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MEETING REVIEW

The International *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis* Conference, "*Bacillus*-ACT05"

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The International *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis* Conference, heretofore known as the "The *Bacillus* ACT Meeting" was held 25 to 29 September 2005 in Santa Fe, N.Mex. The conference represented a union of two meetings previously known as the International Conference on Anthrax and the International Workshop on the Molecular Biology of *Bacillus cereus*, *B. anthracis*, and *B. thuringiensis*. The mission of the *Bacillus* ACT05 Meeting was to bring together researchers involved in scientific research related to the physiology, genetics, molecular biology, and pathogenesis of these closely related bacteria.

Evidence of new research efforts concerning the *B. cereus* group species, fueled in part by global concerns related to bioterrorism, was apparent at the previous joint meeting held in the spring of 2003 in Nice, France. Two and one-half years later, fruits of these efforts were realized. New knowledge of host-pathogen interactions was apparent in many sessions of the 2005 conference. Investigations of the anthrax toxin remained a vital component of the meeting. Reports of toxin-induced physiopathological changes and the innate immune response were emphasized and debated. New information regarding the structure and function of edema toxin, previously overshadowed by studies of lethal toxin, was presented. In addition, data from various animal models of infection generated discussions of species- and strain-specific responses.

While numerous presentations focused on *B. anthracis*, considerable time was also spent on other species of the *B. cereus* group, and important similarities and differences in the species were revealed. The ecology of these species, a long-neglected area of investigation, gained attention at the meeting, which included reports concerning lifestyles of the bacteria in the soil and in the insect gut. The *B. anthracis* genomics explosion has had a major impact on research in all three species. Genomics not only has permitted delineation of phylogenetic trees comprised of *B. cereus* group strains, but has fostered generation of new hypotheses regarding specific physiological differences within the group. The innovative molecular epidemiology tools developed for *B. anthracis* will have broad application for analysis of other bacterial species. Finally, considerable interest in spore structure has emerged. The composition and function of the exosporium, an outer coating specific to the surface of spores of the *B. cereus* group, were queried and deliberated.

Approximately 350 participants representing over 20 countries gathered to discuss 213 oral and poster presentations. This meeting report is limited primarily to highlights of oral presentations.

THE GOOD AND THE BAD OF THE *B. CEREUS* GROUP

The tone of the meeting was set by Jo Handelsman of the University of Wisconsin, who in her keynote address reminded participants that bacteria of the *Bacillus cereus* group have distinctive relationships with eukaryotes, some harmful and some beneficial to humans. *B. anthracis* falls clearly in the harmful category because of its ability to cause animal and human disease as well as its potential role in bioterrorism. *B. thuringiensis* falls typically on the beneficial side of the ledger because of its potent insecticidal activity. *B. cereus* is usually placed in the harmful category, but Handelsman put forth that this placement is unfair to this multifaceted bacterium. She stated that most treatment of *B. cereus* species in the literature strips it of its "colorful and cosmopolitan lifestyle." Famed for its role in food poisoning, *B. cereus* is an abundant inhabitant of the soil and colonizes plant roots, sometimes having profound effects on the structure of the microbial community (19). Certain strains of the species are antagonistic to pathogenic protists of plants known as the oomycetes. Consequently these *B. cereus* strains have tremendous biological control potential. The strains with plant disease-suppressive activity produce the novel and unusual antibiotic, zwittermicin A, which inhibits growth of protists and some gram-negative and gram-positive bacteria (34). In addition to this unusually broad spectrum of antibiotic activity, zwittermicin A also acts as a powerful synergist of *B. thuringiensis* toxin (24). A genomic analysis of a plant-associated strain of *B. cereus* indicates that it differs significantly from many of the food poisoning strains and might represent a separate clade (20). Handelsman expressed hope that if this group is established, it will improve the reputation of *B. cereus*, reminding its fans and detractors alike that *B. cereus* is a complex species that contributes to plant health and human welfare in a variety of ways.

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GENOMICS AND MOLECULAR EPIDEMIOLOGY

Genomic analyses of *B. cereus*, *B. anthracis*, and *B. thuringiensis* strains are in fact leading to a better understanding of similarities and differences within the *B. cereus* group. The genomics and molecular epidemiology of these species have greatly matured in the last 3 years, with unparalleled information about strains and genomic variation currently available. Indeed, these data are being integrated with all aspects of *Bacillus* biology in such a manner that genomics may not be a stand-alone topic at future meetings.

Paul Keim (Northern Arizona University) presented data from a multiple-institution consortium and from six *B. anthracis* whole-genome sequences generated by The Institute for Genomic Research. Variation at >3,500 single nucleotide polymorphisms (SNPs) and 15 variable number of tandem repeats (VNTR) loci were used to define the population structure of this highly clonal pathogen (26). Three major clonal lineages (A, B, and C) were identified and used to reconstruct historical transmission patterns, including a massive evolutionary radiation in the A branch. The A group had a much greater apparent fitness, based upon its global distribution and frequency, associated with a recent bottleneck and expansion. A molecular clock age estimate, based upon the genomic frequency of sSNPs, predicted a mid-Holocene time period for the A-branch radiation. This is consistent with human civilization activities, including domestication of livestock, the development of agriculture, and increased commodity transportation.

