Cytotoxic T-Lymphocyte Epitopes Fused to Anthrax Toxin Induce Protective Antiviral Immunity

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We have investigated the use of the protective antigen (PA) and lethal factor (LF) components of anthrax toxin as a system for in vivo delivery of cytotoxic T-lymphocyte (CTL) epitopes. During intoxication, PA directs the translocation of LF into the cytoplasm of mammalian cells. Here we demonstrate that antiviral immunity can be induced in BALB/c mice immunized with PA plus a fusion protein containing the N-terminal 255 amino acids of LF (LFn) and an epitope from the nucleoprotein (NP) of lymphocytic choriomeningitis virus. We also demonstrate that BALB/c mice immunized with a single LFn fusion protein containing NP and listeriolysin O protein epitopes in tandem mount a CTL response against both pathogens. Furthermore, we show that NP-specific CTL are primed in both BALB/c and C57BL/6 mice when the mice are immunized with a single fusion containing two epitopes, one presented by LA and one presented by DB. The data presented here demonstrate the versatility of the anthrax toxin delivery system and indicate that this system may be used as a general approach to vaccinate outbred populations against a variety of pathogens.

Cytotoxic T lymphocytes (CTL) are essential for immune clearance of many intracellular pathogens, including most viruses and some bacteria (1, 18). CTL recognize infected cells when peptide epitopes (usually 8 to 10 amino acids) derived from these parasites are presented by molecules encoded by the host class I major histocompatibility complex (MHC-I) (17). The epitopes are derived from cytoplasmic proteins cleaved by the proteasome into small peptide fragments. These peptides are then transported into the lumen of the endoplasmic reticulum, where they complex with newly synthesized MHC-I molecules (17). The MHC-I peptide complex is then transported to the cell surface, where recognition by CTL can occur. After recognition of the foreign peptide, CTL lyse the infected cell, depriving the pathogen of its intracellular niche. Cytokines secreted from activated CTL also play a role in the clearance of pathogens (18). After initial exposure to the pathogen, memory CTL are established that are able to respond more rapidly and efficiently upon subsequent exposure. It is these memory CTL that vaccines are designed to elicit, stimulating the effector cells that provide protective immunity to the host.

Because the generation of memory CTL requires intracellular processing of protein antigens and assembly of peptides onto MHC-I molecules, a vaccine system designed to stimulate CTL should deliver protein antigens to the cytosol of host cells. Previous work from this lab has described an epitope delivery system with a modified nontoxic form of anthrax toxin (7, 8). Anthrax toxin consists of three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF). PA binds to the surface of host cells, where it is cleaved by a furin-like protease into its active form of PA63 (10, 19, 24). LF or EF then bind to PA63, and the protein complex is endocytosed via receptor-mediated endocytosis. After endosomal acidification, PA directs the translocation of either LF or EF into the cytoplasm of host cells where they exert their toxic effects (12, 15). Because anthrax toxin is able to direct the translocation of proteins into the cytoplasm of cells, we have investigated its potential to serve as a vehicle for the priming of specific memory CTL. The strategy involves fusing epitopes to the first 255 amino acids of LF, designated LFn. LFn has the ability to bind PA at the cell surface but completely lacks the LF toxic activity (4, 5). In a previous report, a known epitope of the listeriolysin O protein (LLO) from the intracellularly replicating bacteria, Listeria monocytogenes, was genetically fused to LFn to form the fusion protein LFn-LLO91-99 (7). After immunization with LFn-LLO91-99 mixed with PA, BALB/c mice mounted a protective CTL response against L. monocytogenes (7).

In this report we tested broader applications of the anthrax toxin delivery system. By constructing LFn fusion proteins encoding epitopes from the nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV), we demonstrate that vaccination with this delivery system protects mice against subsequent challenge with LCMV. Furthermore, we show that a single fusion protein can be used to prime a CTL response against both LCMV and L. monocytogenes. We also demonstrate that a single toxin fusion can prime a CTL response against LCMV in more than one mouse haplotype. Finally, we conducted experiments designed to test whether the toxin system can be used to deliver a fusion protein containing a substantial portion of the NP protein to the cytoplasm of host cells. The delivery of large protein antigens will be advantageous in outbred populations, where it is not always known which epitope from a given protein will be presented by a particular MHC-I molecule. The data presented here illustrate the versatility of the anthrax toxin delivery system.
TABLE 1. Fusion proteins used in this study

