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Direct Inhibition of T-Lymphocyte Activation by Anthrax Toxins In Vivo

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The causative agent of anthrax, *Bacillus anthracis*, produces two toxins that contribute in part to its virulence. Lethal toxin is a metalloprotease that cleaves upstream mitogen-activated protein kinase kinases. Edema toxin is a calmodulin-dependent adenylate cyclase. Previous studies demonstrated that the anthrax toxins are important immunomodulators that promote immune evasion of the bacterium by suppressing activation of macrophages and dendritic cells. Here we showed that injection of sublethal doses of either lethal or edema toxin into mice directly inhibited the subsequent activation of T lymphocytes by T-cell receptor-mediated stimulation. Lymphocytes were isolated from toxin-injected mice after 1 or 4 days and stimulated with antibodies against CD3 and CD28. Treatment with either toxin inhibited the proliferation of T cells. Injection of lethal toxin also potently inhibited cytokine secretion by stimulated T cells. The effects of edema toxin on cytokine secretion were more complex and were dependent on the length of time between the injection of edema toxin and the isolation of lymphocytes. Treatment with lethal toxin blocked multiple kinase signaling pathways important for T-cell receptor-mediated activation of T cells. Phosphorylation of the extracellular signal-regulated kinase and the stress-activated kinase p38 was significantly decreased. In addition, phosphorylation of the serine/threonine kinase AKT and of glycogen synthase kinase 3 was inhibited in T cells from lethal toxin-injected mice. Thus, anthrax toxins directly act on T lymphocytes in a mouse model. These findings are important for future anthrax vaccine development and treatment.

Anthrax is caused by *Bacillus anthracis*, a large, rod-shaped, spore-forming, gram-positive bacterium (27). Stable *B. anthracis* spores form the basis of potential biological or bioterrorism weapons. The virulence of *B. anthracis* is dependent on the genes carried by two plasmids, pXO1 and pXO2. The genes for the synthesis of an antiphagocytic poly-γ-D-glutamic acid capsule are encoded by pXO2. Plasmid pXO1 contains three genes, *pag*, *lef*, and *cya*, which encode protective antigen (PA), lethal factor (LF), and edema factor (EF), respectively (26). These three proteins form two toxins, edema toxin (EdTx; PA plus EF) and lethal toxin (LeTx; PA plus LF). PA is the receptor-binding component of the anthrax toxins and mediates their entry into host cells. Once PA binds to the receptor, it is cleaved at the N-terminal region by a host cell surface protease (3). The resulting 63-kDa protein heptamerizes and forms a ring structure with competitive binding sites for three molecules of LF and/or EF (28). The toxin complex is then taken up via receptor-mediated endocytosis (5).

The cellular receptors for PA are expressed on a wide variety of cell lines and tissues, including peripheral blood leukocytes, at moderate to low levels (3, 43). EF is a calmodulin-dependent adenylate cyclase that forms cyclic AMP (cAMP) from ATP (23), and LF is a zinc metalloprotease with mitogen-activated protein kinase (MAPK) kinases (MKKs) as the only known substrates (10, 11, 35, 50, 51). The toxins can enter most cells but are specifically cytotoxic to macrophages from certain inbred strains of mice (48).

Anthrax toxins are involved in mediating immune evasion of the bacterium by interfering with innate and adaptive immune responses. LeTx kills or inactivates monocytes, macrophages, and neutrophils (6, 33, 39), whereas EdTx suppresses lipopolysaccharide-induced cytokine production in macrophages (19). Injection of a sublethal dose of LeTx disrupts the adaptive immune response by inhibiting the functions of dendritic cells (2), and in vitro exposure to LeTx or EdTx inhibits the activation of mouse T cells (32).

Currently, extended treatment with antibiotics (e.g., ciprofloxacin) is the recommended therapy for anthrax. Also, the safety of the Anthrax Vaccine Adsorbed vaccine has been in question (44), and as a result, the current focus of many laboratories is to develop new therapies and recombinant subunit vaccines against anthrax (21, 24, 25, 29, 40). Although the role of the humoral immune response in anthrax is well characterized (15, 36), the effect of the bacterial toxins on the adaptive immune response is only partially understood. Here we demonstrate that anthrax toxins directly inhibit the activation of T lymphocytes in a mouse model.

