Computational prediction and identification of HLA-A2.1-specific Ebola virus CTL epitopes

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Abstract

Ebola virus (EBOV) is known to cause a severe hemorrhagic fever resulting in high mortality. Although the precise host defense mechanism(s) that afford protection against EBOV is not completely understood, T cell-mediated immune responses is believed to play a pivotal role in controlling virus replication and EBOV infection. There have been no reports on mapping of MHC Class I-binding CTL epitopes for EBOV till date. In this study, we identified five HLA-A2-binding 9-mer peptides of EBOV nucleoprotein (NP) using computer-assisted algorithm. The peptides were synthesized and examined for their ability to bind to MHC class I molecules using a flow cytometry based MHC stabilization assay. Three of the EBOV-NP peptides tested (FLSFASLFL, RLMRTNFLI and KLTEAITAA) stabilized HLA-A2. The ability of the HLA-A2-binding EBOV-NP peptides to generate peptide-specific CTLs was evaluated in HLA-A2.1 transgenic mice. Epitope-specific CTL responses were confirmed by cytotoxic assays against peptide-pulsed target cells and interferon-γ ELISPOT assay. Each of the EBOV-NP peptides induced CTL responses in HLA-A2-transgenic mice. Interestingly, all the three peptides were conserved in three different strains of Ebola (Zaire and Reston and Sudan). Taken together, these findings provide direct evidence for the existence of EBOV-derived NP epitopes that may be useful in the development of protective immunogens for this hemorrhagic virus.

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Introduction

Ebola virus (EBOV) is a member of Filoviridae family of viruses, which cause hemorrhagic disease in human and nonhuman primates (Bray and Paragas, 2002). The filoviruses are perhaps the least understood pathogenic viruses. They emerge unpredictably in central Africa and cause epidemics of severe hemorrhagic fever with high mortality rates (Sanchez et al., 2001; Bray, 2002). The potential use of these viruses as biowarfare agents is of significant concern (Bray and Paragas, 2002). EBOV has four distinct subtypes: Zaire, Sudan, Ivory Coast and Reston (Sanchez et al., 1996). Infection with the Ebola Reston virus can be fatal in monkeys but there are no reported cases of lethal infections in humans. In contrast, infections with Ebola Sudan or Ebola Zaire subtypes are often fatal in humans (Bray and Paragas, 2002).

A significant pathogenic feature of EBOV infection is its ability to severely impair the functional integrity of the immune system. In EBOV-infected individuals, the failure of early activation of T cells and the subsequent impaired humoral responses are followed by extensive apoptosis of blood leukocytes, phenomena that are closely associated with a fatal outcome (Baize et al., 1999).

No effective therapies or vaccines are currently available for filovirus infections. Results of earlier attempts to develop protective EBOV vaccines in guinea pigs and nonhuman primates have been inconsistent (Lupton et al., 1980; Mikhailov et al., 1994). Recent efforts have focused on the use of DNA vaccine methods to stimulate T cell responses. Variable levels of protection were observed in mice and guinea pigs following EBOV
GP immunization (Vanderzanden et al., 1998; Gilligan et al., 1997; Xu et al., 1998; Mellquist-Riemenschneider et al., 2003).

Although both the induction of humoral and cytotoxic T lymphocyte (CTL) responses to Ebola nucleoprotein (NP) and glycoprotein (GP) have been demonstrated in rodents, the relative contribution of these responses to protection remains unclear (Xu et al., 1998; Vanderzanden et al., 1998; Pushko et al., 2000). Induction of CTL responses to an 8-mer GP sequence was observed in mice immunized with irradiated EBOV or irradiated liposome-encapsulated EBOV (Rao et al., 1999). Wilson et al. (2001) demonstrated that the adoptive transfer of NP-specific CTLs protected naive mice from lethal EBOV challenge. However, passive transfer of polyclonal NP-specific antiserum did not protect these mice. These results suggest that cell-mediated effector mechanisms may play a more important role in protection than humoral responses.

