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Editors

The Challenge of Highly Pathogenic Microorganisms

Mechanisms of Virulence and Novel Medical Countermeasures

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Preface

The OHOLO conferences are sponsored by the Israel Institute for Biological Research and take their name from the site of the first meeting on the shores of Lake Kinnereth. The purpose of these meetings is, as it was at their inception over 50 years ago, "to foster interdisciplinary communication between scientists in Israel, and to provide added stimulus by the participation of invited scientists from abroad".

The core of the organizers of the OHOLO conferences are scientists from the Israel Institute for Biological Research. From time to time a particular OHOLO conference cooperates with an international scientific organization. The present 46th OHOLO Conference marks the resumption of the OHOLO tradition after 8 years of interruption caused by events beyond our control. It is my belief that our uncompromising commitment to excellence in research and development in the various areas of science in Israel is essential to our survival in this troubled region. The OHOLO conference tradition is a reflection of this conviction.

The present 46th OHOLO Conference entitled: *The Challenge of Highly Pathogenic Microorganisms – Mechanisms of Virulence and Novel Medical Countermeasures* intends to address the unique virulence features and host-pathogen interactions of microorganisms constituting emerging biothreat with emphasis on *Y. pestis, B. anthracis, F. tularensis* and Orthopox viruses. Accordingly we selected classical microbiological as well as genomic, proteomic & transcriptomic approaches towards developments of novel prophylactic and post-exposure treatment, as well as updated strategies of diagnostics and bioforensics.

I wish to thank the members of the international Scientific Advisory committee: Elisabeth Carniel, Arthur Friedlander, Paul Keim, Johannes Löwer, Michèle Mock and Anders Sjöstedt who helped us to formulate scientific content of this meeting as well as the local organizers Arie Ordentlich, Baruch Velan and Sara Cohen from IIBR.

Ness-Ziona, Israel — Avigdor Shafferman

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Chapter 1
The Anthrax Capsule: Role in Pathogenesis and Target for Vaccines and Therapeutics

Arthur M. Friedlander

Abstract The polyglutamic acid capsule of *Bacillus anthracis* is a well-established virulence factor, conferring antiphagocytic properties on the bacillus. We have shown that the capsule also confers partial resistance to killing by human defensins. In our research we targeted the anthrax capsule for developing medical countermeasures, first using the capsule as a vaccine, similar to successful efforts with other bacteria, and secondly, by developing a novel therapeutic against the capsule. Our experiments showed that a capsule vaccine is protective in the mouse model and its efficacy could be enhanced by conjugation to a protein carrier. In initial experiments using high challenge doses, a capsule conjugate vaccine was not protective in rabbits but did show some protection in nonhuman primates. This suggests it may be useful as an addition to a protective antigen-based vaccine. We are also developing the use of the *B. anthracis* capsule-depolymerizing enzyme, CapD, as a therapeutic. We demonstrated that in vitro treatment of the encapsulated anthrax bacillus with CapD enzymatically removed the capsule from the bacterial surface making it susceptible to phagocytic killing. Initial experiments in vivo showed that CapD could be used successfully to treat experimental anthrax infections. Such a novel approach to target the capsule virulence factor might be of value in the treatment of infections due to antibiotic-resistant strains.

Keywords Anthrax · Capsule · Vaccine · Therapy · Capsule depolymerase

The recent renewed interest in anthrax after the cases of anthrax that developed in 2001 after the mailing of letters containing anthrax spores has stimulated research to develop new medical countermeasures.

In this review I will give an overview of the anthrax capsule and its role in pathogenesis and describe our efforts to develop a capsule-based vaccine and therapeutic.

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1.1 Overview of Anthrax Pathogenesis

The infectious form of anthrax, the spore, germinates within the infected host to the vegetative bacillus, and in this regard it is similar to the pathogenic dimorphic fungi. *Bacillus anthracis* possesses three major recognized virulence factors: the two exotoxins, lethal and edema toxins and the antiphagocytic polyglutamic acid capsule. In recent years other virulence factors have been identified including several whose deletion results in 2 or more logs of attenuation. These include a capsule depolymerase (CapD) responsible for attachment of the nascent capsule filament to the cell wall peptidoglycan (Candela and Fouet, 2005), a manganese ATP-binding cassette transporter (Gat et al., 2005), anthrachelin siderophore biosynthesis genes (Cendrowski et al., 2004), nitric oxide synthase (Shatalin et al., 2008) and the caseinolytic protease component ClpX (McGillivray et al., 2009). Several other genes have also been identified that contribute to virulence but whose deletion results in less attenuation (<2 logs).

