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Ebola Virus Selectively Inhibits Responses to Interferons, but Not to Interleukin-1β, in Endothelial Cells

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Ebola virus infection is highly lethal and leads to severe immunosuppression. In this study, we demonstrate that infection of human umbilical vein endothelial cells (HUVECs) with Ebola virus Zaire (EZ) suppressed basal expression of the major histocompatibility complex class I (MHC I) family of proteins and inhibited the induction of multiple genes by alpha interferon (IFN-α) and IFN-γ, including those coding for MHC I proteins, 2′-5′ oligoadenylate synthetase [2′-5′(A)$_N$], and IFN regulatory factor 1 (IRF-1). Induction of interleukin-6 (IL-6) and ICAM-1 by IL-1β was not suppressed by infection with EZ, suggesting that the inhibition of IFN signaling is specific. Gel shift analysis demonstrated that infection with EZ blocked the induction by IFNs of nuclear proteins that bind to IFN-stimulated response elements, gamma activation sequences, and IFN regulatory factor binding site (IRF-E). In contrast, infection with EZ did not block activation of the transcription factor NF-κB by IL-1β. The events that lead to the blockage of IFN signaling may be critical for Ebola virus-induced immunosuppression and would play a role in the pathogenesis of Ebola virus infection.

Filoviruses are enveloped, negative-stranded single-stranded RNA viruses with nonsegmented genomes belonging to the family *Filoviridae* in the order *Mononegavirales*. Filoviruses cause a fulminating, febrile hemorrhagic disease that is highly lethal to humans and other primates (2, 12). Ebola virus Zaire (EZ) has the highest mortality rate in humans of all known filoviruses, killing nearly 90% of those infected (19) and was used in this report.

During filovirus infection, the virus grows to high titers in the liver, spleen, lymph nodes, and lungs. These organs are severely damaged during the course of disease (11, 25), but perhaps the most striking observation is that patients with fatal filovirus infections die with very high viremia, an absence of mononuclear phagocytic infiltration into sites of infection, and little evidence of a humoral or T-cell-mediated response (28). In addition, filoviruses are resistant to the effects of the antivirus properties of interferon (IFN) when used prophylactically in infected monkeys (1, 7, 28). The mechanism(s) behind the failure of the body to mount an immune response during infection is unknown.

Immunohistochemistry of biopsies from infected humans and other primates demonstrates that endothelial cells are heavily infected with EZ (13, 14). Although endothelial cells play an important role in the host antivirus response through the expression of a number of immunomodulatory genes that are induced by viruses or cytokines (10, 30), the lack of inflammatory infiltrate near EZ-infected endothelial cells suggests that infection is somehow disrupting the normal host antivirus response. Because IFNs are especially important in the host antivirus response through the induction of many genes, such as those coding for major histocompatibility complex class I (MHC I) proteins, 2′-5′ oligodenylate synthetase [2′-5′(A)$_N$], and IFN regulatory factor 1 (IRF-1), we examined the effect of EZ infection on IFN signaling in endothelial cells.