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Antigen(s)</th>
<th>Epitope(s)</th>
<th>Restricting molecule(s)</th>
<th>Mouse strain tested</th>
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</thead>
<tbody>
<tr>
<td>LFn-NP 118-126</td>
<td>NP from LCMV</td>
<td>NP 118-126</td>
<td>Ld</td>
<td>BALB/c</td>
</tr>
<tr>
<td>LFn-NP 396-404</td>
<td>NP from LCMV</td>
<td>NP 396-404</td>
<td>Dh</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>LFn-LLO 91-99-NP 118-126</td>
<td>LLO from L. monocytogenes</td>
<td>LLO 91-99</td>
<td>Kd</td>
<td>BALB/c</td>
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<tr>
<td>LFn-NP 118-126-NP 396-404</td>
<td>NP from LCMV</td>
<td>NP 118-126</td>
<td>Ld</td>
<td>BALB/c</td>
</tr>
<tr>
<td>LFn-NP 118-126-NP 396-404</td>
<td>NP from LCMV</td>
<td>NP 396-404</td>
<td>Dh</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS

Peptides. Synthetic peptides LLO94-99 (GKYDQNEYI), NP 396-404 (FQFQONGQHI), and NP 118-126 (RPOAVGQYIM) were purchased from Biosearch Technologies (Novato, Calif.).

Animals and cell culture. BALB/c (H-2d) and C57Bl/6 (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). All mice were females between 8 and 12 weeks of age. Mouse thymoma EL-4 (H-2d) and mouse mastocytoma P815 (H-2b) cell lines were maintained at 37°C in 5% CO2 in a medium (RP-10) containing RPMI 1640 and 10% fetal bovine serum as previously described (34). The BALB/c clone 7 (H-2d) fibroblast cell line was maintained at 37°C and 7% CO2 in a medium containing Eagle minimal essential medium supplemented with antibiotics and 10% fetal bovine serum.

Construction of LFn fusion proteins. Five fusion proteins were used in this study: LFn-NP 118-126, LFn-NP 396-404, LFn-LLO 91-99-NP 118-126, LFn-NP 118-126, and LFn-NP 396-404. The construction of LFn-NP 118-126 and LFn-NP 396-404 was performed as described (7). Briefly, an upstream primer homologous to the 5′ end of LF and containing additional sequence encoding an NdeI site was used in combination with a second primer containing sequence homologous to the 3′ end of LFn that contained additional nonhomologous sequence encoding the nine-amino-acid epitope (NP 118-126), two stop codons, and a BamHI site. These primers were used to amplify the fusion sequence by PCR, with the toxin-encoding plasmid from Bacillus anthracis, pXO1, serving as template. The amplified PCR product was cloned into pET15b as described above.

Purification of LFn-NP 118-404. Purification of LFn-NP 118-404 was performed differently in that it required the isolation of inclusion bodies as specified by the manufacturer (Novagen). For inclusion body purification, urea was included in all of the buffers at a concentration of 6 M. After elution of the bound protein (0.5 M imidazole, 6 M urea), the protein was dialyzed against 20 mM Tris (pH 8).

Wild-type PA was isolated from supernatant cultures of an attenuated strain of B. anthracis as previously described (21).

Stimulation of peptide-specific CTL. Mouse splenocytes were harvested, and CTL cultures were established as described earlier (34). Briefly, spleen cells from experimental mice were incubated with and washed once in RP-10. Naive, irradiated (2,000 rads), 2-week-old C57Bl/6 mice were immunized with 10 μg of the cognate peptide, washed with RP-10, and used as stimulator cells. Cultures included one-half of the total splenocytes from the experimental animal and an equivalent number of stimulator splenocytes in a total volume of 20 ml of RP-10. Cultures were incubated upright in a T-25 flask at 37°C in 5% CO2.