Matthew Van Ert (Northern Arizona University) used these phylogenetic and genomic data to develop a strain detection and identification technology that is near the theoretical limits for sensitivity (single molecule) and specificity (single-nucleotide differences) (15). His approach capitalized upon the phylogenetic distribution of the SNPs by identifying canonical SNPs for marking key nodes in the phylogenetic tree (22). He showed how these could be converted into real-time PCR assays specific to a lineage or even a single strain. Three such assays were developed to specifically identify the Ames strain that was involved in the anthrax letter attacks of October 2001. The phylogenetic understanding of this pathogen greatly enhances the confidence in these assays while greatly simplifying analytical problems.

Angelo Scorpio (U.S. Army Medical Research Institute of Infectious Diseases [USAMRIID]) used genomic data to study metabolic trends based upon proteomic analysis of *Bacillus anthracis* during growth under various in vitro and in vivo conditions using a guinea pig model of anthrax infection. Besides the usual virulence factors, there were a surprising number of metabolic genes that were expressed during the infection, especially those involved with fermentation of various sugars, as well as secreted and membrane peptides that may represent future vaccine targets. This study represents a prime example of how genomic sequencing can be used along with a functional analysis of an infection model.

Jacques Ravel (The Institute for Genomic Research) presented data on a new tool: a *B. anthracis* whole-genome tiled custom Affymetrix Gene Chip. This high-density microarray can be used for genotyping and expression analysis; however, the initial goal of SNP typing had not been realized as the tiling of the genome was not dense enough. He mentioned that this has been addressed in a new version of the Gene Chip with a much denser tiling that will hopefully be able to address the SNP typing on a chip. This unique tiling design has been shown to be useful for genotyping unknown isolates as well as expression analysis. This presentation brought to light the new technologies that can be developed based on the bacterial genome sequence and how the *Bacillus* field has been a leader in this arena. This new multifunctional array design allows interrogation of a number of interesting biological questions with one tool.

ECOLOGY AND DETECTION

The ecology of anthrax has long been based upon a model of spore cycling between a mammalian host and a soil reservoir. In each of these major environments, the lifestyle of the bacterium is affected by complex interactions with abiotic and biotic factors.

The amplification of the pathogen in the host is obvious and has been the focus of many studies, but Elke Saile (University of Texas—Houston) presented data on the soil component in her talk. She demonstrated that the rhizosphere of plants could support the growth of *Bacillus anthracis* and that mixed cultures of *B. anthracis* strains could exchange genetic markers (30). While *B. anthracis* growth in the soil may or may not be important for amplifying infectious spores, it clearly offers the opportunity for the transfer of toxin genes and, possibly, the creation of novel pathogenic strains.

Saile's studies were in a model system, while Susan Barns (Los Alamos National Laboratory) surveyed naturally occurring soil and aerosol samples from across the United States for *B. anthracis* and closely related species. Her approach was based upon 16S RNA PCR primers broadly targeting the *B. cereus* group along with more specific PCR primers for the *B. anthracis* toxin genes. This extensive survey of >15,000 samples revealed the presence of *B. cereus* group members and even sequences with very high similarity to *B. anthracis*. These close relatives may not have the ability to cause anthrax, but they may easily be mistaken for bona fide *B. anthracis* in detector assays. In addition, they demonstrate the widespread prevalence of near neighbors to *B. anthracis* in the environment.

Studies based upon unusual human disease cases were presented by Alex Hoffmaster (Centers for Disease Control and Prevention), who described *B. cereus* isolates harboring *B. anthracis* virulence factors (11, 21). These strains were associated with severe and even fatal infections in patients with no obvious underlying conditions. Four independently isolated *B. cereus* strains were described, including one that contained a pXO1 plasmid that was nearly identical to that found in *B. anthracis*. From both the Hoffmaster and Barns presentations, it is clear that the classic *B. anthracis* genes are not restricted to the main *B. anthracis* clades. In most cases, this may be phylogenetically independent of *B. anthracis* and represent lateral gene transfer events. In one case, it is quite possible that the pXO1 plasmid was transferred from the main *B. anthracis* clade into a *B. cereus* relative, which apparently became more virulent than we normally associate with *B. cereus* pathogens.

Plasmids are well recognized as highly mobile genetic elements, and two presentations focused on the plasmid content

of the *B. cereus* group species. The first was by Geraldine Van der Auwera (Universitie Catholique de Louvain) and focused on the evolution of pAW63, a *B. thuringiensis* plasmid that is similar to the pXO2 plasmid from *B. anthracis* (39). The second talk was by David Rasko (The Institute for Genomic Research), who described plasmid sequencing and analysis of the large plasmids from the *B. cereus* group that were most similar to the pXO1 plasmid from *B. anthracis* (29). Both presenters argued that there is a conserved gene pool among the *B. cereus* group. Each demonstrated that the plasmids they had examined show a similar "backbone" structure that is shared with other plasmids found within this group of pathogens. This backbone is exclusive to the *B. cereus* group, and during discussions after the talks were over, many investigators agreed that there may be a chromosomal element that is required for the maintenance and stability of such plasmids. These plasmid presentations are the first genomic studies to focus on the plasmids and show that while there are significant diversity and phenotypic variation derived from the plasmid content, there is also a conserved element that is limited to only this group. While Rasko was conservative in assigning function to plasmid genes, Van der Auwera was not and suggested the presence of a conjugation system that remains to be functionally examined and proven. The origins of replication of the *B. anthracis* virulence plasmid pXO1 and similar plasmids were also discussed. Saleem Khan (University of Pittsburg School of Medicine) presented work indicating a potential site of replication on pXO1; however, the entire replication machinery is not at this location, suggesting that it is possible/probable that the replication machinery has a chromosomal component.

