Ebola Virus Inactivation with Preservation of Antigenic and Structural Integrity by a Photoinducible Alkylating Agent

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Current methods for inactivating filoviruses are limited to high doses of irradiation or formalin treatment, which may cause structural perturbations that are reflected by poor immunogenicity. In this report, we describe a novel inactivation technique for Zaire Ebola virus (ZEBOV) that uses the photoinduced alkylating probe 1,5-iodonaphthylazide (INA). INA is incorporated into lipid bilayers and, when activated by ultraviolet irradiation, alkylates the proteins therein. INA treatment of ZEBOV resulted in the complete loss of infectivity in cells. Results of electron microscopy and virus-capture assays suggested the preservation of conformational surface epitopes. Challenge with 50,000 pfu of INA-inactivated, mouse-adapted ZEBOV did not cause disease or death in mice. A single vaccination with INA-inactivated ZEBOV (equivalent to $5 \times 10^4$ pfu) protected mice against lethal challenge with 1000 pfu of ZEBOV. INA-inactivated virus induced a protective response in 100% of mice when administered 3 days before challenge. Thus, INA may have significant potential for the development of vaccines and immunotherapeutics for filoviruses and other enveloped viruses.

Subunit and vector-based vaccines have been tested in rodent models of filoviral infection [1–7]. Adenoviral- and live vesicular stomatitis virus (VSV)–based vaccines have been successful in monkeys [1, 8–11]. Although these latter strategies offer an important proof of concept, much remains uncertain about them, including acceptable vaccine dose, the impact of prior vector immunity [12], and safety concerns associated with potential human adaptation of live chimeric VSV. As a vector-free approach, viruslike particles (VLPs) of Ebola virus (EBOV) and Marburg virus have been shown to be efficacious both in rodents [13–15] and in monkeys [16].

A protective immune response to filoviral infection requires a combination of cellular and humoral responses. Thus, an inactivated virus that contains all the viral proteins could be advantageous. Vaccinations with EBOV inactivated by heat, formalin, or irradiation have been mostly unsuccessful [4, 13, 17], which may relate to perturbations in the antigenic epitopes. In the current study, we used the photoinducible alkylating com-
pound 1,5-iodonaphthylazide (INA) to inactivate Zaire EBOV (ZEBOV), as a more conservative inactivating approach. INA is a hydrophobic compound that preferentially partitions into lipid bilayers and that has been used to label membrane-embedded cellular [18, 19] and viral proteins [20, 21]. With far-UV irradiation (310–360 nm), INA alkylates the transmembrane domains of the viral proteins, resulting in their inactivation, while maintaining the integrity of the external domains [22, 23]. In this study, we demonstrated that INA treatment renders ZEBOV completely noninfectious without structural perturbation. INA-inactivated ZEBOV was immunogenic and protected mice from lethal challenge.

MATERIALS AND METHODS

Reagents and cells. Vero E6 cells were cultured in Dulbecco’s MEM supplemented with glutamine and 10% fetal bovine serum (FBS) at 37°C in a humidified CO2 incubator. Anti–EBOV glycoprotein (GP) monoclonal antibodies (MAbs; 13C6-1-1-1, 6D3-1-1, and 6D8) were provided by Mary-Kate Hart (US Army Medical Research Institute of Infectious Diseases [USAMRIID], Fort Detrick) [24]. A recombinant green fluorescent protein–expressing ZEBOV (GFP-ZEBOV) [25] was provided by Jason Paragas (USAMRIID).

ZEBOV. ZEBOV was band purified over a sucrose gradient, as described elsewhere [13, 26]. The virus was propagated and counted by standard plaque assay on Vero cells [27]. ZEBOV-infected cells and animals were handled by qualified personnel in a biosafety level 4 laboratory at USAMRIID.

Treatment of virus with INA. Treatment of virus with INA was done as described elsewhere [23]. In brief, INA (100 μmol/L) was incubated with 2 × 106 pfu/mL EBOV in PBS (1 mL) for 30 min at room temperature. Glutathione (20 mmol/L) was added to neutralize residual INA in the aqueous phase. A 100-W ozone-free mercury arc lamp with a collector lens was used to irradiate the sample with UV light through a 310-nm cutoff filter (to allow transmission of mercury emission bands of 313, 334, and 365 nm) and through a water filter (to prevent heating). The light dose was 10 mW/cm2/min. Irradiation was done for 10 min.