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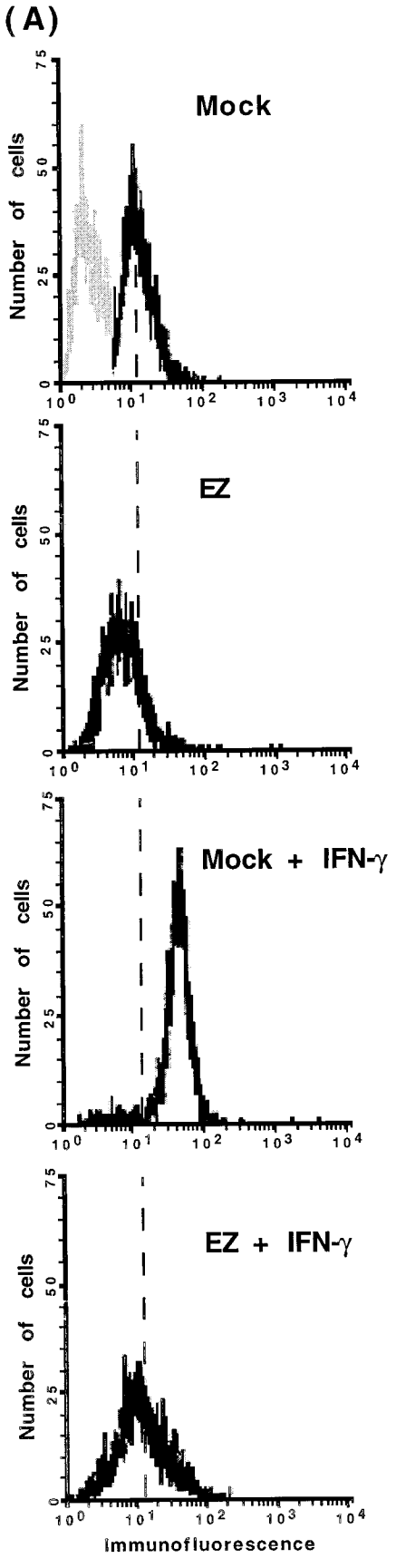
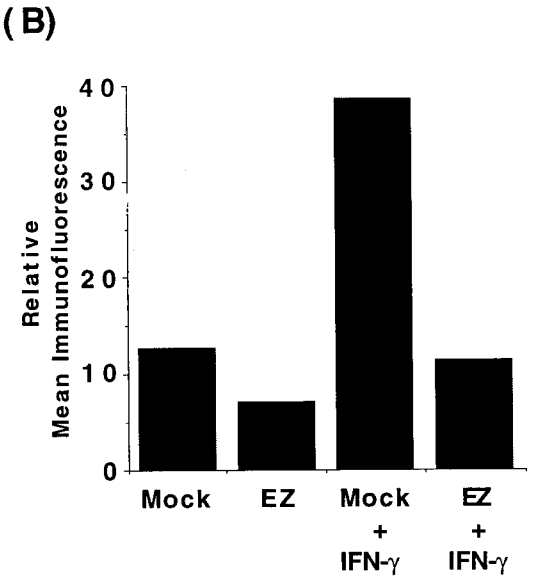
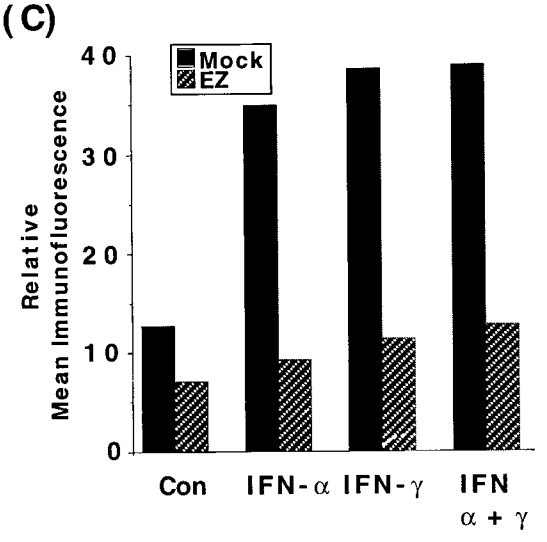
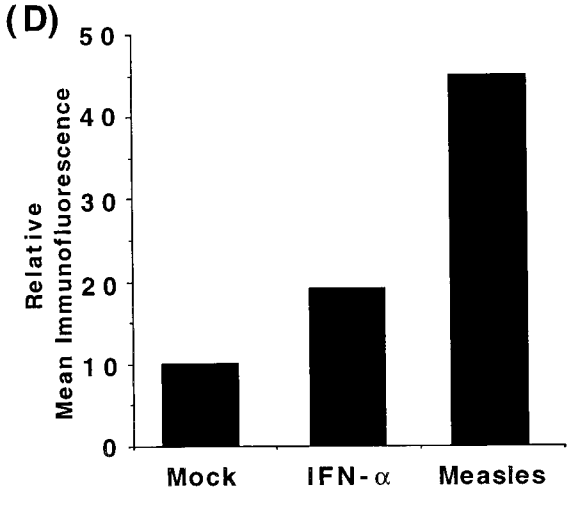
Human umbilical vein endothelial cells (HUVECs) were isolated and grown as previously described (17). HUVECs were infected with EZ at a multiplicity of infection (MOI) of 1.0. MHC I protein levels were measured by flow cytometric analysis as previously described (17). Basal levels of MHC I protein expression remained unchanged 24 h postinfection (p.i.), but expression decreased to approximately 50% of the basal level by 72 h p.i. (data not shown) and remained at this level 96 h p.i. (Fig. 1A and B). Since EZ infection suppressed basal MHC I protein expression, we asked whether infection with EZ also blocked induction of MHC I by IFNs. The cells were infected with EZ for 72 h prior to treatment. This time was chosen because the viral infection of the cells is established, as evidenced by high levels of virion RNA and protein and by the log-phase release of progeny virions (data not shown). Treatment with IFN-γ (Genzyme Diagnostics, Cambridge, Mass.) induced a threefold increase of MHC I protein in mock-infected cells (Fig. 1A and B). Infection with EZ for 72 h prior to the addition of IFN-γ completely blocked this induction and led to a level of MHC protein expression lower than that in the mock-infected uninduced cells. Furthermore, infection with EZ completely blocked induction of MHC I by IFN-α (IFN-alfa-2b was from the Schering Corporation, Kenilworth, N.J.), alone and in combination with IFN-γ (Fig. 1C). We found that infection with EZ for 24 h prior to treatment with IFNs did not block MHC I induction by IFN-α, IFN-γ, or IFN-α and IFN-γ added simultaneously (data not shown). The ability of EZ to suppress basal MHC I protein expression in HUVECs was in stark contrast to that of another negative-stranded RNA virus, measles virus. Infection of HUVECs with the wild-type measles virus strain NJ-2 (16, 29) induced higher levels of MHC I protein expression than did incubation with IFN-α (Fig. 1D). Thus, the decrease in basal expression of MHC I protein and the lack of induction by IFN in EZ-infected cells were unique to EZ and were not a general function of infection with negative-stranded RNA viruses.

