

Sir Arnold Theiler and the discovery of anaplasmosis: A centennial perspective

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Abstract

Sir Arnold Theiler's research in 1908/09 led to the discovery of the first rickettsial pathogen, *Anaplasma marginale*, and set the stage for his development and implementation of an effective live vaccine based on a less virulent strain, *A. marginale* ss. *centrale*. His 1910 report, describing *A. marginale*, is among the classic monographs in infectious disease research, presenting not only observations in exacting detail but also highlighting the deductive reasoning leading to association of a new pathogen with a specific disease. With a centennial perspective and both conceptual frameworks and molecular tools unimaginable in Theiler's time, the significance of several observations in the original report – cyclic bacteremia, strain superinfection, and taxonomic position – is now clear and highlight the broad applicability of key principles of pathogen biology.

Introduction

Arnold Theiler's identification of *Anaplasma marginale* as the microbial agent of a specific disease, which he designated anaplasmosis, was only one of his remarkable achievements carried out in South Africa from the late 19th century through the early decades of the 20th. Equally as impressive was his subsequent recognition of a less virulent strain, which he termed *A. marginale* variety *centrale*, and its implementation as a vaccine – which continues in use today. [Theiler's original taxonomy of this less virulent strain has not been subsequently amended and, according to modern nomenclature, is referred to as *A. marginale* subspecies *centrale*.] On this occasion of the 100th anniversary of both Theiler's original identification of *A. marginale* and the founding of the Onderstepoort Veterinary Institute, I am honoured to have been invited to present this lecture and daunted by the challenge of reviewing Theiler's work from a centennial perspective. To manage this challenge, I will focus on only a few of the prescient observations of Theiler's 1908–1909 report, "*Anaplasma marginale* (gen. and spec. nov.): The marginal points in the blood of cattle suffering from a specific disease".

Discussion

In this landmark paper, Theiler (1910) identified *A. marginale* as the aetiologic agent of a specific disease characterized by fever and anemia resulting in lethargy, anorexia, and a significant case fatality rate. This connection was especially notable in that the punctuate basophilic intra-erythrocytic inclusions that characterize *A. marginale* in stained blood

smears had been reported by Smith & Kilborne (1893) some 15 years earlier as stages of the protozoan *Piroplasma bigeminum* (now *Babesia bigemina*) – a reasonable conclusion given the presence of multiple distinct stages of protozoan parasites.

Theiler's initial suspicion that there were two different etiologic agents involved in what was termed redwater of cattle in South Africa, was based on his view that animals recovered from infection with *B. bigemina* should be immune to further

challenge with this pathogen. Therefore, when animals deliberately inoculated with *B. bigemina* in England and subsequently imported into South Africa underwent a clinically similar disease in the Transvaal, he addressed two alternative hypotheses: (i) the disease was due to a different strain of *B. bigemina* against which the first strain was not protective; and (ii) the disease was a result of infection with a distinct pathogen.

While identification of antibody and T-lymphocytes as primary mediators of protective immunity were still decades in the future, his clear understanding of the specificity of adaptive immunity was evident and key in his discovery of *A. marginale* as the aetiologic agent of the disease. Much of this first report on *A. marginale* describes his

methodical approach to differentiation of *A. marginale* from *B. bigemina* as separable aetiologic agents, each responsible for a specific disease, previously lumped together as redwater or Texas fever. Most impressively Theiler used a combination of approaches including epidemiologic analysis to identify regions where only one of the two diseases were present or where both occurred but at different times of the year, detailed association of microscopic observations with the clinical course of dual and single pathogen infections, and

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linkage of specific clinical signs and post-mortem lesions to the diseases caused by each pathogen. Although Theiler's detailed observations, so characteristic of that era's leading microbiologists, illuminate the first report, his critical review of the existing literature and observations of other scientists, including Smith & Kilborne in the United States, Knuth & Lignieres in Argentina, and Kossel and his co-workers in Europe, provides evidence of his ability to integrate disparate observations into a unifying conclusion.

A final characteristic that stands out from Theiler's initial work on anaplasmosis is the accord paid to the field observations of the farmers themselves that there were often two bouts of disease in cattle occurring at different times of the year – observations that strengthened Theiler's initial belief that two distinct pathogens were responsible and allowed him to correlate microscopic observations with each clinical syndrome. This respect for the insight of farmers is very reminiscent of that which led Smith & Kilborne (1893) to the then unthought-of discovery that arthropods could transmit microbial pathogens.

