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EDITORIAL

What is the evidence that the putative *Mycobacterium lepromatosis* species causes diffuse lepromatous leprosy?

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Introduction

In 2008 a paper published in the American Journal of Clinical Pathology[1] reported that a new *Mycobacterium* species had been isolated from two patients with diffuse lepromatous leprosy (DLL). A subsequent paper[2] elaborated on the initial data without adding additional cases. In this editorial we review the clinical, pathological and molecular biological findings reported in these two papers and discuss whether there is sufficient evidence to support the claim of a new *Mycobacterium* species causing diffuse lepromatous leprosy. Diffuse lepromatous leprosy manifests clinically with diffuse, non-nodular dermal infiltration[3] and pathologically with evidence of mycobacteria in the endothelium and lepromatous granulomatous vasculitis.[4]

The clinical data on the two cases is given in the first paper. Both patients were Mexicans who died in intensive care/burn facilities in Phoenix, Arizona, USA with extensive necrotising skin lesions. Case 1 had multiple purpuric skin lesions with necrosis and vasculitis present. Case 2 had 80% skin necrosis with multiple skin lesions. Biopsies from both lesions showed acid fast bacilli in macrophages with vasculitis. The diagnoses in both cases were compatible with Lucio reactions. DNA and bacteria were isolated from the frozen post-mortem liver of case 1 and the stored paraffin embedded skin biopsy of case two and used for the subsequent molecular studies.

Han *et al.*[1] proposed the name *Mycobacterium lepromatosis* for the disease-causing agent because of the close genetic relationship to *M. leprae* and the apparent association with DLL. Using purified DNA, five genes (*mmaA, hsp65, rpoT, rpoB and rif-rpoS*) were partially sequenced and one gene (*rrs*, 16S RNA) was fully sequenced. All sequences were compared

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with *M. leprae* consensus sequences and a few other mycobacteria to derive genetic relationships. Based on these comparisons the authors argued that the genetic differences were significant enough to propose a novel species. The second report[2] is a more in-depth genetic analysis of the DNA (FJ924) derived from one of the leprosy patients. In this study the investigators analysed full and partial sequences of 20 genes and pseudogenes from the DNA, surveying approximately 0·6% of the theoretical genome. They again concluded that sequence data from FJ924 DNA substantiated the species-level difference with *M. leprae*. Other phylogenetic results attempted to place the proposed new species and *M. leprae* in the context of evolutionary distance from a common ancestor.

NEW *MYCOBACTERIUM* SPECIES?

Han *et al.*[1,2] reported that genetic differences between FJ924 DNA and *M. leprae* are significant enough to propose a novel species. While relatively large sequence differences were reported for some conserved and many less conserved genes, the primary comparison is with full length 16S RNA. This is appropriate because systematic comparisons of DNA sequence are most robust and meaningful when using full-length genes for comparisons.[5]

The authors show that the 16S RNA from *M. leprae* and FJ924 both contain an AT-rich region, not seen in other mycobacteria, and that the overall 16S RNA sequences are 98% identical. While 2% divergence of 16S RNA sequence is not generally sufficient to support species differentiation,[5] this difference is particularly noteworthy when considering the large number of reported *M. leprae* 16S RNA sequences (www.ncbi.nlm.nih.gov/genbank) showing much less diversity. Taking the 16S RNA data along with some 20 other genes that were partially sequenced, raises questions; have the investigators isolated a variant of *M. leprae* that causes leprosy that is clinically indistinguishable from leprosy caused by the type strain, *M. leprae*, or have the investigators isolated DNA from a bacterium very closely related to *M. leprae* that was found associated with leprosy infections but not the cause of the disease?

Infection with environmental mycobacteria in cutaneous lesions in leprosy patients, have been reported[6] and could explain the DNA identified by Han *et al.* Alternatively, the suggestion that diffuse lepromatous leprosy is caused by a bacterium that is very closely related to *M. leprae* but clearly different from the type strain requires further study to authenticate this claim.

ESTABLISHING CAUSATION OF DISEASE

An important milestone in establishing the 'Germ Theory' in medicine was the application of Henle's postulates to establish the causal relationship between a microbe and a disease. Henle's work was later refined by his student Robert Koch, to become known as Henle-Koch's postulates. The postulates state that the following criteria have to be met for an agent to be established as the cause of an infectious disease; (1) the agent must be present in every case of disease (recognising asymptomatic carriers can exist), (2) the agent must be isolated from lesions in pure culture, (3) the agent must be inoculated into a susceptible host reproducing the disease and (4) the agent must be recovered from the lesions.