The selectivity of inhibition of cytokine responsiveness was addressed by examining the effect of EZ on responsiveness to


(A)
Mock
EZ
Mock + IFN-γ
EZ + IFN-γ
Number of cells
immunofluorescence


(B)
Relative Mean Immunofluorescence
Mock
EZ
Mock + IFN-γ
EZ + IFN-γ


(C)
Mock
EZ
Relative Mean Immunofluorescence
Con
IFN-α
IFN-γ
IFN α + γ


(D)
Relative Mean Immunofluorescence
Mock
IFN-α
Measles

interleukin-1β (IL-1β), a cytokine that transduces signals independent of the IFN signaling pathway. IL-6 is not induced by either IFN-α or IFN-γ but is induced by IL-1β (31). IL-6 levels were measured by sandwich enzyme-linked immunosorbent assay as previously described (17). Figure 2 demonstrates that IL-1β (Boehringer Mannheim, Indianapolis, Ind.) induced high levels of IL-6 protein in both mock-infected and EZ-infected cells. Thus, EZ does not inhibit all signal transduction, but selectively inhibits gene induction by IFNs, but not by IL-1β. In addition, the ability of IL-1β to induce IL-6 protein demonstrates that infection with EZ is not blocking de novo protein synthesis.

Northern blot analysis further demonstrated that infection with EZ inhibited induction of a number of genes by IFN, whereas responses to IL-1β appear unaffected. MHC I mRNA was induced to high levels by IFN-α and IFN-γ when added separately or in combination in uninfected cells, whereas infection with EZ inhibited these inductions by greater than 80% (Fig. 3). Induction of other genes by either IFN-α or IFN-γ, including those coding for IRF-1 and 2′-5′$(A)_N$, was also found to be strongly suppressed in EZ-infected cells. In contrast to the ability of EZ to inhibit gene induction by the IFNs, infection with EZ did not inhibit responses to IL-1β, as demonstrated by comparable levels of induction of ICAM-1 and IL-6 in uninfected and EZ-infected cells (Fig. 3, lanes 9 and 10). Comparable levels of EZ glycoprotein gene mRNA were detected in all infected samples, thereby confirming similar levels of infection.

Responses to IFNs occur through activation of transcription factors that recognize and transactivate through IFN-responsive sequences found in the regulatory regions of DNA (3, 6, 9, 20). Type I and II IFNs each have their own signaling pathways using different receptors. For both type and type II IFNs, ligand binding initiates a tyrosine phosphorylation cascade leading to the activation of transcription factors and gene induction. Type I IFN leads to the formation of the transcription factor ISGF-3 (composed of phosphorylated STAT-2 and STAT-1α and DNA binding protein p48), which binds to IFN-stimulated response elements (ISREs), whereas type II IFN leads to the formation of the gamma-activated factor (GAF), which is composed of phosphorylated STAT-1α homodimers, which binds to specific DNA recognition sequences known as gamma activation sequences (GAS) and drives gene transcription. IFN-γ can also induce formation of STAT-1–STAT-1–p48 complexes that bind ISRE (5). Furthermore, within the ISRE is found a related element, the IFN regulatory factor binding site (IRF-E), that competitively binds IRF family members (6), including the transcription factor IRF-1, whose de novo synthesis is induced by both IFN-α and IFN-γ in HUVECs (Fig. 3). In order to determine whether infection with EZ affects the formation of complexes that bind to either ISRE or to GAS elements in response to IFN-α and IFN-γ, gel shift analysis was performed. In mock-infected cells, both IFN-α and IFN-γ induced the formation of several specific complexes that bound to the ISRE (Fig. 4A, lanes 4 and 6, respectively), whereas these complexes were not induced by IFN-α and IFN-γ in cells that were infected with EZ (Fig. 4A, lanes 5 and 7). Although the compositions of the complexes that bound to the ISRE were not characterized, the specificity of these complexes was confirmed by the ability of nonradiolabeled ISRE, but not an unrelated sequence, to specifically compete for binding to the complexes induced by IFN-α and IFN-γ (Fig. 4A, lanes 8 and 9, respectively). IFN-γ, but not IFN-α, induced the binding of a single specific complex to the GAS element in mock-infected cells (Fig. 4B). In EZ-infected cells, this complex was induced by IFN-γ, but at much lower levels. Supershift analysis with an antibody (from Transduction Laboratories, Lexington, Ky.) to STAT-1α revealed that this complex was composed of STAT-1α. Thus, in HUVECs infected with EZ, cells were unable to form complexes that bound to the ISRE in response to either IFN-α or IFN-γ and formed very low levels of complexes that bound to GAS in response to IFN-γ. In contrast to the ability of EZ to inhibit gene induction by IFNs, responses to IL-1β were intact. IL-1β activates the latent transcription factor NF-κB, a transcription factor involved in the induced expression of IL-6 and ICAM-1 (8). Gel shift analysis indicated that IL-1β activated NF-κB to similar levels in either mock-infected or EZ-infected cells (Fig. 4C, lanes 4 and 5, respectively). These data provide further evidence of selectivity in the inhibition of IFN-induced signals, but not IL-1β-induced signals.

