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Anthrax Toxin as a Molecular Tool for Stimulation of Cytotoxic T Lymphocytes: Disulfide-Linked Epitopes, Multiple Injections, and Role of $CD4^+$ Cells

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We have previously demonstrated that anthrax toxin-derived proteins, protective antigen (PA) and the amino-terminal portion of lethal factor (LFn), can be used in combination to deliver heterologous molecules to the cytosol of mammalian cells. In this study we examined the ability of an LFn-peptide disulfide-linked heterodimer to prime cytotoxic T lymphocytes (CTL) in the presence of PA. A mutant of LFn that contains a carboxy-terminal reactive cysteine was generated. This form of LFn could be oxidized with a synthetic cysteine containing peptide to form a heterodimer of the protein and peptide. Mice injected with the heterodimer plus PA mounted a peptide-specific CTL response, indicating that this molecule functioned similarly to the genetically fused forms used previously. We also report the results of an analysis of two aspects of this system important for the development of experimental vaccines. First, CD4 knockout mice were unable to generate a CTL response when treated with PA plus an LFn-epitope fusion protein, suggesting that $CD4^+$ helper responses are essential for stimulating specific CTL with the PA-LFn system. Second, we now show that primary injection with this system does not generate any detectable antibody response to the vaccine components and that prior immunization has no effect on priming a CTL response to an unrelated epitope upon subsequent injection.

Cytotoxic T lymphocytes (CTL) are important immune effector cells in the response to intracellular pathogens, including viruses and some bacteria (1, 10). CTL respond to infected cells following recognition of pathogen-derived epitopes presented at the cell surface by class I major histocompatibility complex (MHC-I) molecules. These epitopes are small peptides (8 to 10 residues) derived from pathogen proteins and are generated through proteasome-mediated cleavage within the cytosol (9, 17). Following recognition of foreign peptide–MHC-I complexes, CTL lyse the target cell and then expand and differentiate. Expansion is important to ensure clearance of other defective cells, and differentiation results in the establishment of memory CTL. These memory CTL provide a more efficient response upon subsequent pathogen exposure. It is the establishment of these specific memory CTL that results in immune protection against these pathogens. For this reason, priming of memory CTL is central to vaccination against these pathogens.

The need for the vaccinating epitope to be delivered to the cytosol has required the development of systems to translocate the molecule across the cell membrane to the interior of the cell, where appropriate processing and MHC-I interaction of the peptide can occur. To overcome this barrier, we have used a modified form of anthrax toxin that is able to enter the cytosol of mammalian cells but is nontoxic (3, 13).

Anthrax toxin is a tripartite bacterial toxin that elicits two toxic effects, edema and lethality (11). Lethal factor (LF) and edema factor (EF) are intracellularly acting proteins, and both require protective antigen (PA) for translocation to the cytosol of mammalian cells. As part of this process, LF and EF compete for binding to a proteolytically activated form of PA (PA_{63}) at the cell surface. Following binding the complex is endocytosed, and after endosomal acidification LF or EF is translocated to the cellular cytosol. Within the cytosol EF expresses its adenylate cyclase activity, generating increased levels of cyclic AMP. The cytosolic activity and the specific target of LF remain undefined. It does appear, however, that LF particularly targets macrophages and induces lethal overproduction of certain cytokines (7, 8).

By eliminating the carboxy-terminal toxic domain of LF, we have generated a form of this protein (the amino-terminal 255 residues [LFn]) that can bind to PA, can be efficiently delivered to cellular cytosol, and is nontoxic. Previously, we have genetically fused specific CTL epitopes to LFn and used these fusions in combination with PA to deliver these epitopes to the interior of cells both in vitro and in vivo (4, 5). We have now expanded this work to examine the ability of this system to deliver an epitope that is disulfide linked to LFn instead of genetically fused. Furthermore, we have examined the role $CD4^+$ T-cell help may play in priming CTL with the PA-LFn system. We have also investigated whether an antibody response is generated following initial immunization and whether this initial vaccination precludes subsequent immunization with different epitopes.

MATERIALS AND METHODS

Peptides. Synthetic peptides $_{cys}LLO_{91-99}$ (CGYKDGNEYI), LLO_{91-99} (GYKDGNEYI), $OVA_{257-264}$ (SIINFEKL), and $NP_{118-126}$ (RPQASGVYM) were purchased from Biosynthesis Incorporated (Lewisville, Tex.).

Animals and cell culture. CD4 knockout C57BL/6J-*Cd4*^{tm1Knw} (*H-2*^{b}), C57BL/6J (*H-2*^{b}), and BALB/cByJ (*H-2*^{d}) mice were obtained from Jackson Laboratory (Bar Harbor, Maine). All mice were females between 8 and 12 weeks of age.

