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TOPIC HIGHLIGHT

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Mycobacterium avium subspecies *paratuberculosis* and its relationship with Crohn's disease

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Abstract

The hypothesis postulating that *Mycobacterium avium paratuberculosis* (MAP) is the cause of Crohn's disease (CD) has been circulating for many years. Advances in molecular techniques, such as polymerase chain reaction and culture methods, have enabled researchers to demonstrate that there is an association between MAP and CD. Recently, genome-wide association studies have identified novel susceptibility genes for CD, which are critical for generation of an adaptive immune response that is protective against intracellular pathogens, including *M. tuberculosis* infection. However, the role of MAP as a cause of CD suffered a setback with the report that administration of antimycobacterial therapy failed to lead to a sustained response in CD patients. Accordingly, this review sought neither to confirm nor refute this, but instead to survey recent literature on the role of MAP in CD.

Key words: *Mycobacterium avium* subspecies *paratuberculosis*; Crohn's diasease; Inflammatory bowel disease

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two related, chronic, remitting and relapsing inflammatory diseases of the gastrointestinal tract, commonly known as inflammatory bowel diseases (IBDs). While the causes of IBDs are unknown, it is thought that inflammation results from an inappropriate chronic activation of the innate and adaptive mucosal immune systems in a genetically susceptible host, and that enteric microflorae play a pivotal role in the initiation and maintenance of disease[1]. The key factors responsible for IBDs are fairly well defined, namely, the environment, genetic makeup, commensal florae, and immune response. Insofar as the components of IBD pathogenesis are concerned, investigation of the role of infectious agents and gut commensal florae is an area in which relatively less progress has been made[2]. One of the most controversial questions is whether or not any given microbe plays a role in promoting disease. The similarities between CD and some forms of infectious enterocolitis are sufficiently evident for numerous specific microbial etiologies for CD to have been proposed over the years, including *Pseudomonas maltophilia*, *Mycobacterium kansasii*, *Chlamydia trachomatis*, *Bacteroides fragilis*, *Listeria monocytogenes, Escherichia coli* and *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Just recently, the status of MAP as an infectious cause of CD-an enduring and controversial topic-suffered a severe setback in the form of a multicenter study which reported that administration of antimycobacterial therapy over 2 years afforded no substantial or prolonged benefits for patients with CD[3]. However, MAP is a recurrent candidate for several reasons: it causes epidemic chronic colitis in cattle and other species, including primates; it is reportedly detectable in the intestinal tissues and blood of many CD patients; antibodies to the organism are often disease-associated; and in some cases,

antimycobacterial drugs ameliorate disease.

Four not necessarily mutually exclusive mechanisms have been proposed to drive pathogenic immunologic responses to luminal microbial antigens: (1) microbial pathogens induce intestinal inflammation, (2) dysbiosis of commensal microbiota, (3) host genetic defects in containing commensal microbiota, (4) defective host immunoregulation. These mechanisms increase exposure of bacterial antigens to mucosal T cells or alter host immune responses to commensal bacteria. The detection of MAP DNA in the blood of CD patients may suggest that this organism is a persistent pathogen in CD. However, these results could be a secondary phenomenon due to increased gut permeability or the inability of macrophages in CD to kill MAP. The increased gut leakiness hypothesis is supported by the more frequent detection of other organisms in CD patients compared with controls and suggests a lack of specificity of MAP detection.

Another recent study supports the possibility that *M. paratuberculosis*, though not itself a very effective pathogen in humans, might induce local suppression of phagocyte function in infected tissue, and that this, in turn, might lead to chronic replication within macrophages of other bacteria, including perhaps the mucosal *E. coli* isolates that have now been found in CD tissue samples by several independent groups[4].