Despite the encouraging results of the studies aimed at developing candidate EBOV vaccines, none of the strategies that protected mice or guinea pigs from lethal infection with this virus have been shown to provide similar protection in nonhuman primates (Geisbert et al., 2002). Recently, Sullivan et al. (2003) reported the development of an accelerated vaccine for Ebola. Single immunization with adenoviral (ADV) vectors encoding the Ebola GP and NP was able to induce highly effective protection against lethal challenge in nonhuman primates. This correlated with the generation of Ebola-specific CTL and antibody responses. Warfield et al. (2003) described the production of virus-like particles (VLPs) of Ebola by coexpressing GP and VP40 proteins. Mice vaccinated with Ebola VLPs developed virus-specific antibodies including neutralizing antibodies and these mice were 100% protected against lethal challenge.

Numerous studies have been undertaken to more clearly define the pathogenic mechanisms associated with EBOV infection as well as the determinants of protective immunity (Gupta et al., 2004). Despite these efforts, many questions regarding the pathophysiology and host defense mechanisms involved in EBOV infection remain unanswered. The paucity of information related to the immunogenic T cell epitopes expressed by EBOV limits the ability to examine in vivo T cell responses. To date, knowledge of such EBOV epitopes has been derived from studies in mice. While these investigations provide insights into the genetic markers of EBOV that stimulate murine effector T cell activation in the context of specific MHC haplotypes, the information derived from such studies may not be applicable to human MHC determinants. In the present study, we used a combination of computational prediction methods together with in vitro and in vivo assays to identify three human HLA-A2.1-restricted CTL epitopes for the nucleoprotein of EBOV.

**Results**

**Computational prediction of EBOV-NP CTL epitopes**

The total protein sequences of six structural EBOV proteins (NP, GP, VP24, VP30, VP35 and VP40) were parsed into peptides of nine amino acids in length, each overlapping by eight amino acids as described in Materials and methods. The HLA-A2.1-specific BIMAS algorithm-scoring pattern of the peptides is depicted in Fig. 1. Most of the peptides scored very low (<1) as shown. Thirty-two peptides with scores >50 were identified as predicted binders. Among these, twelve were components of EBOV NP. The highest ranking predicted EBOV NP epitopes, as determined by BIMAS analysis, are presented in Table 1. Based on these ranking scores, five NP peptides with scores >150 were selected for further study.

**Evaluation of synthetic peptides as candidate CTL epitopes**

Synthetic peptides identified as candidate EBOV epitopes in the context of HLA-A2.1 were evaluated for MHC class I binding using MHC stabilization assay. Three out of the five selected EBOV NP-derived peptides (FLS, RLM and KLT) stabilized the expression of HLA-A2.1 on T2 cells as evidenced by increased MFI profiles when compared with the no peptide control (Fig. 2). An increase in MFI values >200% was noted with the FLS and KLT peptides. This increase was similar to that observed using the positive control peptide (GIL). The RLM peptide caused a 90% increase in MFI over no peptide control as shown in Fig. 2. The other two EBOV NP peptides, AMN and YQG, induced 13% and 9% increases in MFI, respectively.

**Immunogenicity of EBOV-NP peptides in vivo**

In order to gain insight into the functional properties of the high-binding peptides, the three selected EBOV NP peptides...
were evaluated for their ability to elicit CTL responses in HLA-A2.1 transgenic mice. Animals were immunized with peptides as described in Materials and methods. Immune responses were assessed using functional assays including, IFN-\(\gamma\) ELISPOT and CTL assay.

Peptide-specific T cells were enumerated by measuring IFN-\(\gamma\)-producing cells by ELISPOT assay. As shown in Fig. 3, a substantial number of T cells harvested from mice immunized with the selected EBOV NP peptides responded by producing IFN-\(\gamma\). The responses observed indicated a dose-dependent relationship between the concentration of peptide used for stimulation and the number of IFN-\(\gamma\)-producing T cells (Fig. 3).