MATERIALS AND METHODS

Isolation of CD4$^+$ T lymphocytes. Ten- to 12-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were euthanized, and the inguinal, axillary, brachial, and mesenteric lymph nodes were removed by sterile dissection. Lymph nodes were pressed through a 70-μm cell strainer (BD Biosciences, Bedford, MA) to isolate total lymphocytes (46). For cell proliferation assays, CD4$^+$ T cells were positively selected using Dynabeads (Dynal, Oslo, Norway) conjugated with mouse anti-CD4 antibodies followed by treatment with DETACHaBEAD (Dynal) to release the cells from the beads. For interleukin-2 (IL-2) determinations, CD4$^+$ cells were negatively selected using a Dynal CD4 negative isolation kit per the manufacturer's instructions. Both procedures yielded >95% pure CD4$^+$ T cells as determined by flow cytometry. Suspended

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cells were plated at 4×10^6 cell/ml in Dulbecco modified essential medium (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal calf serum (Mediatech), 50 μg/ml of penicillin-streptomycin, and 2 mmol/liter of L-glutamine (Mediatech).

In vitro exposure to toxins. To determine the direct effects of the anthrax toxins on $CD4^+$ T lymphocytes, magnetic bead-isolated cells were incubated with 1 μg/ml of PA and 0.2 μg/ml of LF or with 2.5 μg/ml of PA and 0.625 μg/ml of EF (BEI Resources, Manassas, VA) for 48 h. Titration experiments using the mouse macrophagelike cell line RAW 264.7 indicated that these concentrations of the toxin components resulted in the greatest increases of cAMP and cytolysis in EdTx- and LeTx-treated cells, respectively. Apoptosis of toxin-treated $CD4^+$ T cells was measured by flow cytometry following staining with R-phycoerythrin-labeled anti-CD4 monoclonal antibody (clone CT-CD4; Caltag, Burlingame, CA) and fluorescein isothiocyanate-labeled annexin V (Boehringer Mannheim, Indianapolis, IN) (47). Cell lysis was determined by lactate dehydrogenase release from toxin-treated cells using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega Corporation, Madison, WI) and quantitated by measuring wavelength absorbance at 490 nm.

In vivo exposure of mice to toxins. Agrawal et al. demonstrated that a sublethal injection of LeTx (100 μg of PA and 7.5 μg of LF) inhibited the function of dendritic cells, ultimately affecting antigen-specific B- and T-cell immunity. To investigate the direct effects of in vivo exposure to anthrax toxins on T cells, female BALB/c mice were injected intraperitoneally with 100 μg of PA and 7.5 μg of LF or EF and sacrificed either 1 or 4 days later. Total lymphocytes were isolated and pooled as described above and plated at a density of 4.0×10^6 cells/ml.

Cell proliferation and cytokine assays. For T-lymphocyte functional assays, cells were stimulated with anti-CD3 and anti-CD28 antibodies to mimic the function of major histocompatibility complex class II plus peptide complexes and costimulatory molecules. The proliferative response of T lymphocytes was determined by incubating host cells with 2 μg/ml of anti-CD28 antibody (BD Biosciences Pharmingen, San Diego, CA) in wells precoated with 10 μg/ml of anti-CD3 antibody (BD Biosciences Pharmingen). After 48 h of incubation, cellular proliferation was measured using a tetrazolium MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] cell proliferation kit (ATCC, Manassas, VA). Absorbance at 570 nm was measured with a Bio-Tek Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Statistical differences in $CD4^+$ T-cell proliferation levels were determined by Dunnett's method, and the Student t test was used to determine differences in the T-lymphocyte responses in untreated and toxin-exposed mice.

To determine the cytokine responses of T lymphocytes, cells were plated in 24-well plates in the presence of anti-CD28 and anti-CD3 antibodies and incubated for 48 h. Culture supernatants were harvested, and IL-2 was measured using a BD OptEIA mouse IL-2 enzyme-linked immunosorbent assay set (BD Biosciences). The levels of 18 different cytokines in culture supernatants of T lymphocytes isolated from toxin-exposed mice were measured in triplicate using a Bio-Plex Mouse 18-plex assay (Bio-Rad, Hercules, CA). The statistical significance of differences in IL-2 levels was determined by the Tukey test, and the statistical significance of differences in the Bio-Plex results was determined using Student's t test, comparing the cytokine production of T cells from exposed mice to that of control mice.

