In vivo endogenous spore formation by *Coxiella burnetii* in Q fever endocarditis

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Abstract

Aims—To determine whether *Coxiella burnetii*, the aetiological agent of Q fever, undergoes endogenous spore-like formation, the crucial stage of the developmental cycle, in the infected cardiac valves of patients with chronic Q fever endocarditis.

Methods—Surgically removed valves from three cases of Q fever endocarditis were processed for electron microscopy. Sections were stained with potassium permanganate and uranyl acetate before being extensively examined by transmission electron microscopy.

Results—In all three cases endogenous spore-like formation was seen in the infected cardiac valves.

Conclusions—As the factors that govern sporogenesis in *C. burnetii* are still largely unknown, it is uncertain how important are the implications of the discovery of endogenous spore-like formation in Q fever endocarditis. However, this finding may add new dimensions to current thinking about the treatment of chronic Q fever.

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Coxiella burnetii, the aetiological agent of Q fever, is an obligate intracellular bacterium and its distribution is worldwide. Q fever infection in man is usually acute, ranging from mild to severe. In some people, however, chronic infection may lead to eventual valvular heart disease, hepatitis, splenomegaly, and hepatomegaly.¹ The mortality for chronic endocarditis has been reported to be as high as 65%.² Antibiotic management of acute Q fever is generally successful in non-immunocompromised patients, but treatment of chronic Q fever endocarditis is poor at best.³ Antibiotic treatment of chronic Q fever has not been consistently effective and has been directed toward elimination of the microorganisms from cardiac valve leaflets.³ Even after an apparently good response to antibiotics, relapse frequently occurs once treatment has stopped.³ *C. burnetii* has also been isolated from cardiac valves after years of tetracycline treatment.¹ Valve replacement may become necessary but offers no guarantee of cure as replacement valves frequently become infected. The reason for the persistence of *C. burnetii* following recovery from acute infections is poorly understood.

The biological basis of chronic Q fever disease is also not well understood. The fact that *C. burnetii* has a developmental cycle which consists of both bacterial or vegetative growth by binary fission and unequal cell division consistent with the formation of endogenous-like spores^{4,5} complicates matters. The developmental cycle occurs totally in phagolysosomes of eukaryotic cells, resulting in the formation of different cell types that are morphologically distinguishable.^{4,5} It is believed that one stage of the developmental cycle, endogenous spore-like formation, provides the means of producing a precursor to a very resistant cell.⁵ However, this spore-like formation has only been characterised in vitro (the yolk sac of chick embryonated eggs and cell cultures)^{4,6-9} and not in vivo in either man or animals. Our aim was to undertake an electron microscopic examination of the infected heart valves in three cases of Q fever endocarditis to examine the morphological status of *C. burnetii*.

Methods

Pieces of surgically removed heart valves from cases 2 and 3 were prefixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 24 hours before processing on a LYNX (Leica, Australia) Tissue Processor for electron microscopy. In the tissue processor two changes in 0.1 M cacodylate buffer (pH 7.3) (for five and 25 minutes) preceded postfixation in 1% osmium containing $K_3Fe(CN)_6$ (1%)¹⁰ in 0.1 M cacodylate buffer (pH 7.3). Several washes in 0.05 M maleate buffer (pH 6.0) were followed by 0.05 M maleate buffered 2% uranyl acetate en-bloc staining (30 minutes at room temperature). The blocks were dehydrated through serial dilutions of ethanol. Infected heart valve tissue from case 1, which was received in 10% buffered neutral formalin, was diced and placed in a 0.1 M cacodylate buffer (pH 7.3) wash for two hours before being transferred to cacodylate buffered 3% glutaraldehyde as for cases 2 and 3. The remaining procedure was similar to that carried out in the LYNX processor except that it was carried out manually and that $K_3Fe(CN)_6$ was not used in the osmium after fixation. All blocks were embedded in Spurr Epoxy resin. Ultrathin sections were stained with 1% aqueous potassium permanganate⁴ and 5% uranyl acetate in 50% methanol, before being examined in a Hitachi H-800 transmission electron microscope operated at 100 kV.

