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Natural Exposure to Cutaneous Anthrax Gives Long-Lasting T Cell Immunity Encompassing Infection-Specific Epitopes

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There has been a long history of defining T cell epitopes to track viral immunity and to design rational vaccines, yet few data of this type exist for bacterial infections. Bacillus anthracis, the causative agent of anthrax, is both an endemic pathogen in many regions and a potential biological warfare threat. T cell immunity in naturally infected anthrax patients has not previously been characterized, which is surprising given concern about the ability of anthrax toxins to subvert or ablate adaptive immunity. We investigated CD4 T cell responses in patients from the Kayseri region of Turkey who were previously infected with cutaneous anthrax. Responses to B. anthracis protective Ag and lethal factor (LF) were investigated at the protein, domain, and epitope level. Several years after antibiotic-treated anthrax infection, strong T cell memory was detectable, with no evidence of the expected impairment in specific immunity. Although serological responses to existing anthrax vaccines focus primarily on protective Ag, the major target of T cell immunity in infected individuals and anthrax-vaccinated donors was LF, notably domain IV. Some of these anthrax epitopes showed broad binding to several HLA class alleles, but others were more constrained in their HLA binding patterns. Of specific CD4 T cell epitopes targeted within LF domain IV, one is preferentially seen in the context of bacterial infection, as opposed to vaccination, suggesting that studies of this type will be important in understanding how the human immune system confronts serious bacterial infection. The Journal of Immunology, 2010, 184: 3814–3821.

Detailed understanding of T cell immunity, including the nature of memory and immunodominant epitopes, is lacking for most serious bacterial infections. There is considerable interest in understanding immunity to Bacillus anthracis, the causative agent of anthrax, both as a bioterrorism/biosecurity threat and as a life-threatening infection that is endemic in Iran, Iraq, Turkey, Pakistan, and sub-Saharan Africa, as well as some areas of South and Central America (1, 2).

The bacterium, which is found as spores in the soil and most commonly infects herbivores, typically spreads to human populations through close occupational proximity to infected livestock. In addition, concern has arisen about possible bioterrorist use of anthrax following the release of anthrax through the U.S. mail in 2001 (3, 4).

Anthrax virulence depends on a tripartite exotoxin comprising protective Ag (PA) and two enzymatically active subunits: lethal factor (LF) and edema factor (EF). PA consists of four domains, the proteolytic cleavage of an exposed loop in the 20-kDa N-terminal domain causes the release of this domain, and the spontaneous oligomerization of truncated PA into heptamers that bind to EF and LF, to form edema toxin (ET) and lethal toxin (LT), respectively (5, 6). PA binds the widely expressed, host cell-surface receptors, tumor endothelium marker 8, and capillary morphogenesis protein 2 (7, 8). These receptors interact with LDL-receptor-related protein 6, which is required for ET and LT endocytosis (9). LT is lethal to the host cell (6).

Studies of adaptive immunity to anthrax infection have focused overwhelmingly on serology, with only one study reporting T cell responses to PA (10–13). Data from animal or human vaccination studies show that the induction of LT-neutralizing Ab by vaccines containing PA correlates with protection (14–16). Research has for decades focused on the precept that the protective immune response to anthrax is directed at PA and LF (although with the caveat that in many studies, other targets have not been investigated) (10, 17). Bacterial immunomics highlights other potential immune targets from the B. anthracis proteome (18), although only PA and LF have a track record in conferring both active and passive immunity in response to experimental bacterial challenge (10, 19). Early serological studies of responses from naturally infected individuals in Zimbabwe showed that most individuals generated Abs to PA and LF and a minority also made a response to EF (10). Under in vitro culture conditions, the bacterial output of LF is believed to be around one-sixth that of PA (20).

