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Anthrax toxin-mediated delivery of a cytotoxic T-cell epitope *in vivo*

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ABSTRACT **The protective antigen (PA) component of anthrax toxin mediates entry of the toxin's lethal factor (LF) and edema factor into the cytosolic compartment of mammalian cells. The amino-terminal domain of LF (LFn; 255 amino acids) binds LF to PA, and when fused to heterologous proteins, the LFn domain delivers such proteins to the cytoplasm in the presence of PA. In the current study, we fused a 9-amino acid cytotoxic T-lymphocyte (CTL) epitope (LLO_{91-99}) from an intracellular pathogen, *Listeria monocytogenes*, to LFn and measured the ability of the resulting LFn–LLO_{91-99} fusion protein to stimulate a CTL response against the epitope in BALB/c mice. As little as 300 fmol of fusion could stimulate a response. The stimulation was PA-dependent and occurred with the peptide fused to either the amino terminus or the carboxyl terminus of LFn. Upon challenge with *L. monocytogenes*, mice previously injected with LFn–LLO_{91-99} and PA showed a reduction of colony-forming units in spleen and liver, relative to nonimmunized control mice. These results indicate that anthrax toxin may be useful as a CTL-peptide delivery system for research and medical applications.**

All viruses and some bacterial and protozoan pathogens have evolved the ability to survive and replicate within mammalian cells. Immune recognition of these cytoplasmic pathogens depends upon the cell surface display of peptide antigens derived from pathogen-associated proteins. These peptides are presented in complexes with host class I molecules encoded by the major histocompatibility complex (MHC-I), and cytotoxic T lymphocytes (CTL) are activated following recognition of the foreign peptide in complex with MHC-I (1). Activated CTL lyse the infected cell, secrete cytokines, and then proliferate and differentiate (2, 3). Each of these steps plays an important role in clearing the host of the pathogen. Lysis of the target cell deprives the organism of its replicative niche and exposes the pathogen to elements of the humoral immune system. Secretion of cytokines has many effects, including enhancement of local immune responses. Proliferation of the CTL clone results in expansion of a set of reactive CTL to effect clearance of the pathogen from other infected cells, whereas differentiation provides a set of long-lived memory cells available to respond more quickly and effectively to subsequent challenge. Vaccines that prime these memory cells provide protection of the host upon reexposure to the pathogen.

For a vaccine to mimic infection by cytoplasmic pathogens, it must introduce the target antigen(s) into the cytosol of host cells *in vivo*. This has been accomplished by expressing heterologous antigens by live viral or bacterial vectors, by using adjuvants, or by delivering DNA expression vectors (DNA vaccines) (4–7). Herein we describe a strategy for introducing CTL epitopes into the cytosol of host cells, *in vivo*, using delivery components of an intracellularly acting toxin, anthrax toxin.

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Anthrax toxin is composed of three proteins that act in binary combinations to elicit two toxic effects, lethality and edema (8). Lethal factor (LF) and edema factor (EF) are intracellularly acting proteins, both of which require protective antigen (PA) for their translocation to the cytosol of eukaryotic cells. Initially, LF and EF bind competitively to proteolytically activated PA (PA_{63}) at the cell surface. The protein complex is endocytosed, and LF and EF are translocated to the cytosol following endosomal acidification. There, EF expresses its adenylate cyclase activity, elevating cAMP levels, and LF expresses its as yet undefined activity, inducing lethal overproduction of certain cytokines in its target cells, macrophages (9, 10).

The amino-terminal domain of LF (LFn; 255 residues) has been found to contain the information necessary for binding to PA and mediating translocation. The domain alone lacks lethal potential that depends on the putatively enzymatic carboxyl-terminal moiety (11). Recent results have demonstrated that genetically fusing LFn to heterologous polypeptides enables them to be delivered to the cytosol of cultured mammalian cells in the presence of PA (Fig. 1*A*) (12–14). These findings suggested that CTL epitopes fused to LFn might be delivered to the cytosol and generate a CTL response *in vivo*. Fig. 1*B* shows a hypothetical model of how this might occur. Once in the cytosol, the fusion protein would be processed by the proteasome to peptides, which are transported into the endoplasmic reticulum and complexed with MHC-I. After transport to the cell surface, the peptide–MHC-I complex would be accessible to stimulate specific CTL.