Two cell lines, EL4 (*H-2*^{b}) and P815 (*H-2*d), were used in these studies. These lines were maintained in RP-10 and incubated at 37°C with 7% CO_2 as previously described (4, 5).

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Construction and expression of modified forms of LFn. Four modified forms of LFn were used in this study: LFn($S^{255}\rightarrow C^{255}$), referred to herein as LFn_{cys}, $LFn\text{-}NP_{118\text{-}126}$, $LFn\text{-}LLO_{91\text{-}99}$, and $LFn\text{-}OVA_{257\text{-}264}$.

A DNA fragment encoding LFn_{cys} was constructed by PCR. LFn_{cys} was amplified with an upstream primer which encodes an *Nde*I site and sequence homologous to the 5′ end of the LF gene. The downstream primer was homologous to the sequence encoding the last six amino acids of LFn with a modification that substitutes a single cysteine for serine 255 and also provides two stop codons and a *Bam*HI restriction site. The toxin-encoding plasmid from *Bacillus anthracis*, pXO1, was used as the template. The amplified fragment was restriction digested with *Nde*I and *Bam*HI and ligated into compatible sites within the multiple cloning region of the expression vector pET15b (Novagen). The ligation product was used to transform *Escherichia 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 that the fusion was correct. These clones were then used to transform *E. coli* BL21(DE3) (16) for expression of the mutant protein.

The construction of the $LFn\text{-}OVA_{257\text{-}264}$ and $LFn\text{-}LLO_{91\text{-}99}$ fusion proteins has been described (4, 5). Briefly, an upstream primer homologous to the 5′ end of LF and containing an *Nde*I site was used in combination with a second primer containing sequence homologous to the 3′ end of LFn and encoding $OVA_{257\text{-}264}$, a *Bam*HI site, and two stop codons. These primers were used to amplify the fusion sequence by PCR. The amplified product was cloned and screened as described above. The same approach was used to amplify a product that encodes the $LFn\text{-}NP_{118\text{-}126}$ 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 an Ni^{2+}-charged column. Cultures of BL-21/pET15b (LFn_{cys}, $LFn\text{-}OVA_{257\text{-}264}$, $LFn\text{-}LLO_{91\text{-}99}$, or $LFn\text{-}NP_{118\text{-}126}$) were grown in Luria broth containing ampicillin (50 μg/ml) to an optical density at 600 nm of 0.6 to 0.8, and protein expression was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) 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. LFn_{cys} was isolated in the presence of 10 mM β-mercaptoethanol to prevent oxidation. The protein concentration was determined, and the sample was frozen at −20°C.

Wild-type PA was isolated from supernatant cultures of an attenuated strain of *B. anthracis* according to an established protocol (12).

Disulfide linkage of LFn_{cys} with $_{cys}LLO_{91\text{-}99}$. To generate the disulfide-linked $LFn\text{-}LLO_{91\text{-}99}$, the following protocol was used. Purified LFn_{cys} was buffer exchanged into 20 mM Tris, pH 7.5, by gel filtration on a prepared PD-10 column (Pharmacia). The synthetic $_{cys}LLO_{91\text{-}99}$ peptide was added to the sample at increasing peptide-to-LFn_{cys} ratios of 0, 1, 10, 50, and 100. The mixtures were allowed to incubate for 16 h at 4°C. Samples of these mixtures were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed for heterodimers. The mixture yielding the optimal amount of heterodimer was then passed over a PD-10 column to remove any unlinked peptide. The concentration of this sample was then determined, and aliquots were frozen at −20°C and later thawed for use in the appropriate CTL priming study.

Stimulation of peptide-specific CTL. Mouse splenocytes were harvested and CTL were stimulated as described previously (15), 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 (2,000 rads), syngeneic splenocytes incubated for 1 h with a 10 μM concentration of the appropriate synthetic peptide. The stimulator cells were washed once in RP-10, and cultures containing 3×10^7 stimulator cells and 3×10^7 splenocytes from either immunized or control mice were established. These were incubated upright in a T-25 flask at 37°C in 7% CO_2 in a total volume of 20 ml of RP-10.