Before the advent of molecular biology, sensitivity to gamma phage was used to discriminate between *B. anthracis* and other members of the *B. cereus* group. Whereas most *B. anthracis* strains are gamma phage sensitive, most *B. cereus* and *B. thuringiensis* strains are resistant to gamma phage lysis. Sophie Davison (Institut Pasteur) presented experiments identifying a bacterial protein essential for gamma phage adhesion (GamR, for gamma phage receptor) (13). This is an LPXTG protein that is covalently anchored to the peptidoglycan cell wall by a sortase protein. It appears that the specificity of gamma phage is due the *gamR* gene. Rare *B. cereus* strains sensitive to lysis contain genes highly similar to the *B. anthracis gamR* gene.

CELL STRUCTURE

The *B. cereus* group species, like all members of the genus *Bacillus*, form endospores. As the surface of the spore represents the first point of contact with host defenses and many detection devices, there is much interest in its structure and composition. Spores of the *B. cereus* group are distinguished by the possession of an outermost layer called the exosporium. The overall structure of the exosporium is a paracrystalline basal layer and an external hair-like nap. Several labs have undertaken the task of identifying and characterizing the proteins of the exosporium.

The hair-like nap of the *B. anthracis* exosporium is comprised of a glycoprotein, BclA. The length of the collagen-like central region of BclA is strain specific, and it determines the length of the nap. Several new findings about BclA and other exosporium proteins were presented at the meeting. Jeremy Boydston (University of Alabama at Birmington) described the orientation of BclA within the hair-like nap of the exosporium. The carboxy-terminal domain of BclA forms the distal end of each filament, while the amino-terminal domain appears to be anchored at the basal layer (6). The central collagen-like region of BclA is apparently highly extended. Boydston also reported that the collagenase sensitivity and CD spectroscopy of recombinant BclA reveal a trimeric collagen-like structure. This structure is stabilized by strong interactions between carboxy-terminal domains. The trimers are resistant to high temperature and proteases, suggesting that BclA and particularly its carboxy-terminal domain form a rugged shield around the spore.

Christopher Steichen (University of Alabama at Birmingham) and Patricia Sylvestre (Institut Pasteur) determined independently that the exosporium protein BxpB (also known as ExsFA) plays a role in attachment of BclA to the basal layer of the exosporium (37, 38). According to Sylvestre, a mutant lacking the *bxpB*/*exsFA* gene and the paralogue *exsFB* produces spores that are completely devoid of filaments. Steichen reported that a *bxpB*/*exsFA* mutant germinates earlier than the parent strain, indicating that BxpB/ExsFA suppresses germination. Also, BxpB/ExsFA forms a stable complex with BclA and the basal layer exosporium proteins ExsY and BxpZ (also known as CotY). Steichen's data indicate that exosporium assembly on spores of an *exsY* mutant is aberrant, with assembly arrested after the formation of a cap-like fragment that covers one end of the forespore. The cap contains an irregular basal layer but a normal hair-like nap. Studies of the precise roles of these and other surface proteins in kinetic and spatial assembly of the exosporium are ongoing in both laboratories.

There was much interest and discussion concerning a potential role of the *B. anthracis* exosporium in anthrax disease. A number of investigators suggested that the exosporium was not a significant virulence factor; however, no primary data were reported addressing this issue. The exosporium does not appear to be tightly associated with the underlying spore coat; application of gentle mechanical force can lead to its complete removal from spores. Thus, it is likely that, in addition to the exosporium, components of the underlying spore coat are detected by the host in early stages of infection. Adam Driks and Rebecca Giorno (Loyola University Medical Center) reported studies of the *B. anthracis* orthologues of *B. subtilis* coat proteins, focusing on those with major roles in spore assembly. Their data indicate that the orthologues have different functions in the two organisms. A particularly novel finding was that *B. anthracis cotE* mutant spores have a relatively minor coat defect (in contrast to the *B. subtilis cotE* mutant) but have a significant exosporium defect that leaves many spores without any detectable exosporium. Their data indicate that CotE is involved in the assembly of a structure that spans the interspace and connects the coat and exosporium.

The spore cortex, a thick layer of peptidoglycan lying underneath the spore coat and surrounding the spore core, maintains spore heat resistance and dormancy. Hydrolysis of the cortex is a key process in germination. David Popham (Virginia Tech) presented studies of the *B. anthracis* spore cortex structure. His findings show that the structure of the cortex peptidoglycan of *B. anthracis* is very similar to that of other *Bacillus*

species. However, novel cortex fragments were observed during *B. anthracis* germination, indicating that the types of cortex lytic enzymes may vary between species.

Vegetative cell structure received considerably less attention than spore structure. However, there continues to be considerable interest in the composition, development, and function of the *B. anthracis* capsule. The capsule, which is composed only of poly-γ-D-glutamate, is a major virulence factor of the bacterium. Prior to the meeting, a four-gene operon, *capBCAD* was reported to encode three proteins, CapB, CapC, and CapA, necessary and sufficient for polyglutamate synthesis, and a fourth protein, CapD, which possesses polyglutamate depolymerase activity.