MAP

MAP is a member of the *M. avium* complex. *M. avium* strains are widely distributed in the environment and also inhabit normal animal and human intestines. *M. avium* strains do not usually cause disease unless the host is debilitated or immunocompromised. MAP, in contrast, is a specific pathogen with the ability to cause chronic inflammation of the intestine (Johne's disease) in many species, including ruminants and primates[5]. Moreover, it has been suggested that MAP can exist in animal tissue for years without causing clinical disease. Initial clinical signs follow a prolonged incubation period of 2 to 10 years, depending upon the exposure level and the individual animal's ability to fight the infection[6]. MAP invades macrophages in lymphoid tissue in the ileum, where it inhibits phagosome maturation and induces the recruitment of inflammatory cells, resulting in granulomatous enteritis. Characteristics distinguishing MAP from other *Mycobacterium* spp. include: its extremely slow growth; its inability to produce mycobactin; its possession of the insertion element IS900 that occurs as 14-18 copies within the *MAP* genome[7]. The DNA sequence, IS900, is considered the "gold standard" for differentiating MAP from other mycobacteria[8]. Although other IS900-like elements have been described in environmental mycobacteria, the entire IS900 gene is unique to MAP[9].

In common with other mycobacteria, MAP possesses a thick, waxy cell wall containing 60% lipid, which confers on it the properties of acid fastness (the ability to resist decolorization by acidified alcohol), hydrophobicity, and increased resistance to chemicals (e.g. chlorine) and physical processes (e.g. pasteurization)[7]. At least two potential modes of MAP transmission from the host animal to humans have been hypothesized, i.e. ingestion of either contaminated water or milk[10]. The detection of cell wall-deficient forms, also known as spheroplasts, in tissue cultures from affected humans, and subsequent identification of the agent as positive for the IS900 insertion sequence found in MAP by polymerase chain reaction (PCR) and *in situ* hybridization (ISH), has been the basis for suggesting MAP as a potential cause of CD[11]. Hence, MAP spheroplasts may play a role in the development of these human diseases, as well as in paucibacillary forms of Johne's disease in other species.

DETECTION OF MAP IN CROHN'S DISEASE

The association of MAP with CD is supported by identification of MAP in CD patients but not in appropriate controls. The gold standard for detection of MAP is based on isolation of the organism through culture methods. However, such methods are time consuming, because of the organism's fastidious nature and slow growth. Molecular and serologic methods are widely-used alternatives[12].

MAP CULTURE

Advances in culture methods now enable researchers to grow MAP from intestinal tissue, milk, and blood in CD patients[13]. The introduction of commercially available BACTEC and MGIT liquid culture systems, together with the application of IS900 PCR to such cultures, have led to substantial improvements in the ability to detect subclinical MAP infection in ruminants[14]. Some researchers have suggested that bacterial culture using liquid media has greater analytical sensitivity than that using solid media. Recently, two types of culture media have been shown to determine differential growth of MAP strains. This should be borne in mind when evaluating the detection capabilities of diagnostic tests or interpreting data from molecular epidemiologic studies performed using different type of culture medium[15]. MAP has been cultured from the milk of two women with active CD, who were breastfeeding[16]. This observation has not been replicated by other researchers. In another study[17], MAP was recently cultured in 50% of blood samples from CD patients, 20% of samples from UC patients and 0% of samples from healthy controls. In this study, the observation that MAP could be cultured from CD patients did not correlate with the use of immunosuppressive medications. Finally, MAP has also been cultured in a higher percentage of bowel-pinch biopsies from CD patients (42%) than from controls (9%)[18]. A preliminary report issued by a National Institutes of Health-sponsored blinded study

showed no differences in the culture recovery rates of two independent laboratories, and no detection of MAP 16s rRNA[19].

DETECTION OF THE INSERTION SEQUENCE IS900

The two main methods used to detect the insertion sequence IS900 include PCR and ISH assays. Previous studies have shown that reliable and reproducible detection of *MAP* by PCR applied directly to DNA extracted from human tissue and other samples, is extraordinarily difficult. The use of suboptimal sample-processing procedures results in false-negative results[18]. The results of MAP detection using nucleic acid-based techniques have recently been reported in two meta-analyses. These suggest that there is sufficient evidence for the presence of MAP in the gut of CD patients, regardless of whether CD patients are compared with individuals without inflammatory bowel disease or with UC patients[9,12]. Nevertheless, this association remains controversial and inconclusive. PCR data are open to criticism because the technique assays DNA that could come, either from live bacteria, or merely from the scattered debris of killer organisms and thus be of questionable biologic importance[13].