The spore enters the skin, gastrointestinal tract, or lung. Germination occurs locally extracellularly or in phagocytic cells during or after transport to regional lymph nodes. Some organisms are killed while others remain viable and proliferate. Germination is not synchronous and toxins and capsule are synthesized early after germination. Bacilli located extracellularly or after escape from a phagocyte become encapsulated and resistant to subsequent phagocytosis. Local production of toxins leads to the pathological effects of edema and necrosis. The lethal and edema toxins likely act early in the infectious process, intracellularly and extracellularly, to subvert host innate immune mechanisms by inhibiting phagocytic cells and other cell types (Tournier et al., 2009). The organism spreads from the lymph node, resulting in bacteremia and subsequent toxemia. Death after inhalational anthrax is likely due to lymphatic/vascular obstruction, pulmonary hemorrhage/edema, pleural effusions and toxicity.

1.2 Role of Capsule in Virulence

Since the early 1900s (Preisz, 1909), it has been known that capsule expression is associated with virulence in *B. anthracis* and that strains lacking the capsule are attenuated. When grown with bicarbonate and carbon dioxide *B. anthracis* produces an antiphagocytic capsule. This results in smooth glistening colonies, while capsule-negative mutants, such as Sterne-like strains lacking the capsule encoding pX02 plasmid, appear rough and dry. Capsule can bevisualized by negative staining with India ink. As indicated in Fig. 1.1, the wild-type Ames strain has a typical crinkled appearance while an isogenic, unencapsulated strain is difficult to see.

The five genes necessary for capsule synthesis and formation are encoded in an operon on the pX02 plasmid. CapB and CapC are thought to be responsible for synthesis of the polyglutamate filament, CapA and CapE for translocation across the cell membrane, and CapD for covalent attachment to meso-diaminopimelic acid of the peptidoglycan (Candela and Fouet, 2006; Richter et al., 2009). The capsule is

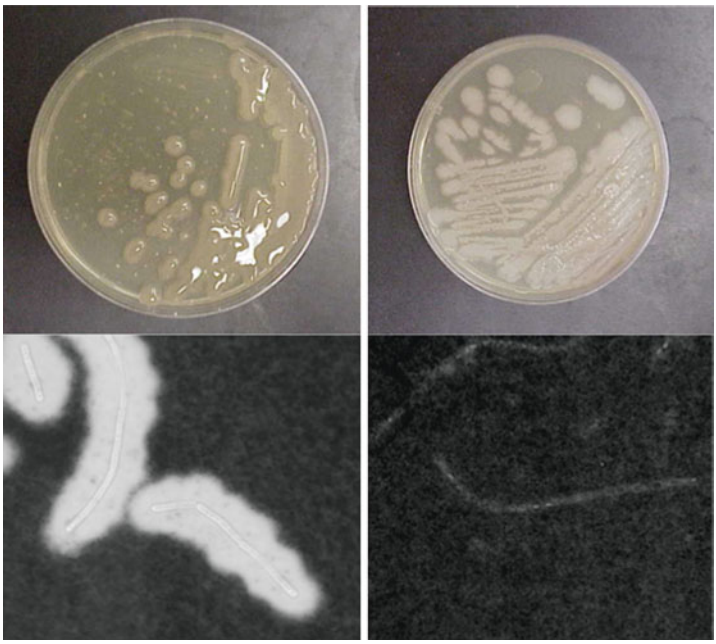

Fig. 1.1 *B. anthracis* grown on NBY/bicarbonate agar with 20% CO_2 at 37°C. The colonial morphology (*top panels*) and India ink preparation (1000×) of bacilli (*bottom panels*) of wild-type encapsulated *B. anthracis* (*left panels*) and an unencapsulated isogenic strain (*right panels*) are shown

located external to the S-layer of the bacillus. As with other extracellular bacterial pathogens, the anthrax capsule has long been known to be antiphagocytic. It also blocks phage attachment (McCloy, 1951) and access of antibodies to underlying bacillus antigens (Mesnage et al., 1998). Synthesis of the capsule is regulated by factors on both pX01 and pX02 and is induced by carbon dioxide and serum. The capsule is a homopolymer of gamma-linked polyglutamic acid composed entirely of the D enantiomer. As such it is resistant to proteases and a poorly immunogenic T-independent antigen. Its mass varies from 100,000 to 1,000,000 daltons.