Thomas Candela (Institut Pasteur) described experiments demonstrating that the polyglutamate is anchored directly to the peptidoglycan and that the bond is covalent (8). His data indicate that CapD is a γ-glutamyltranspeptidase. The work strongly suggests that the enzyme catalyzes the capsule anchoring reaction. Cells of a *capD* mutant are surrounded by polyglutamate material that is not covalently associated with the cell surface. Moreover, Candela showed a fifth gene of the *cap* operon, *capE*, is required for formation of capsule (9). The 47-amino-acid peptide encoded by *capE* is localized in the *B. anthracis* membrane and appears to interact with CapA. Interestingly, in a mouse model employing atoxigenic strains, a *capD* mutant is far less virulent than the parental strain and a *capE* mutant is completely avirulent.

DEVELOPMENT

The first step in the development of a metabolically active vegetative cell from a dormant spore is spore germination. In this process, small molecules and ions serve as indicators of conditions permissive for cell growth. These indicators, called germinants, penetrate to the inner membrane of the spore and initiate a signal transduction process by binding to receptors present in the spore. Generally, a given species possesses multiple receptors that exhibit specificity for various germinants. Anne Moir (University of Sheffield) reported studies of an additional inner membrane protein of *B. cereus*, GerN, and its paralogue, GerT. Genes encoding GerN and GerT are also found in *B. anthracis* and *B. thuringiensis*, but not in *B. subtilis*. GerN is an Na^+/H^+ K^+ antiporter that is required for normal spore germination in response to inosine and for germination in the combination of alanine or inosine plus aromatic amino acids. GerT is less important in spore germination and more important in outgrowth at an alkaline pH, as would be experienced in the insect midgut.

While there has been considerable effort in recent years to pair various germinant receptors of the *B. cereus* group species with specific environmental signals, less attention has been given to mechanisms of sporulation initiation by these species. Andrea White and Marta Perego (The Scripps Research Institute) presented talks describing components of the phosphorelay signal transduction system that controls sporulation initiation in *B. cereus* group isolates. Their data indicate that acquisition of pXO1 seems to require the loss of sporulation sensor histidine kinase activity. The sequences of $pXO1^+$ *B. anthracis* strains indicate that two of the nine sporulation kinase genes harbor frameshift mutations. A frameshift mutation was also noted in one of these genes in the pathogenic pXO1-bearing *B. cereus* strain G9241. The residual sporulation activity observed in the $pXO1^+$ *B. anthracis* Sterne strain is the result of the cooperative function of the remaining seven sporulation histidine kinase proteins. However, one of them, encoded by gene BA2291, may have an especially relevant role due to its ability to act as either an activator or an inhibitor of the phosphorelay, depending on the presence or absence of activating signals, respectively (7).

Perego also reported that a pXO1-encoded Rap phosphatase can inhibit sporulation initiation in the absence of its specific Phr pentapeptide inhibitor (4). Since the production of this pentapeptide is dependent upon an export-import processing pathway, any event that may reduce the concentration of the peptide, such as dilution in the bloodstream, would contribute to the inhibition of the sporulation process. Perego put forth the intriguing hypothesis that the ability of *B. anthracis* to avoid sporulation in the body while retaining the capability to sporulate in the environment has been selectively developed by this organism, perhaps in response to the acquisition of the virulence plasmid pXO1. Poor activation of the sporulation histidine kinases together with lack of inhibition of the Rap phosphatase may synergistically provide the optimal conditions for toxin production and virulence.

Another area that has previously attained little attention is that of motility of the *B. cereus* group. *B. cereus* and *B. thuringiensis*, unlike *B. anthracis*, possess peritrichous flagella and exhibit a specialized form of flagellum-driven motility termed swarming motility that allows cells to move collectively across solid surfaces. In swarming bacteria, the ability to swarm depends on a complex surface-induced differentiation process characterized by the production of elongated and aseptate cells. These cells exhibit a remarkable increase in the number of flagella in comparison with that of the short oligoflagellated swimmer cells. In some microorganisms, the development of swarming motility is linked to increased secretion of virulence factors. Emelia Ghelardi (University of Pisa) presented an update regarding genes required for swarm cell differentiation in *B. cereus* and *B. thuringiensis*. Several nonflagellar genes encoding transmembrane proteins and enzymes are required for the swarming phenotype in these species. One of these, FlhR, appears to modulate protein secretion and in other microorganisms regulates the number and position of flagella on the cell surface. The data indicate that *B. thuringiensis* and possibly *B. cereus* exhibit coordinate secretion of flagellin and virulence-associated proteins.