To test the potential of the anthrax toxin system to mediate presentation of peptides in complex with MHC-I, we chose to fuse LFn to a CTL epitope from *Listeria monocytogenes*. *L. monocytogenes* is a facultative intracellular bacterial pathogen that survives within the cytosol of macrophages. After its entry into the cell by phagocytosis, the bacterium produces listeriolysin O (LLO), which lyses the phagosomal membrane and allows the bacterium to escape to the cytosol. LLO within the cytosol is proteolytically processed, generating short peptides, some of which are presented at the cell surface in complex with MHC-I. Processing of LLO results in the presentation of a nonameric peptide, LLO_{91-99} (GYKDGNEYI), recognized by H-2 K^d-restricted CTLs (15). Adoptive transfer studies have shown that CTLs specific for LLO_{91-99} are sufficient for protection against *L. monocytogenes*, suggesting LLO_{91-99} as a vaccine candidate (16). Herein we report that fusion proteins of LFn and LLO_{91-99}, when mixed with PA, stimulate LLO_{91-99}-specific CTL *in vivo* and partially protect mice against *L. monocytogenes*.

MATERIALS AND METHODS

Peptide Synthesis. The LLO_{91-99} peptide GYKDGNEYI was synthesized on an Applied Biosystems model 432A peptide synthesizer.

Abbreviations: PA, protective antigen; LF, lethal factor; LFn, amino-terminal fragment of LF; CTL, cytotoxic T lymphocyte(s); LLO, listeriolysin O; MHC-I, class one molecule encoded by the major histocompatibility complex; EF, edema factor; cfu, colony-forming unit(s).

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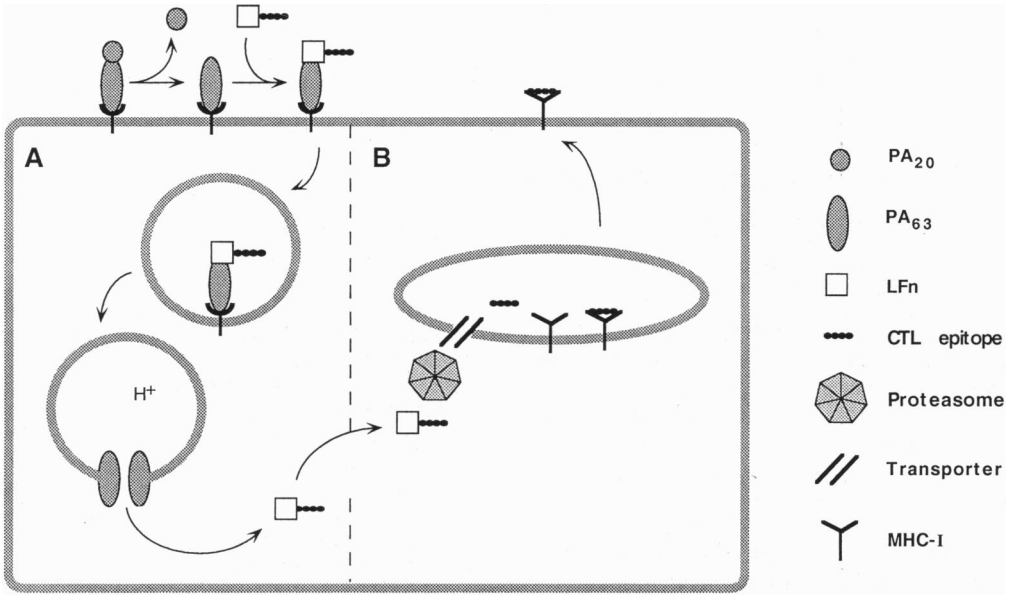