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Results

CASE 1

A 39 year old professional fisherman had noticed exertional dyspnoea in 1979. The following year, moderately severe aortic stenosis was diagnosed following a syncopal episode. His aortic valve was replaced with an allograft and the excised bicuspid valve showed no evidence of endocarditis. Three years later during a febrile illness, an increased phase I titre was demonstrated by complement fixation test for Q fever. This was treated with six weeks of tetracycline.

In 1984 he had another febrile illness and had evidence of severe aortic incompetence. The antibodies against phase I antigen of *C burnetii* were again demonstrated and at surgery, two cusps of the homograft valve were found to be torn, but there were no visible vegetations. The aortic valve was replaced with a St Jude mechanical prosthesis. Microcolonies of organisms consistent with Q fever were demonstrated by light and electron microscopy. He was given high dose tetracycline for 18 months and the antibody titres reduced.

In 1986 he developed cardiac failure and a continuous murmur was noted. Blood cultures were negative and antibodies against *C burnetii* remained low. At echocardiography and subsequent surgery, he was shown to have a false aneurysm of the aortic root which had ruptured into the right atrium. This was successfully repaired. He died in 1991 after a head injury. At necropsy, there was no evidence of active infection of the heart.

The homograft valve cusps showed massive infestation by *C burnetii* (fig 1A). The micro-organism, *C burnetii*, was recognised by its characteristic pleomorphism and the presence of both cell types (small and large cell variants).^{4,6} We also observed in some large vegetative cells within the microcolonies different stages of spore-like formation from the early stage (fig 1B) to the final stage (fig 1C) of development. The spores were located within the polar regions of large vegetative cells and were recognised by their dense core surrounded by layers of membrane-like structures, electron-dense peptidoglycan, and a single trilaminar outer membrane; all of which were similar to those described before by McCaul.⁵

CASE 2

A meat worker developed acute Q fever in 1982. This was treated with a seven month course of tetracycline. Following this he remained well until 1993 when he had severe but asymptomatic aortic incompetence. An echocardiogram revealed a large vegetation attached to the aortic valve leaflets with severe aortic incompetence. He had increased titres against phase I and phase II *C burnetii* antigen. The erythrocyte sedimentation rate was 38 mm/hour and he had mild anaemia. Liver and renal function tests yielded normal results.

At aortic valve surgery, the leaflets showed calcification and fibrosis consistent with previous rheumatic fever, and the right coronary leaflet had been eroded by infection. The valve was replaced with a St Jude mechanical prosthesis. He made a good recovery complicated over the subsequent six months by three transient cerebral ischaemic episodes, thought to be related to difficulty with anticoagulation associated with his treatment with doxycycline and rifampicin.

C burnetii was also recognised by its characteristic pleomorphism and the presence of both cell types, although the number of small cell variants was not as abundant as that recorded for the first case. Spore development was active in one area of the section (fig 2A). Late stage of spore development was also seen in this case (fig 2B). This spore-like structure was recognised by its dense core, surrounded by layers of membrane-like structures, electron-dense peptidoglycan, and a single trilaminar outer membrane.

CASE 3

This patient had acute rheumatic fever at the age of 7 and then had several recurrences. At the age of 20, while working in an abattoir, he developed mild exertional dyspnoea and severe haemoptysis. He was found to have severe mitral stenosis and mild aortic incompetence. He underwent mitral valve replacement with a xenograft and made an uneventful recovery. No serology for Q fever infection was undertaken before surgery.

Histopathological examination of the excised valve revealed calcification and fibrosis consistent with post-rheumatic scarring.

Figure 1 Electron microscopy of the excised aortic homograft tissue (case 1). (A) Microcolonies of bacteria consistent with *C burnetii* (B) and (C) endogenous spore-like formation within the polar region of the cell of *C burnetii*; (B) Early stage of development of the endogenous spore; (C) late stage of endogenous spore formation occurring within the mother cell. The endogenous spore contains a dense core (C) surrounded by layers of membrane-like structures (M), electron-dense peptidoglycan (P), and a single trilaminar outer membrane (O). O_M = outer membrane of mother cell. Bars = (A) 2 μ m; (B), (C) 100 nm.

