The evidence for *Mycobacterium paratuberculosis* in Crohn's disease

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Purpose of review

Though long hypothesized, the putative link between *Mycobacterium avium paratuberculosis* and Crohn's disease remains neither confirmed nor refuted. This article reviews published contributions that directly or indirectly address this question.

Recent findings

Epidemiologic studies, looking for *M. avium paratuberculosis* DNA in Crohn's tissue, show a strong association between the agent and this disease. Supporting data, however, are presently inconclusive on a causal role. Genetic studies provide indirect support for a role of mycobacteria in Crohn's disease, by identifying susceptibility genes that encode proteins implicated in innate immunity to intracellular bacteria. Clinical trial data support at least a short-term benefit for antimycobacterial therapy in Crohn's disease, but the microbial specificity of this response is presently unknown.

Summary

There appears to be a strong association between *M. avium paratuberculosis* and Crohn's disease, but the causality of this association is unknown. Consequently, the therapeutic implications of this association require further study. A number of critical questions about the biology of *M. avium paratuberculosis* remain unanswered. Data from studies of this organism, and its interaction with the immune system, can help address proposed reasons for or against a role of *M. avium paratuberculosis* in the etiology of Crohn's disease.

Keywords

Crohn's disease, *Mycobacterium avium paratuberculosis*

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Introduction

Two decades prior to the description of Crohn's disease as a clinical entity [1], Dalziel [2] had drawn an association between cases of chronic enteritis in humans and pseudotuberculosis (now known as paratuberculosis) in cattle. In the ensuing decades, several dozen published studies, reviews and workshop proceedings have provided evidence either to claim or dispute an association between the causative agent of paratuberculosis, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and Crohn's disease. Overall, three-quarters of a century of debate has yielded more heat than light, a circumstance that we largely attribute to a lack of available tools or paucity of resources committed to address this question with suitable scientific rigor. Fortunately, recent advances in the field have enabled the formulation of studies to test this hypothetical link and help resolve this longstanding debate.

We present here a brief review of the current evidence for or against a link between MAP and Crohn's disease. We next highlight priority research areas and detail specific questions that need to be answered to formally test this hypothesis to resolve this nearly century-old question with important clinical implications for the treatment of patients with Crohn's disease.

What is *Mycobacterium paratuberculosis*?

Mycobacterium paratuberculosis is a member of the mycobacterial genus, which includes the agents of tuberculosis (*M. tuberculosis*) and leprosy (*M. leprae*). At the species level, *M. paratuberculosis* is a subspecies of *M. avium*, and is hence referred to as *M. avium* subsp. *paratuberculosis* or MAP. Although MAP belongs within *M. avium*, genomic study has uncovered tremendous variability between *M. avium* organisms [3]. Hence, the name *M. avium* confers precision similar to the term *Escherichia coli*, potentially obscuring important phenotypic differences among *M. avium* subsets.

The extent to which these *M. avium* variants phenotypically differ is largely unknown. While a few characteristics of MAP are accepted, others have been more tenuously assigned. For instance, MAP is slow growing and depends on addition of the siderophore mycobactin J to permit

in-vitro growth. These two properties are not, however, linked. Adding mycobactin J to culture broth permits growth of bovine strains of MAP (so-called MAP Cow). While ovine strains (MAP Sheep) are probably not mycobactin-dependent, they are, however, more challenging to grow in the lab [4•]. An epidemiologically relevant property is whether MAP is a ubiquitous environmental mycobacterium, or rather a host-associated pathogen. Both microbiologic and epidemiologic studies argue for the latter. Microbiologic studies have documented finite survival outside of the host, such that de-stocking of livestock can be used to render a pasture MAP-free [5]. Epidemiologic support that MAP is not a ubiquitous environmental organism comes from the striking paucity of MAP isolates among the many cases of AIDS-associated *M. avium* bacteremia (just one case report). While tempting to attribute this lack of reporting to under-detection by hospital laboratories, it is noteworthy that unknown mycobacteria were first detected and named in this clinical setting (e.g. *M. genavense*), and novel species continue to be described (e.g. *M. saskatchewanense*). As clinical labs in developed countries detect *M. avium* as one of the most common isolates, the failure to routinely find MAP argues against it being a ubiquitous organism that immunocompromised hosts encounter on a regular basis.