FIG. 1. Proposed steps by which a heterologous CTL peptide epitope fused to LFn is delivered to the cytosol by PA, processed to peptide form, and delivered to the cell surface in complex with MHC-I molecules. (*A*) Cytosolic delivery of the LFn–peptide by PA. PA binds to its cell surface receptor, and a furin-like protease removes a 20-kDa fragment from its amino terminus, leaving PA_{63} bound to the receptor. LFn with its fused 9-residue CTL epitope then binds to PA_{63}, and the complex then enters the cell by receptor-mediated endocytosis and is delivered to the endosome. PA_{63} oligomerizes, inserts into the endosomal membrane, and forms a channel under the influence of low endosomal pH, and the LFn–peptide is translocated to the cytosol. (*B*) Processing and presentation of CTL- reactive peptide. The LFn–peptide within the cytosol is processed into small peptides by the multicatalytic proteasome, and the peptides are transported by specific transporters into the endoplasmic reticulum, where the peptide binds to newly synthesized MHC-I molecules. These complexes then traverse the Golgi and the trans-Golgi network and are presented at the cell surface, where recognition by CTL occurs.

Construction, Expression, and Purification of Fusion Proteins. DNA fragments encoding the LFn–LLO_{91-99} and LLO_{91-99}–LFn fusion proteins were constructed using PCR. LLO_{91-99}–LFn was amplified using an upstream primer that included (5′ to 3′) an *Nde*I site, an ATG start codon, sequence encoding the LLO_{91-99} epitope, and sequence homologous to the 5′ region (3′ to the LF secretory sequence) of the LF gene. The downstream primer was homologous to the sequence encoding the last 6 amino acids of LFn and included (downstream of the homology) sequence encoding stop codons and a *Bam*HI site. LFn–LLO_{91-99} was amplified using an upstream primer that included (5′ to 3′) an *Nde*I site and sequence homologous to the 5′ region of the LF gene. The downstream primer was homologous to the sequence encoding the last 6 amino acids of LFn and included (downstream of the homology) sequence encoding the LLO_{91-99} epitope, sequence encoding stop codons, and a *Bam*HI site. pXO1, the toxin-encoding plasmid from *Bacillus anthracis* Sterne strain, was used as template. The *Nde*I–*Bam*HI fragment was ligated into compatible sites within the multiple cloning region of pET15b (Novagen) and used to transform *E. coli* XL1-Blue (Stratagene). For each clone, the plasmid DNA was amplified, purified, and screened for the appropriate insert by restriction analysis. Clones containing inserts were locally sequenced to confirm the fusion was correct. These clones were then used to transform *Escherichia coli* BL21(DE3) (17) for expression of the fusion protein.

Recombinant proteins expressed in pET15b contain a His_6 tag at the amino terminus of the protein. This tag allows for a one-step affinity purification of the expressed protein using a Ni^{2+} charged column. Cultures of BL-21/pET15b(LFn–LLO_{91-99}) or BL-21/pET15b(LLO_{91-99}–LFn) were grown in Luria broth containing ampicillin (50 μg/ml) to an OD_{600} of 0.6–0.8 unit and protein expression was induced by addition of 1 mM isopropyl β-D-thiogalactoside for approximately 3 h. Cells were then pelleted and disrupted by sonication. The sonicate was centrifuged, and the supernatant was passed over an equilibrated Ni^{2+} charged column. The bound fusion protein was removed with 0.5 M imidazole according to the manufacturer's instructions (Novagen). The eluted protein was then equilibrated in 20 mM Tris·HCl (pH 7.5). The protein concentration was determined and the sample was frozen at −20°C.

PA was isolated from supernatant cultures of an attenuated strain of *B. anthracis* according to an established protocol (18).

Stimulation of LLO_{91-99}-Specific CTL. All mice used in this study were female BALB/cByJ (The Jackson Laboratory), H-2^d, between 8 and 12 weeks of age. Mouse splenocytes were harvested and CTL-stimulated as described (19), with the following modifications. Spleen cells from immunized and control mice were isolated and washed once in RP-10. Cells used as stimulators were naive irradiated (2000 rad; 1 rad = 0.01 Gy), syngeneic splenocytes treated with 10 μM sterile LLO_{91-99} peptide. The stimulator cells were incubated 1 h in the presence of peptide and then washed once in RP-10. Cultures contained 3×10^7 stimulator cells and 3×10^7 splenocytes from either immunized or control mice. These were incubated upright in a T-75 flask at 37°C in 7% CO_2 in a total volume of 20 ml of RP-10.