Figure 2 Electron microscopy of the excised aortic tissue: (A), (B) case 2; (C) case 3 (a). An area within the microcolony of *C burnetii* showing two vegetative large cells undergoing sporogenesis (arrows). (B) Endogeneous spore (arrow) within the mother cell. (C) Endogenous spore within the polar region of the mother cell showing the membrane-like structures (M) extending into the mother cell. Bars = (A) 250 nm; (B), (C) 100 nm.

There was no endocarditis. After six years, the xenograft became regurgitant and after a further two years a mitral valve replacement was again performed. This time a St Jude mechanical prosthesis was inserted. Pre-operative serology for phase II antibodies to *C burnetii* was positive (1/128). Phase I serology was not performed. Histological examination of the xenograft cusps revealed an infiltrate of lymphocytes and macrophages. A Giemsa stain showed microcolonies of bacteria consistent with *C burnetii*. He was discharged on a regimen of tetracycline and made a good recovery.

C burnetii was also recognised by its characteristic pleomorphism and the presence of both cell types. Despite the high number of small cell variants seen in this xenograft, the number of large vegetative cells undergoing sporogenesis was not as high as that described for the first two cases. In one instance membranous layers that circumscribed the endogenous-like spore were also found to extend into the mother cell (fig 2C), which was similar to that described for the formation of endogenous-like spore.⁵

Discussion

The endogenous-like spores, described for the first time in Q fever endocarditis, have already been identified and characterised in vitro (in the yolk sac of chick embryonated eggs and cell cultures⁴⁻⁹), but have not been reported before in vivo in either man or animals. The endogenous spore is a product of a developmental cycle which provides the basis for the formation of different cell types that must withstand physiological, biochemical, and immunological variations in their habitats, ranging from external environment (the air surrounding us), and intracellular environment such as the phagolysosomes of the host cells.⁵ The ability of the micro-organism to survive in the external environment has posed problems for the disinfection of objects and the pasteurisation of dairy products contaminated by this agent. The resistance of *C burnetii* to physical and chemical stress, raised temperatures, desiccation, osmotic shock, and chemical disinfectants has been well illustrated.⁶⁻¹¹⁻¹⁵ One element of the developmental cycle, the formation of the endogenous spores, produces a precursor to a very resistant cell.⁵

In other bacterial spore formers sporulation is an ordered process whereby a complex sequence of physiological and morphological changes is set in motion in responses to conditions of nutrient depletion. Sporulation also provides the basis for producing cells that have higher chances of survival in a very unsettled environment. But the factors that govern sporogenesis in *C burnetii* are still largely unknown and at this stage it is uncertain what are the important implications of the discovery of endogenous spore-like formation in Q fever endocarditis.

The finding of endogenous spore-like formation may, however, add new dimensions to

current thinking about the treatment of chronic Q fever. Genetically distinct *C burnetii* isolates showing different antibiotic susceptibilities have been shown to be associated with different disease manifestations including acute and chronic diseases.^{3–16} Chronic isolates, for example, exhibited dramatic antibiotic resistance when compared with acute isolates in vitro.¹⁷ This differential antibiotic susceptibility has led to problems in choice of suitable chemotherapy. Treatment of chronic Q fever is complex and requires long term antibiotics, and in cases of endocarditis, often heart valve replacement. At the present time, neither an optimal antibiotic combination nor the duration of treatment is known. Apparently, no treatment, not even combined antibiotic treatment, can eradicate Q fever endocarditis within two years.² An alternative means of an effective treatment is therefore warranted. As *C burnetii* was observed to undergo sporogenesis in the infected heart valves, despite the patients being given antibiotic treatment, the question that needs to be answered is whether chemically blocking the pathway of sporogenesis would lead to the eradication of the pathogen during the sensitive or vegetative stage of the development. The knowledge of the underlying mechanisms of developmental gene regulation in *C burnetii* may provide the means of identifying the determinative factors of the complex differentiating process which may lead to an effective control against chronic Q fever.

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