin no. 2, ABI PRISM 7700 sequence detection system, revision A, 11 December 1997, p. 3–15). To determine the most rapid and accurate method for quantifying cytokine and chemokine mRNA levels in this study, two of the methods, the relative standard curve method and the comparative Ct method, were evaluated using RNA samples isolated from alveolar macrophage cultures from experiment 1.

For the relative standard curve method, each sample was quantitated by using linear regression analysis from a standard curve. The standard curve was established over a range of RNA concentrations (75, 25, 10, 1, and 0.1 ng) from a designated calibrator sample (total RNA from 12-h LPS-stimulated alveolar macrophages). RNA from 12-h LPS-stimulated macrophages was chosen as the calibrator sample because previous experiments had indicated that cytokine and chemokine target RNAs were expressed at high levels at this time. The Ct value (y axis) was then plotted against the log of the mass of total RNA (x axis). RNA transcription levels of cytokine and chemokine genes were determined by division of the concentration of the cytokine or chemokine RNA of interest (interpolated from the standard curve for that cytokine or chemokine RNA) by the concentration of the endogenous reference RNA (GAPDH; interpolated from the GAPDH standard curve).

To use the comparative Ct method to quantitate RNA transcription, a validation experiment was performed in order to demonstrate that the amplification efficiencies of the target cytokine and chemokine RNA and the endogenous reference RNA (GAPDH) primer sets were approximately equal. The amplification efficiencies were determined using data generated from an examination of the relative standard curve method. For the comparative Ct method to be valid, the absolute value of the slope of log input amount versus Ct should be less than 0.1. The slope was calculated by plotting the ΔCt value (y axis) (where ΔCt is the average of the cytokine Ct value minus the average of the GAPDH Ct value) against the log nanograms of total RNA (x axis) using linear regression analysis. If the slope is ≤0.1, then the formula 2−ΔΔCt can be used to determine the relative quantities of target RNA present; the ΔΔCt value is defined as the ΔCt of the sample of interest minus the ΔCt of the calibrator sample. In this instance, the ΔCt of the sample is defined as the average of the cytokine Ct value minus the average of the GAPDH Ct value for the same time point (averages were derived from experiments performed in triplicate). The calibrator sample in

### Table 1. Sequences of PCR primers and sequence-specific probes for macaque cytokines

| Cytokine | Primer or probe | Sequence | Corresponding cDNA sequence | Product length (bp) |
|----------|----------------|----------|-----------------------------|---------------------|
| IL-1β<sup>a</sup> | Forward primer | 5’TCACGACTGCACTTCCG3<sup>α</sup> | 361–380 | 101 |
| | Reverse primer | 5’TGCTCAGAGTCCGTCCG3<sup>α</sup> | 442–461 | 396–430 |
| | Probe | 5’FAM-AGGCTTGGGTGACGTCGTTCAATGAACTGAAG-TAMRA-3<sup>α</sup> | | |
| IL-8<sup>a</sup> | Forward primer | 5’AGATGGGACACACTGTC3<sup>α</sup> | 217–236 | 138 |
| | Reverse primer | 5’ATGATAGTTGTTCCGACCC3<sup>α</sup> | 334–354 | 300–322 |
| | Probe | 5’FAM-TGATCCGACAGGGGTTGAGGAA-TAMRA-3<sup>α</sup> | | |
| IL-6<sup>a</sup> | Forward primer | 5’CAACAGCCACGGACCCG3<sup>α</sup> | 125–144 | 137 |
| | Reverse primer | 5’TTCGGCAGCTCTGCTTTGC3<sup>α</sup> | 242–261 | |
| | Probe | 5’FAM-CACGCTCCTCTGTCCTGACCTT-TAMRA-3<sup>α</sup> | 166–191 | |
| TNF-α<sup>a</sup> | Forward primer | 5’CAACAGCGCACTGTCG3<sup>α</sup> | 127–146 | 143 |
| | Reverse primer | 5’TTCGCTGACTGCTCCTGAC3<sup>α</sup> | 248–269 | |
| | Probe | 5’FAM-CACGCTCCTCTGTCCTGACCTT-TAMRA-3<sup>α</sup> | 162–187 | |
| MIP-1α<sup>a</sup> | Forward primer | 5’ACTACTTGAGACAGACGGC3<sup>α</sup> | 86–106 | 114 |
| | Reverse primer | 5’CGCTGACATTTGGACTGACCC3<sup>α</sup> | 179–199 | |
| | Probe | 5’FAM-CTCCGACGCGGTGTCATCCTTCTCTAATTAMRA-3<sup>α</sup> | 111–136 | |
| IL-10<sup>a</sup> | Forward primer | 5’CTTGCTGAGGACTTATTAGGA3<sup>α</sup> | 214–234 | 116 |
| | Reverse primer | 5’GCTCCATGACTGCTGTCG3<sup>α</sup> | 310–329 | |
| | Probe | 5’FAM-CTCCGACGCGGTGTCATCCTTCTCTAATTAMRA-3<sup>α</sup> | | |
| IFN-β<sup>a</sup> | Forward primer | 5’CAACATGCACCAACAAGTGTCCTC3<sup>α</sup> | 24–46 | 122 |
| | Reverse primer | 5’TTCGAGGACCAAGGAAGTTGACG3<sup>α</sup> | 120–145 | |