Vaccines have been widely administered in a biodefense setting over several decades, notably the U.K.-licensed anthrax vaccine precipitated (AVP) and the U.S.-licensed anthrax vaccine adsorbed...
(AVA, BioThrax), and more recently, rPA vaccines have completed phase II clinical trials (1, 21). The AVP and AVA vaccines are prepared from a filtrate of the culture supernatant of the Sterne strain, containing PA and smaller, variable amounts of LF and EF. An Ab response to both PA and LF is induced in AVP recipients (15). With respect to CD4 T cell response of vaccinees, only immunity to PA has been analyzed and found to be long lasting, encompassing Th1 and Th2 cytokine responses (22, 23). Although these early vaccines were developed empirically, there is increasing appreciation of the potential to string together a number of epitopes eliciting both B cells and CD4 T cell help to achieve a rationally designed vaccine with multivalent potential across several bacterial species. The paucity of information on the natural history of T cell immunity to bacterial infection is in stark contrast to our detailed understanding of several viral infections (24). Furthermore, although most viral infections will be resolved through host immunity leading to an ensuing program of long-term immunological memory, most serious primary bacterial infections are curtailed by antibiotic intervention juxtaposed on the contribution of natural immunity. In common with many viral infections, anthrax is of particular interest because of its ability to subvert immunity (25–33). Anthrax toxins exert multiple effects contributing to immune dysregulation at the level of innate immunity, Ag presentation, and T cell and B cell signaling. Once in the cytosol, LF cleaves and inactivates MAPK kinases, preventing the activation of MAPKs and stress-activated kinases, with multiple downstream consequences, including apoptosis of macrophages and dendritic cells. In T cells, anthrax LT and ET inhibit the MAPK and stress kinase pathways, inhibiting activation of NFAT and AP-1, thus blocking proliferation and cytokine gene expression (33). It thus might be predicted that survivors of natural infections with B. anthracis would have markedly impaired T cell memory of the pathogen, in comparison with vaccinees, and it was of specific interest to determine whether anthrax infection is able to confer long-term T cell memory. In this paper, we report detailed characterization of CD4 immunity in naturally infected individuals from rural Turkey; all showed strong CD4 T cell responses, most notably to LF, as well as to PA, in some cases several years after the infectious event.

Materials and Methods

Patient samples

Patients treated for cutaneous anthrax in the Department of Infectious Disease at Erciyes University Hospital (Kayseri, Turkey) within the past 8 y were invited to participate in the study. Cases were defined as mild if they demonstrated a cutaneous lesion (<4 cm in diameter) surrounded by a narrow erythema and no systemic symptoms. Severe cutaneous anthrax was defined as the presence of a large cutaneous lesion (>4 cm), with bullous reaction and extensive edema, and systemic symptoms including fever, tachycardia, and tachypnea. Toxemic shock was defined as the presence of a large cutaneous lesion (>4 cm), with bullous reaction and extensive edema, and systemic symptoms including fever, tachycardia, and tachypnea, acute mental changes, and hypotension.

Blood was taken from each patient (n = 9) for HLA genotyping and T cell analysis, with full informed consent (Erciyes University Ethical Committee). Blood was taken also from 10 volunteers routinely vaccinated every 12 mo for a minimum of 4.5 y with the U.K. AVP vaccine (U.K. Department of Health) under approval by the Convention on Biological Diversity Independent Ethics Committee for the U.K. Ministry of Defense. The date of first and last immunizations and the number of booster doses that each subject had received were recorded at the time of sampling. Responses were compared with those of 10 age-matched healthy controls with no known exposure to anthrax Ags (Research Ethics Committee reference number 08/H07077/173).

PBMCs were prepared from sodium heparinized blood, using Accupsin tubes (Sigma-Aldrich, Dorset, U.K.), and centrifuged at 800 × g for 30 min, after which the cells were removed from the interface and washed twice in AIM V serum-free media (GIBCO Invitrogen, Carlsbad, CA).