Detection of virus infectivity by use of GFP-ZEBOV. Vero E6 cells were infected with GFP-ZEBOV at an MOI of 5. Infected cells were harvested at various time points, fixed in 10% buffered formalin, and analyzed by flow cytometry using an FL1 channel.

Real-time polymerase chain reaction (PCR) for detection of the EBOV genome. RNA was extracted from test samples by use of the TRIzol LS reagent (Invitrogen), in accordance with the manufacturer’s instructions. Primers specific for ZEBOV GP were synthesized by use of an ABI 394 DNA/RNA synthesizer (ABI Biosystems). Primer sequences were as follows: forward primer, 5′-GCTTCCACAGTTATCTACCGAGG-3′; reverse primer, 5′-CTCTCTCAAGGGTGTTGAC-3′; and probe, FAM-5′-TTTCGTCAGGTTGTTGCA-3′-Tamra. A real-time reverse-transcription (RT) PCR assay was used, combining Superscript reverse transcriptase with Platinum Taq polymerase and a TaqMan probe assay (Invitrogen). The RT and PCR were done in a single tube on the Light Cycler (Roche) as follows: 1 cycle at 50°C for 15 min and 1 cycle at 95°C for 2 min, followed by 45 cycles at 95°C for 10 s and at 60°C for 30 s. Reactions were monitored by recording the fluorescence emission at each PCR cycle. The PCR cycle–threshold method was used for comparison of samples. Standard curves showing plots of cycle-threshold values versus the log of plaque-forming units or genome copy number were obtained by use of plasmid DNA or spiked samples with titered virus at a predetermined value for plaque-forming units.

Virus-capture assay. Anti-GP MAbs (5 μg/well) were immobilized on 96-well plates at 4°C. The wells were blocked with 5% nonfat dried milk in PBS for 3 h. ZEBOV was added and incubated for 2 h at room temperature. The wells were washed 3 times with PBS, and lysis of bound virus was done by addition of Triazole reagent. RNA was prepared from the captured virus and subjected to real-time PCR. The efficiency of virus capture was quantified as percentage captured in comparison with anti-GP 6D8 antibody, which recognizes a linear epitope.

Vaccinations. Female C57BL/6 mice, 6–8 weeks old, were vaccinated by intramuscular injection with different doses of INA-inactivated, mouse-adapted ZEBOV (MA-ZEBOV) [28] once or twice at a 3-week interval. Control mice received PBS. Serum samples were obtained from each mouse on days 0 and 28 after vaccination. Mice were challenged 4 weeks after the second vaccination, by intraperitoneal injection with 1000 pfu (~30,000 LD50) of MA-ZEBOV [28] and were observed at least twice daily for signs of illness. Mice were housed in microisolator cages and were provided autoclaved water and chow ad libitum.

Research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and experiments involving animals and adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals [29]. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, MD).

Antibody titers. Levels of EBOV-specific antibodies were determined by an ELISA using whole inactivated virus as antigen, as described elsewhere [14, 15]. End-point titers were established as the inverse of the last dilution in which the optical density of the sample was at least 0.2 units greater than that in the corresponding control wells (irrelevant antigen).

Determination of T cell response to INA-inactivated ZEBOV, by intracellular interferon (IFN)–γ staining. Two
Figure 1. Inactivation of Ebola virus (EBOV) by photoinduced alkylation. Green fluorescent protein (GFP)-expressing Zaire EBOV was irradiated with UV light with or without 1,5-iodonaphthylazide (INA) treatment and was used to infect Vero E6 cells. After 96 h, flow cytometry was used to analyze cells for GFP expression.