<sup>a</sup> Based on sequences deposited in GenBank. Accession numbers: IL-1β, U19845; IL-8, U19849; IL-6, AB000554; TNF-α, AB000513; IL-10, L26029; IFN-β, AJ011909.

<sup>b</sup> Based on an unpublished sequence obtained from Francois Villinger.
this instance was the $\Delta C_T$ of the T0 mock-infected RNA sample. Target mRNA transcription in samples from experiments 2, 3, and 4 was determined by the comparative $C_T$ method. All samples were normalized to the calibrator T0 mock-infected $\Delta C_T$ value, which was assigned as 1. Ranges given for samples were determined by evaluating the expression $2^{-\Delta\Delta C_T} = s$, where $s$ is the standard deviation of the $\Delta C_T$ value. The standard deviation of $\Delta C_T$ is defined as the square root of the sum of the standard deviations of the average $C_T$ values for the cytokine or chemokine and GAPDH individually squared.

IFN-β RT-PCR assay. RT-PCR assays were performed with an Applied Biosystems EZ rTth RNA PCR kit as instructed by the manufacturer. Thermocycling conditions for the IFN-β primer set were as follows: 55°C for 45 min, 94°C for 1 min, and 40 cycles at 94°C for 15 s and 60°C for 30 s. Final concentrations used in the 50-μl reaction mixture were as follows: 0.5 μM each primer, 5 U of rTth enzyme, 300 μM each deoxynucleotide triphosphate, 1× EZ buffer, and 2.5 mM manganese acetate. One hundred nanograms of total RNA was used in IFN-β RT-PCR assays. All assays were performed with a PTC 100 thermocycler (Applied Biosystems).

Direct analysis and detection of PCR-amplified products on agarose gels. Five-microliter aliquots of the RT-PCR products were analyzed by electrophoresis on 4% agarose gels (NuSieve; FMC BioProducts, Rockland, Maine) in Tris-borate buffer containing 1 μg of ethidium bromide/ml. The DNA bands were visualized with an Alpha-Innotech imaging system.

Sequencing of PCR-amplified products. To verify the identities of PCR products, amplicons from RT-PCRs were sequenced by using dye-labeled terminators and cycle sequencing (Taq Prism kit; ABI, Foster City, Calif.). Products from sequencing reactions were analyzed on an ABI 377 sequencer. The sequences were determined using the National Center for Biotechnology Information basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST/).

Flow cytometry. To characterize the cell surface expression of MHC class I and class II molecules, alveolar macrophages were analyzed by fluorescence-activated cell sorting. Cells were collected by gently scraping the wells at 0, 2, 12, and 24 h postexposure. Cold RPMI 1640 (4°C) containing 0.5% BSA was added to each well. Single-cell suspensions were obtained by vigorous mixing with a vortexer. Cells were pelleted by centrifugation at 240 × g for 5 min, medium was removed, and 20 μl of each fluorescein isothiocyanate (FITC)-conjugated antibody (anti-HLA-A, B, and C [Pharmingen, San Diego, Calif.] and anti-HLA-DR [Pharmingen]) was added to individual tubes. Antibody staining was performed at 4°C for 45 min. At the end of the 45 min, RPMI 1640 containing BSA was added to each tube, and cells were again pelleted by centrifugation at 240 × g for 5 min. Medium was aspirated, and the cell pellet was fixed in 10% NBF (4% paraformaldehyde) at 4°C for 72 h before reading was done by flow cytometry with a FACSort instrument operating with CELLQuest software (Becton Dickinson). Dead cells and debris were excluded from the analyses. Each histogram plot was generated from at least 10^4 events.

Statistical Analysis. Data obtained from ELISAs and fluorogenic 5′-nuclease assays were evaluated for significant differences by the Kruskal-Wallis analysis of variance. For each time point examined, data from alveolar macrophages isolated from individual monkeys were normalized by transformation to the logarithmic scale, analyzed, and ranked. Ranked samples were then analyzed, and significant differences were determined by comparison to data for mock-infected cells at each time point. A significant difference was determined when the $P$ value was <0.05.