Ags

Recombinant full-length LF and LF domains were produced in an Escherichia coli expression system, as previously described (15). In brief, a biologically inactive form of LF, in which glutamic acid replaces cysteine at position 687, and the individual domains of LF on the basis of the crystal structure (34) were cloned and expressed from E. coli as recombinant N-terminal histidine-tagged proteins, using a commercially available expression system, pQE30 (Qiagen, Hamburg, Germany). Owing to the high AT nucleotide content of LF, the corresponding gene sequences were codon optimized for expression in E. coli (GenScript, Piscataway, NJ). The recombinant proteins were expressed according to the manufacturer's instructions (Qiagen). To purify the proteins, bacterial pellets were disrupted using a French press, and target protein was recovered by centrifugation at 45,000 × g at 4˚C for 20 min. The resulting supernatant was incubated with Talon metal affinity resin (Clontech, Mountain View, CA) to bind the N-terminal histidine tag. After washing, protein was recovered at 4˚C, using elution buffer comprising 150 mM imidazole, 50 mM sodium phosphate, and 300 mM NaCl (pH 7). Finally, the protein was dialyzed against HEPES buffered 150 mM NaCl, 50 mM NaOH (pH 7.5), using a 6000 Da dialysis cassette (Pierce, Thermo Fisher Scientific, Waltham, MA). Good manufacturing practice-grade rPA was kindly provided by Aveca Vaccines (Billingham, Cleveland, U.K.) and had endotoxin levels <1 EU/mg, whereas endotoxin levels of LF and its domains were <4 EU/mg. All were used at a final concentration of 25 µg/ml.

A peptide panel scanning the full-length sequences of LF (accession number P15917) comprising 20 mers overlapping by 10 was commercially produced (Abgent, San Diego, CA); these were HPLC purified to a purity of 98%. The peptides were resuspended in DMSO at a concentration of 25 mg/ml; they were then prepared in a matrix, with six peptides per pool, so that each peptide occurred in two pools but no peptides occurred in the same two pools. Each peptide was at a final concentration of 25 µg/ml in the pool, and the total peptide concentration per pool was 150 µg/ml. The choice of 25 µg/ml represented the lowest concentration at which CD4 responses could be reproducibly obtained. The use of peptide pools in preference to assaying each individual peptide was necessitated by the limited blood samples that could be collected from anthrax convalescent donors.

ELISpot

T cell responses to the anthrax recombinant proteins and peptides were quantified by IFN-γ ELISpot (Diaclone, Manchester, U.K.). In brief, 96-well hydrophobic polypyrilene difluoride membrane-bottomed plates (MAIP S 4; Nunc, Porvoo, Bedford, MA) were coated with 200 µl of a 4% solution of peptide in 0.1% gelatin (pH 4), blocked with 2% skimmed milk, and washed with PBS before adding Ag to the wells, each peptide represented in two separate triplicates. After the addition of Ab to the plates, they were frozen at −80˚C until use. All ELISpot plates used in this study were prepared simultaneously with the same batches of Ags. PBMCs were added to the wells at a known concentration, ~2 × 10^5 cells/well (range, 1.6 × 10^5–2.1 × 10^6 cells/well), and the plates incubated for 18 h at 37˚C with 5% CO2. Postincubation, 50 µl supernatant was removed from the negative control and the LF- and PA-stimulated wells. The supernatant from the triplicate wells was pooled, and the samples were stored until required at −80˚C. Samples from the naturally infected cohort were shipped on dry ice and then stored at −80˚C. The ELISpot plates were next washed with PBS Tween 20 (0.1%) and incubated with biotinylated anti–IFN-γ Ab. Incubation was followed by a further wash and the addition of streptavidin-peroxidase conjugate at room temperature. After a final wash, plates were developed with the addition of 100 µl 4-bromo-4-chloro-3-indolyl phosphate and NBT was added. Spots were counted using an automated ELISpot reader (AID, Strassberg, Germany). The results were expressed as spot-forming cells (SFC) per 10^6 PBMCs (SFC/10^6 PBMCs responding – SFC per 10^6 PBMCs in negative control). Results were considered positive if the mean SFC was more than 2 SD above the negative control and ≥50 spots.