With the leprosy bacillus the steps requiring growth of the agent have been impossible to meet because the bacterium cannot be grown in culture. The same is apparently true of

the agent FJ924 though only a few attempts at cultivation have been made. As a surrogate for cultivation, DNA obtained from bacteria found in leprosy lesions have been repeatedly shown to be essentially identical to the type strain of *M. leprae*.[7,8] In addition, *M. leprae* has been isolated from human lesions, passaged in mice and armadillos and recovered from infected tissues. These passaged bacteria have been shown to cause characteristic granulomatous lesions with nerve invasion in the armadillo, the hallmark of *M. leprae* infection in man. Similar studies have not been forthcoming from Han's group using the agent FJ924. These kinds of studies are essential to establish the linkage between the suspect agent from which DNA has been isolated from a small number of patients and the claim that the causative agent is separate and distinct from *M. leprae*.[9]

CLINICO-PATHOLOGICAL FEATURES OF DLL

A century ago, leprologists were debating whether or not different organisms or strains of *M. leprae* existed and caused the differing clinical and histological appearances of the leprosy types in the Ridley-Jopling spectrum. They also asked whether different organisms might cause the different leprosy 'reactions' in patients – ENL, RR, or Lucio's. All of these differences and complications have since been shown to be the result of individual host responses to *M. leprae* antigens, with the possible exception of the Lucio reaction, which consists of ischemic infarcts of the skin, the mechanism of which is still very poorly understood. Thus, the possibility that a strain of *M. leprae* might encode unique characteristics that could elicit the Lucio reaction is attractive and seems plausible, although highly speculative and challenging to prove, since genetic clonality and the absence of overt toxic affects in animal models seem to be major characteristics of *M. leprae*.

The 'new organism' described in the initial report was obtained from lesions of two patients with the Lucio reaction, but the authors then generalised beyond the Lucio phenomenon and asserted that this new organism was responsible for diffuse lepromatous leprosy (DLL). DLL is a clinical variant of lepromatous leprosy described by Latapi.[10] In DLL diffuse infiltration of the skin is observed, without nodular lesions that occur in lepromatous leprosy. Histological features of this variant overlap with those of more common, nodular lesions, and DLL is usually diagnosed retrospectively in persons who develop the Lucio Reaction.[4] To associate a new strain or species of organism with this clinical variant of LL seems highly premature with so few cases reported. Moreover, since the different clinical manifestations of tuberculoid and lepromatous disease are elicited by one organism, it seems unlikely that such a clinical variant of LL would be the result of a different strain or species of organism.

The two cases underpinning this are both deceased and so it is very tenuous to ascribe a specific clinical condition that was not recognised in life to a new *Mycobacterium* species. It can be said that FJ924 is associated with two cases of Lucio's phenomenon. It is not possible to say that FJ924 caused the disease in these two cases. While it is theoretically possible for different *M. leprae* strains to cause different disease types, so far no evidence has been found to support this hypothesis and the pathological model in which the immune response of the host is paramount in determining disease outcome still holds good. There is solid evidence for minor DNA changes in *M. leprae* at the strain level and these differences have been used for molecular epidemiological studies[11] and drug resistance.[12]

EXPERIMENTS NEEDED TO VERIFY CLAIM OF NEW *MYCOBACTERIUM* SP CAUSES LEPROSY

Whether Han *et al.* have isolated DNA from a new *Mycobacterium* species or a variant of *M. leprae*, prospective studies must be performed to confirm the claim that this agent causes leprosy. The work to attain this milestone is not trivial and will require the collaboration of many clinical and research sites. First, bacteria from patients harbouring the suspect agent will have to be isolated. Every attempt should be made to grow and isolate the agent using culture with standard mycobacterial media. If this approach is unsuccessful, mouse foot pads should be inoculated with biopsy material from patients suspected of harbouring the agent so that 'pure' collections of the bacteria can be grown and further analysed. Growth in the mouse foot pad assumes that the suspected agent can replicate, as does *M. leprae*, in the foot pads of mice. Because this is uncertain, multiple attempts will have to be carried out using different conditions to confirm absence or presence of mycobacterial growth. If the bacteria can be passaged successfully in mice, samples of infected tissues must be collected at each stage of passage to monitor the genetic characteristics of the bacteria. The agent may be found in association with *M. leprae* and passage in the mouse may be permissive for one bacterium over the other causing potential changes in proportion of agents over time. Because of this potential enrichment artifact, multiple, successful expansions of 'M. lepromatosis' in the mouse will be needed.

Upon successful expansion and genetic characterisation showing 'purity' of the bacteria, expansion in nude mice would help prepare large numbers of the bacteria needed for the next phase of characterisation. Nude mouse-derived bacteria would be harvested and injected into naïve armadillos to monitor the bacteria's ability to infect nerves, the hallmark pathology associated with leprosy. Other than man, armadillos are the only known naturally susceptible host for *M. leprae* and nerve infection is seen with all animals infected with *M. leprae*.[11] Expansion of the infection in the armadillo with subsequent nerve invasion by the suspect agent would confirm that the bacterium is capable of causing a leprosy-like infection. If nerve invasion is not observed, the bacteria cannot be considered a new *Mycobacterium* species causing leprosy.

SUMMARY

Han *et al.*[1,2] have made a retrospective isolation of DNA from two patients with fatal Lucio's phenomenon. This DNA does have some molecular differences to *M. leprae* and may constitute a variant of *M. leprae*. However the experiments and data needed to confirm that this is a new leprosy-causing species have not yet been done. We have outlined the work that does need to be done. For the moment the assertion that '*M. lepromatosis*' is a new leprosy-causing species is not proven.

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