RESULTS

Ebola virus replication in alveolar macrophages. To evaluate the ability of Ebola virus to infect and replicate in an in vitro culture of alveolar macrophages, freshly isolated cells from cynomolgus monkeys were infected with Ebola virus (Zaire) at an MOI of 1.0. Viral infection was characterized by plaque assays, IHC analysis, ISH, and light microscopy. Culture medium was removed at 0, 6, 12, and 24 h postexposure and was titrated by plaque assays on Vero E6 cells. As shown in Fig. 1C, Ebola virus (Zaire) productively infected the alveolar macrophages, showing a 10-fold increase in titer in macrophages from experiments 1 and 2 and a 100-fold increase in titer in macrophages from experiments 3 and 4 over a 24-h
period. Under light microscopy, Ebola virus caused visible cytotoxic effects in cell cultures during the 24-h period. By 12 h postexposure, cells in Ebola virus-infected wells began to detach from the culture plates, and by 24 h, 50 to 60% of the cells were detached and floating in the culture medium. At 12 h postexposure, alterations in cellular morphology which are indicative of irreversible cell injury were observed in Ebola virus-infected alveolar macrophages. Alterations such as membrane blebbing were present throughout the cultures. At 24 h postexposure, numerous plasma membranes were disrupted (blebbing was prominent), there was prominent karyorrhexis, and cells were vacuolated. Large amounts of cellular debris were present in Ebola virus-infected cultures, and cytolysis was evident. In contrast, alveolar macrophages in mock-infected and LPS-treated wells remained firmly attached to the bottoms of wells during the 24-h incubation period. Under light microscopy, mock-infected cells appeared normal. Cells stimulated with LPS appeared to increase in size and take on the characteristics of activated macrophages.

IHC analysis and ISH were used to further characterize Ebola virus infection in alveolar macrophage cultures. Compared to the results seen at T0, at T6 there was a notable increase in the amounts of Ebola virus RNA and protein in the cytoplasm of infected cells. Throughout the course of infection (Fig. 1), the numbers of cells staining positive in IHC analysis and ISH increased steadily. By 24 h postexposure, the cytoplasm of 80 to 90% of infected cells stained intensely for viral protein and RNA.

Additionally, fluorogenic 5'-nucleotide assays were performed to further confirm the presence or absence of viral RNA in infected alveolar macrophage cultures. Positive Ct values and the visualization of a 143-bp band by agarose gel electrophoresis indicated the presence of viral RNA. RT-PCR detected viral RNA throughout the course of infection in Ebola virus-infected cells but not in LPS- and mock-infected cells.

Flow cytometry. Flow cytometry was performed to characterize the alveolar macrophage population and determine the state of cellular activation after infection with Ebola virus (Zaire). Alveolar macrophages were analyzed for MHC class I and MHC class II expression after exposure to medium (mock-infected controls), Ebola virus (MOI 1.0), and LPS (20 μg/ml). Figure 2 shows MHC class I and II expression on alveolar macrophages from a single cynomolgus monkey in a representative experiment. Ebola virus infection did not induce the expression of HLA-A, HLA-B, HLA-C, or HLA-DR in alveolar macrophages compared to the results seen for mock-infected controls at the same time points. At 24 h postexposure, MHC class I cell surface expression was decreased in Ebola virus-infected cells compared to mock-infected controls (Fig. 2).

Alveolar macrophage supernatant cytokine and chemokine levels. Cytokines and chemokines play important roles in both innate and acquired immune responses to viral pathogens. Secretion of these soluble mediators by alveolar macrophages is key in establishing an effective antiviral pulmonary response. The accumulation of TNF-α, IL-1β, IL-8, IL-6, and MIP-1α in aliquots of supernatants from alveolar macrophages exposed to medium (mock-infected controls), Ebola virus (MOI 1.0), and LPS (20 μg/ml) was examined. The results are shown in Table 2. Alveolar macrophages incubated in medium alone failed to produce significant levels of TNF-α, IL-1β, IL-8, IL-6, and MIP-1α during 24 h of incubation. In contrast, at 6, 12, and 24 h poststimulation with LPS, there were significant increases in cytokine and chemokine production compared to the results seen for mock-infected controls (P < 0.05). IL-1β reached maximum levels of production at 12 h after LPS stimulation. IL-6, IL-8, TNF-α, and MIP-1α protein levels rose steadily during the 24-h period after LPS stimulation (Table 2). At 24 h postexposure, alveolar macrophages infected with Ebola virus showed significantly increased levels of IL-1β and TNF-α (P < 0.05); however, IL-6, IL-8, and MIP-1α protein levels in Ebola virus-infected cultures were similar to those in mock-infected controls at all time points examined (Table 2).

Comparison of relative standard curve method versus comparative Ct method for quantitation of cytokine and chemokine mRNA levels. To determine the most rapid and accurate method for quantitating cytokine and chemokine mRNA levels, total RNA isolated from alveolar macrophages from experiment 1 was tested by using two methods: the relative standard curve method and the comparative Ct method. The relative standard curve method was performed as described in Materials and Methods.