Quantification of cytokines in cell culture supernatants

The levels of IL-2, IL-5, IL-9, IL-10, IL-13, IL-17, and TNF-α were measured in supernatants removed from the negative control and the LF- and PA-stimulated wells of the ELISpot assay. The supernatants were diluted 1:1 with AIM V media, and the cytokines were quantified using a Bioplex assay (Bio-Rad, Hercules, CA). In brief, this is a bead assay based on a capture sandwich immunoassay. A mixture of Abs targeting specific cytokines, coupled to fluorescently labeled beads, was incubated with the samples. An Ab coated to remove unbound protein, biotinylated detection Abs were added. Streptavidin-FITC was then added, which bound to the detection Abs on the bead surface. Data from the reaction were acquired using the Luminex 200 system (Luminex, Austin, TX). The individual dyed bead populations, as well as the fluorescent signal on the bead surface, were detected. This allowed identification of each cytokine and reported the level of target protein in the well, expressed from the standard curve generated. Analysis of each cytokine produced by the naturally infected individuals, AVP-vaccinated cohort, and healthy controls in response to both LF and PA recombinant proteins were compared using a two-way ANOVA with Bonferroni post hoc testing.
Intracellular cytokine staining

PBMCs were extracted from sodium heparinized blood from a subset of the AVP-vaccinated cohort (n = 5), as described above. The cells were resuspended in AIM V media and incubated with 1.25 × 10^6 per well in a 24-well plate. The cells were stimulated with media only (negative control), 5 μg/ml ConA (positive control), 25 μg/ml LF or LF domain IV, or a pool of the LF peptides most frequently recognized by AVP-vaccinated subjects (25 μg/ml total = 2.08 μg/ml each peptide): LF41–60, LF101–120, LF337–356, LF417–436, LF457–466, LF547–566, LF574–593, LF634–643, LF674–693, and LF694–713. The cells were cultured with the Ag for 24 h, in the presence of Golgi Stop (BD Biosciences, Oxford, U.K.) (1 μl/ml) for the last 8 h of incubation. The cells were washed with PBS (10% FCS) and stained with the following Abs: PE-conjugated anti-CD3, PerCP conjugated anti-CD8, V450-conjugated anti-CD4, and Alexa 647-conjugated anti-CD56 (BD Biosciences). For intracellular staining of cytokine, cells were stained with FITC-conjugated anti–IFN-γ Abs (BD Biosciences) or isotype controls according to the manufacturer’s protocols. All flow cytometry was performed on a FACSaria II (BD Biosciences), and data were analyzed using FlowJo (Treestar, Ashland, OR).

HLA-DR peptide binding assay

Peptide binding to HLA-DR molecules was assessed by competitive ELISA, as previously reported (35). HLA-DR molecules were immunopurified from consanguineous EBV-transformed lymphoblastoid B cell lines by affinity chromatography. A biotinylated reporter peptide was incubated with a dose range of LF peptides and the appropriate HLA-DR molecule. After 24–72 h, the supernatants were transferred into ELISA plates previously coated with L243 mAb and incubated at room temperature for 2 h. The presence of biotinylated peptide/HLA-DR complexes was revealed using streptavidin-alkaline phosphatase conjugate (GE Healthcare, Saclay, France). Emitted fluorescence was measured at 450 nm postexcitation at 365 nm on a Gemini Spectramax Fluorimeter (Molecular Devices, St. Gregoire, France). Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment. Peptide concentration that prevented binding of 50% of the labeled peptide (IC_{50}) was evaluated. Data were expressed as relative affinity: ratio of IC_{50} of the peptide to IC_{50} of the reference peptide. Sequences of the reference peptide and their IC_{50} values were the following: HA 306–318 (PKYVKQNTLKLAT) for DRB1*0101 (4 nM), DRB1*0401 (8 nM), and DRB1*1101 (7 nM); YKL (AAYAAA-KAAALAA) for DRB1*0701 (3 nM); A3 152–166 (EAEQLRAYLDGTGVE) for DRB1*1501 (48 nM); MT 2–16 (AKTIAYDEEARRGLE) for DRB1*0301 (100 nM); and B1 21–36 (TERVRLVTRHIYNREE) for DRB1*1301 (37 nM).