The antiphagocytic nature of the capsule is evidenced by the fact that there is minimal binding of encapsulated bacilli to macrophages without addition of anti-capsule antibodies. There is also some evidence that low molecular weight capsule fragments are released from the bacillus and may contribute to virulence (Makino et al., 2002). Evidence from our laboratory suggests that the capsule provides some protection against the bactericidal effects of some of the antimicrobial peptides, including human beta defensins. The defensins are cationic peptides with intramolecular disulfide bonds and are an important part of the innate immune system. Beta defensins occur in the epithelium of the skin, lung, gastrointestinal, and genitourinary tracts.

We found that the beta defensins 1, 2, and 3 have some antimicrobial activity against the encapsulated Ames strain, but that they are significantly more active

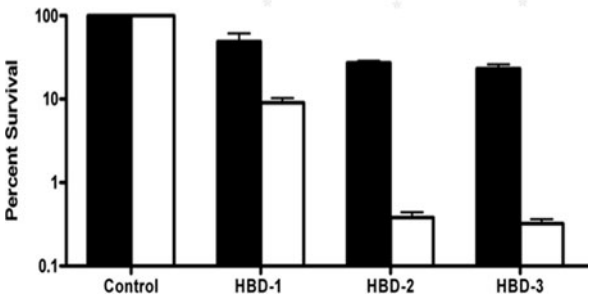

Fig. 1.2 Antimicrobial activity of human beta defensins against encapsulated and unencapsulated *B. anthracis* strains. Bacilli were incubated with human beta defensins-1, 2, or 3 (HBD-1,2,3) at a final concentration of 20 μg/ml at 37°C in 5% CO_2 for 2 h and bacteria were then plated for cfu. Survival percentages were calculated by comparing the cfu at 2 h to a control containing no defensins. Results are expressed as the mean + SEM of triplicate samples. Black bars represent wild-type and white bars represent unencapsulated bacilli. The differences between encapsulated and unencapsulated strains were significant for all defensins tested ($P < 0.0001$)

against an unencapsulated isogenic strain (Fig. 1.2) (O'Brien et al. manuscript in preparation). The mechanism of this inhibition is under study but preliminary data suggest the capsule on the surface of the bacillus may bind some of the antimicrobial peptide and thus prevent it from binding to its presumed target on the cytoplasmic membrane.

1.3 Capsule as a Vaccine Target: Development of a Capsule Vaccine

Essentially all licensed vaccines against extracellular bacteria are based upon the bacterium's capsule. However no work had been done with the anthrax capsule until research in our laboratory demonstrated for the first time that the capsule is effective as a vaccine against challenge with an encapsulated nontoxinogenic *B. anthracis* strain that is virulent for the mouse (Chabot et al., 2004). Mice vaccinated with two doses of capsule were protected against subcutaneous challenge with approximately 100 LD_{50} with 7/12 animals surviving compared to 0/12 controls (Table 1.1, $P = 0.014$). The capsule vaccine was unable to protect against challenge with the fully virulent encapsulated and toxinogenic Ames strain as was protective antigen by itself, as has been reported previously. However, the combination of capsule and protective antigen was protective (9/11 surviving) in mice suggesting that capsule and protective antigen might be synergistic. In an attempt to convert the capsule from a T-independent to a T-dependent antigen resulting in a more mature IgG response and immunological memory, as has been done with polysaccharide homopolymer antigens, we conjugated the capsule to an immunogenic protein carrier (bovine serum albumin). This resulted in the expected increased IgG response but did not protect mice, likely because the carbodiimide conjugation procedure

Table 1.1 Efficacy of capsule vaccine against anthrax infection in the mouse[a]

Experiment	Vaccine	No. survivors/no. challenged
1. Challenge with *B. anthracis* delta Ames (cap+, tox-)	Capsule	7/12
	PBS	0/12
2. Challenge with *B. anthracis* Ames (cap+, tox+)	Protective antigen	1/12
	Capsule	0/12
	Protective antigen+capsule	9/11
	PBS	0/12
3. Challenge with *B. anthracis* Ames (cap+, tox+)	Capsule conjugate	9/9
	Alum control	1/10

[a]Mice were vaccinated with two doses of vaccine and challenged subcutaneously 4–8 weeks later with 116 LD_{50}, 78 LD_{50} or 20,000 spores in Experiments 1, 2, or 3, respectively. Data and details for experiments 1 and 2 were from Chabot et al. 2004 and for experiment 3 from Joyce et al. 2006. The differences in survival between capsule and PBS in experiment 1, protective antigen+capsule and PBS in experiment 2, and capsule conjugate and alum control in experiment 3 were all significant ($P = 0.014$, $P < 0.001$, and $P < 0.001$, respectively, Fisher's exact test).

