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SYMPOSIUM

From Structure to Solutions: The Role of Basic Research in Developing Anthrax Countermeasures

Microbiology Graduate Program Seminar: Anthrax Toxin

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Dr. John Collier traced the discoveries that elucidated the structure and function of the anthrax toxin in his talk "Anthrax Toxin," which was part of the Microbiology Graduate Program Seminar Series at Yale School of Medicine on February 23, 2012. Dr. Collier, Professor of Microbiology and Immunobiology at Harvard University, began by noting the advantages to studying anthrax pathogenesis in a biosafety level-1 lab. This designation does not merely facilitate his research, but also reflects a larger trend of basic research being leveraged to develop translational applications. Basic research on toxin structure has led to the development of a vaccine by Dr. Collier's group. Next-generation prophylactics also may stem from recent discoveries uncovering a role for cellular cofactors that mediate toxin function. Finally, basic research into the toxin substructure has facilitated efforts to change the receptor tropism to target dysregulated cells for therapeutic purposes. The urgency around biodefense agents makes the choice of research priorities a salient issue. As such, this author submits that basic research occupies a unique and lucrative niche driving clinical applications.

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†Abbreviations: AT, anthrax toxin; AVA, anthrax vaccine absorbed; BSL, biosafety level; cAMP, cyclic AMP; CopI, coatomer protein I; DTA, Diphtheria Toxin fragment A; EF, edema factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IL-2, interleukin-2; LF, lethal factor; LFn, LF N-terminal domain; MAPK-K, mitogen-activated protein kinase-kinase; NIAID, National Institute for Allergy and Infectious Disease; NIH, National Institutes of Health; PA, protective antigen; PA63, protective antigen 63-kDa fragment; rPA, recombinant PA.

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Hazard suits, biocontainment labs, and public controversy. Even though Dr. John Collier, Professor of Microbiology and Immunobiology at Harvard University, has worked exhaustively on *Bacillus anthracis*, the causative agent of anthrax [1], one is hard pressed to find any of these in his lab. Dr. Collier has devoted much of his career to the study of bacterial toxins of diseases of great public health importance. Instead of being relegated to the bench, his work on how the anthrax toxin (AT†) is able to cause disease has yielded profound translational contributions. As such, his review of the field during his talk "Anthrax Toxin," part of the Yale Microbiology Graduate Program Seminar Series on February 23, 2012, serves as a powerful case study of how basic research on biodefense agents can be an effective approach to devising clinically relevant strategies to combat disease.

How three individual components combine to form the AT has been an area of intense interest for Dr. Collier. Even though the *B. anthracis* bacterium is classified as a Category A Priority Pathogen by the National Institute for Allergy and Infectious Disease (NIAID) [2], Dr. Collier noted that his work is classified as biosafety level (BSL)-1. Because he works on individual components of the toxin, which are nontoxic until combined, he enjoys the relative ease of conducting research without the additional regulation and resources required at higher BSL levels.

This fact merits a closer inspection of why this claim is important. Indeed, this author supports the implicit assertion that research on biodefense agents, even and especially at the BSL-1 level, can have profound translational consequences. Basic research on AT has led to the development of a more efficacious vaccine, the identification of cellular cofactors that may assist with toxin translocation, and the engineering of novel therapeutics that utilize AT as a platform for drug delivery. In light of the recent controversies and recalibrations over high-risk research, the importance and relevance of fundamental research becomes an especially relevant issue.

TOXIN-BASED VACCINE DEVELOPMENT

Although the bacterium cannot spread from human to human, *B. anthracis* is an attractive choice of bioweaponeers, due to its formation of hardy spores and relatively high morbidity and mortality rates [3,4]. Thus, countering a potential anthrax attack using vaccines and prophylactic countermeasures has been a biodefense priority for several decades [5,6]. In order to survive and replicate in an infected individual, *B. anthracis* must evade host defenses. One mechanism is to selectively kill off cells of the innate immune system [7,8]. To accomplish this, *B. anthracis* delivers components of the multimeric AT into the bloodstream, where they can encounter host immune cells [9].

AT is comprised of 83-kilodalton (kDa) Protective Antigen (PA), which serves as a pore [10,11] to translocate the other two components, 89-kDa Edema Factor (EF) and 90-kDa Lethal Factor (LF). Once delivered to the cytosol, EF acts as an adenylate cyclase [12], adding a cyclic AMP (cAMP) molecule onto critical proteins in neutrophils, leading to inactivation of these cells. The targeting of endothelial cells also results in fluid leakage and excess fluid build-up, known as edema. LF similarly inactivates host protein function via its protease domain, which irreversibly cleaves members of the mitogen-activated protein kinase-kinase (MAPK-K) family [13,14]. The impaired immune response combined with edema promotes bacterial replication in the bloodstream, leading to septic shock and possibly death.