To use the comparative Ct method, the amplification efficiencies of each cytokine and chemokine primer set and the reference set (GAPDH) were analyzed (user bulletin no. 2, ABI PRISM 7700 sequence detection system). The amplification efficiency of each primer set was evaluated by establishing standard curves with the calibrator sample (12-h LPS-stimulated alveolar macrophage RNA) and then plotting the ΔCt value (y axis) against the log nanograms of total RNA (x axis). The slope of the line was calculated using linear regression analysis. When the amplification efficiencies of the cytokine and chemokine primer sets were compared to those of the GAPDH reference set, only two sets (IL-8 and MIP-1α) had amplification efficiencies similar to those of the reference set (GAPDH) (slope was <0.1). Analysis of all other primer sets indicated that the amplification efficiencies were not equal (slopes were not <0.1). However, data collected by both methods of quantitation yielded results which were essentially indistinguishable. Data calculated by the relative standard curve method most often fell within the range of data calculated by the comparative Ct method. Thus, we chose the comparative Ct method to analyze samples from all alveolar macrophage experiments.

IL-1β, IL-8, IL-6, MIP-1α, and TNF-α mRNA transcription in Ebola virus-infected alveolar macrophages. The mRNA transcription of IL-1β, IL-8, IL-6, MIP-1α, and TNF-α in alveolar macrophages exposed to medium alone, Ebola virus, and LPS was monitored at 0, 6, 12, and 24 h postexposure. mRNA transcription in each of the cultures was expressed as the percent increase over the values for the T0 mock-infected samples. The data shown in Table 3 represent the mean and the standard error of the mean for all four experiments. Statistical analysis was performed by using the Kruskal-Wallis analysis of variance by ranks. Observations were considered statistically significant when the P value was <0.05. Cytokine and chemokine mRNA expression in mock-infected cells did not significantly increase at any time during the experiment compared to the values for the T0 mock-infected cells. Cells stimulated with LPS produced significantly greater amounts of all cytokines and chemokines examined with the fluorogenic
MHC Class I

Time 0 hours

Time 12 hours

Time 24 hours

5'-nuclease assays at 6, 12, and 24 h poststimulation. Alveolar macrophages from rhesus monkeys exposed to medium (mock-infected control), Ebola virus (EBO) (MOI, 1.0), or LPS were harvested at 0, 12, and 24 h postinfection. Cells were left untreated or were treated with monoclonal antibodies to HLA-DR (MHC class II) or to HLA-A, B, and C (MHC class I), washed, fixed in 4% paraformaldehyde, and analyzed by fluorescence-activated cell sorting. A total of 10^4 cells per condition were analyzed. Relative fluorescence intensity for HLA-DR and for HLA-A, HLA-B, and HLA-C proteins is displayed on the horizontal axis. These data are from a representative experiment with cells from one of the four rhesus monkeys.

MHC Class II

Time 0 hours

Time 12 hours

Time 24 hours

FIG. 2. HLA-A, B, C, and HLA-DR expression by alveolar macrophages. Alveolar macrophages from cynomolgus monkeys exposed to medium (mock-infected control), Ebola virus (EBO) (MOI, 1.0), or LPS were harvested at 0, 12, and 24 h postinfection. Cells were left untreated or were treated with monoclonal antibodies to HLA-DR (MHC class II) or to HLA-A, B, and C (MHC class I), washed, fixed in 4% paraformaldehyde, and analyzed by fluorescence-activated cell sorting. A total of 10^4 cells per condition were analyzed. Relative fluorescence intensity for HLA-DR and for HLA-A, HLA-B, and HLA-C proteins is displayed on the horizontal axis. These data are from a representative experiment with cells from one of the four cynomolgus monkeys.

IFN-β expression in Ebola virus-infected alveolar macrophages. One of the most important and early components of the host defense against viral infections is the production of IFN-α/β. IFN-α/β are synthesized in response to viral infections or the presence of double-stranded RNA (dsRNA) species (14, 17). In an attempt to determine whether Ebola virus induced IFN-α/β production, the mRNA expression of IFN-β was monitored at 0, 2, and 6 h postexposure in alveolar macrophages exposed to medium alone (mock-infected controls), Ebola virus, and LPS. In two of the four experiments, at T0 IFN-β was expressed at higher levels in Ebola virus-infected cells than in mock-infected cells (Fig. 3). However, by 2 h after exposure to Ebola virus, there was a significant reduction in the amount of IFN-β mRNA present in alveolar macrophages (Fig. 3). The IFN-β response was absent in macrophages from the other two cultures. At 6 h postexposure, equal amounts of IFN-β were observed in both mock-infected and Ebola virus-infected cells. IFN-β production was not detected in cells stimulated with LPS at 6 h postexposure (Fig. 3).