From a centennial perspective, with 100 years of investigation generating a conceptual framework of pathogenic microbiology and immunology unavailable to Theiler as well as the then unimaginable tools of electron, fluorescence and confocal microscopy, PCR and whole genome sequencing, and high-throughput proteomics, it is remarkable how well Theiler's conclusions have stood the test of time. Furthermore, buried in the 1910 manuscript are several observations which can now be understood – a true compliment to Theiler in that his observations were so clearly presented. The first of these is his observation that there were cyclic peaks of *A. marginale* bacteremia. Theiler noted: "Marginal points on 32nd day...62nd day with re-appearance of marginal points." Unlike African trypanosomes, for which Ross & Thomson (1910) reported cyclic waves of parasitemia, light microscopy has insufficient sensitivity to reliably track the fluctuations in *A. marginale* bacteremia, except in the acute and very early post-acute periods observed by Theiler.

Although Theiler clearly understood that *A. marginale* persisted in infected animals and that the pathogen levels were much lower during this phase, the basis for this persistence would remain poorly understood until more sensitive detection methods were developed. Initially, Southern hybridization (Kieser, Eriks & Palmer 1990) and then PCR (French, McElwain, McGuire & Palmer 1998) allowed definitive detection of the cyclic waves of bacteremia between 102–107 organisms per ml of blood during persistent infection. These observations, in turn, led to the hypothesis that persistence was mediated by

outer membrane protein antigenic variation and subsequent discovery of the immunodominant and antigenically-variable Major Surface Protein (Msp)-2 (reviewed in Palmer, Brown & Rurangirwa 2000).

Research over the past decade has elucidated how the small (1.2 Mb) *A. marginale* genome generates the tremendous number of variants needed to evade immune clearance for long-term persistence. This mechanism, termed segmental gene conversion (Palmer & Brayton 2007), uses a combination of recombination of complete donor alleles and oligonucleotide segments of these alleles into a single expression site to generate 103–104 unique Msp-2 variants. Most interesting is that there is compelling evidence that variants generated by recombination of complete donor alleles have a strong *in vivo* fitness advantage (Palmer, Futse, Leverich, Knowles, Rurangirwa & Brayton 2007). These variants arise preferentially in acute infection (during which bacteremia reaches $\geq 10^8$ *A. marginale* per ml) and in the initial bacteremic peak in the post-acute period (Futse, Brayton, Knowles & Palmer 2005). This *in vivo* fitness results in bacteremia levels during the first post-acute peak that exceed 10^7 *A. marginale* per ml and thus can be observed microscopically. In contrast, subsequent bacteremic cycles during persistent infection are increasingly composed of organisms with less fit variants generated from multiple donor alleles and peak at levels just below reliable microscopic detection (Futse *et al.*, 2005; Palmer *et al.*, 2007). This understanding is consistent with both the timing and levels observed by Theiler (1910) – detection of the acute and first postacute bacteremia followed by long-term persistence with retention of infectivity but below levels of microscopic detection. This understanding of the interplay between antigenic variation and pathogen fitness is relevant not only to *A. marginale* but numerous persistent microbial pathogens, including the Human Immunodeficiency Virus (HIV) (Goulder & Watkins 2004; Kent, Fernandez, Dale & Davenport 2005).

A second observation made by Theiler that can now be explained is the occurrence of strain superinfection. At several places in his original 1910 monograph, he notes that re-infection with a milder course of disease occurs and raises the question as to whether this may be attributable to *A. marginale* strain differences: "An animal may be successfully inoculated more than once, although the second inoculation will only cause a slight reaction. This second reaction might be due to a difference in the strain of the anaplasma, the primary reaction gives sufficient ground immunity to protect an animal against severe lesions and death from a subsequent infection." This observation formed the basis for his sub-