The anthrax exotoxin is a critical virulence factor for *B. anthracis* pathogenesis [15,16]. Because of the requirement for the toxin in disease progression, developing a formulation targeting the toxin has been a priority for pharmaceutical companies interested in a vaccine. The federal government established Project BioShield in 2004, which sought to invest in vaccines and prophylactic agents to employ for defensive purposes. BioShield contracted for 29 million doses of Anthrax Vaccine Absorbed

(AVA) [17], an efficacious first-generation vaccine. AVA is comprised of an impure preparation of cultured supernatants from an avirulent *B. anthracis* strain.

The PA subunit alone was known to be sufficient for protection against *B. anthracis* challenge [18,19]. Thus, second-generation vaccines were developed that utilized recombinant approaches in a subunit vaccine. Vaccines based on recombinant PA (rPA) have been shown to successfully induce robust antibody-mediated protection [20]. However, next-generation vaccines also must address technical challenges such as reducing the high rate of dosing and improving stability over time and a range of temperatures.

To differentiate themselves from other rPA formulations, Dr. Collier's group took a different approach based on their research to develop a third-generation anthrax vaccine. Dr. Collier and colleagues noted that rPA could be optimized to increase immunogenicity and safety during human administration. Their variant takes advantage of structural and functional studies that have elucidated the fundamental organizing principles of the AT pore. The pore is comprised of either a heptameric [21] or octameric [22] circular complex of PA proteins. Under conditions of low pH, as occurs in the endosome, the PA "prepore" spontaneously converts into a bonafide membrane-spanning pore [23]. This is accomplished by the movement of loops that face outward from the pore lumen, moving down into the membrane and forming a 100Å linear pore [24]. This pore is capable of keeping substrates in an unfolded conformation and stimulating their translocation through the membrane into the cytosolic compartment.

Critical to pore functioning is the action of residue Phe427, which resides on the loops that swing into a pore conformation at low pH. Phe427 forms a ring-like complex called the Φ clamp with other Phe residues in the pore [25]. The Φ clamp is thought to function as a hydrophobic seal that preserves the local proton gradient in the pore, maintaining the force of directed diffusion during translocation [26]. Charge reversal mutations near the Φ clamp act as a dominant negative inhibitor of pore function. Dr. Collier's construct, which contains a double K397D D425K mutation, is capable of binding to other PA subunits but unable to translocate LF or EF through the pore [27]. The dominant-negative PA is more immunogenic than native PA, potentially due to increased affinity by immune recognition proteins.

Dr. Collier has translated this finding into a proprietary rPA formulation rooted in the dominant negative PA strategy. Initial tests in a rat model of LF toxicity showed protection when immunized with the mutated PA proteins [28]. Early thermostability studies have been successful [29], a primary consideration in its ability to be stockpiled. As it may be more efficacious than other vaccines, the vaccine may require fewer administrations than the six AVA currently requires to achieve protection. The vaccine is being commercialized by Soligenix, Inc. through a licensing agreement with Dr. Collier. As of press time, Dr. Collier's vaccine is being tested as SGX204 for both preventive and prophylactic indications. According to Soligenix, Inc., the proof of concept and animal testing phases were completed, with the compound currently in phase 1 clinical trials [30]. Thus, this compound has immediate translational applicability to developing medical countermeasures for anthrax infection. Dr. Collier's basic research into the critical residues in the PA pore led to the solution-identification of dominant negative mutations, opening up immediate commercial and translational applications.

IDENTIFYING CELLULAR CONSPIRATORS OF DISEASE

Cellular cofactors of anthrax toxicity may represent an additional area that can be translated for prophylactic purposes. Recent evidence shows that while anthrax toxin is sufficient to form a membrane pore and direct the toxic proteins LF and EF into the cell, this process may be enhanced by cellular cofactors. This is compelling not only for the previously underappreciated role for cel-

lular proteins in AT translocation, but also because these cellular proteins represent attractive novel targets for possible therapeutic intervention.