reduced the size of the capsule and destroyed antigenic epitopes. However, using a novel controlled conjugation procedure to couple the capsule to the outer membrane protein complex of *Neisseria meningitidis* serotype B as the carrier, the high molecular mass and antigenic epitopes on the capsule were preserved (Joyce et al., 2006). This capsule conjugate was now able to protect mice against challenge with the fully virulent encapsulated toxinogenic strain (Table 1.1). Note that this degree of protection in mice was greater than what is observed with the protective antigen.

To further investigate the effectiveness of the capsule conjugate, we tested the vaccine against an aerosol challenge with the encapsulated toxinogenic Ames strain in the rabbit and nonhuman primate models. The vaccine was highly immunogenic in both species and induced opsonic anticapsule antibodies. Rabbits vaccinated with two doses of the conjugate vaccine and challenged with 680 LD_{50} were not protected, with none of 10 animals surviving. However, significant protection was seen in the nonhuman primate challenged with 123 LD_{50} with 3/5 and 2/5 animals surviving after two or one dose of the vaccine compared to 0/5 controls ($P = 0.02$ and $P = 0.04$, respectively) (Chabot et al., 2009). The results suggest that including the capsule might increase the potency of a protective antigen-based vaccine. Such a multicomponent vaccine might be of value against strains resistant to vaccination with one antigen and as the mouse experiments suggest, there may be synergy between the capsule and protective antigen components targeting different virulence factors.

1.4 Capsule as a Therapeutic Target

Vaccines against bacterial capsules function by inducing antibodies that are opsonic and enable phagocytic killing as described above for anthrax. Antibodies against the capsule have also been developed as a therapeutic and have shown some efficacy in experimental animals (Kozel et al., 2004; Joyce et al., 2006). A novel alternative approach we have taken is to convert the encapsulated bacillus to a form susceptible to phagocytic killing by removing the capsule from the bacterial surface using a capsule-degrading enzyme. This builds on the concept of using microbial enzymes as antibacterials developed many years ago, and subsequently demonstrated with the use of capsule degrading enzymes to treat with varying success, pneumococcal (Avery and Dubos, 1931), *E. coli* (Mushtaq et al., 2005) and cryptococcal infections (Gadebusch, 1960). Another approach recently explored is the development of drugs that inhibit capsule attachment (Richter et al., 2009).

As indicated above, *B. anthracis* expresses CapD, which is required for covalently attaching the nascent polyglutamic acid filament to the peptidoglycan and CapD null mutants are attenuated (Uchida et al., 1993; Candela and Fouet, 2005). However, this enzyme, a gamma glutamyltranspeptidase, when added to purified capsule, can carry out a hydrolytic reaction and degrade the high molecular weight polyglutamate to low molecular species using water or amino acids as an acceptor as shown in Fig. 1.3.

We used this property to show that purified CapD added externally to encapsulated *B. anthracis* is capable of removing the capsule from the surface (Fig. 1.4), making the bacillus susceptible to phagocytosis and neutrophil killing. Pretreating encapsulated bacilli with CapD before incubation greatly enhanced their adherence

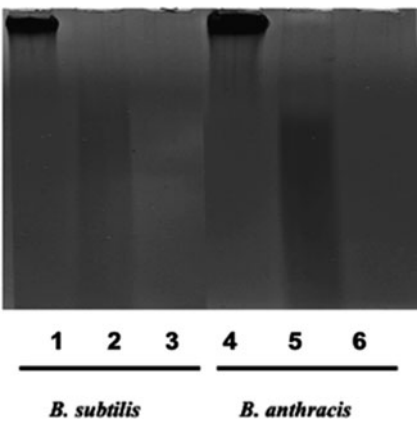

Fig. 1.3 Degradation of capsules from *B. anthracis* and *B. subtilis* by CapD. Capsule purified from *B. subtilis* (lanes 1–3) or *B. anthracis* (lanes 4–6) were incubated with CapD and examined by SDS-polyacrylamide gel electrophoresis. CapD was present at the following concentrations: lanes 1 and 4, 0.35 ug/ml; lanes 2 and 5, 3.5 μg/ml; and lanes 3 and 6, 35 μg/ml together with capsule at 200 μg/ml. Data adapted from Scorpio et al., 2007