GENE REGULATION AND GENETIC TOOLS

As was true for the previous meeting, there was significant discussion of the global regulator *plcR*, which controls expression of numerous virulence-associated genes in *B. thuringiensis* and *B. cereus*. Michel Gohar (Institut National de Recherche Agronomique [INRA]) used bioinformatics and transcriptome analysis to update the *plcR* regulon and revise the PlcR target sequence (PlcR box). In batch culture, the regulon includes 30 genes located downstream of 22 PlcR boxes. In addition to known and putative virulence factors, these genes encode proteins involved in cell wall turnover, detoxification, and motility. Christina Neilson-Roux (INRA and the Institut Pasteur) re-

ported tissue-specific expression of *plcR*-regulated genes during *B. cereus* infection of *Galleria mellonella* larvae (5). Histopathological analysis of infected larvae revealed multiplication, adhesion, and colonization of the intestinal epithelium for isogenic parent and *plcR* mutant strains. However, only infection with the parent strain resulted in cell damage, necrosis, and hemocoel invasion. Neilson-Roux also showed results of an in vivo expression technology (IVET)-based approach which revealed in vivo activation of genes encoding proteins involved in bacterial metabolism, protein, gene regulation, and virulence.

Monika Ehling-Schulz (Technical University of Munich) described the genetic basis of cereulide synthesis in *B. cereus*. This emetic toxin is produced via modular enzyme complexes called nonribosomal peptide synthases (NRPS). The 23-kb *ces* (cereulide synthetase) gene cluster is located on a plasmid in emetic *B. cereus* strains and is flanked by sequences showing significant homology to plasmids of the other *B. cereus* group species. The *ces* cluster includes an open reading frame (ORF) predicted to encode an ABC transporter which is cotranscribed with the structural *ces* genes. Ehling-Schulz speculated that the transporter confers self-resistance to the toxin (16).

Tjakko Abee and Willem Van Schaik (Wageningen University) presented talks describing the roles of various extracytoplasmic function (ECF) and other sigma factors on *B. cereus* gene expression in stress conditions associated with food processing and preservation. Transcriptome and proteome analyses indicate roles for these genes in biofilm formation, sporulation, and other stress responses (14, 40). Van Schaik focused on the stress response sigma factor SigB. A *sigB* mutant was affected for high- and low-temperature adaptation, metabolism of nitrogen sources, germination, and sporulation. Of particular interest is the fact that a unique regulator, RsbY, plays a key role in *B. cereus* SigB activation. The RsbY phosphatase has an amino-terminal CheY-like response regulator domain indicating a structural difference from regulators of SigB activity established in other gram-positive bacteria.

An ECF sigma factor of *B. anthracis* was implicated in expression of the β-lactamase genes *bla1* and *bla2* of *B. anthracis*. Cana Ross (University of Texas—Houston) reported that deletion of ORFs BA2502 and BA2503, predicted to encode an ECF sigma/anti-sigma factor pair, from a penicillin-resistant clinical isolate abolished *bla* gene expression. Deletion of these ORFs in a prototypical penicillin-susceptible strain had no effect on *bla* expression. Complementation of the deletion mutants with the ORFs from the penicillin-resistant strain conferred β-lactamase activity upon the recombinant strains, while complementation of the mutant strains with the corresponding ORFs from the penicillin-susceptible strain did not result in enzyme activity. Differences in ECF/anti-ECF function were attributed to nucleotide differences in the ECF/anti-ECF genes from the penicillin-resistant and -susceptible strains.

TOXINS

Studies of the function and structure of the anthrax toxin proteins were a mainstay of the meeting. Many presentations provided new insights on the physiopathological changes induced by anthrax edema toxin (ET; protective antigen and edema factor [EF]) and lethal toxin (LT; protective antigen and lethal factor [LF]) in the host and the roles of host defenses during infection.

Two talks concerned the interaction of protective antigen (PA) with the two cell surface receptors ANTXR1/TEM8 and ANTXR2/CMG2. John Young (Salk Institute for Biological Studies) presented the unexpected finding that receptor type can dictate the pH threshold of anthrax toxin pore formation. Specifically, the pore is formed at pH values around 6.2 when PA is bound to the ANTXR1/TEM8 receptor, but at pH 5.2 when it is bound to ANTXR2/CMG2 (28). This result is likely to have important implications for understanding precisely where in the cellular endocytic pathway the pore is formed when PA is bound to either receptor. Young also presented evidence that complete receptor dissociation might be a component of the toxin pore-forming mechanism and that LF can be translocated into cells under mildly acidic pH conditions. In addition, he described an altered allele of PA that binds specifically to ANTXR2/CMG2 and that supports lethal toxin killing of rats, thus illustrating the physiological importance of this receptor. He presented data demonstrating that soluble receptor decoys are effective inhibitors of anthrax lethal toxin killing in cultured cells and in Fisher rats (31).

Wensheng Wei (Stanford University) put forth a new model in which a novel cell surface protein serves as an additional essential component for anthrax toxin lethality. Antibody directed against epitopes in the extracellular domain of the protein provided specific and extensive protection from LT-induced lethality in cultured macrophages. Wei's data indicate that this additional component interacts with ANTXR1/TEM8 and ANTXR2/CMG2. He showed that entry of complexes containing these proteins into cells is triggered by exposure to PA.