CTL Assay. For target cells, mouse mastocytoma P815 (H-2^d) cells were incubated with 10 μM LLO_{91-99} synthetic peptide and labeled with sodium [^{51}Cr]chromate (100 μCi; 1 Ci = 37 GBq) for 1 h. The cells were then washed three times with medium to remove unbound peptide and extracellular radionuclide. Ten thousand radiolabeled cells either treated with peptide or untreated (negative control) were then added to stimulated effector cell dilutions. The total volume in each assay well was 200 μl. Spontaneous and complete lysis of target cells was determined by incubating target cells with either RP-10 or 1% Triton X-100, respectively. Following 4 h of incubation at 37°C, the 96-well plates were centrifuged at 2000 × *g*, and 100 μl of the supernatant was counted for release of ^{51}Cr. Percent specific lysis was determined as 100 × (CTL release − spontaneous release)/(maximum release − spontaneous).

***In Vivo* Protection Experiments.** Mice (six per group) were injected intraperitoneally (i.p.) with 30 pmol of LFn–LLO_{91-99} plus 6 pmol of PA (vaccinated group) or PBS (control group) in a volume of 300 μl. Challenge experiments were carried out 28 days following injection.

For the challenge study, *L. monocytogenes* strain 10403S was grown in trypticase soy broth at 37°C with shaking, and both vacccinated and control mice were injected intravenously (i.v.) with 1×10^4 colony-forming units (cfu) of *L. monocytogenes* in a volume 300 μl. Seventy-two hours after challenge, spleens and livers were harvested and homogenized in either 5 or 10 ml of 0.2% Nonidet P-40, respectively. Dilutions of the homogenates were made in 0.2% Nonidet P-40 and plated on TSB agar plates containing streptomycin (10 μg/ml). The plates were incubated overnight at 37°C, and each dilution was assayed for cfu.

RESULTS

To determine if CTL epitopes fused to LFn are able to stimulate epitope-specific CTL, we first constructed plasmids encoding the 9-amino acid LLO_{91-99} CTL epitope fused to either the amino terminus or the carboxyl terminus of LFn (generating LFn–LLO_{91-99} and LLO_{91-99}–LFn, respectively). Mice (five per group) were injected i.p. with 30 pmol of either fusion protein mixed with 6 pmol of PA. Control groups of mice were injected with LFn–LLO_{91-99} alone, LLO_{91-99}–LFn alone, PA alone, LLO_{91-99} alone, or PA plus LLO_{91-99}. At 14 days the animals were sacrificed, and 3×10^7 splenocytes were stimulated on syngeneic spleen cells coated with the LLO_{91-99} peptide. After 5 days of stimulation *in vitro*, the cells were assayed for ability to lyse H-2^d mastocytoma (P815) cells that had been coated with LLO_{91-99}.

As shown in Fig. 2, lysis of peptide-coated P815 was substantially higher than lysis of P815 cells alone, indicating that the mice had mounted an LLO_{91-99}-specific CTL response. Priming occurred regardless of whether LLO_{91-99} was fused to the carboxyl terminus or the amino terminus of LFn. None of the controls stimulated a LLO_{91-99}-specific CTL response (data not shown), indicating that this is an LFn-mediated PA-dependent event.