Two of the EBOV NP peptides, RLM and KLT, were found to generate strong peptide-specific T cell responses by virtue of their ability to induce increased frequencies of IFN-\(\gamma\)-producing cells as compared to the other high-binding peptide, FLS. The frequency of IFN-\(\gamma\)-producing cells in the RLM- and KLT-primed splenocyte population was found to be 535 and 338 per one million cells, respectively, when cells were stimulated with 100 \(\mu\)M peptide (Fig. 3). In contrast, the frequency of IFN-\(\gamma\)-producing cells generated in response to FLS at the 100-\(\mu\)M peptide concentration was found to be 155/10^6 cells. This response decreased with decreasing peptide concentrations showing a dose-dependent profile.

Splenocytes from peptide-primed HLA-A2.1 transgenic mice were tested for their cytolytic activity by measuring their ability to serve as effector cells that kill peptide-pulsed T2 target cells expressing HLA-A2.1. As shown in Fig. 4, effector cells generated after 5 days of culture with specific peptides killed peptide-pulsed target cells in a dose-dependent manner. Cells stimulated with the RLM peptide exhibited the highest level of specific lysis (42.41%) at an E:T ratio of 40:1. This was followed by the KLT and FLS peptides, showing specific lysis of 34.51% and 19.87%, respectively, at the 40:1 E:T ratio (Fig. 4).

**Discussion**

Cellular and humoral immunity each play pivotal roles in host defenses against viral pathogens. Previous studies aimed at elucidating immune mechanisms associated with protective
responses against EBOV have demonstrated the critical role of the cellular arm of the immune system. Protective humoral responses to EBOV have also been demonstrated and shown to result from B cell responses to EBOV GP (Jahrling et al., 1996). In contrast, both EBOV GP and NP are known to induce T cell responses (Sullivan et al., 2000; Xu et al., 1998). A greater understanding of the nature of protective immune responses is necessary to facilitate the development of future vaccines against this virus. To date, there have been no reports showing CTL responses induced by EBOV-derived peptides specific for human class I alleles. The present study was undertaken to identify candidate CTL epitopes present in the NP of EBOV. Synthetic EBOV NP peptides with computationally predicted class I-binding motifs were studied for their ability to up-regulate and stabilize HLA-A2.1 molecules expressed by T2 cells.

Computational methods combined with in vitro/in vivo studies have proven to be very useful in the identification of immunogenic T cell epitopes from defined antigens and pathogens (Huang et al., 2004, Turcanova and Hollsberg, 2004; Boesen et al., 2005; Simmons et al., 2004). Many groups have demonstrated the utility of the matrix-based algorithms such as BIMAS and SYFPEITHI in predicting viral and tumor antigen-specific CTL epitopes (Huang et al., 2004; Lu and Celis, 2000; Sun et al., 2000; Turcanova and Hollsberg, 2004; Vonderheide et al., 1999). Using the matrix-based algorithm, BIMAS, we were able to predict five MHC class I-binding NP peptides within the structural proteins of EBOV (Table 1).

To validate the ability of these predicted epitopes to ligate HLA-A2.1, we used a quantitative flow cytometric assay to measure surface induction of class I molecules on TAP-deficient cells. This commonly used method measures the relative binding affinity of different peptides to the same class I allele (Pamer and Cresswell, 1998). Three of the five selected EBOV NP-derived peptides (FLS, RLM and KLT) stabilized the expression of HLA-A2 molecules expressed on T2 cells. T2 cells treated with the other two NP-derived peptides (AMN and YQG) did not show any appreciable class I stabilization. Each of the EBOV peptides capable of binding HLA-A2 on T2 cells contains a primary anchor residue at the 2nd position (L). In the case of nonbinding peptide AMN, the presence of other HLA-A2 anchoring residues, namely, M at the 2nd position and V at the 9th position, did not correlate with any appreciable HLA-A2 stabilization. Therefore, despite the presence of anchor residues known to correlate with HLA-A2 binding, AMN failed to bind to this class I allele. It has been shown that the success rate of computational algorithms in predicting MHC class I binding can range from 30% to 70% depending on the class I allele and peptide motif under study (Feltkamp et al., 1994; Sette et al., 1994). Other factors that contribute to the success or failure of these prediction algorithms to map MHC class I-binding epitopes include peptide length, spacing between required anchoring residues, and the presence of other amino acids which may act as secondary anchor residues (Eberl et al., 1993; Ruppert et al., 1993; Zhou et al., 1992). Combinations of these factors may also influence the ability of peptides to bind MHC class I molecules. Thus, it is difficult to accurately explain the failure of some of the EBOV peptides identified computationally as candidate epitopes to stabilize HLA-A2.1 molecules.