The classical paradigm in the field was that cellular receptors have a role only at the initial steps in AT trafficking. After PA is secreted from *B. anthracis*, it can bind to ubiquitous cellular receptors ANTXR1 [31] or ANTXR2 [32]. PA binding to ANTXR1 or ANTXR2 renders it susceptible to proteolytic cleavage by furin proteases [33], yielding a 63-kDa peptide (PA63) that is capable of binding LF and EF substrates [34]. After substrate binding is initiated, the PA63 prepore [11] and bound substrate are endocytosed through a clathrin-dependent mechanism [35,36]. Events downstream of these initial actions have been assumed to be largely independent of cellular cofactors.

The traditional model posited that substrate denaturation and translocation were driven solely by only changes in pH [23] in the endosomal microenvironment or electric potential in artificial systems [37,38]. Once environmental changes partially denature substrates [39], unfolding and protein "racheting" through the pore was thought to be self-sustaining. This is likely mediated through the Φ and α clamps, which may act to preserve the proton gradient driving translocation [25] and support an unfolded conformation by non-specific binding [40], respectively. Indeed, support for this was shown that in artificial lipid bilayers containing only phospholipids and assembled AT, translocation was observed with no further requirements for exogenous cellular proteins [25,37]. However, recent work has raised the possibility that although AT components are both necessary and sufficient for substrate translocation, cellular proteins may further optimize this process.

Recently, cellular proteins have been implicated in both the early process of substrate unfolding and the late process of protein refolding after a substrate has completed translocation. The N-terminal domain of LF (LFn) contains the PA-binding domain and is used as a model peptide for stimulating efficient translocation [41,42]. Diptheria Toxin fragment A (DTA) is often used as a model non-native substrate for the PA pore. This catalytic domain contains ADP-ribosylation activity, which causes death in susceptible cells. When LFn is fused to DTA (LFnDTA), translocation can be measured as a function of cell death resulting from ADP-ribosylation of cellular proteins [43]. Using this approach, Tamayo and colleagues investigated the role for cellular proteins that enhance the native unfoldase activity of the PA pore [44]. Using a trypsin protease protection assay, they identified the eukaryotic chaperone BiP (GRP78) as acting to unfold LFnDTA. Importantly, siRNA-mediated knockdown of BiP prevented intoxication of J774 macrophages by LF and EF. Further, cellular extracts containing ß-Cop, a component of the eukaryotic coatomer protein I (CopI) complex, were found to enhance translocation of LFnDTA compared to basal levels of native LF [45]. As before, antibody-mediated immunodepletion of ß-Cop from cellular extracts impaired this translocation.

This study led to the characterization of another cellular cofactor involved in the distal steps of substrate binding to the PA pore. After proteins complete translocation, they exit the pore in a linearized, unfolded state. Chaperone proteins must bind to the translocated substrates to mediate refolding into an active conformation. Dmochewitz and colleagues recently reported that CypA and Hsp90, well-characterized cellular chaperones, are required for the restoration of LFnDTA ADP-Ribosylation activity [46]. Interestingly, downregulating these proteins with pharmacological inhibitors protected cells from intoxication.

Thus, for cellular proteins that may enhance both early and late steps during intoxication, robust protection is observed when these proteins are downregulated through various mechanisms. Because of the preliminary experimental validation, these proteins may be attractive targets for future therapeutic interventions in human studies. Indeed, characterization of the anthrax toxin receptor has led to subsequent therapeutic interventions in combating angiogenesis

[47]. Taken together, these results may serve as the basis for future applications to target these proteins as a therapeutic approach to countering anthrax infection.

ENGINEERING THE TRANSLOCON AS A PEPTIDE DELIVERY SYSTEM

While AT co-opts host machinery to effect cellular dysfunction, researchers are seeking to co-opt the activity of AT for beneficial purposes. Dr. Collier's group is completing work using the finely calibrated translocation system to target and deliver proteins of therapeutic benefit to a specific subset of cells. Given the robust assembly and delivery of substrates by PA, Collier and colleagues are investigating whether they can manipulate this system to selectively target and kill dysregulated human cells in various disease contexts.

In the final portion of his talk, Dr. Collier offered insights into current work in the lab focusing on engineering AT to deliver toxic proteins exclusively into specified cells. His team has fused the epidermal growth factor (EGF) protein to the previously characterized C-terminus of the PA receptor domain [48]. His group is investigating if this fusion can change the receptor specificity of AT to bind the EGF receptor (EGFR). As certain types of cancers have upregulated EGFR at the cell surface [49], AT bound to EGF would specifically target cancerous cells for intoxication. Because the receptor tropism is altered, this could function in a precise manner, homing in on dysregulated cells while leaving healthy cells intact. Indeed, Dr. Collier described preliminary experiments indicating that the chimeric fusion competes with free EGF for binding to EGFR, whereas native AT does not. Future work will continue in the lab on this promising translational application, which has seen many benefits using a similar paradigm with other fusion proteins.