Assay for CTL responses. Mouse thymoma EL-4 (*H-2^b*) or mouse mastocytoma P815 (*H-2^d*) target cells were incubated with a 10 μM solution of the appropriate synthetic peptide and 20 μl of sodium [^{51}Cr]chromate (600 Ci/ml; 1 Ci = 37 GBq) for 1 h. The cells were then washed three times with medium to remove unbound peptide and extracellular radionuclide. Radiolabeled cells (10,000), either treated with peptide or untreated (negative control), were then added to stimulated effector-cell dilutions in a 96-well assay plate. 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. After 4 h of incubation at 37°C, the 96-well plates were centrifuged at 2,000 × *g*, and 100 μl of the supernatant was analyzed for release of ^{51}Cr. Percent specific lysis was determined as 100 × [(CTL release − spontaneous release)/(maximum release − spontaneous release)].

RESULTS

Disulfide linkage of LFn_{cys} and $_{cys}LLO_{91\text{-}99}$. To determine if a disulfide-linked heterodimer of LFn and a synthetic peptide could be generated, we constructed a mutant form of LFn that expressed a single reactive cysteine. This modified form of LFn, LFn_{cys}, was oxidized with increasing amounts of the synthetic peptide $_{cys}LLO_{91\text{-}99}$. As shown in Fig. 1, at low peptide-to-LFn_{cys} ratios there are three LFn-containing reaction products. Based on relative electrophoretic migration of the products, these correspond to an unlinked form of LFn_{cys}, an LFn_{cys}, homodimer, and an $LFn_{cys}\text{-}_{cys}LLO_{91\text{-}99}$ heterodimer. As the amount of $_{cys}LLO_{91\text{-}99}$ is increased there is no detectable LFn_{cys} homodimer and the protein species become largely $LFn_{cys}\text{-}_{cys}LLO_{91\text{-}99}$ heterodimers. Estimates from relative band intensities suggest that more than 80% of the LFn_{cys} is linked to the synthetic peptide. Further increases in time of incubation or amount of synthetic peptide did not improve the yield of heterodimer.

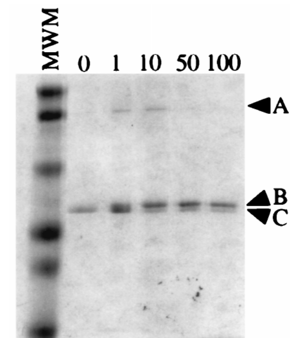

FIG. 1. $LFn_{cys}\text{-}_{cys}LLO_{91\text{-}99}$ heterodimer formation. LFn_{cys} and $_{cys}LLO_{91\text{-}99}$ were incubated at 4°C for 16 h at increasing ratios of synthetic peptide to LFn_{cys}. The reactions were then screened for heterodimer (band B) formation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes show, from left to right, molecular weight markers (MWM); LFn_{cys} alone in 10 mM β-mercaptoethanol (0) and 1:1, 10:1, 50:1, and 100:1; ratios of $_{cys}LLO_{91\text{-}99}$ to LFn_{cys}. Band A represents LFn_{cys} homodimers. Band C represents unlinked LFn_{cys} molecules. $_{cys}LLO_{91\text{-}99}$ molecules not linked to LFn_{cys} are too small to be seen clearly on this gel.

In order to determine the ability of the $LFn_{cys}\text{-}_{cys}LLO_{91\text{-}99}$ to prime specific CTL, BALB/c mice (five mice per group) were injected with 30 pmol of the heterodimer and 6 pmol of PA; a control group of mice were injected with the heterodimer in the absence of PA. Two weeks after injection, splenocytes were harvested and stimulated for 5 days on syngeneic $LLO_{91\text{-}99}$-coated splenocytes. Following stimulation, the cultures were assayed for $LLO_{91\text{-}99}$-specific CTL. As shown in Fig. 2, mice injected with the heterodimer and PA mounted a CTL re-

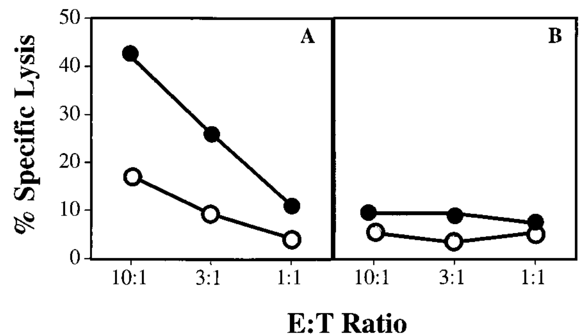

FIG. 2. $LLO_{91\text{-}99}$-specific CTL activity following immunization with an epitope disulfide linked to LFn. Mice were injected i.p. with $LFn_{cys}\text{-}_{cys}LLO_{91\text{-}99}$ with PA (A) or without PA (B). After in vitro stimulation, samples were assayed for their ability to lyse ^{51}Cr-labeled P815 cells coated with $LLO_{91\text{-}99}$ peptide (solid circles) or not coated (open circles). Targeting was evaluated by ^{51}Cr release. E:T, effector-to-target-cell ratios. Similar levels of lysis were observed in each of five replicates. One example is shown.