Results

Naturally infected individuals show strong CD4 responses to PA and LF

Immunity was analyzed in nine individuals living and working in the Kayseri region of Turkey who had been treated at Ericyves University.

FIGURE 1. T cell responses to anthrax PA and LF in naturally infected, AVP-vaccinated, and healthy control individuals. T cell IFN-γ release in response to the toxin components LF and PA were quantified using ELISpot in naturally infected (○), vaccinated (□), and healthy control (△) cohorts. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs. *Denotes responses that are significantly (p < 0.001) elevated in comparison with those detected in healthy controls (Mann-Whitney U test).

FIGURE 2. The IFN-γ response to LF proteins and peptides is predominantly derived from CD4 T cells. PBMCs from a subset (n = 5) of AVP-vaccinated individuals were stimulated for 24 h with LF, LF domain IV, or a pool of LF peptides. A. Statistically significantly elevated levels of IFN-γ were produced in the stimulated lymphocytes compared with the negative control samples (Wilcox signed rank test). B. A representative flow cytometry plot, showing the response of one subject to the LF peptide pool, clearly demonstrates that the IFN-γ is being produced by the CD4 T cells (CD3+ CD4+ CD8− NK1.1−), as opposed to the CD8 T cells (CD3+ CD8+ CD4− NK1.1+) or the NK cells (CD3− NK1.1+). C. The dominance of CD4 T cells in the release of IFN-γ can clearly be seen in response to rLF protein, rLF domain IV only, and the LF peptide pool.
Hospital for clinically confirmed cutaneous anthrax. All had contracted the infection through contact with infected livestock. These infections had occurred between 0.4 and 7.5 y prior to the study and had been treated with acute antibiotic therapy (Supplemental Table Ia). We compared this group with healthy, unexposed individuals (Supplemental Table Ic) and, as a positive control cohort, individuals who had been hyperimmunized on the standard U.K. schedule with 7–14 doses of the AVP vaccine over 3.5–10 y (Supplemental Table Ib). Fig. 1 shows IFN-γ ELISpot responses of these individuals to anthrax LF and PA Ags. Both naturally infected donors and vaccinees make robust T cell responses to LF. Although naturally infected individuals mount significant responses also to PA, this response in the vaccinees was not significantly different from that seen in naive donors. Thus, whereas natural infection induced significant T cell immunity to PA and LF, AVP vaccinees showed significant immunity only to LF. This finding is unexpected, given that LF is produced by the bacterium at considerably lower levels than is PA and is a minor component of the AVP vaccine (15). However, it may reflect the differential processing of these Ags during infection and vaccination—PA and LF secreted from the bacterium being intracellular, compared with the extracellular PA and LF presented during vaccination. Although it would have been highly unlikely that our 20-mer peptides could have bound to HLA class I peptide binding grooves and so activated CD8 cells, we sought to confirm that our ELISpot analysis indeed reflected IFN-γ responses of CD4 cells. Cells from a subset (n = 5) of the AVP-vaccinated cohort were stimulated with LF, LF domain IV, or LF peptides and intracellular IFN-γ staining was performed to establish the cell populations responding to these Ags. It can clearly be seen (Fig. 2) that the IFN-γ response is overwhelmingly predominated by CD4 T cells.