CTL assay. EL-4 (H-2d) or P815 (H-2b) target cells were incubated with a 1 μM concentration of the peptide to be tested and 100 μCi of sodium [3H]chiorpromate (NE-101, Boston, Mass.) for 1 h at 37°C. Control cells without peptide were also incubated in 4°C. The cells were washed three times to remove bound peptide and extracellular radionuclide. Ten thousand radiolabeled cells, either treated with peptide or left untreated (negative control), were then added to effector cell dilutions in a 96-well assay plate. The highest dilution represents one-sixth of the stimulated culture or approximately one-twelfth of the total splenocytes from the experimental mouse. The total volume in each well was 200 μl. Spontaneous and maximum lysis of target cells was determined by incubating target cells with either RP-10 or 1% Triton X-100, respectively. After 4 h of incubation at 37°C and 7% CO2, the plates were centrifuged at 1,000 rpm, and 100 μl of the supernatant was counted on a Wallac 1470 gamma counter to determine the release of 3H. The percent specific lysis was determined as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Ex vivo CTL assay. Ex vivo CTL assays were essentially as described above with the following modifications. BALB/c clone 7 (H-2d) target cells were incubated with a 100 nM solution of NP 118-126 peptide and labeled with 10 μCi of sodium [3H]chiorpromate (NE-101, Boston, Mass.) for 1 h at 37°C. Control cells without peptide were also incubated in 4°C. The cells were washed three times to remove bound peptide and extracellular radionuclide. Ten thousand radiolabeled cells, either treated with peptide or left untreated (negative control), were then added to effector cell dilutions in a 96-well assay plate. The highest dilution represents one-sixth of the stimulated culture or approximately one-twelfth of the total splenocytes from the experimental mouse. The total volume in each well was 200 μl. Spontaneous and maximum lysis of target cells was determined by incubating target cells with either RP-10 or 1% Triton X-100, respectively. After 4 h of incubation at 37°C and 7% CO2, the plates were centrifuged at 1,000 rpm, and 100 μl of the supernatant was counted on a Wallac 1470 gamma counter to determine the release of 3H. The percent specific lysis was determined as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

RESULTS

CTL are primed after immunization with anthrax toxin fusion proteins. To determine if the anthrax toxin system could be used to stimulate CTL specific for two different pathogens, LCMV and L. monocytogenes, we constructed a series of plasmids encoding fusion proteins between the first 255 amino acids of LF (designated LFn) and the specific CTL epitopes shown in Table 1. The fusion proteins are described in the Materials and Methods section.

Control groups of mice were injected with the LFn fusion proteins in the absence of PA. Two weeks postimmunization the mice were sacrificed and splenocytes were stimulated on irradiated syngeneic spleen cells which had been previously incubated with the cognate peptide. After 5 days of in vitro
stimulation, the cultures were assayed for the ability to lyse peptide-coated P815 (H-2d) or EL-4 (H-2b) cells in a chromium release assay.

As shown in Fig. 1, cultures from animals immunized with each of the fusion proteins containing either single or double nine-amino-acid epitopes were able to lyse peptide-coated P815 or EL-4 cells to a significantly greater extent than cells not treated with peptide, thus demonstrating that epitope-specific CTL had been primed. No specific lysis was seen in cultures from mice immunized with these fusion proteins in the absence of PA (data not shown). Cultures from animals immunized with LFn-NP118–404 were able to lyse peptide-coated P815 or EL-4 cells to a significantly greater extent than cells not treated with peptide, demonstrating that this longer fusion also was able to prime epitope-specific CTL (Fig. 2). Interestingly, cultures from either BALB/c or C57BL/6 mice immunized with LFn-NP118–404 in the absence of PA demonstrated NP-specific CTL activity (see Discussion).

BALB/c mice immunized with LFn-LLO91–99-NP118–126 mounted a CTL response against both LCMV and L. monocytogenes (Fig. 1C), demonstrating that a single fusion protein could prime CTL against two different pathogens. Also shown are data demonstrating that a single fusion protein (LFn-NP118–126-NP396–404 or LFn-NP118–404) could prime an NP-specific response in two different haplotypes of mice (Fig. 1D, Fig. 2). These data suggest that the anthrax toxin system could be used in outbred populations such as humans by fusing peptides which bind to different MHC-I molecules to a single LFn molecule in series.

Immunization with anthrax toxin fusion proteins results in enhanced recall response and protective immunity against LCMV challenge. Previous work from this lab has demonstrated that mice immunized with PA plus LFn-LLO91–99 were partially protected against a subsequent challenge with L. monocytogenes (7). Although CTL are important effectors of immunity to L. monocytogenes, they are not the only cell type involved in the resolution of either primary or secondary infections (26). In contrast, CD8+ T cells are the dominant effector population involved in the resolution of infection and subsequent immunity to LCMV (13). There has also been extensive characterization of the immunodominant CTL epitopes presented during LCMV infection (9, 25, 37). We
therefore examined the protective capacity of anthrax toxin fusions containing LCMV epitopes. Immunization with an LFn fusion protein encompassing the immunodominant epitope from the nucleoprotein of LCMV, NP118–126, was investigated. This epitope is presented by the Ld MHC-I molecule (30, 37).