Peptide-induced stabilization of MHC class I molecules is directly correlated with their ability to induce CTL response in vivo (Sette et al., 1994). We therefore tested the ability of HLA-A2.1-binding EBOV peptides FLS, RLM and KLT to stimulate CTL responses in HLA-A2.1 transgenic mice. The moderate-binding peptide RLM was found to induce the strongest CTL responses as compared with the high-binding peptides tested (FLS and KLT). RLM was also markedly more efficient than the high-binding peptides in its ability to induce peptide-specific IFN-γ-secreting cells.

Identification of EBOV epitopes that induce CTL responses in humans previously infected with EBOV will advance our ability to develop vaccine strategies for this pathogen. Gupta et al. (2004) have demonstrated the critical importance of CD8+ T cells in virus clearance. Relatively few studies have addressed the subject of EBOV CTL epitopes focusing on the identification of murine T cell epitopes. Rao et al. (1999) reported the identification of a murine EBOV GP CTL epitope. Studies by Warfield et al. (2003, 2005) using lipid-encapsulated particles containing EBOV GP and matrix viral protein (VP40) have shown that this approach is a highly efficacious method of immunization against EBOV infection. Other studies have used EBOV subunit vaccines to identify the major EBOV determinants capable of conferring immune protection against this pathogen in mice and guinea pigs (Wilson et al., 2001; Olinger et al., 2005). A recent study attempted to map murine CTL epitopes of EBOV-NP using overlapping peptides (Simmons et al., 2004). They identified three EBOV-NP epitopes specific for H-2Aβ as well as H-2Kβ-restricted class I molecules.

Knowledge of the immunogenic motifs that induce EBOV-specific CTL responses will facilitate the development of effective vaccination strategies. Our approach to mapping and
testing selected human MHC class I allele-specific EBOV motifs has confirmed the existence of three epitopes capable of generating peptide-specific CTL responses. To our knowledge, this is the first report describing such EBOV epitopes. Future studies using nonhuman primates to examine these candidate immunogenic epitopes should be undertaken to confirm their ability to stimulate EBOV-specific CTLs capable of protecting animals from challenge with live virus. In addition, studies to explore the utility of these mapped EBOV epitopes as diagnostic tools in monitoring and assessing T cell responses following infection or vaccination will also be useful.

Materials and methods

Computational prediction of candidate CTL epitopes for Ebola

A well-known matrix-based prediction algorithm, BIMAS (http://bimas.dci.nih.gov/molbio/hla_bind/), was used for the prediction of EBOV peptides binding to HLA-A2.1 (Parker et al., 1994). The amino acid sequences of six structural proteins of Ebola Zaire strain (NCBI accession no. NC_002549) were individually tested using these algorithms. The total sequences of each of this protein were parsed into peptides of nine amino acids in length, each overlapping by eight amino acids. The selected peptides were then screened for similarity to the human genome using the NIH Blast server (http://www.ncbi.nlm.nih.gov/blast/). Peptides showing homology with regard to the human proteome were eliminated for further study.

Cell lines and peptides

TAP-deficient T2 cells expressing HLA-A2 were obtained from American Type Culture Collection (Manassas, VA) and maintained in IMDM (Invitrogen, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) at 37 °C with 5% CO2. All peptides used in this study were synthesized commercially (Sigma-Genosys, Woodlands, TX) and documented to be >95% pure. Peptides were dissolved either in water or DMSO and diluted to desired concentrations in phosphate buffered saline (PBS).

