Plasmids, Pasteur, and Anthrax

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Few microbial pathogens have had as great an impact on the development of the science of medical bacteriology as *Bacillus anthracis.* This organism is the etiological agent of anthrax (Greek; "coal, carbuncle, pustule"), a historical disease of considerable economic importance. Anthrax-like infections are described in ancient literature, including the biblical Book of Exodus, which describes the disease as the fifth plague of Egypt. Anthrax epizootics were responsible for enormous domestic livestock losses in Europe from the seventeenth through the nineteenth century. The first infections in animals in the United States were documented in the Louisiana Territory in the early eighteenth century. The disease spread throughout the South and Northeast, and in the 1820s the first human cases of anthrax in this country were reported in Kentucky. Since the early 1900s, there has been a gradual decline in the incidence of anthrax in humans in the United States. This is attributed to three major factors: (i) the use of a vaccine for individuals deemed at risk, (ii) better working conditions for those exposed to wool, hides, and animal products, and (iii) less exposure to imported contaminated animal products (2).

History of Anthrax Research

Although anthrax dates back more than 2,000 years, it was not recognized as a disease until the eighteenth century. Maret in 1752 and Fournier in 1769 both described the "malignant pustule" in humans, and Chabert in 1780 described the disease in animals (19). However, no connection between the two diseases was made for the next 80 years. In 1853, Barthelemy demonstrated transmission of the disease by inoculating blood from infected animals into healthy animals (18), and 15 years later the anthrax bacillus was observed microscopically by Delafond (2). During the 1850s and 1860s, numerous studies established that (i) blood from anthrax-infected animals contained large, nonmotile bacilli, (ii) inoculation of healthy animals with blood or tissue containing these bacilli engendered the disease, and (iii) the "malignant pustule" in humans contained bacilli similar to those found in animals with anthrax. Yet it was not until 1877 that Robert Koch conclusively proved that there was a causal relationship between the large nonmotile bacilli and the disease of anthrax (18). In so doing Koch became the first to demonstrate that a specific bacterium was responsible for a specific disease. This work resulted in the formulation of Koch's famous postulates, which established the framework of theory and practice for the development of the science of medical microbiology.

In France in the late 1870s anthrax was a severe disease that destroyed flocks of sheep throughout the French countryside. This destruction influenced Louis Pasteur to direct his attention to the study of anthrax. Pasteur had just developed a regimen for heat attenuation of

the causative agent of chicken cholera, and he used a similar approach in his studies of virulent anthrax bacilli (18). Cultures of the organism grown at elevated temperatures were shown to be decreased in virulence. In historic field trials at Pouilly-le-Fort in 1881, Pasteur showed that these heat-attenuated organisms were capable of producing immunity against later challenge with virulent strains of the bacillus in animals. Pasteur is therefore given credit for developing the first vaccine effective in the prevention of anthrax (18).

Mention must be made, however, of W. S. Greenfield, Professor Superintendent of the Brown Animal Sanatory Institution in England (1878-81). Greenfield, like Pasteur, anticipated the results of growing cultures of the anthrax bacillus at high temperature and actually performed experiments similar to Pasteur's some months before the trials at Pouilly-le-Fort (16). However, recognition for his scientific achievements was not forthcoming, partly owing to financial and political considerations of the time. Therefore, Greenfield may actually deserve the credit for developing the first anthrax vaccine, a point of contention perhaps best left to the medical historians and presented here only for the sake of historical accuracy.

The Anthrax Toxin and Vaccine Development

In the 100 years that followed these landmark studies, much was learned about the physiology and pathobiology of this unique organism. It is now generally accepted that to be fully virulent, *B. anthracis* strains must possess both a polyglutamic acid capsule and a protein exotoxin composed of three factors. The production of this toxin in vivo was suggested by the work of Cromartie et al. in 1947 (3a), and its presence was conclusively proven in 1955 (13). By the early 1960s it was recognized as a complex toxin or toxic mixture composed of three protein factors: edema factor (EF), protective antigen (PA), and lethal factor (LF) (1, 14). Although generally

considered innocuous, the PA component has been reported to effect transient alterations in neural and cardiovascular function in challenged hosts (17), whereas neither EF nor LF alone is biologically active. However, PA in combination with EF or LF produces localized edema or death, respectively, in experimental animals (8). Many investigators have tried to elucidate the role of the holotoxin in the disease process, in the belief that understanding the toxin's mechanism of action would aid in developing a safe, effective, and long-lasting human vaccine.