Evaluation of the cytokine profile produced in response to LF and PA

It was possible that the lack of IFN-γ T cell responses to PA in AVP-vaccinated individuals was a reflection of the Th2-biasing nature of the vaccine, and that the response in these individuals would be apparent in other cytokines. To assess this, cytokine levels in the cell

FIGURE 3. The deficiency in response to PA seen in AVP-vaccinated individuals is not restricted to Th1 cytokines. Cytokine levels were quantified in supernatants recovered from the LF- and PA-stimulated ELISpot assays from naturally infected individuals (black bars), AVP-vaccinated individuals (gray bars), and unexposed controls (white bars). The levels are expressed as Δpg/ml ± SEM (pg/ml in stimulated well – pg/ml in negative control well). The vaccinated individuals have a suppressed Th2 response, as seen by IL-5 and IL-13 levels, to both LF and PA when compared with naturally infected individuals. There is also a diminution of the IL-2, IL-9, and IL-10 levels in the vaccinated cohort compared with those exposed to the Ags in the context of natural cutaneous infection.
culture supernatants recovered from ELISpot assays were determined (Fig. 3). The T cell responses to both PA and LF in naturally infected individuals variably encompassed Th1, Th2, Th9, and regulatory cytokines; that is, there was significant induction of IL-2, IL5, IL13, IL-9, and IL10. Analysis of cytokine responses in vaccinees revealed a marginal IL-5 and IL-9 response, confirming that such T cell memory as prevails may indeed be Th2/Th9 skewed.

Magnitude of the T cell response is related to duration of infection

Although all cutaneous anthrax patients showed CD4 T cell responses to PA and LF, the magnitude varied substantially, with some individuals showing responder T cell frequencies that were 30 times higher than in others (Fig. 1). We predicted that those with the most severe and long-lasting infections might show the most impaired responses. On the contrary, we found that, with respect to responses to PA (but not LF), there was a significant positive correlation between magnitude of response and duration of infection (Fig. 4). Interestingly, the subject who developed toxemic shock during the course of infection, and thus theoretically had the highest level of exposure to anthrax toxins/Ag, had the highest level of responses to both PA and LF.

T cell immunity to LF is strongly focused on domain IV

In light of the ability of LF to subvert Ag processing, it was important to establish whether this had an impact on preferential immune recognition of the individual domains of LF. Domain I contains the PA docking site, domain II is structurally similar to the ADP-ribosylating toxin of Bacillus cereus, domain III inserts into domain II and is believed important for LF activity (deletion of one of its four tandem imperfect repeats makes LT nontoxic), and domain IV has structural homology to thermolysin and encompasses the catalytic core (34). The catalytic binding site into which the N terminus of MAPK kinases fits involves components of domains II, III, and IV. From immunization studies of HLA class II transgenic mice with isolated LF domains and peptides, we know that immunogenicity is primarily within domains II and IV, although there are HLA-DQ restricted responses to domain III and a number of cryptic epitopes in domain I (K. Chu, S. Ascough, R. Ingram, L. Kim, E.D. Williamson, B. Mailleire, S. Sriskandan, D. Altmann, manuscript in preparation). We found that in naturally infected human donors, the T cell response was overwhelmingly focused on domain IV (Fig. 5); responses in naturally infected donors were 30-fold greater than in healthy controls (p < 0.0001), whereas responses of vaccinated individuals to the same domain were 10-fold greater than in healthy controls (p = 0.0039). No significant response to domains I, II, and III was observed.

CD4 epitope mapping of the response to LF domain IV

A number of human antiviral T cell responses have been shown to constitute “public specificities,” or common, dominant responses to an individual epitope involving a conserved TCR sequence (36). To determine if this was the case for anthrax, we epitope mapped the CD4 immune response to LF domain IV (Fig. 6A–C). We found an extremely heterogeneous response spread across the entire domain IV sequence. Some epitopes, such as peptides 554–573, 654–673, and 674–693, were predominantly a feature of the response of vaccinees, whereas others, such as the response to 714–733, were presented in a number of the infected donors but were rarely recognized by T cells from either vaccines or healthy controls. Epitope 714–733 overlaps the catalytic center of the metalloprotease. Given that this center is conserved across this class of metalloproteases and that they represent virulence factors in other bacterial pathogens,
this may constitute an example in which the immune response has focused attention on an epitope for which the microbial fitness cost of mutation would be too high.