In order to determine whether protective immunity against LCMV could be generated in mice immunized with LFn fusion proteins containing NP118–126, groups of BALB/c mice (five per group) were immunized with 1 μg of the LFn fusion proteins listed in Table 1 mixed with 2.7 μg of PA. At 8 weeks postimmunization the mice were challenged i.p. with $1.8 \times 10^6$ PFU of LCMV Armstrong. At 93 h postchallenge, the mice were sacrificed and the viral titers in the spleens and lungs were determined. As shown in Fig. 3, mice immunized with each of the fusion proteins containing NP118–126, showed protection after challenge with LCMV, as demonstrated by the lower viral titers in the organs of vaccinated animals compared to the control animals. Control BALB/c mice immunized with the haplotype-mismatched LFn-NP396–404 fusion protein were not protected against LCMV challenge. These data demonstrate that NP118–126-specific CTL were primed in vaccinated animals and functioned in a protective capacity.

In order to further characterize the protective capacity of CTL primed with the LFn-NP118–126 fusion protein, we challenged immunized BALB/c mice with a variant of LCMV Armstrong, designated clone 13. Clone 13 suppresses the CTL response in immunocompetent naive mice and thereby causes a persistent infection, compared to the acute, rapidly cleared infection caused by LCMV Armstrong (28). Mice immune to LCMV however, are able to mount a vigorous secondary CTL response and control infection of clone 13. The ability to control infection of clone 13 therefore serves as a indicator of memory CTL (32). Groups of BALB/c mice (four per group) were immunized i.p. with 1 μg of LFn-NP118–126 mixed with 2.7 μg of PA. At 7 weeks postimmunization the mice were challenged intravenously (i.v.) with $2 \times 10^6$ PFU of clone 13. Control groups of BALB/c mice were immunized with 1 μg of the haplotype-mismatched fusion protein LFn-NP396–404 mixed with 2.7 μg of PA. At days 7 and 14 postchallenge, serum was collected from the mice, and viral titers were measured. As shown in Fig. 4, mice immunized with LFn-NP118–126 had significantly lower viral titers of LCMV compared to control animals. At 14 days after infection five of seven immunized mice had no detectable infectious virus. These data provide...
We demonstrate that protective immunity against a viral pathogen, LCMV, is generated in BALB/c mice immunized with LFf fusion proteins encoding the immunodominant LFn-\(H-2d\) protein can prime a CTL response against two different pathogens, LCMV and L. monocytogenes. BALB/c mice immunized with LFf-LLO91–99-NP118–126 plus PA mounted a specific CTL response against both the LLO and NP epitopes. Cultures of spleen cells from immunized mice were able to lyse peptide-coated cells to a significantly greater extent than untreated controls. This indicates that the LFf fusion protein can successfully prime specific memory CTL that are capable of protecting mice against subsequent LCMV challenge.

Another LCMV model of infection utilizes an isolate of Armstrong designated clone 13. Clone 13 suppresses the CTL response in naive, immunocompetent mice, and infection with this isolate results in a chronic lifelong LCMV infection (2, 23). In mice where LCMV-specific memory CTL have been primed, there is an effective secondary CTL response which is determined predominantly by the ability of the host to mount a rapid and effective CTL response, it serves as an excellent model for testing experimental vaccines designed to elicit CTL responses (37). BALB/c mice immunized with various anthrax toxin fusion proteins containing the LFf-NP118–126 epitope showed protection against challenge with LCMV Armstrong as demonstrated by reduced viral titers in the lungs and spleens compared to control animals. In addition, BALB/c mice immunized with PA plus LFf-NP118–126 showed an enhanced recall response to LFf-NP118–126 as demonstrated by an ex vivo CTL assay 3 days after challenge with LCMV Armstrong.

**DISCUSSION**

Vaccines designed to stimulate CTL should be able to deliver protein antigens to the cytoplasm of host cells for processing and presentation by MHC-I. Many systems have been exploited to accomplish cytoplasmic delivery, including viral and bacterial vectors and DNA vaccines (7, 8, 14, 22, 27, 33, 36). There have also been reports demonstrating that antiviral recognition by CTL is not mediated by the presence of the antigen in the cytoplasm of the cell. Instead, the data demonstrate that the recognition of the antigen by CTL is mediated by the presence of the antigen in the cytoplasm of the cell. This report describes the use of a nontoxic, truncated form of anthrax toxin as an epitope delivery system without the use of adjuvant.