Phosphorylation of signaling molecules in T lymphocytes isolated from anthrax toxin-exposed mice. Female BALB/c mice were injected with LeTx or EdTx. Total lymphocytes were isolated after 24 h and pooled as described above. T cells were stimulated with 5 μg/ml of hamster anti-CD3 and 10 μg/ml of goat anti-hamster immunoglobulin G (Caltag, San Francisco, CA) at 37°C for 5 min. Cells were then lysed with a Bio-Rad cell lysis kit, and phosphorylated signaling proteins were measured with a Bio-Plex phosphoprotein assay. The Tukey test was used to determine the statistical significance of differences in phosphorylation levels. The presence of equal amounts of protein in the various samples was determined by Western blotting using antibodies against beta-actin as a probe.

Animal experiments. All animal experiments were performed in accordance with the regulations of the UTMB Institutional Animal Care and Use Committee and the NIH Office of Laboratory Animal Welfare. The mice were housed in facilities that are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. A total of three mice were used per group.

RESULTS

Anthrax toxins directly inhibit the activation of $CD4^+$ T lymphocytes. To determine the effects of the anthrax toxins on adaptive immune responses, we isolated $CD4^+$ T cells from mice by antibody-mediated bead isolation. The bead- and antibody-free $CD4^+$ T cells were stimulated in the presence of LeTx or EdTx. Both toxins inhibited T-cell proliferation in a dose-dependent manner. At the highest concentrations of LeTx (1.0 μg/ml of PA and 0.2 μg/ml of LF) and EdTx (2.5 μg/ml of PA and 0.625 μg/ml of EF), activation of T cells was completely inhibited. In the presence of a 1,000-fold-lower concentration of the toxins, T lymphocytes responded normally to stimulation with anti-CD3 and anti-CD28 antibodies (Fig. 1).

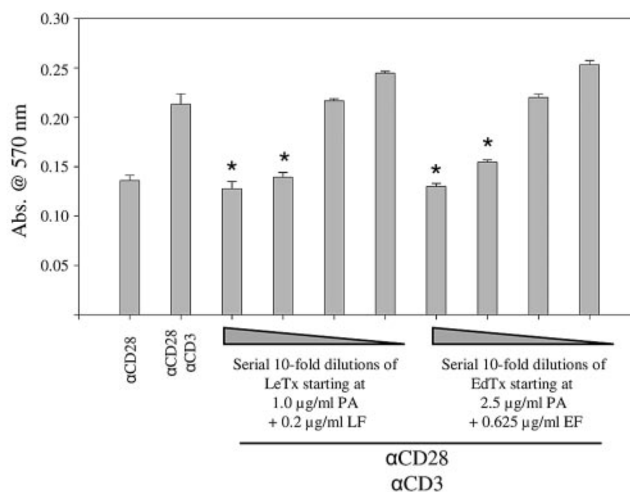

FIG. 1. Proliferation of $CD4^+$ T cells in the presence of LeTx or EdTx. $CD4^+$ T cells were isolated from female BALB/c mice and stimulated with anti-CD3 (αCD3) and anti-CD28 (αCD28) antibodies in the presence of 10-fold serial dilutions of LeTx (starting at 1 μg/ml PA and 0.2 μg/ml of LF) or EdTx (starting at 2.5 μg/ml of PA and 0.625 μg/ml of EF). After 48 h of incubation at 37°C and 5% CO_2, cellular proliferation was measured using the MTT cell proliferation assay described above. Bars represent means of triplicates ± standard errors. The data are from one experiment representative of three independent repetitions. Asterisks denote a statistically significant difference between untreated and toxin-treated cells ($P < 0.05$ by Dunnett's test). Abs., absorbance.

To determine whether LeTx and EdTx also inhibited the production of IL-2 in $CD4^+$ T cells, lymphocytes were negatively selected to eliminate $CD8^+$ T cells, B cells, dendritic cells, and macrophages. The $CD4^+$ T cells were stimulated with anti-CD28 and anti-CD3 antibodies in the presence of LeTx or EdTx, and the IL-2 concentration in culture supernatants was measured. Cells stimulated in the absence of anthrax toxins secreted 408 pg/ml of IL-2, whereas those stimulated in the presence of LeTx or EdTx secreted only 4 or 6 pg/ml, respectively ($P < 0.001$ by the Tukey test) (Fig. 2). In addition, we found no evidence of increased apoptosis or cytolysis in toxin-exposed cells, as measured by annexin V staining or lactate dehydrogenase release, respectively (data not shown).