Many oral and poster presentations concerned studies of host responses to the anthrax toxins. Jeremy Mogridge (University of Toronto) examined the effect of LT on cytokine levels in human endothelial cells using quantitative real-time PCR (3). He found that interleukin-8 (IL-8) mRNA was significantly reduced after toxin treatment, leading to reduction in the amount of secreted cytokine. The reduction in IL-8 mRNA was due to a decreased half-life of IL-8 mRNA, an effect that was mapped to an AU-rich LT-responsive element in the 3′-untranslated region of this transcript. This effect of LT was not restricted to IL-8 mRNA because IL-1β mRNA, which also contains an AU-rich element, was also destabilized by the toxin in a human monocytic cell line. Since there are over 900 human mRNAs that contain similar AU-rich elements, it seems likely that LT will cause the destabilization of a number of other cellular mRNA transcripts. The effect of LT on mRNA stability may be explained by cleavage of mitogen-activated protein kinase kinases (MAPKKs) because pharmacological inhibitors of the ERK, p38, and JNK pathways also decreased stability of IL-8 mRNA. Mogridge's work demonstrates a novel mechanism by which a pathogen interferes with host cytokine responses.

The possible role of LT on vascular dysfunction, a component of systemic anthrax pathogenesis, was investigated by Felice D'Agnillo (U.S. Food and Drug Administration). D'Agnillo's talk focused on LT effects on human endothelial barrier function, adhesion molecule expression, and chemokine production (36, 41). Primary human lung microvascular endothelial monolayers exposed to LT showed a dose- and

time-dependent decrease in barrier function which was not dependent on cell death. The data were consistent with changes in endothelial morphology that included cellular elongation, interendothelial gap formation, actin stress fiber formation, and vascular endothelial (VE) cadherin redistribution. LT was also shown to modulate key endothelial inflammatory functions that included the enhancement of cytokine-induced vascular cell adhesion molecule 1 (VCAM-1) expression and the inhibition of IL-8 production. This inhibitory effect of LT on chemokine production probably involves the LT-mediated cleavage of mitogen-activated protein kinase kinases (MEKs) as a causative mechanism. D'Agnillo highlighted the potential relevance of these findings to the vascular pathologies of anthrax, such as hemorrhages, vascular leakage, and vasculitis.

Fiorella Tonello (University of Padua) described a set of studies showing that both EF and LF are strong inhibitors of the activation and proliferation of T cells and importantly that there is a strong synergism between EF and LF (25). These results point to the importance of the combined effects of both lethal and edema toxins and indicate that the toxins effects on T cells could play a major role in determining the long recovery period in cases of cutaneous anthrax, which seems to depend on the production of anti-PA, -EF, and -LF antibodies.

Nicholas Duesbery (Van Andel Research Institute) is attempting to exploit anthrax lethal toxin as a tumor-specific agent based on the ability of LF to cleave and perturb the function of multiple MAPKKs. In vitro observations indicate that LF can inhibit proliferation of several cancer types, notably melanoma, but also leukemias, non-small cell lung cancer, colon cancer, and renal and prostate cancers. Duesbery reported that LF can block and even revert growth of human tumor-derived tumors (melanoma) in xenograft experiments using athymic nude mice (1). Toxin-treated tumors showed a marked lack of vascularity, appearing anemic and staining poorly for endothelial markers. To test whether LF can be used to treat endothelial tumors such as Kaposi's sarcoma (KS), he used human herpesvirus 8 (HHV8) vGPCR-expressing murine endothelial cells. HHV8 vGPCR is an upstream activator of multiple MKK pathways, and its expression is sufficient to drive the formation of vascular lesions resembling KS in mice. In vitro, LF treatment caused decreased proliferation of these cells and blocked their ability to release vascular endothelial growth factor. In xenograft models, treatment with LF caused these tumors to dramatically regress in size. These results indicate that LF may be an effective therapeutic agent in the treatment of KS as well as other cancers.

Although anthrax toxin discussions are often dominated by reports of LT function, a number of investigators presented new information regarding ET. Aaron Firoved (National Institute of Allergy and Infectious Disease) presented a detailed characterization of ET-induced pathology in a murine model (17). Intraveneously introduced ET was lethal to BALB/cJ mice. A time course analysis revealed extensive tissue damage, changes in serum chemistry and cytokine profile, and cardiac abnormalities; however, the meningitis and pleural edema commonly observed during lethal inhalation anthrax were not observed. Although the correlation between the pathology and the levels of ET produced in the animal during an infection and those observed in this intoxication model remains unknown, the study yielded important clues on the nature of ET-associated toxicity.

Wei-Jen Tang (University of Chicago) reported the molecular structure of calmodulin-bound EF (32). He also presented the revised catalytic mechanism of this enzyme, which is a "two-metal-ion catalysis" commonly utilized by most DNA and RNA polymerases. A clinically approved anti-hepatitis B virus drug, adefovir, which targets virus-specific DNA polymerase, can effectively block the ET-mediated alteration of cellular functions (33).

BACTERIUM-HOST INTERACTIONS

In presentations at previous meetings, most research addressing *B. anthracis* pathogenesis focused on the activities of purified anthrax toxin proteins and the effects of the toxins in cell culture and animal models. Whereas studies of host response to purified toxins continue to reveal important information regarding anthrax disease (as reviewed above), studies employing animal models of infection are growing. At this meeting, a number of investigators presented results of studies revealing bacterial and host response to infection with *B. anthracis*.