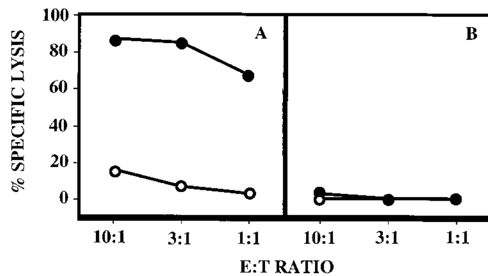

FIG. 3. Peptide-specific CTL responses in CD4 T-cell-deficient mice. $CD4^{-/-}$ mice or control $CD4^{+/+}$ mice were injected i.p. with LFn-$OVA_{257\text{-}264}$ plus PA. After in vitro stimulation, samples were assayed for their ability to lyse ^{51}Cr-labeled EL-4 cells coated with $OVA_{257\text{-}264}$ peptide (solid circles) or not coated (open circles). Targeting was evaluated by ^{51}Cr release. E:T, effector-to-target-cell ratios. (A) CTL response following immunization of $CD4^{+/+}$ mice; (B) CTL response following immunization of $CD4^{-/-}$ mice. Similar levels of lysis were observed in each of five replicates. One example is shown.

sponse specific to $LLO_{91\text{-}99}$. As in previous studies, PA was required for CTL priming: controls not injected with PA were unable to stimulate a response.

CTL responses in CD4 knockout mice. Both the experimental $CD4^{-/-}$ and control C57BL/6 mice were immunized as previously described with PA and LFn-$OVA_{257\text{-}264}$. Mice were injected intraperitoneally (i.p.) with 30 pmol of the fusion protein mixed with 6 pmol of PA. Fourteen days after injection, splenocytes were harvested from the immunized mice and assayed for specific CTL responses. As seen in Fig. 3, the CD4 knockout mice did not mount a detectable CTL response, suggesting that CD4 help is required to generate a response with this system.

Influences of primary injections upon subsequent treatments with PA-LFn. In these experiments, two LFn-peptide fusions were used, the previously described LFn-$LLO_{91\text{-}99}$ fusion protein and another fusion protein, LFn-$NP_{118\text{-}126}$. We have constructed LFn-$NP_{118\text{-}126}$ to study immunization against the model pathogen lymphocytic choriomeningitis virus (unpublished data). To determine whether initial vaccination precludes a subsequent immunization with this system, two groups of mice (seven mice per group) were injected with either LFn-$NP_{118\text{-}126}$ plus PA or LFn-$LLO_{91\text{-}99}$ plus PA. Twenty-eight days after the initial injection, two mice from each group were sacrificed, serum was collected, and splenocytes were stimulated and tested for evidence that a CTL response against the antigen was primed. The remaining five mice in each group were injected with the reciprocal protein: mice initially injected with LFn-$NP_{118\text{-}126}$ were injected with LFn-$LLO_{91\text{-}99}$ plus PA, and mice previously injected with LFn-$LLO_{91\text{-}99}$ were injected with LFn-$NP_{118\text{-}126}$ plus PA. Two weeks after the second injection, splenocytes were harvested from each mouse, stimulated in vitro, and assayed for CTL activity against both epitopes.

As shown in Fig. 4, mice initially injected with either fusion plus PA mounted an epitope-specific CTL response, and this response did not prevent subsequent toxin-mediated priming of CTL against a different epitope. Immunoblot analysis with serum taken from mice 28 days after immunization did not reveal an antibody response to PA, LFn, or the fused peptide (data not shown).