weeks after vaccination of the mice, splenocytes were isolated and cultured at 37°C for 5 h in the presence of 1–5 mg of peptide(s) or phorbol myristate acetate (25 ng/mL) and ionomycin (1.25 µg/mL), in 100 mL of RPMI–Eagle’s Hanks’ amino acids supplemented with 10% FBS, 2 mmol/L glutamine, 10 µg/mL gentamicin, 5 mmol/L HEPES, human interleukin (IL)–2 (10 U/mL; National Cancer Institute), 0.05 mmol/L β-mercaptoethanol, and 10 mg/mL brefeldin A. Cells were blocked with a MAb for FcγRIII/II receptor and were stained with anti-CD44 fluorescein isothiocyanate and either anti-CD8 or anti-CD4 Cy-Chrome (Pharmingen) in staining wash buffer (PBS, 2% FBS, 0.01% NaN₃, and 10 µg/mL brefeldin A). The cells were fixed in 1% formaldehyde, made permeable with staining wash buffer containing 0.5% saponin, and stained with anti–IFN-γ phycoerythrin. Data were acquired by a FACSCalibur flow cytometer and analyzed with CELLQuest (Becton-Dickinson). Samples were considered positive if the percentage of CD8⁺, CD44⁺, and INF-γ-positive cells was ≥2-fold higher than the background percentage. Background was determined by addition of an irrelevant peptide from Lassa virus N (RPLSA-GVYMGNLSSQ) or no peptide, in a solution that contained equivalent amounts of the DMSO used to dilute peptides.

Electron microscopy (EM). Live or INA-inactivated ZEBOV was applied to 300-mesh, nickel EM grids precoated with formvar and carbon, treated with 1% glutaraldehyde in PBS for 10 min, rinsed in distilled water, and negatively stained with 1% uranyl acetate. For immuno-EM, virus was processed as described elsewhere [30]. In brief, samples were applied to grids and immersed for 45 min in dilutions of antibodies. Grids were washed with TRIS buffer and then were incubated for 45 min with gold-conjugated goat anti–mouse IgG, washed in PBS, and fixed in 1% glutaraldehyde. After being rinsed in water, the grids were negatively stained with 1% uranyl acetate. Stained grids were examined with a JEOL 1200 EX transmission electron microscope at 80 kV.

RESULTS

Inactivation of ZEBOV by treatment with INA followed by UV irradiation. To examine whether EBOV could be inactivated by INA, we initially used a recombinant ZEBOV engineered to encode for GFP [25]. GFP-ZEBOV was treated with 100 µmol/L INA for 30 min at 4°C, followed by UV irradiation. As a control, virus was UV irradiated in the absence of INA. These virus samples were used to infect Vero E6 cells, and infected cells were visualized by flow cytometry, at multiple time points. Although UV irradiation alone had no effect on virus infectivity, UV irradiation of INA-treated virus resulted in complete loss of infectivity (figure 1).

Figure 2. Results of photoinduced inactivation of Ebola virus (EBOV), as determined by real-time polymerase chain reaction (PCR). Zaire EBOV was treated for 10 min with UV irradiation alone (control) or was pretreated with 100 µmol/L 1,5-iodonaphthylazide (INA) before UV irradiation. Vero E6 cells were infected at an MOI of 10. Viral replication was monitored by use of a real-time PCR assay. Genome copy nos. are shown from triplicate samples.
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Figure 3. Infectivity in mice challenged with 1,5-iodonaphthylazide (INA)–inactivated Zaire Ebola virus (ZEBOV). Mouse-adapted ZEBOV was treated with INA alone, UV irradiation alone, or with INA followed by UV irradiation, and 1000 pfu of virus was used to infect groups of 10 C57BL/6 mice. Two other control groups were infected with untreated virus diluted in PBS. Survival was monitored over 20 days.

Figure 4. Ebola virus (EBOV) morphology and immunoreactivity of surface glycoprotein (GP) after treatment with 1,5-iodonaphthylazide (INA). A, Electron-microscopy images of live and INA-inactivated, mouse-adapted Zaire EBOV. Results of negative staining (top row) and immunostaining with an anti–EBOV GP MAb specific for a conformational epitope (middle and bottom rows). B, Virus-capture assay with live and INA-inactivated EBOV. Mouse MAbs against EBOV GP (6D3-1-1, 13C6-1-1-1, and 6D8) or control anti-Myc and anti-VP40 (AE11) MAbs were analyzed for efficiency of binding to live or INA-inactivated EBOV. Captured virus was quantified by means of a real-time polymerase chain reaction assay. The efficiency of capture for conformational antibodies is shown as percentage of total virus captured by the linear-epitope antibody 6D8.