Two talks featured elegant techniques for identification of factors contributing to *B. anthracis* virulence. William Day (USAMRIID) described the use of a novel mutagenesis technology termed TraSH (transposon site hybridization) and competitive growth selection microarray analysis to obtain a better understanding of bacterial and host responses to infection in a guinea pig model. *B. anthracis* genes that are expressed predominantly in vivo and that are required for bacterial survival and pathogenesis were identified. The overall gene expression patterns indicated that *B. anthracis* uses carbon sources other than carbohydrates in the animal. The up-regulated genes included those associated with (i) enhanced uptake of peptides, fatty acids, and iron; (ii) increased biosynthesis of amino acids and heme; and (iii) increased protein and fatty acid catabolism. Metabolic and physiologic changes in the host that correlated with progression to a moribund state included pronounced hypothermia and decreased levels of glucose. Notably, Day's results were in general agreement with Angelo Scorpio's proteomic analysis of *B. anthracis*-infected guinea pigs (described above).

The application of in vivo-induced antigen technology (IVIAT) to *B. anthracis* was presented by Sean Rollins (Massachusetts General Hospital). A *B. anthracis* protein expression library was screened using sera from convalescent anthrax-infected humans and nonhuman primates. By using sera that had been preadsorbed against the bacteria grown in batch culture conditions, antigens specifically induced in vivo were detected. This approach led to the identification of 17 genes, including a number of *N*-acetylmuramoyl-L-alanine amidases.

Questions about the early innate immune response and the roles of macrophages and polymorphonuclear neutrophils (PMN) in the control of infection were addressed in two oral presentations followed by much discussion. Hao Shen (University of Pennsylvania School of Medicine) showed that PMNs were found to play an essential role in the early control of infection in a murine model employing intranasal infection with spores of the attenuated Sterne vaccine strain (Tox^+

Cap$^-$). PMN depletion resulted in overwhelming outgrowth and lethal infection of Sterne-resistant mice, whereas treatments augmenting the level of PMNs enhanced survival of susceptible A/J mice. Intranasal Sterne immunization (INSI) rapidly induced an innate immune response highlighted by activated dendritic cells. This resulted in synthesis of Th1-specific cytokines and mobilized T cells leading to production of PA-specific memory T cells. Shen's finding offers a possible means of immediate prophylaxis. However, whether or not INSI will provide long-term immunity against fully virulent *B. anthracis* remains a major question.

Christopher Cote (USAMRIID) reported a detailed analysis of the roles of macrophages and PMNs in BALB/c mice infected with the fully virulent Ames strain of *B. anthracis* (12). Experiments employing intraperitoneal and aerosol delivery of spores were presented. Cote assessed the susceptibility of mice to infection after treatments with macrophage or PMN depletion agents or, correlatively, after augmenting PMN or macrophage populations before spore challenge. His results provided evidence that macrophages have an essential role in early host defenses against infection by *B. anthracis* fully virulent spores, whereas PMNs, though necessary, appear to be secondary to macrophages.

Differences in *B. anthracis* infection models such as mouse strain, *B. anthracis* strain (pXO2$^+$ or pXO2$^-$), spore dose, and route of infection highlight critical questions of host-pathogen interactions that require further study. In some cases, various models employed by different groups of investigators revealed similar results. For example, data from multiple participants revealed lack of significant spore germination in the lung. In other cases, results of different groups were in conflict. Results in a poster presentation by Amy Herring-Palmer (University of Michigan) indicated no significant PMN influx in murine lungs up to 3 days following infection, whereas Shen reported a large PMN influx peaking at 12 to 24 h and detectable to 48 h. Clearly, such differences and similarities indicate that caution must be taken in making conclusions based upon one specific infection model.

VACCINES AND THERAPEUTICS

The current human anthrax vaccines licensed in the United States and United Kingdom are alum-precipitated or aluminum hydroxide-adsorbed culture supernatants from toxigenic (pXO1$^+$) noncapsulated (pXO2$^-$) *B. anthracis* strains. Although the vaccines are considered to be safe and effective, the need for frequent booster vaccinations and reports of occasional local reactogenicity are driving the quest for development of a second generation vaccine. Many studies have indicated that antibodies to PA are crucial for protection against virulent *B. anthracis* in animal models. Presentations included studies of PA- and non-PA-centered approaches for new vaccines and therapeutics.

Robert Mabry (University of Texas at Austin) showed that antibody fragments engineered to recognize PA with high affinity and conjugated to polyethylene glycol for prolonged circulation half-life, conferred significant protection (50 to 60%) against inhalation of *B. anthracis* spores (250× to 625× 50% lethal dose [LD_{50}] of the Vollum strain) in the guinea pig model, despite their lack of Fc regions (23). Because these antibody fragments can be produced quickly and relatively inexpensively, this approach might have a distinct advantage in developing antibody-based therapeutics for the treatment of anthrax and other emerging infectious diseases. In addition, immunoassays were developed to detect toxin in the systemic circulation of animals exposed to both Vollum and Ames spores. By applying an anti-PA antibody fragment as the capture ligand, a sandwich enzyme-linked immunosorbent assay (ELISA) approach was used to detect free PA in guinea pigs (0.07 to 24.32 μg/ml) and rabbits (82 to 102 μg/ml), both before and after the onset of symptoms. Using PA_{63} as the capture ligand for ELISA, the investigators were able to detect 10.8 to 15.2 μg/ml of LF in rabbits at the time of death. These studies provide important new insights into the levels of toxin which are associated with death.