If Dr. Collier is successful, he will have optimized a system he conceived in the early 1980s. He previously described a fusion protein consisting of DTA fused to EGF as a technique to downregulate EGFR overexpressing cells [50]. With this strategy, the domain containing the ADP-riboslylation activity of DT is delivered specifically to pathogenic, EGFR-overproducing cells. Subsequent work by Dr. John Murphy's group in the 1990s saw commercial success with the replacement of the receptor binding domain of DT with interleukin-2 (IL-2) to yield chimeric DT-IL2 [51]. This fusion protein operates in an analogous manner to that of Dr. Collier's group, whereby the ADP-ribosylating activity of DT would intoxicate cells expressing IL-2 receptors at the surface. A similar fusion expressing the DT catalytic domain (DT389) fused to IL-2 is marketed as Ontak® (Denileukin diftitox) by Eisai Corporation, having gained full FDA approval in 2008 [52]. It is indicated for the treatment of cutaneous T-cell lymphoma, characterized by high IL-2 receptor expression in cancerous cells. Thus, Dr. Collier's work seems poised to take advantage of the promising field of toxin fusion therapeutics as a platform for selective drug delivery.

Drug delivery is the most immediate application of repurposing toxins for therapeutic use, but other creative approaches may provide additional platforms for molecular delivery. Dr. Collier noted in the seminar that the PA pore is capable of binding a broad array of peptides in addition to its native LF and EF substrates. That is, LF and EF contain a polybasic leader sequence [53] that is recognized by the pore or auxiliary cellular elements. However, this recognition is promiscuous, where the PA pore can translocate substrates with multiple amino acids combinations in the leader sequence. This is likely due to the function of the α clamp, which binds peptide helices non-specifically and could confer broad specificity to recognize multiple substrates [40].

Dr. Collier observed that LFn fused to an N-terminal His-6 tag was competent for translocation [37]. Further, an N-terminal Lys-6 sequence fused to the model non-native substrate DTA was found to translocate, albeit inefficiently [54]. Thus, as leader se-

quences containing exclusively basic residues are competent for translocation, it is an attractive possibility that the adaptability of the PA translocon could be leveraged. Perhaps compounds of interest could hijack the AT translocation machine to gain access to cellular cytosolic compartments. In the future, AT will likely be engineered to both deliver toxic molecules to pathogenic cells as well as deliver beneficial compounds to a broad range of human cells.

PERSPECTIVES

The cost and ease of research remain salient considerations as biomedical research budgets are stretched thin. These factors also represent a major difference between basic and applied research approaches.

Although intrinsically valuable, live-pathogen work, often consisting of "applied" research approaches, has limitations not observed in subunit research. According to a Sandia National Laboratories survey [55], 38 percent of investigators reported diverting research funds to comply with security protocols. At the federal level, the estimated biodefense budget for the National Institutes of Health (NIH) in 2005 allocated 8.9 percent of total expenditures specifically for the construction of biosafety laboratories [56]. Further, as additional administrative responsibilities accrue, the productive time that can be devoted to research is reduced. This effect can be quantified as a measure of publications produced in a given year normalized by the amount of federal funding in that year [57]. After select agent requirements were enhanced in 2002, the number of *B. anthracis* publications per million dollars reduced 5-fold compared to the output before 2002 [57]. These indirect costs and consequences must be considered in the total cost effectiveness of live-pathogen, select agent research.

Basic biodefense research, in contrast, is largely insulated from these accessory costs and loss of research efficiency. Indeed, the federal government continues to include basic research in its portfolio of biodefense research priorities. The 2005 NIH biodefense budget provided for $550.2 million for basic research, representing almost one-third of total biodefense expenditures [56]. These resources appear to be a sound investment when basic anthrax toxin research, for example, provides a source for translational applications.

Indeed, the elucidation of how substrates are recognized and translocated through the AT pore has achieved profound relevance to countermeasures for this disease. Dr. Collier's work has been, and will likely continue to be, leveraged in the development of efficacious vaccines and prophylactic compounds against anthrax infection. There are also new insights into repurposing AT as a molecular delivery system that will selectively target dysregulated cells in the context of overproliferation.

When basic research is included in the portfolio of approaches against our most dangerous pathogens, it yields results while remaining independent of the constraints of applied biodefense research. As such, basic biodefense research represents a lucrative area for discovering next-generation translational applications that are necessary for the continued protection of the general population.

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