The mechanism by which EZ inhibits responses to both classes of IFNs remains to be determined, but the inhibition

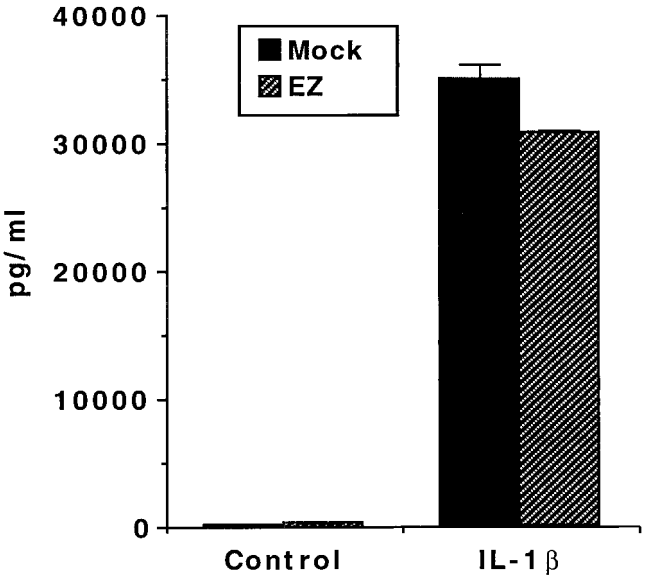


FIG. 2. Effect of infection with EZ on IL-6 protein induction. Conditioned medium was collected from HUVECs that had been treated with IL-1β (100 U/ml) for the final 24 h of a 96-h incubation with cells undergoing mock infection (solid bar) or EZ infection (stippled bar). IL-6 was measured by sandwich enzyme-linked immunosorbent assay. Values are represented as means ± standard deviations. Samples were tested in duplicate.

FIG. 1. Effect of infection with EZ on MHC I protein expression. The levels of MHC I protein expression were measured by flow cytometric analysis in HUVECs infected at an MOI of 1.0 with EZ (17) and harvested at 96 h p.i. (A) Histograms of MHC I induction. Mock, mock infection for 96 h (MHC I shown in black and nonreactive antibody [antimouse immunoglobulin G] shown in gray); EZ, EZ infection for 96 h; Mock + IFN-γ, mock infection for 96 h with 150 U of IFN-γ per ml for the final 24 h; EZ + IFN-γ, EZ infection for 96 h followed by treatment with 150 U of IFN-γ per ml for the final 24 h. The dotted line represents the mean immunofluorescence for MHC I protein in cells mock infected for 96 h. (B) Graphical representation of the relative mean immunofluorescence of MHC I protein from panel A. (C) Relative mean immunofluorescence for MHC I surface protein induction by IFN-α (1,000 U/ml), IFN-γ (150 U/ml), or both, when IFN was present for the final 24 h of a 96-h incubation with cells undergoing mock infection (solid bars) or EZ infection (stippled bars). Con, control. (D) HUVECs were infected for 24 h with measles virus at an MOI of 1.0. MHC I protein levels were detected by flow cytometry, and values are given as relative mean immunofluorescence. The relative mean immunofluorescence is a relative number representing the fluorescence intensity of fluorescein isothiocyanate presented on a linear scale.