Mice

Six- to eight-week-old C57BL/6J transgenic mice expressing HLA-A2.1 [C57BL/6-TgN (HLA-A2.1) 1Enge] were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in our animal facility under specific pathogen-free conditions.

MHC stabilization assay

The ability of peptides to bind to HLA-A2.1 molecules was evaluated using an MHC class I stabilization assay (Nijman et al., 1993). Briefly, T2 cells were grown overnight at 37 °C in IMDM supplemented with 20% FBS. Cells were harvested and washed twice with serum-free AIM-V medium (Invitrogen) and then incubated (5 x 10^7/250 μl) with 50 μM of individual peptides at 37 °C for 18 h in AIM-V medium. Following incubation, cells were washed twice with staining buffer (PBS containing 3% FBS) and stained for 30 min at 4 °C with the FITC-conjugated anti-HLA-A2.1 antibody (Pharmingen, San Diego, CA) at a concentration of 500 ng per reaction in 100 μl of staining buffer. Stained cells were washed twice with staining buffer and resuspended in PBS. HLA-A2.1 expression was measured flow cytometrically using a BD LSRII flow cytometer (BD Biosciences, Mountain View, CA) and the data analyzed using FlowJo software (Tree Star, Ashland, OR). A synthetic EBOV peptide capable of causing a >150% increase in MFI values over the ‘no peptide’ control was considered as a “high binder”. The peptide GILGFVFTL, a well-known HLA-A2.1 epitope derived from influenza matrix antigen, was used as a positive control (Micheletti et al., 2002). The peptide IAGNSAYEY, a known HLA-A2.1 nonbinder, was used as a negative control (Bhasin et al., 2003).

Generation of CTLs in HLA-A2.1 transgenic mice

Eight-week-old HLA-A2.1 transgenic female mice were immunized with 100 μg of individual EBOV test peptides together with 140 μg of an HBV-derived helper epitope (128-140AA). The peptides were emulsified in SEPPIC adjuvant-Montanide ISA 70 (Seppic, France) at a ratio of 1:1 and injected subcutaneously. Mice were boosted once a week following initial immunization and the splenocytes were harvested one week after boosting to assess CTL responses.

Stimulation of CTL effector cells in vitro

Single-cell suspensions were prepared from splenocytes of immunized mice. RBCs were lysed using ACK lysis buffer and the cells were used immediately for ELISPOT assay. Alternatively, cells were cultured for 5 days in RPMI medium supplemented with 10% FBS, 10 mM HEPES and 10 μM T-stim (Pharmding) and 50 μM 2-mercaptoethanol, 10% T-stim (Pharmding) and 10 μM of EBOV peptides. Nonadherent CTLs were harvested at the end of the culture period and used as effector cells in the CTL assay described below.

ELISPOT assay

Peptide-specific T cells were enumerated using ELISPOT assays (Lycke and Coico, 1996). ELISPOT plates (Pharmding) were coated overnight at 4 °C with 5 μg/ml of monoclonal antimouse IFN-γ antibody. After blocking the wells for 2 h with RPMI medium supplemented with 10% FBS, 100 μl of cell suspension (2 x 10^4/ml) with different peptide concentrations (0.01 μM–100 μM) were added to each well. A positive control (5 μg/ml, Con A) and a ‘no peptide’ negative control were included in all assays. The plates were incubated overnight at 37 °C in a 5% CO2 humidified incubator. Following incubation, the wells were washed twice with deionized water and three times with wash buffer (PBS + 0.05% Tween-20). Biotinylated
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temperature. Following three washes, Streptavidin
specific cell lysis was calculated using the following formula:
using an ELISA reader (Bio-Rad, Hercules, CA). The percent-
chromogenic substrate. Absorbance was measured at 495 nm
Fifty microliters of culture supernatants was then harvested
T2 target cells at different effector: target cell ratios ranging
μ2
target cells and were pulsed overnight with individual test
μ2
M). Peptide-stimulated effector cells from
immunized mice were incubated with these peptide-loaded
μ2
spontaneous) / [target maximum – target spontaneous].

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