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sequent development of a less virulent strain, *A. marginale* ss. *centrale*, as a vaccine to prevent severe morbidity and death upon challenge with highly virulent *A. marginale* (Theiler 1911; 1912). However, the basis for this strain superinfection remained unexplored until genome sequencing of multiple *A. marginale* strains (Brayton, Kappmeyer, Herndon, Dark, Tibbals, Palmer, McGuire & Knowles 2005; Rodriguez, Palmer, Knowles & Brayton 2005) began to provide clues as to how a second strain could evade the immunity induced against an already established primary strain infection. During infection with the primary strain, immunity is sequentially developed against the MSP2 variants expressed by recombination of complete donor alleles and subsequently by segmental recombination. Consequently, a second strain must evade this immunity and does so only by encoding at least one distinct variant allele in its genome (Futse, Brayton, Dark, Knowles & Palmer 2008). Among highly similar strains with shared variant alleles strain superinfection either does not occur or is rare – the latter perhaps occurring in a narrow window following infection with the initial strain, but before broad immunity to the initial variant repertoire has been induced. This same mechanism appears to underlie the use of the *A. marginale* ss. *centrale* vaccine as the repertoire of this vaccine strain (Shkap, Molad, Brayton, Brown & Palmer 2002) is distinctly different from the *senso stricto* *A. marginale* strains examined to date. Understanding the basis for the strain superinfection first observed by Theiler has a broader impact for infectious diseases. The pressures for genomic divergence among microbial pathogens where host immunity against a dominant strain limits new transmission, are not unique to *A. marginale* – similar pressures appear to manifest in pathogens as taxonomically distinct as HIV, Hepatitis C virus and *Trypanosoma brucei* (Blackard & Sherman 2007; Hutchinson, Picozzi, Jones, Mott, Sharma, Welburn & Carrington 2007; Piantadosi, Chohan, Chohan, McClelland & Overbaugh 2007). The strain structure of *A. marginale* is remarkably diverse and serves as an illustrative model as to how pathogens emerge under conditions of natural transmission and specifically how diversification occurs in, unlike the RNA viruses, a relatively genomically stable organism.

A final observation now viewed through the prism of time is Theiler's discussion of the taxonomy of *A. marginale*. Theiler's clear statement that this was a specific disease agent, apart from *B. bigemina*, was highly significant as it led to development of specific vaccines and, later, specific treatments for these diseases – similar in several clinical features but with very different etiologies and epidemiology. Theiler's choice of the genus name *Anaplasma* reflected his observations that the inclusion lacked any observable cytoplasm, distinct from the other intra-erythrocytic protozoa in which the basophilic nucleus and eosinophilic cytoplasm could be distinguished microscopically: "They differ from any known blood parasite

by the absence of a protoplasmic body and consist only of chromatin substance, thus resembling to a certain extent, the bacteria. They represent, in my opinion, a new genus of protozoa which I propose to call *Anaplasma* and the species under consideration *Anaplasma marginale*." Although *A. marginale* would be variously classified as a virus or protozoa, the initial observations by Theiler are consistent with its correct classification as a novel bacterial genus. *Anaplasma marginale* was the first rickettsial pathogen to be identified, although it was not correctly placed in the Order Rickettsiales until the 7th edition of *Bergey's manual* in 1957. The genus name has remained and, based on precedent and the guiding principles of bacterial nomenclature, now also gives its name to one of the two families that compose the Order Rickettsiales, Anaplasmataceae (the other being the Rickettsiaceae). Interestingly, Cowdry (1925) working in South Africa would identify the first pathogen representative of a second genus, *Ehrlichia*, in the Family Anaplasmataceae with his discovery of *E. ruminantium* as the cause of African heartwater. [Cowdry placed the newly discovered organism in the genus *Rickettsia*. This was later placed in a novel genus, *Cowdria*, honouring his discovery. Recent taxonomic re-organization (Dumler *et al.*, 2001) has unified highly related organisms in the genus *Ehrlichia*.] The genera *Anaplasma* and *Ehrlichia* include the tick-transmitted pathogens within the Family Anaplasmataceae and it is notable that pioneering work in South Africa established the fundamentals of transmission, pathogenesis and immunity that have now been

applied to a greatly expanded number of animal and human pathogens in these genera. Dumler's highly cited paper (2001), revising the taxonomy of the Family Anaplasmataceae, has stood the test of complete genome sequencing, confirming the relationships among the members, and has led to better understanding of the biology of these organisms and their consequent diseases. Thus today, research initiated by Theiler has a widespread impact on understanding pathogen biology far beyond that envisioned a century ago.

Anaplasma marginale still holds many challenges for investigation and for improvement of disease control. Among these, three stand out: (i) development of safe, standardized and effective vaccines; (ii) understanding the basis for age and breed innate resistance; and (iii) understanding transmission dynamics and virulence leading to severe outbreaks. Despite Theiler's development of the *A. marginale* ss. *centrale* as a live vaccine in 1910/11, disease control remains suboptimal and, along with other tick-borne infections, exacts a disproportionate burden on small-holder farmers in resource poor countries (Minjauw & McLeod 2003). Addressing these challenges is incumbent on the research community to both continue the Theiler tradition of disease investigation leading to improvement of animal health and to meet our global responsibilities to enhance human development and well-being.

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