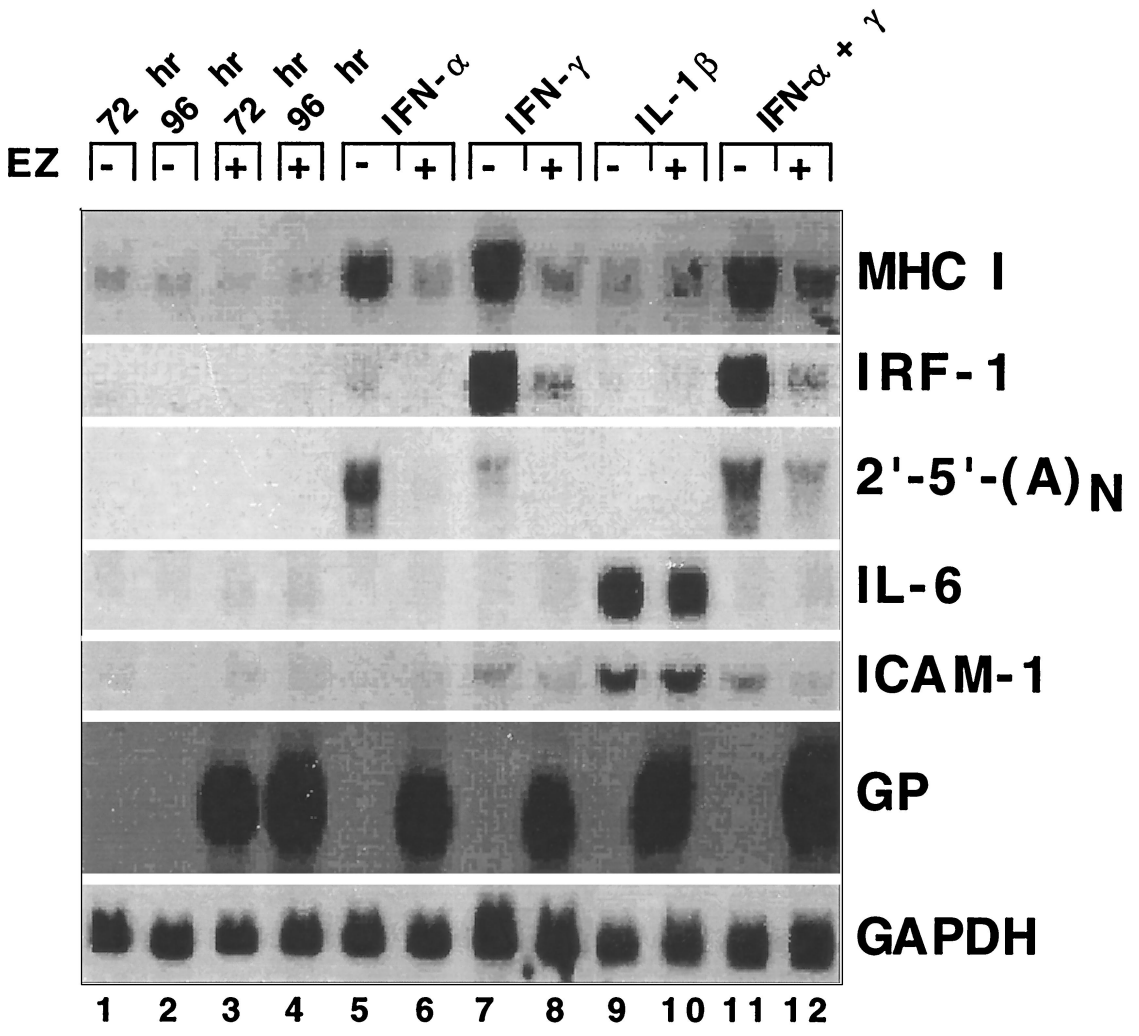
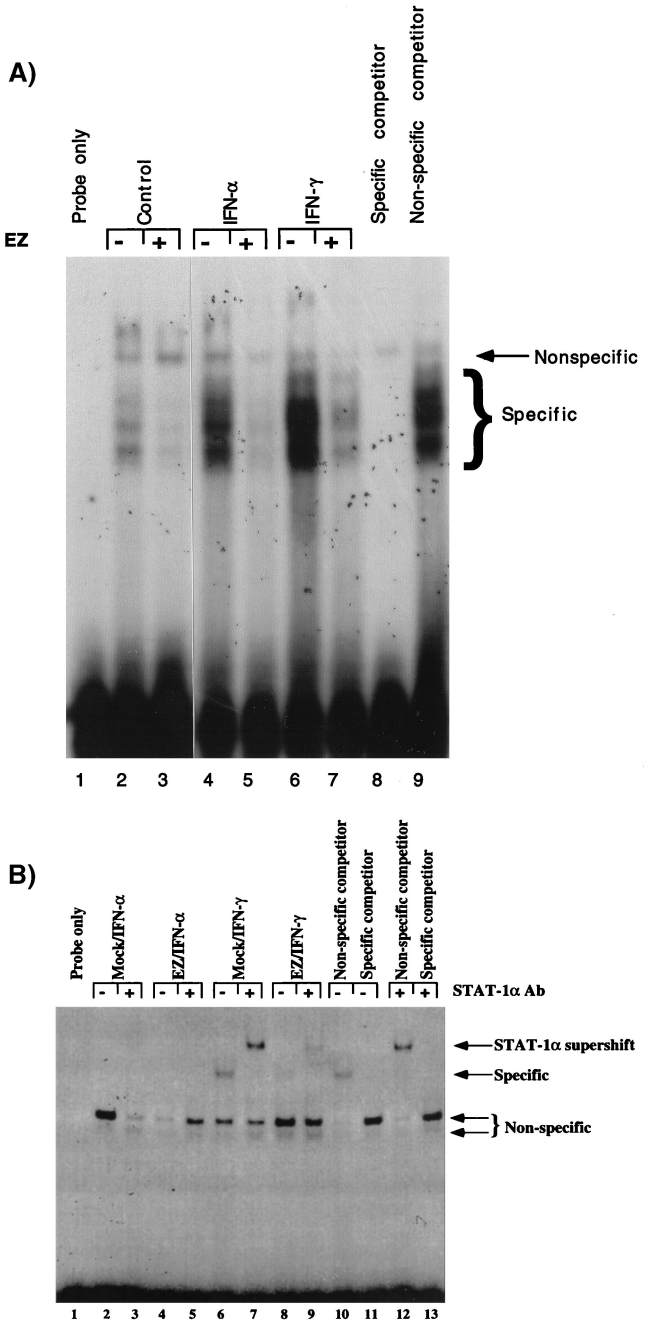