Injection of mice with anthrax toxins blocks the ability of T lymphocytes to respond to antigenic activation. Given the profound inhibitory effect of EdTx and LeTx on $CD4^+$ cells in vitro, we determined the ability of T cells isolated from LeTx- or EdTx-exposed mice to respond to stimulation. Therefore, female BALB/c mice were injected with 100 μg of PA and 7.5 μg of LF, and lymphocytes were isolated after 1 or 4 days. T cells were stimulated with anti-CD28 and anti-CD3 antibodies.

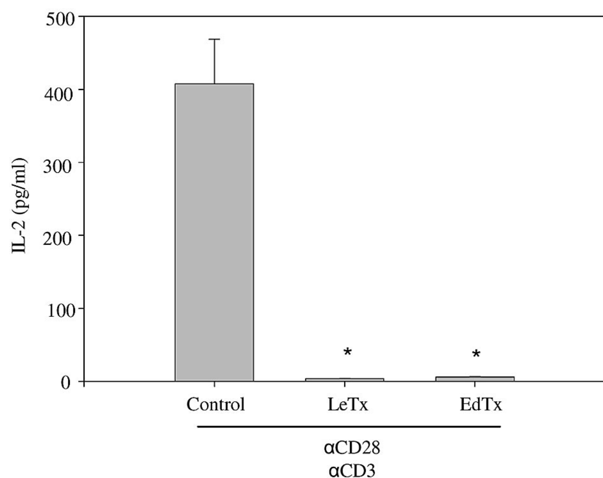

FIG. 2. Secretion of IL-2 by $CD4^+$ T cells in the presence of LeTx or EdTx. Isolated $CD4^+$ T cells were incubated with anti-CD28 (αCD28) and anti-CD3 (αCD3) in the presence of LeTx (1 μg/ml PA and 0.2 μg/ml of LF) or EdTx (2.5 μg/ml of PA and 0.625 μg/ml of EF). After 48 h of incubation at 37°C and 5% CO_2, IL-2 was measured in culture supernatants. Bars represent means of triplicates ± standard errors. The data are from one experiment representative of three independent repetitions. Asterisks denote a statistically significant difference between untreated and toxin-treated cells ($P < 0.05$ by the Tukey test).

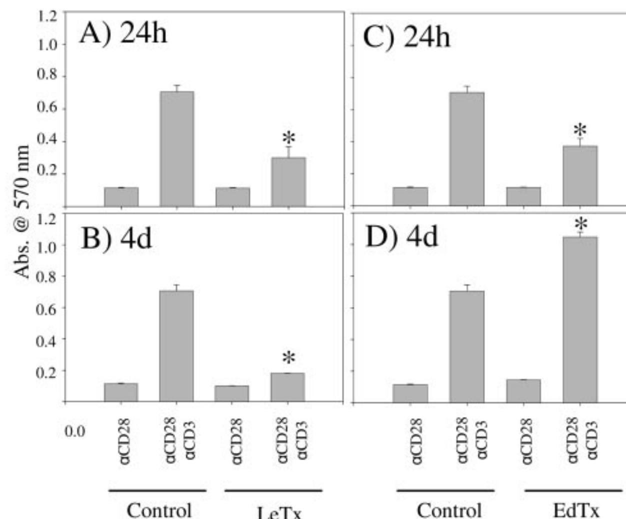

FIG. 3. Cellular proliferation of T cells isolated from mice injected with LeTx or EdTx. Female BALB/c mice were injected with 100 μg of PA and 7.5 μg of LF or EF. Total lymphocytes were isolated after 24 h (A and C) or 4 days (B and D). T cells were incubated with anti-CD28 (αCD28) and anti-CD3 (αCD3) antibodies for 48 h at 37°C and 5% CO_2. Proliferation was measured using the MTT cell proliferation assay described above. Bars represent means of triplicates ± standard errors. The data are from one experiment representative of three independent repetitions. Asterisks indicate a statistically significant difference between stimulated cells from untreated and toxin-injected mice ($P < 0.05$ by Student's t test).