A licensed vaccine against anthrax, which appears to afford some protection from the disease, is currently available for human use (3). The vaccine contains alum-precipitated supernatant material from fermenter cultures of an avirulent *B. anthracis* strain (V770-NP1-R). Although. an acceptable human vaccine now exists, there are reasons to believe it can be improved. Fish et al. (4) reported that purified PA is a much more effective protective immunogen than crude PA, yet the current vaccine consists of a crude supernatant precipitate consisting primarily of PA. Mahlandt et al. (9) reported that LF was a highly effective protective immunogen, protecting rats against both toxin and spore challenge and guinea pigs against spore challenge, and Stanley and Smith (15) reported that the combination of EF and PA afforded better protection than PA alone. There is also strong evidence that immunity to anthrax is dramatically increased by administering first the PA vaccine and then a booster of live attenuated bacteria (6).

The goal of ongoing research with the anthrax bacillus is to produce a more efficacious human vaccine, with the PA moiety used as the central element. Modern techniques in molecular biology and recombinant DNA technology have been used toward this end, with the hope that such studies might provide a molecular explanation of the successes achieved by Pasteur and Greenfield a century ago.

Plasmid Studies

Cell-free supernatant fluids from broth cultures of two nonencapsulated toxigenic strains of *B. anthracis* (Sterne and V770-NP1-R) and an encapsulated, toxigenic strain (Vollum 1B) grown in a chemically defined medium (12) at 37 (optimal) and 42.5°C (heat-treated) have been assayed for lethal toxin (1, 5) and edema production (13). Both activities were readily detectable in parent strain supernatants, whereas neither activity was found in culture supernatants from the heat-treated strains. Furthermore, loss of virulence was noted in cells subjected to heat treatment.

Single plasmid species that differed in molecular weight were detected in each parental *B. anthracis* strain. Plasmid DNA, however, could not be detected in the heat-treated isolates, suggesting a correlation between the presence of plasmid elements and the ability of the *organism to produce the toxin or regulate its production. Definitive evidence of plasmid-associated toxin production was obtained by transforming heat-treated cells with plasmid DNA purified from the parent strain. Biological activities in the supernatant were restored, and plasmid DNA similar in molecular weight to that of the parent strain was isolated from cultures of the transformants (10).

The locus of the PA structural gene was identified by recombinant DNA techniques (M. H. Vodkin and S. H. Leppla, submitted for publication). Restriction fragments of plasmid DNA purified from the Sterne strain of *B. anthracis* were cloned into an *Escherichia coli* K-12 pBR322 host vector system. Two 'clones were identified as producing a protein which reacted with antibody- to PA and was indistinguishable from purified PA when analyzed by gel electrophoresis and Western blotting techniques. A biological assay showed that this protein was a functional PA gene product (7).

Through recombinant DNA technology, a strain of *E. coli* was thus constructed which contained a subgenomic fragment of plasmid

DNA from *B. anthracis.* This recombinant molecule contained the structural gene(s) for PA. This protein can now be harvested from nonpathogenic bacterial cultures free of the other two components and indeed free of any other contaminating *BaciZZus* proteins. Future manipulations to specifically enhance PA production can now be planned. These accomplishments have led to the threshold of the primary objective — the development of a safe, more efficacious human anthrax vaccine.

During the course of his studies on anthrax, Pasteur asked how virulence could be lost during 8 days at 43°C (11). A reasonable answer to this question can now be given and can also explain the observations made by Pasteur and Greenfield. In addition to the studies already described, two Pasteur vaccine strains of *B. anthracis* (ATCC 4229 and ATCC 6602) were examined for biological and serological toxin activity and for the presence of plasmids. These strains contained no detectable plasmid elements. Furthermore, they resembled the heat-treated strains in lethal toxin, edema-producing, and serological activities. It is likely, therefore, that Pasteur and Greenfield attenuated the anthrax bacillus by using heat to cure the strains of temperature-sensitive plasmid elements encoding one if not all of the toxin proteins.

Science is the process of answering seemingly simple questions, such as that posed by Pasteur. It is indeed rare to have the opportunity to continue work initiated more than a century ago. Many individual advances — electron microscopy, the decoding of the structure, components, and behavior of DNA, the development of genetic engineering, and sophisticated assay techniques — were required before a simple answer to Pasteur's simple question could be found.

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