FIG. 3. Effect of infection with EZ on gene induction. HUVECs were either mock infected or EZ infected at an MOI of 1.0 for 72 h prior to treatment with IFN-α (1,000 U/ml), IFN-γ (100 U/ml), IFN-α and -γ simultaneously, or IL-1β (100 U/ml) for 24 h. Total cellular RNA (20 μg) was size fractionated and analyzed by Northern blot analysis (17). All bands coincided with the known size of the mRNA of each gene. The GAPDH probe confirmed similar RNA loading between lanes.

clearly occurs before the formation of nuclear complexes that specifically bind to ISRE or GAS. IFN-α and IFN-γ share several signaling molecules, including Jak-1 and STAT-1α (6, 20). It is possible that EZ infection eliminates or inhibits the function of either Jak-1 or STAT-1α, which would effectively block most, if not all, IFN signal transduction, but other mechanisms of inhibition are also possible. Because IL-1β does not transduce signals through any components of the Jak/STAT pathway (4), an inhibition of this pathway would have no effect on gene induction by IL-1β. The time lag associated with the inhibition of IFN signaling suggests that EZ inhibits these functions through an active process involving either the production of an inhibitory RNA or protein species or the modulation of a cellular gene.

IFN is a mediator of the antiviral response of a cell through its ability to induce the MHC I, PKR, and $2'\text{-}5'(A)_N$ proteins and those coded for by other genes. Because the IFN signaling pathway is ultimately detrimental to virus survival, many DNA and RNA viruses have evolved mechanisms to eliminate either specific IFN-inducible gene function or the IFN signaling pathway itself. Most of the targets for disruption by viruses have been IFN-induced effector proteins. For example, adenovirus, vaccinia virus, poliovirus, influenza virus, reovirus, and human immunodeficiency virus have evolved means of disrupting PKR function (for review, see references 18 and 21). PKR is a serine/threonine protein kinase that upon activation by double-stranded RNA, leads to the inhibition of protein synthesis (21) and is thought to lead to the activation of the transcription factor NF-κB through the phosphorylation of its inhibitor, IκB (22, 26). These activities help contain viral infection. Adenovirus (27) and cytomegalovirus (32) are well-known suppressors of MHC I expression. Suppression of MHC I interferes with the generation of cytotoxic T-lymphocyte responses, because MHC I is a surface protein that presents antigen to $CD8^+$ cytotoxic T cells (15). In addition to disrupting IFN-inducible effector proteins, adenovirus E1A protein also disrupts IFN signal transduction by decreasing the amount of available STAT-1α and p48 (23, 24). Ebola virus can now be added to the list of viruses that disrupt the IFN signaling pathway. Although the site of inhibition has not been determined, it occurs prior to the formation of nuclear complexes that recognize ISRE, GAS, or IRF-E sequences. EZ infection of HUVECs globally affects responses to IFN-α and IFN-γ by