Trypan blue exclusion analysis revealed no difference in the number of viable lymphocytes from control animals and that from injected animals (data not shown). The proliferation of T lymphocytes from mice injected with LeTx 24 h before stimulation with antibodies against CD3 and CD28 was reduced by 57% compared to the response from control mice (Fig. 3A). This inhibition was even more pronounced when T cells were isolated and stimulated 4 days after toxin injection (Fig. 3B).

Bio-Plex analysis of cell culture supernatants of T lymphocytes from LeTx-injected mice showed a cytokine profile altered from that of the control group. Table 1 lists the levels of T-cell-secreted cytokines in lymphocyte cultures isolated from toxin-injected and control animals after T-cell receptor-mediated stimulation. T cells stimulated with anti-CD3 and anti-CD28 antibodies 24 h after injection of mice with LeTx secreted much lower levels of cytokines than did T cells from untreated control mice. When T cells were isolated 4 days after LeTx injection, their ability to respond to T-cell receptor-mediated stimulation had partially recovered. Whereas secretion of IL-2 and tumor necrosis factor alpha (TNF-α) remained fully suppressed, IL-5 and gamma interferon (IFN-γ) secretion levels were higher than those for control lymphocyte cultures at this time point (Table 1).

We also determined the effect of EdTx injection on T-cell activation. One day after injection of mice with 100 μg of PA and 7.5 μg of EF, edema was evident in the peritoneal cavity but had subsided by day 4. Also, there was no significant difference between the number of viable lymphocytes in the EdTx-treated mice and that for controls. One day after injection with EdTx, T lymphocytes responded to anti-CD3 and anti-CD28 antibody stimulation with significantly reduced proliferation, showing only 53% of the proliferation level observed in controls (Fig. 3C). The levels of most T-cell-secreted cytokines were diminished in cultures of lymphocytes from EdTx-treated mice, with the exception of the levels of IL-2 and IL-6, which were significantly higher than those in lymphocyte cultures from control mice (Table 1). The increase in IL-2 production in T lymphocytes from EdTx-treated mice was in contrast to the findings for $CD4^+$ T cells from untreated mice (Fig. 2). Despite the increase in IL-2, T cells isolated 24 h after injection of EdTx showed a decrease in cellular proliferation.

The inhibitory effect of EdTx on T-cell proliferation was not observed when lymphocytes were harvested 4 days after injection of the mice, and proliferation following stimulation was significantly enhanced compared to that observed in T lymphocytes isolated from control mice (Fig. 3D). Four days postinjection, only the secretion levels of TNF-α, IFN-γ, granulocyte-macrophage colony-stimulating factor, and IL-17 remained lower in cultures from treated mice (Table 1).

T lymphocytes from mice injected with anthrax toxins exhibit altered activation of signaling proteins. To determine the mechanism by which in vivo exposure to anthrax toxins inhibited the activation of T lymphocytes, we injected mice with LeTx or EdTx and isolated lymphocytes 24 h later. We then stimulated T cells for 5 min with anti-CD3 and anti-CD28 antibodies. In T lymphocytes from LeTx-injected mice, the phosphorylation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2), the stress-activated kinase p38, the activating transcription factor 2 (ATF-2), the serine/threonine kinase AKT, and the glycogen synthase kinase 3α and -β (GSK-3α and β) was significantly decreased. For all measured signaling proteins, phosphorylation was significantly lower in nonstimulated cells from LeTx-injected mice than in nonstimulated cells

TABLE 1. Cytokine profiles of anti-CD3- and anti-CD28-stimulated T cells from mice injected with LeTx or EdTx or left untreated[a]