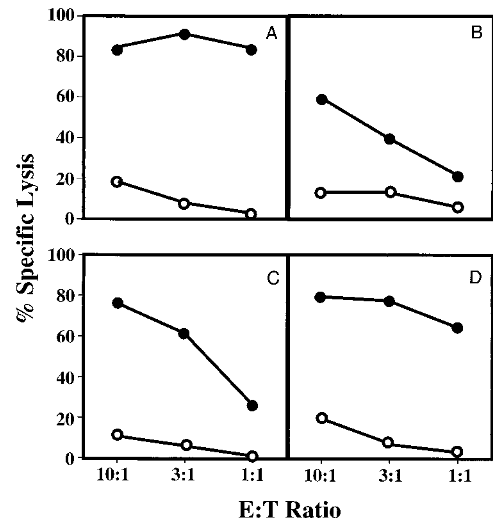

FIG. 4. Peptide-specific CTL responses in mice immunized sequentially with different toxin fusions. Mice were injected i.p. with LFn-$LLO_{91\text{-}99}$ plus PA or LFn-$NP_{118\text{-}126}$ plus PA. After 28 days the mice were injected i.p. with the reciprocal protein; 14 days later they were sacrificed and the response to both CTL epitopes was analyzed. Splenocyte cultures were stimulated in vitro and assayed for their ability to lyse ^{51}Cr-labeled P815 cells coated with either $LLO_{91\text{-}99}$ or $NP_{118\text{-}126}$ peptide (solid circles) or not coated (open circles). Targeting was evaluated by ^{51}Cr release. E:T, effector-to-target-cell ratios. (A) LLO-$_{91\text{-}99}$-specific CTL response in mice injected with LFn-$LLO_{91\text{-}99}$ plus PA followed by injection with LFn-$NP_{118\text{-}126}$ plus PA; (B) $NP_{118\text{-}126}$-specific CTL response in mice injected with LFn-$LLO_{91\text{-}99}$ plus PA followed by injection with LFn-$NP_{118\text{-}126}$ plus PA; (C) $LLO_{91\text{-}99}$-specific CTL response in mice injected with LFn-$NP_{118\text{-}126}$ plus PA followed by injection with LFn-$LLO_{91\text{-}99}$ plus PA; (D) $NP_{118\text{-}126}$-specific CTL response in mice injected with LFn-$NP_{118\text{-}126}$ plus PA followed by injection with LFn-$LLO_{91\text{-}99}$ plus PA. Similar levels of lysis were observed in each of five replicates. One example is shown.

DISCUSSION

In previous studies as well as in part of this study, we have used genetic fusion to generate LFn-peptide hybrid molecules. Here we investigated disulfide linkage as another method of generating LFn-peptide molecules. A mutant form of LFn, containing a carboxy-terminal cysteine residue, was oxidized with a synthetic form of $LLO_{91\text{-}99}$ to form an LFn-peptide heterodimer. The molecule appears to function similarly to molecules created by genetic fusion, since specific CTL can be primed by the heterodimer in the presence of PA. Although more detailed analysis is required, these initial results suggest that disulfide bonds in this form do not block translocation. We can envision this system being used in broader applications in which a given molecule with a reactive cysteine is linked to this form of LFn and delivered to the cytosol of cells by PA.

$CD4^+$ T-helper cells have generally been considered important in the establishment of productive antibody responses. In addition, $CD4^+$ helper responses may also be required for the generation and establishment of the CTL response. However, in some systems it has been demonstrated that CTL can be primed in animals that have depleted populations of $CD4^+$ cells. This has been especially true in studies of CTL responses to certain viruses. Buller et al. (6) clearly demonstrated that mice depleted of $CD4^+$ cells were able to mount specific CTL responses to ectromelia virus. Furthermore, the $CD4^+$-cell-depleted mice were protected against establishment of the disease. Studies by Ahmed et al. (2) have shown that depletion of $CD4^+$ cells leads to abrogation of antibody responses to lymphocytic choriomeningitis virus but does not lead to a decline in the number of CTL directed toward the virus. Finally, work by Sauzet et al. (14) has shown that while help from $CD4^+$ cells may not be necessary for establishment of specific CTL, it may be important for long-term maintenance of the

response. The studies here suggest that $CD4^+$ T cells are essential for induction of specific CTL following immunization with toxin fusions. The mechanism by which $CD4^+$ help is stimulated is not clear. It is possible that individual polypeptides may be taken up by antigen-presenting cells and presented to $CD4^+$ helper cells by MHC-II. As a result of these findings, we are presently investigating whether approaches that improve $CD4^+$ help may lead to more efficient CTL priming. We are focusing on methods which enhance this helper response while keeping antibody responses to the vaccinating proteins at a minimum.

As a general approach, vaccination against a wide variety of pathogen-infected or defective cells may require multiple injections. For this reason, we are interested in whether initial vaccination prevents the use of this system in subsequent treatments. The results presented in this report suggest that initial injection with the PA-LFn system does not preclude secondary immunization directed toward priming CTL unrelated to the first injection. As part of this experiment, the test animals were assayed for antibody responses to PA, LFn, or the CTL-priming peptide. Even though $CD4^+$ responses were important, we were unable to detect antibody responses to any of the proteins used in this system. An appealing aspect of the PA-LFn vaccination system is the wide range of pathogens for which it could be applied. This system might even be used to vaccinate against other defective cells, such as cancers.

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