Findings that directly implicate *M. paratuberculosis* in Crohn's disease

Crohn's disease shares certain clinical and histopathological similarities with Johne's disease, a chronic inflammatory enteritis of ruminants caused by MAP. Prompted by these observations, a number of epidemiologic studies have aimed to detect MAP infection in Crohn's disease and control patients. Difficulties with isolating this fastidious organism in pure culture mean that the most common of these studies are those using in-house polymerase chain reaction (PCR) directly on tissue. Before considering the results of these studies, it is worth noting that in-house PCR in the tuberculosis lab has been shown prone to error, in part because of the many methodologic variances between labs (direct PCR vs. nested PCR, differing number of cycles and extension times, etc.) [6]. Additionally, despite the theoretical promise that PCR might be able to detect as little as one genome in a sample, the experience with detection of *M. tuberculosis* in sputum has been that PCR is less sensitive than culture [7]. At least two considerations have hampered the sensitivity of PCR for mycobacterial infection: (i) the cell wall is very thick and hard to lyse, and (ii) the organism is buoyant and therefore resists concentration by centrifugation.

In the specific case of PCR-based detection of MAP in Crohn's disease, one further hurdle limits our ability to interpret findings from these studies. For *M. tuberculosis*, when nucleic acid amplification assays became available, an acceptable gold standard was available for comparison (culture), permitting validation of tests. In studies seeking MAP in Crohn's disease, positive cultures have been exceedingly rare, so validation is problematic. While one control is amplifying a MAP genetic target from purified genomic DNA, the technical challenge with tissue PCR is detection of MAP DNA within the mycobacterium, within the macrophage, within the tissue. To verify this, one should prevalidate a protocol with MAP-infected tissue, ideally where the bacterial burden is low (e.g. paucibacillary Johne's disease in sheep). To verify specificity, one should determine that tissue infected with another mycobacterium fails to amplify MAP DNA using the designated protocol. The authors are unaware of a PCR-based study of Crohn's disease where the assay was validated on a panel of tissues infected with MAP and other mycobacteria prior to testing human samples.

With these caveats in mind, the most recent advance in this field is the collection of this disparate literature in the form of a meta-analysis [8•]. Despite a high degree of heterogeneity between studies, the association between MAP and Crohn's disease has been reported by a number of different independent laboratories, leading to an overall odds ratio (OR) of 7.0. Notably, even studies finding a strong association have reported varying proportions of MAP in control groups: Bull and colleagues [9] detected MAP in 26% of controls, while Autschbach and colleagues [10] detected MAP in 2% of ulcerative colitis and 5% of noninflammatory bowel disease controls. The importance of this difference may not matter when looking for an association, but when trying to understand the causal role of an organism in a disease, it is important to know whether the organism is commonly present in control subjects and enriched in the disease, or rather whether its presence is very specific for disease. The inconsistency of findings across different studies, using methods that are prone to technical variability, continues to be a major impediment to a definitive assessment of the role of MAP in Crohn's disease.

Considerations that indirectly support a role of *Mycobacterium paratuberculosis* in Crohn's disease

Independent of studies looking for MAP in Crohn's disease, recent findings from several fields of investigation have generated results that are supportive, but not necessarily indicative, of a mycobacterial etiology in Crohn's disease. Like mycobacterial diseases, Crohn's disease is increasingly considered to involve some form of immunodeficiency, based on immunologic studies of biopsy sites [11•] and therapeutic benefit with administration of granulocyte macrophage-colony stimulating factor [12]. This notion has gained further support from the findings of whole genome association studies that are generating an ever-expanding list of Crohn's disease susceptibility genes. A common thread among these Crohn's disease susceptibility genes is defective innate immunity

Table 1 Crohn's susceptibility genes and mycobacterial resistance

Gene	Role in mycobacterial immunity	References
NOD2	Mononuclear cells from patients with permissive *NOD2* alleles have decreased recognition of MAP *in vitro*	[14•]
IRGM1	Mice disrupted for *Irgm1* (also known as LRG-47) have decreased resistance to *M. avium*	[15]
IL23R	Mutations of the interleukin-12 p40 subunit, which is shared with interleukin-23, predispose to disseminated mycobacterial infection in infants. In murine models, interleukin-23 has an important role in generation of antimycobacterial $CD4^+$ T-cell responses.	[16,17]

to intracellular bacteria [13••]. As shown in Table 1, for three Crohn's disease susceptibility genes (*NOD2*, *IL23R*, *IRGM1*), there are already data implicating the gene in mycobacterial resistance [14•,15–17].