Cytokine[b]	Control profile	Profile (at indicated time postinjection) for mice injected with[c]:			
		LeTx (24 h)	EdTx (24 h)	LeTx (4 days)	EdTx (4 days)
IL-2	378 ± 23	124 ± 40*	3,970 ± 276*	95 ± 27**	3,020 ± 141**
IL-3	648 ± 112	49 ± 4**	171 ± 18**	229 ± 12**	1,779 ± 257**
IL-4	83 ± 7	25 ± 4**	49 ± 11*	62 ± 7*	691 ± 294*
IL-5	177 ± 3	59 ± 4**	98 ± 12**	236 ± 18*	625 ± 43**
IL-6	2,656 ± 424	83 ± 11**	3,908 ± 424*	532 ± 65**	2,276 ± 159
IL-10	305 ± 71	10 ± 3**	167 ± 16*	170 ± 19*	957 ± 56**
IL-17	>5,000	233 ± 6**	205 ± 19**	2,049 ± 375**	2,237 ± 376**
TNF-α	119 ± 3	8 ± 0**	17 ± 2**	13 ± 2**	19 ± 5**
IFN-γ	2,290 ± 213	760 ± 25**	1,637 ± 103**	2,860 ± 269*	1,781 ± 63*
GM-CSF	960 ± 127	63 ± 2**	149 ± 11**	333 ± 16**	650 ± 81*

[a] Values are given in pg/ml. The data are expressed as means ± standard deviations of triplicates and are representative of three independent experiments. Analysis was done using Student's *t* test.
[b] GM-CSF, granulocyte-macrophage colony-stimulating factor.
[c] * denotes a statistically significant difference from control values with a *P* value of ≤0.005, and ** indicates a *P* value of ≤0.001.

from control mice (data not shown). Treatment of mice with EdTx did not affect the phosphorylation of these kinases following T-cell stimulation. However, the phosphorylation of the stress-activated c-Jun NH_2-terminal kinase (JNK) was inhibited by treatment of mice with EdTx but not by treatment with LeTx (Fig. 4).

DISCUSSION

The toxins produced by *B. anthracis* contribute to its virulence by helping the bacterium evade the immune system. Our data showed that incubation of $CD4^+$ T cells with LeTx or EdTx inhibited T-cell activation (Fig. 1 and 2). These data are consistent with findings by Paccani et al. (32) and Fang et al. (14). However, the question of how anthrax toxins affected adaptive immune responses in vivo still remained. It has been demonstrated that injection of LeTx inhibits antigen-specific T-cell proliferation by the inactivation of dendritic cells (2), but no data on direct effects of anthrax toxins on T cells are available. Here we demonstrate that sublethal doses of either LeTx or EdTx injected into mice blocked the ability of T lymphocytes to respond to stimulation through the T-cell receptor. Our data further indicated that LeTx directly disrupted the activation of T lymphocytes by blocking cellular signaling. To our knowledge, this is the first report demonstrating direct effects of anthrax toxins on T lymphocytes in vivo. To date, no studies on the effects of a *B. anthracis* infection on T-cell function have been reported.

These findings are important for understanding the mechanisms of immune evasion by *B. anthracis*. To mount an effective adaptive immune response resulting in long-term protection mediated by memory lymphocytes, T cells have to be activated by antigen-presenting cells. Anthrax toxins inhibit both antigen presentation and T-cell signaling, thus rendering an adaptive immune response against *B. anthracis* impossible.

The roles of the toxins in a *B. anthracis* infection differ among species, and evidence suggests that they are not the major virulence factor in the murine model (53). In fact, mice, unlike rabbits and nonhuman primates, cannot be consistently protected from challenge with the current anthrax vaccine Anthrax Vaccine Adsorbed, in which the major immunogen is PA (20, 37, 52). However, antigen presentation and T-cell signaling mechanisms are conserved across species, which suggests that the observed inhibition of T cells by anthrax toxins in mice is relevant to other species. Moreover, Fang et al. showed that treatment of human $CD4^+$ cells with LeTx blocked MAPK kinase-dependent IL-2 production in vitro (14).

In our study, 24 h after injection of LeTx, T cells were severely impaired in their ability to secrete cytokines upon T-cell receptor stimulation. EdTx had a lesser effect on cytokine secretion. However, both toxins drastically blocked proliferation of T cells in response to stimulation. It is important to point out that we injected individual anthrax toxins. In combination, LeTx and EdTx might be expected to have an inhibitory effect on T-cell function even greater than that of either toxin alone, and synergy between the toxins as it relates to the T-cell function is under investigation. Our studies are also focused on studying the effects on T-cell function in vivo during infection with anthrax spores.