DISCUSSION

In this study, we developed and characterized an in vitro model of alveolar macrophages in which the host response to...
Filovirus infection can be studied. This is the first documented evidence of Ebola virus infection and replication in alveolar macrophages in an in vitro system.

Data from this study indicate clearly that nonhuman primate alveolar macrophages from four different cynomolgus monkeys in four separate experiments. Data are for alveolar macrophages from four different cynomolgus monkeys in four separate experiments.

The ability of viruses to replicate efficiently and to a high titer inside the target cell largely depends upon their ability to evade the host immune response. One of the most important and early components of the host defense against viral infections is the production of IFN-α/β and other cytokines that inhibit viral replication (TNF-α and IL-1β). IFN-α/β and proinflammatory cytokines are synthesized in response to viral infections or the presence of dsRNA species. dsRNAs are intermediates that are generated during the replication cycle of many viruses (14, 17). For negative-stranded RNA viruses (filoviruses, paramyxoviruses, and orthomyxoviruses), dsRNA intermediates arise during the production of positive-stranded progeny. In response to dsRNA, IFN-α/β signal through a common receptor to induce the transcription of antiviral proteins such as dsRNA-dependent protein kinase R and 2′, 5′-oligoadenylate synthetase (17). These antiviral proteins then act to inhibit viral replication by blocking viral RNA and protein synthesis and inducing apoptosis (19). In addition, dsRNA induces the transcription of many proinflammatory cytokines, such as IL-6, TNF-α, and IL-1β.

Either the virus itself or the dsRNA species produced by the

### Table 2. Effects of Ebola virus infection and LPS stimulation on cytokine and chemokine protein production in nonhuman primate alveolar macrophages

| Time | Treatment | Mean ± SEM concn (pg/ml) of: | IL-1β | IL-6 | IL-8 | MIP-1α | TNF-α |
|------|-----------|-------------------------------|-------|------|------|--------|-------|
| T0   | Mock infected | 0 | 0 | 1.71 ± 1.11 | 0 | 1.11 ± 1.11 |
|      | Infected    | 0 | 8.19 ± 8.19 | 24.20 ± 24.20 | 0 | 0 |
| T6   | LPS         | 94.06 ± 29.38b | 55.51 ± 23.51b | 8,598.61 ± 1,025.66b | 1,940.59 ± 476.56b | 3,070.28 ± 870.74b |
|      | Mock infected | 0 | 0 | 440.01 ± 155.20 | 30.28 ± 26.88 | 0 |
|      | Infected    | 0 | 1.73 ± 1.42 | 842.92 ± 306.37 | 112.66 ± 57.69 | 1.44 ± 1.44 |
| T12  | LPS         | 216.69 ± 139.31b | 665.59 ± 314.41b | 269,088.57 ± 3,218.03b | 18,600.80 ± 5,287.57b | 5,625.12 ± 1,633.96b |
|      | Mock infected | 0 | 0.04 ± 0.04 | 912.31 ± 394.92 | 209.04 ± 122.67 | 29.01 ± 24.95 |
|      | Infected    | 0 | 0 | 1,079.49 ± 368.21 | 141.92 ± 113.45 | 1.44 ± 1.44 |
| T24  | LPS         | 212.95 ± 117.15b | 1,240.08 ± 666.67b | 37,840.58 ± 2,704.53b | 36,735.02 ± 4,850.02b | 5,966.06 ± 1,532.75b |
|      | Mock infected | 0 | 23.73 ± 23.73 | 676.83 ± 287.05 | 158.45 ± 127.00 | 0.00 |
|      | Infected    | 16.02 ± 7.60b | 7.24 ± 7.24 | 2,762.62 ± 1,076.25 | 218.65 ± 143.03 | 102.51 ± 56.35b |

a Data are for alveolar macrophages from four different cynomolgus monkeys in four separate experiments.
b Value was significantly different (P < 0.05) from value for mock-infected cells at the same time point.

### Table 3. Effects of Ebola virus infection and LPS stimulation on cytokine and chemokine RNA production in nonhuman primate alveolar macrophages