To ensure that INA treatment also could inactivate authentic (nonrecombinant) virus, ZEBOV was treated with UV irradiation alone or with INA plus UV irradiation and was used to infect Vero E6 cells. RNA was purified from the supernatants after 24, 48, or 72 h, and viral genome copy numbers were quantified by means of a real-time PCR assay. Although viral replication was evident in the control samples as early as 48 h after infection, INA-inactivated virus did not show any viral replication: the genome copies detected in culture samples remained at the level of the initial input (figure 2).

We then examined the lack of infectivity of INA-inactivated ZEBOV in mice. When mice were infected with 1000 pfu of MA-ZEBOV pretreated with INA plus UV irradiation, all the mice survived with no sign of disease (figure 3). In contrast, virus treated with INA or UV irradiation alone remained lethal (figure 3). A low level of inactivation after INA treatment alone may be related to the ambient UV irradiation present in a room’s light. Clearly, our results demonstrate that INA can effectively abolish EBOV infectivity.

Retention of normal morphology and the antibody reactivity of GP in INA-inactivated ZEBOV EM studies were done to determine whether treatment with INA plus UV irradiation had any gross effect on virus morphology or on the conformational epitopes of GP. As shown in figure 4A, INA-inactivated virus was morphologically indistinguishable from live virus. Staining with the conformational anti–EBOV GP MAb 13C6 [24] showed strong reactivity to both live and INA-inactivated virus (figure 4A).

To further examine the question of preservation of conformational epitopes, the binding of live and inactivated ZEBOV to 2 conformational anti-GP MAbs was examined by use of a virus-capture assay. Different MAbs against EBOV GP or control antibodies AE11 (anti–EBOV VP40) and 9E10 (anti-Myc) were used, and the efficiency of binding was compared to 6D8, a mouse anti-GP MAb that recognizes a linear epitope [24]. The assay was performed as described in Materials and Meth-
Induction of antibody response by vaccination with INA-inactivated ZEBOV. To investigate whether vaccination with INA-inactivated EBOV can confer protective immunity against lethal challenge, groups of mice were vaccinated with 1 or 2 intraperitoneal injections (at a 3-week interval) of a dose of INA-inactivated ZEBOV equivalent to $5 \times 10^4$ pfu. Two weeks after each vaccination, serum samples were collected, and antibody levels were measured by ELISA. A single vaccination resulted in anti-EBOV–specific titers of $\sim 1:300$ (figure 5A). Titers increased significantly after the second vaccination, reaching an average of $\sim 1:3200$ (figure 5A).

Induction of CD8+ T cell–specific epitopes within ZEBOV proteins, by vaccination with INA-inactivated ZEBOV. Protective cellular responses, described elsewhere [31], were detected in both C57BL/6 mice and BALB/c vaccinated mice. To investigate whether INA-inactivated ZEBOV could induce specific T cell responses, a range of peptides derived from ZEBOV proteins were examined by use of IFN-γ intracellular staining, as described in Materials and Methods. C57BL/6 (H-2b) mice vaccinated with INA-inactivated ZEBOV showed a CD8+ T cell response to EBOV GP WIPYFGPAAEGIYTE (GP531) and to nucleoprotein (NP) epitopes VYQVNNLEEIC (NP44) and DAVLHYHMM (NP663) (table 1). The strongest response detected was to the VP35 epitope RNIMYDHL (VP35225). Finally, a response to the VP40 epitope LRIGNQALQLQVL (VP40150) was detected in vaccinated mice. BALB/c mice had detectable CD8+ T cell responses to 2 previously described ZEBOV peptides [31] derived from GP and VP24 (table 1). CD8+ T cell responses to NP peptide SFKAALSSL (NP279) and VP24 epitope PGPAKFSLL also were detected (table 1). Considered together, these data indicate that vaccination with INA-inactivated virus induces cellular immune responses.