Importantly, these putative associations between Crohn's disease susceptibility genes and mycobacteria do not indicate bacteriologic specificity: *Nod2* and *Irgm1*-disrupted mice have impaired handling of *Listeria* infection [18,19] and infants deficient in interleukin-12 p40 (and hence interleukin-23) are at risk of *Salmonella* infection [16]. Therefore, genomic studies of Crohn's disease should serve to encourage further experimentation with candidate intracellular bacteria, MAP included.

Arguments against a role for *Mycobacterium paratuberculosis* in Crohn's disease

Two commonly expressed arguments against a role for MAP in Crohn's disease are: (i) farmers (and people in rural settings) should be at increased risk of a livestock-associated pathogen, but there is no evidence that they have increased rates of Crohn's disease [20]; and (ii) if Crohn's disease was a chronic mycobacterial infection, tumor necrosis factor (TNF)-α suppressive therapies should be associated with increased rates and severity of mycobacterial disease, rather than improvement [21].

An occupational risk is observed for infectious diseases where spread is by aerosols (e.g. tuberculosis, brucellosis) or direct contact (cutaneous anthrax, hepatitis B). In contrast, an occupational risk of livestock-associated food-borne pathogens in farmers is not the norm. One study of 12 327 *Campylobacter* cases from Norway found no clustering of disease by counties and no association between rates of human disease and grazing density [22]. A study of 8598 *Escherichia coli* O157 cases by the Centers for Disease Control reported direct contact with animals in only 11 cases [23]. Therefore, in the case of MAP, without knowing where and how humans may be exposed (water vs. food vs. direct contact), we consider it difficult to project which occupations should or should not be at higher risk. Although the optimal study design to determine risk may involve challenge experiments in humans that are unethical or not feasible, there may be certain natural experiments (e.g. people who drink unpasteurized milk, vegetarians) that could help address this epidemiologically. Already, it has been shown by Abubakar and colleagues [24] that consumption of pasteurized milk was associated with a reduced risk of Crohn's disease (per kg/month: OR = 0.82) and that meat intake was associated with an increased risk (per kg/month: OR = 1.40).

In the case of TNF-α inhibition, to our knowledge, there is no published evidence in either the clinical or experimental setting that establishes whether MAP infection is exacerbated by antibodies against TNF-α. One option is to generate an expected outcome by extrapolating from data on *M. tuberculosis*, based on the premise that immunity to mycobacteria is uniform and independent of species. Unfortunately, the published literature suggests a qualitatively different role for TNF-α during infection with *M. tuberculosis* as compared with *M. avium*. In human series, the association between infliximab and tuberculosis has been striking, both in number of reported cases and in the severity of disease (disseminated tuberculosis). In contrast, the number of case reports on PubMed of *M. avium* disease after infliximab or eternacept has been low, although the actual rate may be underestimated as some series did not speciate mycobacterial isolates [25]. As *M. avium* is not a reportable pathogen; these results may represent some degree of publication bias, nevertheless, the data are derived from pharmacological databases monitoring adverse events and thus represent reasonable estimates. Moreover, the rate of *M. avium* disease after TNF-α inhibition pales in comparison to the rate of *M. avium* disease in other at-risk patient groups, such as AIDS patients prior to the advent of highly active antiretroviral therapy (up to 70%), arguing that TNF-α has a less important role in *M. avium* infection. Further support for a differential role for TNF-α across human mycobacterial infections comes from a recent report where infliximab was used in the treatment of leprosy [26].

Data from experimental murine infections also support a qualitatively differential role of TNF-α in the control of *M. tuberculosis* infection, when compared with *M. avium*. In the case of *M. tuberculosis*, neutralization of TNF-α using monoclonal antibodies (simulating infliximab) in early infection results in prompt morbidity and mortality, and blockade during chronic infection also results in progressive demise [27]. In contrast, the impact of antibody treatment during early infection with *M. avium* has been variable, with no evidence of accelerated mortality [28,29]. Unfortunately, we could not find data on the effect of anti-TNF-α antibodies during chronic *M. avium* infection. Importantly, while *M. tuberculosis* is not *M. avium*, it is vital to remember that MAP is itself an