The efficiency of this delivery system was examined by injecting a fixed amount of PA (6 pmol) with variable amounts of LFn–LLO_{91-99} (0.003 pmol to 0.3 pmol). Splenocytes were harvested after 14 days and assayed for CTL activity after 5 days of stimulation *in vitro*. As shown in Fig. 3, priming was

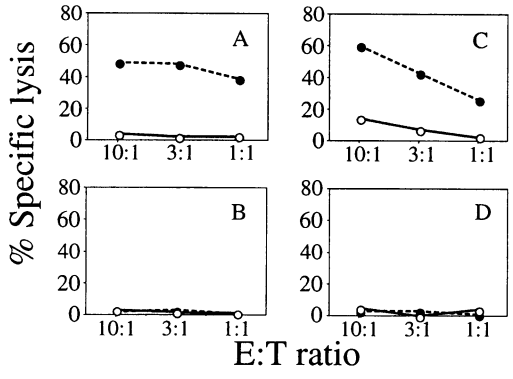

FIG. 2. CTL-mediated lysis of LLO_{91-99}-peptide-coated P815 cells. Mice were immunized with LFn–LLO_{91-99} plus PA (*A*), LFn–LLO_{91-99} without PA (*B*), LLO_{91-99}–LFn plus PA (*C*), or LLO_{91-99}–LFn without PA (*D*). After *in vitro* stimulation, samples were assayed for their ability to lyse ^{51}Cr-labeled P815 cells coated with LLO_{91-99} peptide (solid circles) or not coated (open circles). Targeting was evaluated by ^{51}Cr release. Effector-to-target cell ratios (E:T ratios) examined were 10:1, 3:1, and 1:1. Similar levels of lysis were observed in each of five replicates.

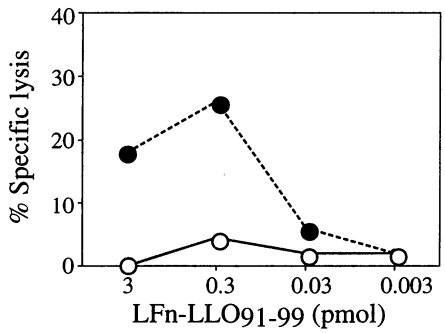

FIG. 3. Efficiency of stimulation as a function of LFn–LLO_{91-99} concentration. Mice were immunized with 6 pmol of PA mixed with 3 pmol, 0.3 pmol, 0.03 pmol, or 0.003 pmol of LFn–LLO_{91-99}. After *in vitro* stimulation, the cells were assayed for their ability to lyse ^{51}Cr-labeled P815 cells coated (solid circles) or not coated (open circles) with peptide.

achieved with as little as 0.3 pmol of LFn–LLO_{91-99}. Increasing the concentration of PA 10-fold, to 60 pmol, did not enhance the efficiency of delivery (data not shown).

Experiments were performed to determine whether mice vaccinated with the LFn–LLO_{91-99} fusion protein are protected against challenge with *L. monocytogenes*. Mice were immunized with 30 pmol of LFn–LLO_{91-99} plus 6 pmol of PA, and 4 weeks after immunization the mice were challenged i.v. with $2 \times LD_{50}$ (1×10^4 cfu) of *L. monocytogenes*. Forty-eight hours later the mice were sacrificed, and their spleens and livers were harvested. As shown in Fig. 4, the vaccinated group showed an average of 30-fold fewer bacteria in the liver and an average of 20-fold fewer in the spleen, compared with control mice (PBS alone). No protective effect was observed in control mice immunized with purified LFn and PA (data not shown).

DISCUSSION

The use of anthrax toxin to deliver epitopes to the cytosol of cells represents a novel approach for overcoming problems associated with stimulating a CTL response *in vivo*. Previous methods have involved using bacteria, viruses, adjuvants, or liposomes to introduce foreign molecules into the cell (6, 7, 20, 21). By taking advantage of the membrane translocation capability of anthrax toxin (modified to eliminate toxicity), we have developed a new and convenient tool for delivery of CTL epitopes *in vivo*.