Protection against lethal EBOV infection by vaccination with INA-inactivated ZEBOV. Groups of mice were immunized either once or twice, at a 2-week interval, with 50,000 pfu of INA-inactivated MA-ZEBOV. Three weeks after the last immunization, mice were challenged with 1000 pfu of MA-ZEBOV. As shown in figure 5B, vaccination with either 1 or 2 doses of INA-inactivated virus in the absence of adjuvant conferred $>80\%$ protection from lethal challenge.

We had previously demonstrated that treatment of mice with Ebola VLPs but not with irradiated virus conveys protection against lethal infection when administered 1–3 days before challenge [32]. This effect is mediated primarily by activation of NK cells [32]. To examine whether INA-inactivated ZEBOV can induce a protective innate response, mice were injected intraperi-
toneally with 50,000 pfu of INA-inactivated MA-ZEBOV or with PBS as a control and were challenged 3 days later. All the mice treated with INA-inactivated virus survived infection, whereas all the control succumbed to infection (figure 5B).

Antibody titers in these animals also were determined after challenge. Titers after challenge were found to be 1–2 orders of magnitude higher than titers before challenge (figure 5A). The mice vaccinated 1–3 days before challenge also showed a strong antibody response after challenge, suggesting that virus was not entirely cleared by the innate response but that activation of the innate response resulted in a protective adaptive response.

DISCUSSION

The most common approach for inactivating virus is the cross-linking of surface GPs by formaldehyde. Although this approach results in efficient inactivation, covalent cross-linking of surface proteins can severely distort the structure of immunogenic epitopes [33]. Thus, there is a need for inactivation approaches that retain the structural integrity of the virus. Here, we report that EBOV can be safely inactivated by photoinduced alkylation using INA. INA-inactivated virus was noninfectious and retained virus structure and the immunoreactivity of GP. INA-inactivated ZEBOV was highly immunogenic, eliciting both innate and adaptive responses that resulted in protection from lethal challenge. These data suggest that INA-induced inactivation of EBOV and potentially of other enveloped viruses represents a potential attenuation approach for vaccine applications.

Several groups have studied the effect of cross-linking and other denaturing approaches on the immunogenicity of protein antigens. Koch et al. [33] demonstrated that heat denaturation of ovalbumin results in formation of higher-order oligomers. Although cross-linking with formaldehyde did not induce oligomerization, it was shown to be less immunogenic in animals with a much lower epitope density [33]. Several non–cross-reactive antibodies were identified between the native and denatured forms of the antigen [33]. Inactivation of Pasteurella multocida toxin by formalin reduced the immunoreactivity of specific epitopes in vitro and immunogenicity in vivo [34]. Compared with formaldehyde, INA offers clear advantages. First, INA is an inert compound under normal conditions and is activated only when excited by far-UV light [18]. Second, INA partitions rapidly into lipid bilayers, with a partition coefficient of 163,000 [35], and is virtually absent in aqueous medium. In the lipid bilayer, INA can be triggered by UV irradiation, to alkylate the acceptor moieties present therein. Furthermore, any residual INA in the aqueous medium can be inactivated by glutathione. This approach results in preservation of surface epitopes that could be critical for an effective immune response. We had shown previously that 3 broadly neutralizing anti–human immunodeficiency virus (HIV) antibodies were able to bind efficiently to INA-inactivated virus [21]. Similarly, in this study, we showed that the binding of INA-inactivated ZEBOV to several conformational antibodies remained intact. Furthermore, EM studies showed that the virions maintained their normal morphology. These results are particularly intriguing in view of the fact that vaccination with irradiated EBOV in the absence of adjuvant did not protect mice from lethal challenge [13] and that vaccination with irradiated lipid A–encapsulated virus, although protective in mice, was not protective in monkeys [4].