SEROLOGIC STUDIES OF MAP

Another approach to studying the possible role of MAP in the etiology of CD is to evaluate CD patients for the presence of antibodies reactive against MAP antigens[20]. Serologic tests for diagnosis of paratuberculosis, such as agar gel immunodiffusion, enzyme-linked immunosorbent assay and complement-fixation, are relatively easy to perform but suffer from a lack of sensitivity[10]. In a recent meta-analysis[12], the prevalence of MAP antibodies was higher in CD patients than in controls in most of the studies but there were high levels of inter-study heterogeneity. For the studies using serologic markers, the sources of heterogeneity remained unclear: confounding factors, bias and differences in study populations are all likely to have contributed to heterogeneity[12]. The p35 and p36 antigens were the most frequently used, and several studies have shown that CD patients display specific reactivity to the p35 and p36 antigens[21]. However, both p35 and p36 are present in *M. avium* subsp. *avium*, suggesting that a similar reaction would be obtained with the *M. avium* subsp. *avium* p35 and p36 homologs[20]. Accordingly, though positive tests were more common among CD patients than among UC patients and controls, this is not necessarily because of MAP infection but may be attributable to other MAP-like bacteria.

MAP AND GENETIC SUSCEPTIBILITY TO CROHN'S DISEASE

The simultaneous discovery, by two groups using positional cloning and candidate-gene approaches, of Nucleotide Oligomerization Domain 2/Caspase Recruitment Domain 15 (NOD2/CARD15*)* as a susceptibility gene for CD[22], provided specific support for the long-held theory that a genetically dysregulated host immune response to luminal bacteria results in CD. Muramyl dipeptide, a component of bacterial peptidoglycan, is recognized by the NOD2 receptor; nonetheless, the exact mechanism whereby NOD2 polymorphisms contribute to increased propensity to develop CD is still not completely understood. NOD2/CARD15 deficiency induces abnormal development and function of Peyer's patches, characterized by an exaggerated immune response and increased permeability[23]. Thus, patients carrying CARD15/NOD2 mutations are unable to control bacterial infections, which results in an inadequate innate response to bacterial invasion and enables bacteria to accumulate[24]. CARD15/NOD2 mutations are associated with defective clearance of invasive *Salmonella* infection in epithelial cells. Hence, an attractive explanation linking CARD15/*NOD2* to CD is that of ineffective clearance of intracellular MAP infection: indeed, a recent clinical study has shown that the mononuclear cells of CD patients, which are mutant for NOD2, display defective recognition of MAP bacteria[25].

However, no association between MAP serologies and NOD2 polymorphisms was observed in a large population-based study conducted in Manitoba[26]. Elsewhere, in a small-scale Sardinian study[27], the possibility of an interaction between MAP positivity and NOD-2 gene mutations was raised, with the authors suggesting that there might be a trend towards an association between the presence of CARD15/NOD2 mutations and MAP-positive status. However, this association was only present when CD subjects were compared against controls who had CARD15/NOD2 mutations, whereas having NOD2 mutations had no impact on MAP status among the CD subjects[27]. The same team failed to observe any association between SLC11A1 polymorphisms in the Sardinian population and MAP infection[28].

A genome-wide association study identified the interleukin-23 receptor (IL-23R) as a novel susceptibility gene for CD[29]. Mounting evidence suggests that IL-23, which is similar to IL-12, is critical for generation of an adaptive immune response that is protective vis-à-vis intracellular pathogens, including *M. tuberculosis* infection[30]. Recently, two studies demonstrated an association between CD and a coding variant of autophagy-related-16-like 1 (ATG16L) gene[31] and IRGM gene[32], thereby implicating the autophagy pathway of the innate immune system. The autophagy trafficking pathway is critical in inhibiting *M. tuberculosis* survival in infected macrophages[33]. Accordingly, infection of a subset of CD patients with intracellular killing defects caused by ATG16L1, IRGM, IL-23, NCF4, or any other as yet unreported gene, needs to be investigated[34].