We found no examples of “missing epitopes” of the type we had expected, that is, ones present in the context of vaccination but lost upon infection. If anything, the suggestion was that live infection unveils cryptic epitopes (714–733) not commonly recognized in the model response to injection of protein Ag, as has been proposed for other infectious diseases (37).

**HLA binding of immunodominant epitopes**

The pattern of LF peptides recognized by T cells in cutaneous anthrax patients does not lend itself readily to definition of the presenting HLA class II molecules owing to the very large number of different class II molecules represented in that cohort. However, some important clues regarding the breadth of HLA binding (and thus, the relevance to responsiveness in other individuals) can be obtained from direct HLA binding affinity studies. Four of the key anthrax LF CD4 epitopes we had defined—574–593, 654–673, 674–693, and 714–733—were analyzed for binding to a range of common HLA-DR alleles (Table I). Each of them showed relatively broad binding to several alleles. Indeed, 574–593, 654–673, and 674–693, all of which had been identified as common epitopes in responses of vaccinees, showed binding to each of the HLA class II products assayed (DR1, DR3, DR4, DR7, DR11, DR13, and DR15). Because these alleles together cover the vast majority of the population, responses to these epitopes would be expected in a large proportion of any given immunized human population. The epitope that we had picked up predominantly in the response of infected individuals, LF 714–733, also bound to HLA-DR1, 4, 7, 11, and 15, whereas there was no detectable binding to HLA-DR3 or 13. Binding of this peptide to HLA-DR15 is of a very high affinity. It is of note that all of the DR4+ subjects within the naturally infected cohort respond to peptide 714–733.

**Discussion**

Sixty years of therapeutic dependence on antibiotic treatment of bacterial infection have taken their toll on our conceptual understanding of the nature of adaptive immunity to bacterial pathogens. With a very small number of exceptions, including *Mycobacterium tuberculosis* and *Listeria monocytogenes* (38, 39), detailed analysis of the relationship between the adaptive response and specific, bacterial Ags, epitopes, and pathogenic mechanisms is limited. This omission becomes increasingly troubling as concerns about multidrug resistance increase and greater interest arises in the development of new epitope, multivalent (multiepitope and multipathogen) vaccines.
Through the National Institutes of Health–National Institute of Allergy and Infectious Diseases Immune Epitope Database, there has been an international initiative to map and characterize epitopes from a wide range of category A–C bacterial and viral pathogens of concern in the developing world and with respect to biowarfare threats (40). The current investigation benefited from being able to marry the resources and methodologies of the Immune Epitope Database to a valuable cohort of patients: individuals in the Kayseri region of rural Turkey who had been diagnosed with and recovered from cutaneous anthrax (41). Even several years after antibiotic-treated anthrax infection, strong T cell immunity was detectable. These data for strong memory back up clinical experience with anthrax in this endemic area where we have seen no cases of re-infection.

Most attention and indeed most vaccine strategies have focused on PA, the major target of T cell immunity in both infected and vaccinated individuals was LF, particularly domain IV. Infected individuals had strong CD4 T cell responses to both PA and LF, whereas vaccinated individuals had a significant response to LF only. This observation held true not only for the Th1 response; there was also a significant reduction in Th2 and Th9 responses to PA in vaccinated individuals. Further evidence that infection induces superior cellular immune response to anthrax in comparison with vaccination comes from the results of trials of live spore vaccines (42), in which it was shown by skin test positivity that cellular immune responses rapidly declined after vaccination. In contrast, the same research previously demonstrated that >90% of cutaneously infected individuals remained skin test positive a year postinfection (43).