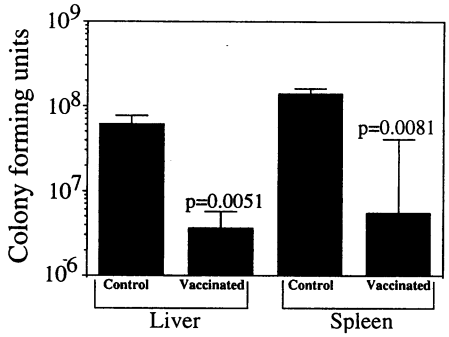

FIG. 4. Protection against *L. monocytogenes* after immunization with LFn–LLO_{91-99} plus PA. Mice were vaccinated with LFn–LLO_{91-99} plus PA. Control groups were given injections of PBS. Four weeks later mice were challenged i.v. with $2 \times LD_{50}$ of *L. monocytogenes*. Forty-eight hours after infection, livers and spleens were harvested, and the number of *L. monocytogenes* cfu per organ was determined. Significance was calculated by Wilcoxon's rank sum analysis.

Anthrax toxin-mediated delivery of LLO_{91-99} worked equally well regardless of the position of the epitope relative to LFn, and it proved to be highly efficient, with as little as 300 fmol (≈10 ng of protein) priming a peptide-specific CTL response. The dependence on PA for priming, with the requirement that the peptide be fused to LFn, strongly implies that cytosolic delivery is necessary. The possibility that PA might function as an adjuvant independent of translocation is unlikely, as injections with PA plus the unfused LLO_{91-99} peptide did not prime a peptide-specific CTL response. Also, the fact that delivery occurred *in vivo*, and with such minute quantities of protein, makes it highly unlikely that external loading of MHC-I molecules may have occurred after protein degradation. This latter possibility is difficult to exclude in two other toxin-based peptide delivery systems that have been reported to function in cell culture (22, 23).

Recently, the adenylate cyclase toxin from *Bordetella pertussis* has been used to stimulate a peptide-specific CTL response in mice (24). Unlike the anthrax system presented herein, however, efficient delivery by adenylate cyclase toxin required the presence of adjuvant. Additionally, whereas the adenylate cyclase toxin system required approximately 280 pmol of fusion protein to prime a CTL response, the anthrax toxin system stimulated CTL with 1000-fold less. Finally, while the adenylate cyclase system does show CTL stimulation, it has not yet been reported whether this CTL response affords protection *in vivo*.

The reduction in *L. monocytogenes* cfu isolated from vaccinated compared with nonvaccinated mice suggests that the anthrax toxin delivery system may have potential in vaccine development. In general, toxin-based systems may hold significant advantages over other systems for cytoplasmic delivery of CTL epitopes, including ones involving viruses, direct DNA injection, intracellular bacteria, or various adjuvants. Both the bacterial- and virus-based delivery systems use live infectious agents that although attenuated, still raise concern. Additionally, in virus- or DNA-based systems, there is the potential for a recombination event involving the exogenous DNA and host genome. While the use of adjuvant mixtures is satisfactory in mice and other nonhuman systems, it is not clear whether most adjuvants will be well tolerated in humans. Besides avoiding many of the complications associated with non-toxin-related epitope delivery systems, the anthrax toxin system also presents certain advantages over other toxin-related systems. Potential interference in toxin folding by a fused peptide is minimized by the fact that only the folding of a single domain, LFn, is in question; and we know that heterologous proteins or peptides may be fused to either terminus of LFn without affecting its activity. Also, because toxic activity in the anthrax system requires combining two separate proteins, which we produce in different strains of bacteria, governmental regulatory restrictions on cloning toxins are avoided.

The anthrax toxin CTL epitope delivery system may have potential for treatment of diseases in which CTL responses are required for protection. CTL epitopes have been characterized for a number of pathogenic viruses and bacteria, including cytomegalovirus, human immunodeficiency virus, and *Yersinia enterocolitica*, and each of these epitopes represents a candidate for LFn-mediated peptide vaccination against the corresponding microbial disease (19, 25, 26). In addition, several cancer-related CTL epitopes have recently been identified, and such epitopes may serve as the basis for development of anthrax toxin-based antitumor therapies and vaccines.

Note Added in Proof. Independent work by K. R. Klimpel, T. J. Goletz, N. Arora, S. H., Leppla, J. M. Keith, and J. A. Berzofsky demonstrates that LFn fusions can be used to target cells *in vitro* for lysis by CTL (personal communication).

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