| Time | Treatment | Mean ± SEM % increase in template RNA fora: | IL-1β | IL-6 | IL-8 | MIP-1α | TNF-α |
|------|-----------|---------------------------------|-------|------|------|--------|-------|
| T0   | Mock infected | 1 | 54.16 ± 34.93b | 1.87 ± 0.22b | 3.91 ± 0.59b | 1.19 ± 0.41 |
|      | Infected    | 1.9 ± 0.47b | | | | |
| T6   | LPS         | 52.35 ± 6.00b | 19,533.49 ± 6,432.60b | 24.81 ± 5.50b | 98.30 ± 17.24b | 60.10 ± 23.94b |
|      | Mock infected | 0.47 ± 0.05 | 0.71 ± 0.15 | 0.20 ± 0.03 | 3.52 ± 2.70 | 1.11 ± 0.86 |
|      | Infected    | 0.83 ± 0.31 | 0.03 ± 0.11 | 0.41 ± 0.05b | 3.34 ± 1.37 | 0.28 ± 0.07 |
| T12  | LPS         | 41.82 ± 6.23b | 24,990.04 ± 8,976.29b | 25.71 ± 6.50b | 144.23 ± 58.92b | 162.31 ± 127.46b |
|      | Mock infected | 1.53 ± 1.20 | 245.14 ± 229.05 | 0.35 ± 0.27 | 9.02 ± 7.37 | 5.51 ± 5.15 |
|      | Infected    | 0.44 ± 0.19 | 1.04 ± 0.39 | 0.51 ± 0.08 | 3.80 ± 1.14 | 3.32 ± 2.63 |
| T24  | LPS         | 18.57 ± 5.10b | 14,218.51 ± 9,022.08b | 12.69 ± 4.30b | 67.60 ± 28.6b | 58.87 ± 53.02b |
|      | Mock infected | 0.04 ± 0.02 | 2.53 ± 1.15 | 0.01 ± 0.00 | 0.60 ± 0.14 | 0.05 ± 0.05 |
|      | Infected    | 6.44 ± 4.19b | 5,864.48 ± 5,851.41b | 5.49 ± 2.90b | 25.38 ± 17.58b | 67.54 ± 63.64b |

a Data are for treated cells relative to T0 mock-infected cells over time in four separate experiments with alveolar macrophages from four separate cynomolgus monkeys.
b Value was significantly different (P < 0.05) from value for mock-infected cells at the same time point.
virus during replication induce the transcription of IFN-α/β and proinflammatory cytokines. Many viruses have developed survival mechanisms that enable them to counteract dsRNA-induced cellular responses (2a). Of the viruses that have developed these mechanisms, those of particular relevance to the current study are members of viral families (the family Paramyxoviridae and the family Orthomyxoviridae) that are closely related to the family Filoviridae. Influenza virus A (11), simian virus 5 (31), measles virus (23), and Sendai virus (12) have all developed distinct mechanisms for subverting the dsRNA-induced IFN-α/β response. In addition, recently published data indicate that Ebola virus, like many other negative-stranded RNA viruses, is capable of suppressing the IFN-α/β response. More specifically, VP35, a phosphoprotein encoded by the Ebola virus genome, acts as an IFN-α/β antagonist by blocking dsRNA- and virus-mediated induction of the IFN-β promoter (1). In this study, infection of alveolar macrophages with Ebola virus appeared to completely block or suppress IFN-β mRNA production. Alveolar macrophages infected with Ebola virus either responded with an initial burst of IFN-β RNA production which was later suppressed or failed to produce any IFN-β (Fig. 3). Many important effector molecules (i.e., MHC class I and cytokines) involved in the host antiviral response are dependent on IFN-α/β expression. Therefore, the ability of VP35 to inhibit IFN-α/β production may explain why Ebola virus replicated to a high titer but failed to induce sustained expression of critical mediators (proinflammatory cytokines and chemokines and MHC class I and II expression) involved in the host antiviral response in alveolar macrophages (Tables 2 and 3 and Fig. 2).

The mechanisms responsible for VP35 inhibition of IFN-α/β production have not been fully elucidated. However, like other viral proteins (NS1 of influenza virus A), VP35 may inhibit the dsRNA-induced activation of transcription factors needed for IFN-α/β gene induction. For example, the NS1 protein of influenza virus A blocks IFN-β production by inhibiting the activation of both IFN regulatory factor 3 and nuclear factor κB (NF-κB), two factors known to be important in induction of the IFN-β promoter (26, 30). If, in fact, VP35 blocks the dsRNA-induced expression of IFN-α/β by inhibiting the activation of transcription factors such as NF-κB, this action could account for the lack of sustained proinflammatory cytokine expression observed in this system. Both TNF-α and IL-1β contain elements in their promoters that are responsive to induction by NF-κB, and the expression of both is known to be induced by dsRNA (13, 28). Furthermore, IFN-α/β are important stimulators of MHC class I expression in virus-infected cells. In this study, we did not observe an upregulation of MHC class I expression after viral infection. At 24 h postexposure, when a large proportion of cell death was observed in infected cultures, MHC class I expression was markedly decreased in Ebola virus-infected cells. These data are similar to those of an earlier report in which Ebola virus was shown to suppress dsRNA induction of IFN regulatory factor 1, MHC class I, IL-6, and 2'-5'-oligoadenylate expression in endothelial cells (14).