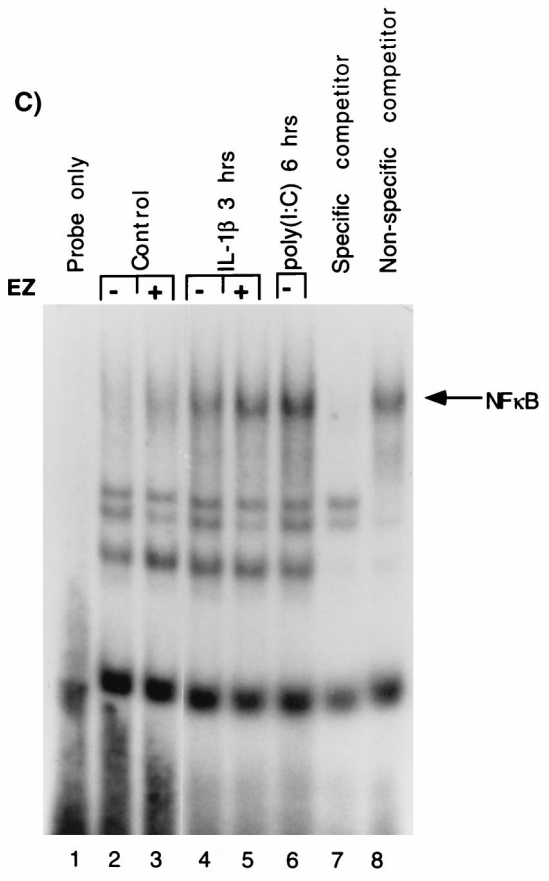


FIG. 4. Effect of EZ infection on binding to the ISRE, GAS, and NF-κB elements. HUVECs were either mock infected or EZ infected at an MOI of 1.0 for 75 h and treated with either IFN-α, IFN-γ, or IL-1β for the final 3 h. Gel shift analysis was performed as previously described (17, 33) with nuclear extracts by using either the ISRE from the ISG54 gene (A), the GAS element from the IRF-1 gene (B), or the NF-κB binding site from the IL-6 promoter (C) as a probe. Both the specific and nonspecific competitor DNAs [IL-6 NF-κB site in panel A, the mouse immunoglobulin kappa light chain gene NF-κB site in panel B, and $(IRF\text{-}E)_2$ in panel C] were added in 30-fold excess and tested by using the mock IFN-α sample in panel A, mock IFN-γ sample in panel B, and mock-infected sample treated with 100 μg of poly(I-C) per ml (Pharmacia Biotech, Piscataway, N.J.) for 6 h in panel C. Supershift analysis (B) was performed with a monoclonal antibody (Ab) to the N-terminal region of STAT-1α. Binding complexes were resolved by 4% nondenaturing polyacrylamide gel electrophoresis and visualized by autoradiography (17, 33).

disrupting induction of IFN-responsive genes, and as a consequence, host antiviral defenses are subverted.

The ability of EZ to replicate and produce progeny virions in endothelial cells while suppressing basal MHC I expression and inhibiting expression of antiviral genes in response to IFNs could play a role in the pathogenesis of disease caused by EZ infection. Because viral infection does not cause the induction of MHC I, the cell is unable to signal the immune system to mount an immune response to EZ infection. In addition, by inhibiting IFN signaling, IFN is not able to upregulate genes, such as those coding for PKR and $2'\text{-}5'\text{-}(A)_N$, that are vital to the antiviral defense of the infected cell. Disruption of these antiviral pathways could contribute significantly to the pathogenesis of disease and to the immunosuppression seen in fatal cases of infection with Ebola virus.

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