ANTIMYCOBACTERIAL ANTIBIOTICS FOR CROHN'S DISEASE

Another approach to identifying disease causation is the use of chemotherapeutic agents to eliminate the infectious agents. The most irrefutable evidence of the fact that a microbial agent causes a given disease is long-term remission of clinical manifestations and an altered natural history of disease following clearance of the infection. In common with other atypical mycobacteria, MAP has characteristics that limit the number and type of potentially effective antibiotics[35]. *In vitro* sensitivity analyses show that clinical isolates of MAP are not responsive to traditional anti-*M. tuberculosis* agents, and so isoniazid, ethambutol, and rifampicin are not effective. Clarithromycin and azithromycin are considered to be the most effective drugs for treatment of MAP. In 2000, a meta-analysis[36] suggested that antimycobacterial treatment may be effective in maintaining remission achieved by corticosteroids. Treatment of CD with antimycobacterial therapy does not seem to be effective without a course of corticosteroids to induce remission. However, because of the small number of studies included in this meta-analysis and the heterogeneity of the trials, which used a wide range of antibiotic combinations administered for variable periods, these results should be interpreted with caution. Lastly, the largest study, a well-designed, randomized, placebo-controlled trial of clarithromycin, rifabutin and ethambutol, failed to show a sustained response in CD patients. Although the antibiotics registered a short-term benefit at 16 wk, in addition to the effect of the corticosteroid therapy, the study showed no prolonged advantage of the antibiotic combination, whether during the 2-year treatment phase or, more importantly, after the therapy had been halted[3].

An argument against a role for MAP in CD is that, if CD were indeed a chronic mycobacterial infection, then immunosuppressive therapies (corticosteroids, thiopurine drugs and tumor necrosis factor (TNF)-α suppressive therapies) should be associated, not with improvement, but rather with increased rates and severity of mycobacterial disease[35]. Currently, there is no published evidence, clinical or experimental, to establish whether or not MAP infection is exacerbated by TNF-α antibodies[37]. In one study[38], corticosteroid therapy was associated with lower MAP detection rates. However, in the case of the most commonly used immunosuppressive drugs used to treat CD symptoms, such as thiopurine drugs, e.g. azathioprine, and their metabolites, e.g. 6-mercaptopurine, inhibit the growth of MAP *in vitro*[39]. It is possible that intracellular cell deficient (spheroplast) MAP may not replicate well despite immunosuppression[35]. A recent study[40] showed that antimycobacterial and thiopurine drugs used in concert may produce an interactive effect. The apparent bacteriostatic effects of 6-mercaptopurine on *M. paratuberculosis* rendered the organism less susceptible to the bactericidal effects of antibiotics. These findings should also influence the design of therapeutic trials aimed at evaluating antibiotic treatments of CD: thiopurine drugs may confound interpretation of data on therapeutic responses for both antibiotic-treated and control groups.

EPIDEMIOLOGIC EVIDENCE FOR MAP AS A CAUSE OF CD

Other arguments against a role for MAP in CD are based on the difficulty of reconciling some of the epidemiologic features of CD with a causative role for chronic MAP infection[41]. First, farmers (and persons in rural settings) should be at increased risk of a livestock-associated pathogen, yet there is no evidence that they have increased rates of CD[42]. Second, environmental conditions, such as poor sanitation and overcrowding which should favor transmission of an infection, actually appear to protect against CD. Third, there is a remarkable paucity of evidence for vertical or horizontal transmission of CD[41]. Finally, detection of MAP in CD is neither disease- nor bacterium-specific. Detection of bacterial DNA in the granulomas of intestinal CD is not specific to MAP, in that other forms of bacterial DNA are also present[43]. However, a study which reviewed epidemiologic models of disease causation, has concluded that the current epidemiologic evidence strongly supports the conjecture (especially among those who believe in the theory of inductivism) that CD is caused by MAP[5]. Inductivism is one of the major philosophical doctrines about causation. This doctrine holds that science proceeds from observation to theory, beginning with observations derived from experiments, and extrapolating from these to general laws[5].

CONCLUSION

MAP is the causative agent of Johne's disease. It seems likely that chronic infection with MAP does occasionally occur in humans. MAP is widely present in our food chain and the DNA of this organism can be recovered from the intestine of CD patients. Studies have shown that a high percentage of subjects with CD are infected with MAP, though whether the association of this bacterium and CD is causal or coincidental is not known. Epidemiologists have gathered enough information to indicate an association between MAP and CD. Nonetheless, the role of MAP in CD etiology is not known, and may be determined from consistent results of studies using improved methods of isolation and detection of MAP bacilli and/or MAP-elicited immune responses in the host.

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