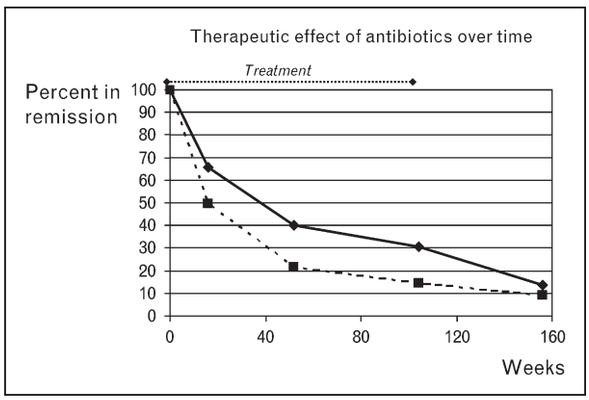
immunologically unique subset of *M. avium*. Therefore, just as the *M. tuberculosis* experience cannot accurately predict clinical outcomes with *M. avium*, data for the latter organism should not be blindly applied to predict outcomes with MAP where there are no data that we are aware of. Information on whether neutralization of TNF-α in animals chronically infected with MAP results in worsening (or improvement) of disease would be valuable to address this question.

Avenues forward

Broadly speaking, one can envision three avenues forward to help address the impasse in the current literature: epidemiologic, clinical trials and/or improved fundamental understanding of both MAP and Crohn's disease. Epidemiologic studies could be informative on whether a consistent association is observed across centers between presence of MAP and Crohn's disease. Prior to such studies being initiated, however, an assay is needed that has been validated to be both reproducible (different labs get the same result) and accurate (different labs get the right result). Without this precondition, we caution that little or no progress will be made in tissue studies of Crohn's disease patients and controls. Fortunately, through the efforts of teams of MAP investigators, in the USA and Europe, quality-assured cultures and panels of tissue can be made available to researchers interested in this question to ensure that laboratory methods are empirically validated prior to epidemiologic study. Of note, the development of immunologic tests based on MAP-specific antigens also requires a gold-standard microbiologic assay, as novel immunologic assays for tuberculosis were first validated in patients with culture-proven tuberculosis.

A second approach would involve treatment trials, looking at clinical benefit with antimycobacterial agents, and optimally, for evidence of clearing of MAP infection on therapy. A recently published study, using an anti-*M. avium* cocktail, reported transient benefit (increased remission at 16 weeks), but no further benefit beyond this time [30•]. Of note, the subset of patients in remission at 16 weeks was analyzed for outcomes at later timepoints, even though randomization had occurred at week 0. By comparing the two treatment groups over time, as an intention-to-treat analysis, one observes a statistically significant absolute benefit of 15–20% during treatment that is lost when antibiotics are stopped (Fig. 1). Nonetheless, without a specified target of antimicrobial therapy, a major issue with antibiotic trials is that this approach cannot really address the role of a single microbe in this disease, but rather answers the more clinically relevant question of whether antimicrobial therapy can make patients better. An advance would come if such studies can embed microbiologic studies to determine whether clinical amelioration is associated with elimination of a candidate microbe. To do this, however, requires a robust and reliable assay, returning us to the problem of the preceding paragraph.

Figure 1 Effect of antibodies against Crohn's disease in [30•]



A recently published clinical trial showed that addition of antibiotics to prednisolone led to increased remission compared with prednisolone plus placebo. Those in remission at 16 weeks were followed to determine whether the benefit extended to 52, 104 and 156 weeks. By analyzing percentage remission as a function of all patients randomized to antibiotics (solid line) or placebo (dashed line) at the beginning of the study, one obtains this figure, showing that the 15–20% absolute benefit with antibiotics is seen at 52 weeks ($P = 0.003$) and 104 weeks ($P = 0.005$). Data obtained from Selby and colleagues [30•].

A third approach involves an improved fundamental understanding of both MAP and Crohn's disease. At the microbial end, we need a better understanding of how MAP differs from other *M. avium* organisms, especially in experimental infections. We need to better understand the role of *NOD2* and other Crohn's disease susceptibility genes in disease pathogenesis, as individuals whose *NOD2* genotype predicts an elevated risk of Crohn's disease are still much more likely to be healthy than have Crohn's disease. Finally, we need to test for host-pathogen interactions, between intracellular pathogens and Crohn's disease susceptibility genes. For instance, mouse knockouts of the orthologs of Crohn's disease susceptibility genes can be used to determine whether challenge with candidate pathogens produces disease conditional upon gene and microbe.

Conclusion

Despite decades of efforts to address the role of MAP in Crohn's disease, the hypothesis remains neither proven nor refuted. With the availability of newly developed tools and reagents as well as the availability of human and bacterial genome data, opportunities now exist to extend analyses beyond epidemiologic studies and confront this question through alternative approaches. For patients and their treating doctors, a clear answer, one way or the other, will be an important first step towards a more definitive plan to manage this debilitating disease.

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References and recommended reading

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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