Although it has long been established that immunity to either PA or LF can be protective in animal models, interest has focused overwhelmingly on PA, as the binding unit of an-A toxin complex. In the context of AVA vaccination, LF is a minor component relative to the major, active component PA, and indeed, during live bacterial infection, the bacterium produces (at least during in vitro culture) around one sixth as much LF as PA (20). However, a significant correlation was found between the duration of infection and T cell immunity to PA, but not to LF. Certainly, the immune responses of cutaneous anthrax patients confirm that production of LF is important in actual infection of humans with *B. anthracis*.

It is unclear at present why the CD4 memory response to anthrax infection should be so focused on domain IV of LF. We probed a number of epitope-predictive algorithms to determine whether the domain IV sequence might simply be more epitope rich. This is obviously not the case: with adjustment for the size of domains, domains I and IV (which differ markedly in actual magnitude of elicited response) each contain around the same number of predicted epitopes across common HLA types, whereas domain II would be predicted to contain considerably more. In pathogenesis, the LT complex endocytosed, and it is thought that the PA component remains in the endosome, releasing LF into the cytosol. Thus, an alternative possibility is that inherent differences exist between accessibility of the LF domains to Ag-processing enzymes. This might also explain the observed increased development of T cell immunity to the less available PA, with duration of infection.

The most surprising aspect of our study was the finding of robust memory responses in recovered anthrax patients. The observation in murine models had been that expansion of CD4 helper cells after immunization with LT-treated Ag-pulsed APC was severely impaired (25). In that setting, initial T cell activation takes place, followed by failure to develop normal effector memory phenotypes, possibly resulting in anergy (33). In model systems, profound defects in cell signaling and activation are seen both at the level of the APC and with respect to signaling downstream of TCR engagement (25, 33). The fact that the responses observed by us in the previously infected donors were not only of large magnitude, as judged by frequency, but were also associated with effector function, as judged by IFN-γ release, makes it unlikely that anergy had been induced. It may be that during the early stages of a cutaneous infection, when levels of LF are relatively low and not all of the PA and LF have yet been taken up by APC in the form of toxin, LF can still be taken up and processed as a conventional Ag. In the early stage of infection, both Ag processing and immune suppression may be present, but as the severity of the infection increases, immune cell death may increase owing to the large-scale production of LT. Even this view is not easily reconciled with the fact that we observed the highest T cell response in the person who had suffered the most extreme systemic infection, as judged by toxemic shock. There is a degree of dichotomy between predictions from the in vitro models and clinical observations: Effective immunity to cutaneous anthrax must commonly develop, as the majority of cutaneous anthrax cases are nonlethal and self-limiting. Furthermore, the immunosuppressive effects demonstrated for LT in murine models are somewhat dependent on the model and strain investigated (44). For example, the effects of LT are more pronounced on naïve than on mature dendritic cells (32).

Of specific CD4 T cell epitopes targeted within domain IV, one appears to be seen predominantly in the context of bacterial infection, as opposed to vaccination. Cases of this type have been termed “infection-specific cryptic epitopes,” the possibility being that the infection has in some way skewed those epitopes that are preferentially processed or recognized. For example, a CTL epitope from HIV reverse transcriptase has been described, its generation being dependent on the IFN-γ-mediated induction of proteosome subunits (34). It will be important to determine which analogous processes might operate during anthrax infection.

In summary, this study is a rare example in which detailed mapping of the T cell response several years after serious bacterial infection has been undertaken, in this case, for anthrax, a disease about which there is considerable current concern. The fact that some key T cell targets were primarily identified in the context of infection suggests that studies of this type will be important for the epitope vaccine strategies being developed for a wide range of serious bacterial infections.

**Disclosures**

The authors have no financial conflicts of interest.

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