Interestingly and in contrast to the ability of EdTx to completely block IL-2 secretion in $CD4^+$ T cells when present during activation, cultures of T lymphocytes isolated from mice 24 h after injection with EdTx contained much higher levels of IL-2 than did cultures of T cells from control mice. This observation was unexpected, because increased cAMP levels inhibit IL-2 production and T-cell activation by activating protein kinase A type I (1). Despite increased levels of IL-2 in the medium, the proliferation of T cells recovered from EdTx-treated mice 24 h after injection was strongly inhibited. When T cells were isolated 4 days after EdTx-treatment, stimulation with anti-CD3 and anti-CD28 antibodies resulted in enhanced levels of IL-2 and the T-helper type 2 cytokines, IL-4, IL-5, and IL-10. Since we treated the mice with only a single injection of EdTx, degradation of the toxin by cellular proteases and hydrolysis of intracellular cAMP by phosphodiesterases may have progressively reduced the effects of the toxin. Thus, levels of cAMP may have changed from inhibitory to immunoenhancing. Moderate increases in cAMP have previously been demonstrated to increase the production of IL-4 and IL-5 in T cells (31). Further, cAMP-inducing agents increased the production of IL-6 and IL-10 in UVB-irradiated human keratinocytes (16).

The proposed mechanism of action of LeTx includes cleav-

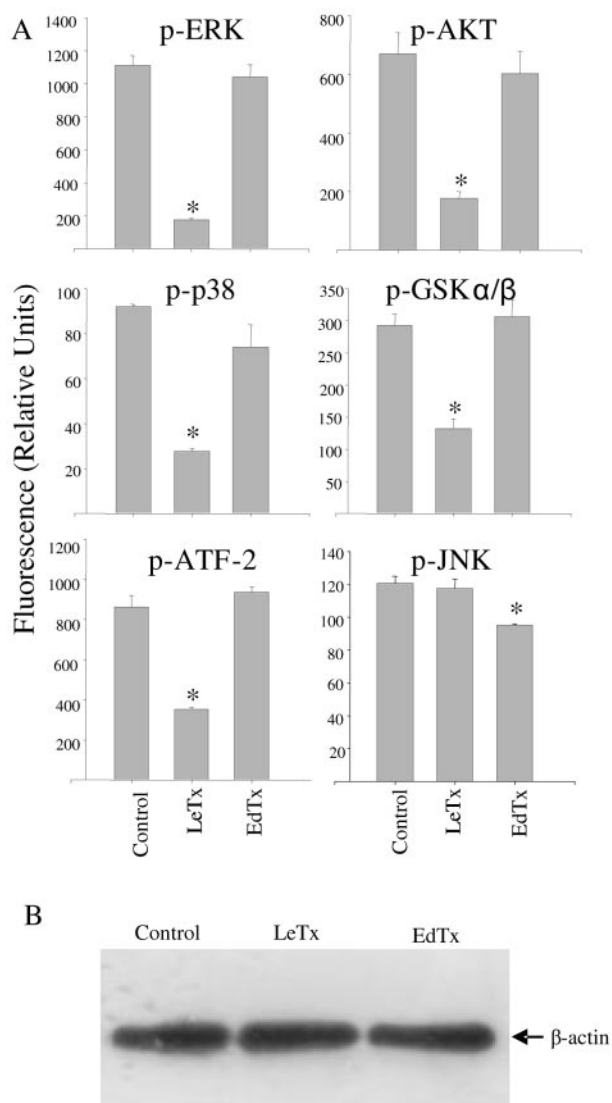

FIG. 4. (A) Phosphorylation of proteins defining various signaling pathways in T-cell receptor-stimulated T cells isolated from toxin-injected mice. Mice were injected with 100 μg of PA and 7.5 μg of LF or EF, and lymphocytes were isolated 24 h later. The T cells were stimulated with anti-CD3 antibody and cross-linked with a secondary antibody for 5 min. The cells were lysed, and phosphorylation (p-) of ERK 1 and 2, p38, ATF-2, AKT, GSK-3α and β, and JNK was measured. Values are given as relative fluorescence units, and bars represent means of duplicates ± standard errors. The data are from one experiment representative of three independent repetitions. Asterisks indicate a statistically significant difference ($P < 0.05$ by the Tukey test). (B) The presence of equal amounts of protein in the samples was ascertained by Western blotting with antibodies against beta-actin as a probe.