We demonstrate that protective immunity against a viral pathogen, LCMV, is generated in BALB/c mice immunized with LFf fusion proteins encoding the immunodominant LFf-NP118–126 epitope. Because the outcome of LCMV infection is determined predominantly by the ability of the host to mount a rapid and effective CTL response, it serves as an excellent model for testing experimental vaccines designed to elicit CTL responses (37). BALB/c mice immunized with various anthrax toxin fusion proteins containing the LFf-NP118–126 epitope showed protection against challenge with LCMV Armstrong as demonstrated by reduced viral titers in the lungs and spleens compared to control animals. In addition, BALB/c mice immunized with PA plus LFf-NP118–126 showed an enhanced recall response to LFf-NP118–126 as demonstrated by an ex vivo CTL assay 3 days after challenge with LCMV Armstrong.

Another LCMV model of infection utilizes an isolate of Armstrong designated clone 13. Clone 13 suppresses the CTL response in naive, immunocompetent mice, and infection with this isolate results in a chronic lifelong LCMV infection (2, 23). In mice where LCMV-specific memory CTL have been primed, there is an effective secondary CTL response which is capable of clearing clone 13 infection and preventing the establishment of a persistent infection. In the experiments presented here, the majority of BALB/c mice immunized with PA plus LFf-NP118–126 were able to clear an LCMV clone 13 infection by day 14 postchallenge, while control mice had established a persistent infection with average serum titers of \(10^5\) PFU/ml at the same time point. These data demonstrate that immunization with the anthrax toxin delivery system successfully primes specific memory CTL that are capable of protecting mice against subsequent LCMV challenge.

We also showed that immunization with a single LFf fusion protein can prime a CTL response against two different pathogens, LCMV and L. monocytogenes. BALB/c mice immunized with LFf-LLO91–99-NP118–126 plus PA mounted a specific CTL response against both the LLO and NP epitopes. Cultures of spleen cells from immunized mice were able to lyse peptide-coated cells to a significantly greater extent than untreated controls.
macrophage cells are internalizing particulate LFn-NP118–404. It is possible that these specialized latex beads are presented by MHC-I on certain specialized MHC-II molecules, it has been previously reported that endogenous antigen taken up by cells is processed and presented by highly represented MHC-I molecules could be linked in tandem to LFn and used to vaccinate human populations.

The data presented in this study demonstrate that multiple epitopes from the same protein that bind to different MHC-I molecules, or epitopes derived from different pathogens, can be linked in tandem and used to vaccinate mice. Although we have demonstrated the ability to link multiple epitopes to our delivery system, sequential immunizations may be necessary to protect against the wide variety of pathogens encountered. The use of a protein-based vaccine raises the possibility that the host will generate an antibody response against the delivery system itself, preventing the use of the system for subsequent immunizations. In fact, PA is known to generate an antibody response that is capable of preventing the intoxication of animals after exposure to B. anthracis. However, our previous work has demonstrated that immunization with the small amounts of protein used in these experiments does not prevent the use of this system for subsequent vaccination. In the same report we also show that no antibody response against the toxoid molecule was detected in mice immunized with this system (6). Taken together, these data suggest that the anthrax delivery system may be versatile enough to induce protective immunity against multiple pathogens in the human population.

Finally, we investigated whether anthrax toxoid can deliver larger protein antigens to the cytoplasm of host cells. Since different peptide epitopes from a given protein will be presented on MHC-I from an outbred population, cytoplasmic delivery of larger antigenic proteins is ideal for candidate vaccines designed to prime CTL. Although we report the ability of LFn-NP118–404 to prime a protective CTL response in immunized mice, priming of CTL was not PA dependent. A few explanations exist for this observation. One possibility is that the LFn-NP118–404 fusion protein is capable of entering cells in a PA-independent manner, although this explanation seems unlikely since neither LFn or NP has been reported to enter cells in the absence of PA. Another possibility is that LFn-NP118–404 is being alternatively processed. Although most exogenous antigen taken up by cells is processed and presented by MHC-II molecules, it has been previously reported that peptides from antigens coupled to particulate structures such as latex beads are presented by MHC-I on certain specialized macrophage cells (16, 20). It is possible that these specialized macrophage cells are internalizing particulate LFn-NP118–404 protein and presenting peptides from this protein in the context of MHC-I. In mice vaccinated in the presence of PA it is likely that both intracytoplasmic processing of peptide fragments and alternative processing by certain macrophage cells is resulting in the priming of CTL. Experiments are currently underway to determine the mechanism by which LFn-NP118–404 primes a CTL response in the absence of PA.

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REFERENCES