The apparent suppression of IFN-β production after infection with Ebola virus is consistent with recently published data showing that the VP35 phosphoprotein of Ebola virus inhibits IFN-β gene induction (1) and that Ebola virus is capable of inhibiting dsRNA induction of genes important in orchestrating an early antiviral state (14). The lack of an early antiviral response in macrophages is significant because these cells are the earliest and most heavily affected targets for Ebola virus replication (7, 9, 16). In addition, the variability observed among primates with respect to their ability to mount an early IFN response to Ebola virus may provide some indication as to why some individuals remain asymptomatic and others succumb to infection after exposure to Ebola virus (20). It is possible that some individuals are capable of subverting the VP35-induced suppression of the IFN-α/β response and therefore are able to control viral replication and mount an effective immune response early in the course of the disease. In fact, asymptomatic individuals exposed to Ebola virus had very low viral titers. These individuals produced an early and sustained inflammatory response with high levels of IL-1β, TNF-α, IL-6, and MIP-1α (20).

The data presented here provide further evidence to support the hypotheses that Ebola virus is capable of suppressing the dsRNA-induced host antiviral response and that the suppression of this response may play a key role in the ability of the virus to replicate and induce profound immunosuppression.

FIG. 3. IFN-β expression by alveolar macrophages, as analyzed by RT-PCR. One hour after exposure to Ebola virus (lane 3), cells expressed IFN-β mRNA at significantly increased levels over mock-infected cells. However, by 2 h postexposure, the expression of IFN-β was markedly reduced (lane 6) (observed in two of four monkeys). In the other two monkeys, Ebola virus infection failed to stimulate any IFN-β production (data not shown). GAPDH was used as an internal control. Lane 1 and 12, molecular weight markers; lane 2, T0 mock-infected cells; lane 3, T0 Ebola virus-infected cells; lane 4, T2 LPS-stimulated cells; lane 5, T2 mock-infected cells; lane 6, T2 Ebola virus-infected cells; lane 7, T6 LPS-stimulated cells; lane 8, T6 mock-infected cells; lane 9, T6 Ebola virus-infected cells; lane 10, LPS-stimulated peripheral blood mononuclear cells (positive control); lane 11, no-template control (NTC).
during the course of the disease. Further characterization of the early immune response to Ebola virus infection may provide insight into the mechanisms by which the virus evades and suppresses the host immune system.

In conclusion, our data demonstrate that in an in vitro system of alveolar macrophages, Ebola virus infection induced a transient, early increase in proinflammatory cytokine and chemokine mRNA expression. The ability or inability to produce a cytokine response immediately following Ebola virus infection may be a critical factor in determining the prognosis of a patient. Recently published data suggest a link between the clinical status of a patient and the inflammatory response. Persons who remain asymptomatic after exposure to Ebola virus are capable of mounting an early, sustained proinflammatory cytokine response, and those who become clinically ill are not (20). Cytokine responses associated with terminal cases of Ebola virus infections (29) occur late in the course of disease (following damage to the gastrointestinal tract, endotoxin release, and endothelial damage) and therefore are not likely to play a key role in determining the prognosis of Ebola virus-infected patients. Therefore, the fluorogenic 5'-nuclease assays developed and evaluated in this study are of questionable use for early diagnostic purposes but, if used immediately following exposure, may prove to be valuable as prognostic indicators for individuals exposed to Ebola virus. If applied immediately after exposure, these assays could be used to predict which individuals will be more likely to remain asymptomatic as opposed to those who will be more likely to develop clinical signs and eventually succumb to the virus.