age and thus inactivation of the upstream kinases, MKKs 1, 2, 3, 4, 6, and 7, thus preventing the activation of MAPKs (10, 35, 50, 51). Our experiments revealed that injection of mice with LeTx inhibited the phosphorylation of several kinases crucial for T-cell activation (Fig. 4). When T lymphocytes from LeTx-treated mice were isolated 24 h after injection and stimulated via the T-cell receptor, phosphorylation of ERK1/2, p38, ATF-2, AKT, and GSK-3 was less than 50% of that in T cells from untreated control mice (Fig. 4). MKK1 and 2 phosphorylate ERK1 and 2, and MKK3 and 6 phosphorylate p38 (4). ERK1 and 2 are important in T-cell proliferation (7), and the p38 pathway promotes Th1 differentiation and the production of IFN-γ (42, 56). The transcription factor ATF-2 is activated by p38 and JNK (18) and induces immediate inflammatory genes (41). Our data indicate that LeTx may prevent adaptive immune responses during infection with *B. anthracis* by blocking the activation of p38 and ERK 1/2, thus directly affecting T-cell activation.

The phosphorylation of the serine/threonine kinase AKT was also inhibited in T cells from LeTx-injected mice. The effect of LeTx on AKT has not been previously observed. AKT is activated by the phosphoinositide signaling pathway and in turn activates a variety of substrates, including GSK-3 (22), which was also poorly phosphorylated in T cells from LeTx-injected mice. Our data are consistent with previous in vitro studies demonstrating differential expression of genes under the regulation of GSK-3 following LeTx treatment of macrophages (49).

No difference in the phosphorylation of JNK was found between LeTx-treated and control mice. JNK is activated by MKKs 4 and 7 (4), known targets of LeTx (50), and the inability of the toxin to inhibit its phosphorylation was unexpected. However, blocking of ERK1/2 and p38 is sufficient to prevent T-cell activation, because pathways mediated by these kinases activate, among other transcription factors, c-Fos and c-Myc, which promote T-cell survival and proliferation, respectively (34).

EdTx treatment did not interfere with the activation of most of the signaling kinases measured in T cells stimulated 24 h after injection of mice but moderately affected the phosphorylation of JNK. The activation of p38 and ERK 1/2 was not affected. Paccani et al. reported that in vitro treatment of T cells with EdTx inhibited the activation of JNK and ERK but not of p38 (32). The cAMP-dependent protein kinase A inhibits the activation of ERK by phosphorylating its upstream kinase, Raf-1 (8), but does not affect the activation of ERK by B-Raf, which is stimulated by an increase in cAMP (55). Thus, these two pathways may counteract each other. The lack of an effect of EdTx treatment on subsequent T-cell receptor-mediated phosphorylation of ERK1/2 also suggests that the inhibition of proliferation observed in T cells stimulated 24 h after EdTx exposure of mice was probably due to inhibition of JNK phosphorylation.

During a *B. anthracis* infection, the host is exposed to an array of virulence factors. The electronegative charge of the capsule helps the organisms evade phagocytic cells and is essential for dissemination of the bacteria in the mouse model (9, 13). Also, a hemolysin, termed anthrolysin, has been described and may play a role in virulence (45). The anthrax toxins produced by the bacteria play an important role in the pathologies associated with human anthrax cases (e.g., hemorrhage and massive edema) (17). The toxins, like the capsule, enable *B. anthracis* to evade the host immune response. LeTx inhibits the antibacterial activity of human monocytes and causes them to undergo apoptosis (38). EdTx also alters the activity of monocytes by inhibiting lipopolysaccharide-induced production of TNF-α (19). In addition, both toxins also effect the function of neutrophils (12, 30, 54).

Here we have demonstrated that injection of anthrax toxins in mice directly inhibited the subsequent activation of T cells by T-cell receptor-mediated stimulation. While immunosuppression during infection has not been reported, these data suggest that the toxins have the capacity to disable both the adaptive and innate immune responses. It has been established that the anti-PA antibody titer is the defining factor in survival (37). Inhibition of memory immune cells in vaccinated individuals with amounts of circulating antibodies insufficient to counteract the toxins would allow the organism to overwhelm the host. The inhibitory effect of the anthrax toxins on T-cell activation is of profound importance for anthrax vaccine development and for the treatment of infection with *B. anthracis*.

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