Donald Chabot (USAMRIID) reported that including pXO2, the 96-kb plasmid bearing the capsule biosynthetic genes, in a live PA-expressing *B. anthracis* spore vaccine (ΔAmes-pPA102) dramatically increased protection in a guinea pig intramuscular challenge model (10). Chabot suggested that the encapsulated vaccine strain provided a stronger immune response to PA because of an adjuvant effect of capsule and/or increased persistence. A single dose of ΔAmes-pPA102 was highly efficacious in the guinea pig model and in a rabbit aerosol challenge model, even with challenge doses of over 1,000× LD_{50}. Indeed, a single dose was more efficacious than two doses of AVA (the U.S.-licensed vaccine) or rPA-alum (recombinant PA precipitated with alum) in the guinea pig model. Although the encapsulated vaccine was more efficacious than AVA or rPA-alum, the latter vaccines induced stronger anti-PA responses. Thus, other antigens, such as spore antigens and capsule, appear to contribute to protection.

Ian Glomski (Institut Pasteur) presented the results of extended studies showing that vaccination with formalin-inactivated *B. anthracis* spores induces protective immunity in a murine subcutaneous model against a nontoxinogenic encapsulated strain. Protection could be transferred with immune spleen cells but not serum. The vaccine was still effective in antibody-deficient mice, but mice depleted of CD4 T lymphocytes were sensitized to infection. Thus, immunity induced by formalin-inactivated spores, in contrast to that induced by PA-based vaccines, is mediated by cellular immunity involving CD4 T lymphocytes and not by antibodies.

In another presentation, Orit Gat (Israel Institute for Biological Research) reported studies with a PA-producing, nontoxinogenic, nonencapsulated *B. anthracis* spore vaccine that had been shown to protect guinea pigs against a subcutaneous virulent spore challenge when given by the subcutaneous route (2). Her data demonstrate that oral immunization with this spore vaccine could protect guinea pigs against a virulent subcutaneous challenge. The protection was observed in approximately 30 to 40% of animals and correlated with a threshold level of PA-neutralizing antibody. This protection is long lived and requires actively metabolizing organisms. The results suggest that oral immunization is possible and that approaches to optimize protection are warranted.

Avigdor Shafferman (Israel Institute for Biological Research) described a computational analysis approach which led to the selection of ~200 vaccine candidate ORFs of *B. anthracis*. High-throughput screening methodologies were developed

and applied, based on either immunoreactivity of PCR-amplified full-length ORF DNA product or serological and proteomic analyses of *B. anthracis* surface proteins. The functional screens revealed several promising novel immunogens. One of these is MntA, a solute-binding component of a manganese ion ABC transporter (18). An *mntA* deletion results in complete loss of MntA expression, resulting in impaired growth in rich medium (alleviated by manganese supplementation), increased sensitivity to oxidative stress, and delayed release from cultured macrophages. The *mntA* deletion mutation results in severe attenuation (a 10^4-fold drop in LD_{50}), and yet the mutant expresses all of the anthrax-associated classical virulence factors, LT, ET, and capsule (in vitro as well as in vivo). Virulence is restored almost completely by transcomplementation from a plasmid expressing MntA.

Pierre Goossens (Institut Pasteur) reported on innate defenses against anthrax, showing that group IIA secreted phospholipase A2 (sPLA2-IIA) efficiently kills both germinated *B. anthracis* spores and encapsulated bacilli and that sPLA2-IIA-dependent anthracidal activity was found in guinea pig alveolar macrophage supernatants and in bronchoalveolar lavage fluids from acute respiratory distress syndrome patients (27). *B. anthracis* LT reduces sPLA2-IIA production from macrophages, suggesting a bacterial countermeasure against this host defense. He also showed that transgenic mice expressing human sPLA2-IIA are protected against anthrax, and local administration of recombinant human sPLA2-IIA significantly protects mice against *B. anthracis* infection. Thus, sPLA2-IIA may be considered a novel therapeutic agent to be used in adjunct with current therapy for treating anthrax, especially since its anthracidal activity would be effective even against strains resistant to multiple antibiotics.

SUMMARY

The topics of the meeting represented broad areas related to the *B. cereus* group species and reflected the increasing diversity and expertise of the participants. Nevertheless, a large number of the presentations included information relevant for host-pathogen interactions. Thus, it was most fitting that Harry Smith of The University of Birmingham (United Kingdom) concluded the meeting with an insightful summary. Smith is among the first microbiologists to emphasize the importance of investigating bacterial activities in the context of the host. In 1954, with J. Keppie, Smith reported the first evidence for a toxin produced by *B. anthracis* in vivo (35). In his meeting summary, Smith noted the diverse and sometimes incongruous observations of investigators exploring the interplay of the *Bacillus* species with hosts. While many inroads have been made, clearly metabolic aspects of infection require more attention. Apart from the germination of spores in macrophages, Smith stated that we know little regarding the nutrients required for germination of spores and growth of vegetative cells in vivo and whether nutrients vary in availability from one host to another. Such variation may explain differences in susceptibility to anthrax which are apparent from epidemiology.

Smith also noted the expanded interest in *B. cereus* group spore structure and development, areas which had received significantly less attention at past meetings. He commented on the rapid advances in genomics and gene regulation, lessons learned from environmental studies, and the impact on microbial physiology. Research in these areas has exceptionally benefited from the union of the International Anthrax and *B. cereus* Group Molecular Biology meetings. The next *Bacillus* ACT Meeting is scheduled for 17 to 21 June 2007 in Oslo, Norway.

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