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REFERENCES

1. Basler, C. F., X. Wang, E. Muhlbberger, V. Vellekov, J. Paragas, H. D. Klenk, A. Garcia-Sastre, and P. Palese. 2000. The Ebola virus VFP5 protein functions as a type I IFN antagonist. Proc. Natl. Acad. Sci. USA 97:12289–12294.
2. Brehutin, N. B., E. F. Belanov, V. A. Spiridonov, A. V. Poitenko, N. A. Krivenchuk, S. A. Krotov, N. I. Omelchenko, A. I. Tereshchenko, and V. V. Khomichev. 1992. The effect of the methods for producing an experimental Marburg virus infection on the characteristics of the course of the disease in green monkeys. Vopr. Virusol. 37:153–156.
3. Biron, C. A., and G. C. Sen. 2001. Interferons and other cytokines, polyclonal responses. J. Interferon Cytokine Res. 21:311–331.
4. Bulletin of the World Health Organization. 1978. Ebola haemorrhagic fever in Sudan, 1976. Report of a WHO/International Study Team. Bull. W. H. O. 56:247–270.
5. Bulletin of the World Health Organization. 1978. Ebola haemorrhagic fever in Zaire, 1976. Bull. W. H. O. 56:271–293.
6. Centers for Disease Control and Prevention. 1990. Update: filovirus infections among persons with occupational exposure to nonhuman primates. Morb. Mortal. Wkly. Rep. 39:266–267.
7. Centers for Disease Control and Prevention. 1995. Update: outbreak of Ebola viral hemorrhagic fever—Zaire, 1995. Morb. Mortal. Wkly. Rep. 44:468–469, 475.
8. Connolly, B. M., K. E. Steele, K. J. Davis, T. W. Geisbert, W. M. Kell, N. K. Jaax, and P. B. Jahring. 1999. Pathogenesis of experimental Ebola virus infection in guinea pigs. J. Infect. Dis. 179(Suppl. 1):S201–S217.
9. Dalgard, D. W., R. J. Hardy, S. L. Pearson, G. J. Puca, R. V. Quander, P. M. Zack, C. J. Peters, and P. B. Jahring. 1992. Combined simian hemorrhagic fever and Ebola virus infection in cynomolgus monkeys. Lab. Anim. Sci. 42:452–457.
10. Davis, K. J., A. O. Anderson, T. W. Geisbert, K. E. Steele, J. B. Geisbert, P. Vogel, B. M. Connolly, J. W. Huggins, P. B. Jahring, and N. K. Jaax. 1997. Pathology of experimental Ebola virus infection in African green monkeys. J. Infect. Dis. 176:2208–2214.
11. Feldmann, H., H. Bugany, F. Mahner, H. D. Klenk, D. Drenckhahn, and H. J. Schnittler. 1996. Filovirus-induced endothelial leakage triggered by infected monocytes/macrophages. J. Virol. 70:2208–2214.
12. Garcia-Sastre, A., A. Egorov, D. Matasov, S. Brandl, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 252:324–330.
13. Garcia, D., P. Latorre, and D. Kolakofsky. 1999. Sendai virus protein encoded in the interferon-mediated induction of an antiviral state. J. Virol. 73:5559–5565.
14. Gendelman, H. E., R. M. Friedman, S. Joe, L. M. Baca, J. A. Turpin, G. Dvksler, M. S. Meltzer, and C. Diedffenbach. 1990. A selective defect of interferon alpha production in human immunodeficiency virus-infected monocytes. J. Exp. Med. 172:1433–1442, (Erratum, 173:277, 1991.)
15. Harcourt, B. H., A. Sanchez, and M. K. Offermann. 1998. Ebola virus inhibits induction of genes by double-stranded RNA in endothelial cells. Virology 245:178–188.
16. Jaax, N., P. Jahring, T. Geisbert, J. Geisbert, K. Steele, K. McKee, D. Nagley, E. Johnson, G. Jaax, and C. Peters. 1995. Transmission of Ebola virus (Zaire strain) to uninfected control monkeys in a biocountermeasure laboratory. Lancet 346:1669–1671.
17. Jaax, N. K., J. J. Davis, T. J. Geisbert, P. Vogel, G. P. Jaax, M. Topper, and P. B. Jahring. 1996. Lethal experimental infection of rhesus monkeys with Ebola-Zaire (Mayinga) virus by the oral and conjunctival route of exposure. J. Virol. 70:1540–1555.
18. Jacobs, B. L., and J. O. Landlang. 1996. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology 219:339–349.
19. Johnson, E. N., N. Jaax, J. White, and P. Jahring. 1995. Lethal experimental infections of rhesus monkeys by aerosolized Ebola virus. Int. J. Exp. Pathol. 76:227–236.
20. King, P., and S. Goodbourn. 1998. STAT1 is inactivated by a caspase. J. Biol. Chem. 273:8699–8704.
21. Leroy, E. M., S. Baize, V. E. Vellekov, S. P. Fisher-Hoch, M. C. Georges-Courbot, J. Lansoud-Soukate, M. Capron, P. Debre, J. B. McCormick, and J. A. Georges. 2000. Human asymptomatic Ebola virus infection and strong inflammatory response. Lancet 355:2210–2215.
22. Martini, G. A., and R. Siegert. 1971. Marburg virus disease, p. 1–230. Springer, New York, N.Y.
23. Moe, J. B., R. D. Lambert, and H. W. Lupton. 1981. Plaque assay for Ebola virus. J. Clin. Microbiol. 15:791–793.
24. Nakich, D. A., Y. H. D. Eto, M. Manchester, R. M. Friedman, and M. B. Oldstone. 2000. Evasion of host defenses by measles virus: wild-type measles virus infection interferes with induction of alpha/beta interferon production. J. Virol. 74:7478–7484.
25. Pananska, J. R., R. Merolla, N. A. Rebert, S. P. Hoffmann, P. Titivit, N. M. Cirino, R. H. Silverman, and J. A. Rankin. 1995. Respiratory syncytial virus induces interleukin-10 by human alveolar macrophages. Suppression of early cytokine production and implications for incomplete immunity. J. Clin. In-\n\n\n\n\n
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