Several studies have revealed residual infectivity in formalin-inactivated viruses [36]. In 1955, many cases of poliomyelitis that resulted from residual infectivity in vaccine preparations were reported [37, 38]. Outbreaks of foot-and-mouth disease in Europe in the 1980s were caused by improperly inactivated virus [36]. A formaldehyde-inactivated Venezuelan equine encephalitis vaccine was suspected to have caused outbreaks in Central America during 1969–1972 [36]. More studies are needed in order to rigorously examine the safety of INA-inactivated viruses. However, the results of our study strongly suggest a high degree of reliability. Although 1 pfu of EBOV is considered to be equivalent to 30 LD50 [28], up to 50,000 pfu of INA-inactivated EBOV was completely noninfectious in mice. This finding suggests that INA treatment may provide an alternative to formalin, with higher levels of safety.

The mechanism of EBOV inactivation by INA treatment remains to be determined. Inhibition of the fusion ability of HIV and simian immunodeficiency virus by INA treatment has been described elsewhere [21, 23]. EBOV GP, like HIV GP, consists of receptor-binding (GP1) and fusion (GP2) subunits.

### Table 1. Zaire Ebola virus (ZEBOV)–specific cellular responses detected after vaccination.

<table>
<thead>
<tr>
<th>Mouse strain, ZEBOV protein</th>
<th>Epitope</th>
<th>Amino acid position</th>
<th>IFN-γ ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c</td>
<td>GP</td>
<td>VSTGTGPAGDFAHK</td>
<td>141–155</td>
</tr>
<tr>
<td></td>
<td>VP24</td>
<td>PGPAKFSSL</td>
<td>214–222</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>GP</td>
<td>WIPYGFGPAEIGYTE</td>
<td>531–545</td>
</tr>
<tr>
<td></td>
<td>NP</td>
<td>VYOVNLEELIC</td>
<td>44–52</td>
</tr>
<tr>
<td></td>
<td>NP</td>
<td>DAVLYYHMM</td>
<td>663–671</td>
</tr>
<tr>
<td></td>
<td>VP24</td>
<td>RNI MYDHL</td>
<td>225–233</td>
</tr>
<tr>
<td></td>
<td>VP40</td>
<td>LRGNOAFLQEOFVLP</td>
<td>150–165</td>
</tr>
</tbody>
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**NOTE.** Mice vaccinated with 1,5-iodonaphthylazide–inactivated ZEBOV were assessed ex vivo for cellular responses to ZEBOV epitopes in glycoprotein (GP), nucleoprotein (NP), VP24, VP30, VP35, and VP40, described elsewhere [31]. Mice received a booster vaccination on day 14. Splenocytes were collected 7 days later. ICC, intracellular cytokine; IFN, interferon.

* Splenocytes were used ex vivo for identification of peptides that induced IFN-γ-expressing CD8+ T cells. Data shown represent intracellular IFN-γ data after 5 h of restimulation with peptide. Data are expressed as the peptide-induced percentage positive/background percentage positive.
GP2 has a structure similar to HIV gp41, consisting of a central triple-stranded coiled coil domain [39, 40]. Fusion appears to be critically dependent on conformational changes in the structure of the fusion domain. Thus, on the basis of results with HIV [21, 23], we hypothesize that INA inhibits EBOV infection by blocking GP fusion activity.

Successful vaccination against EBOV infection requires both cellular and humoral immune responses [41]. Although GP is the primary target for an antibody response, T cell responses can be mounted against all viral proteins. Thus, a vaccine that would contain all the viral proteins could provide a major advantage. In this study, we showed that INA-inactivated virus can induce strong antibody and T cell responses. Specific responses against GP, NP, VP35, VP40, and VP24 were detected. Furthermore, complete protection against challenge at 3 days after vaccination with INA-inactivated EBOV indicates that INA-treated virus is a strong stimulus to the innate immune system. Our previous findings indicated that VLP-induced rapid protection is dependent on NK cells. Whether INA-inactivated EBOV also stimulates NK cells remains to be determined. In summary, this study provides strong evidence that inactivation of enveloped viruses by photoinduced alkylation of the transmembrane domain of GP is an efficient method for inactivation of the virus and may provide a novel approach to the development of a new generation of whole virus–based vaccines.

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