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REVIEW

Mycobacterium avium subspecies *paratuberculosis* in the etiology of Crohn's disease, cause or epiphenomenon?

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Abstract

The origin of inflammatory bowel disease is unknown. Attempts have been made to isolate a microorganism that could explain the onset of inflammation, but no pathological agent has ever been identified. Johne's disease is a granulomatous chronic enteritis of cattle and sheep caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and shows some analogies with Crohn's disease (CD). Several studies have tried to clarify if MAP has a role in the etiology of CD. The present article provides an overview of the evidence in favor and against the "MAP-hypothesis", analyzing the methods commonly adopted to detect MAP and the role of antimycobacterial therapy in patients with inflammatory bowel disease. Studies were identified through the electronic database, MEDLINE, and were selected based on their relevance to the objective of the review. The presence of MAP was investigated using multiple diagnostic methods for MAP detection and in different tissue samples from patients affected by CD or ulcerative colitis and in healthy controls. On the basis of their studies, several authors support a close relationship between MAP and CD. Although increasing evidence of MAP detection in CD patients is unquestionable, a clear etiological link still needs to be proven.

Key words: *Mycobacterium avium paratuberculosis*; Crohn's disease; Inflammatory bowel disease; Johne's disease; Mycobacterial protein tyrosine phosphatase

Core tip: The etiology of inflammatory bowel disease (IBD) is unknown. Some analogies between Crohn's disease (CD) and Johne's disease, a granulomatous chronic enteritis of cattle and sheep caused by *Mycobacterium avium* subspecies paratuberculosis (MAP) have been identified. Several studies have tried to clarify if MAP has a role in the etiology of CD. However, the involvement of MAP in CD is still debatable. The present article provides a literature review of the evidence in favor and against the "MAP-hypothesis", the methods commonly adopted to detect MAP and the role of antimycobacterial therapy in treating IBD patients. In particular, new mechanistic findings seem to encourage the CD-MAP relationship.

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INTRODUCTION

Inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) represent a group of

chronic inflammatory alterations, whose etiology is still unknown and is notoriously multifactorial. According to the most accepted hypothesis, CD and UC are the result of an inappropriate and deregulated intestinal immune response against the normal bacterial flora. This is due to a complex interaction between environmental, genetic and immune factors. The above-mentioned factors are all necessary, but none of them, if singularly considered, is enough to explain the etiopathogenesis of IBD. We will focus our attention on analyzing the available data on this latter theory, mainly on the possible involvement of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in IBD.

On the basis of analogies between CD and some forms of infective enterocolitis, the hypothesis of a specific mycobacterial etiology has been proposed. In particular, some authors have studied the potential role of MAP in CD. MAP is the responsible agent in Johne's disease (JD), a chronic granulomatous enteritis affecting cattle and sheep, and could be implicated in the etiopathogenesis of disease in humans, according to clinical and histological similarities between the two diseases[1,2]. The MAP hypothesis has aroused great scientific interest since the 1980s, following data reported by Chiodini *et al*[3,4]. There is now a renewed interest regarding the association between CD and MAP, due to the improvements in microbiological and genetic methods[3,4]. However, published data on this issue are still contradictory. Two main hypotheses exist: MAP could represent the "primum movens" in causing CD or, alternatively, it could constitute an epiphenomenon of the disease, later colonizing the already inflamed mucosa in these patients[5].

INFECTIVE THEORY

Many attempts have been made to isolate a microorganism that could act as the "primum movens" in the etiology of IBD (Table 1), however, to date no pathogen has been convincingly identified. Numerous bacterial pathogens such as *Listeria monocytogenes*[6], *Pseudomonas maltophila*[7], *Mycobacterium kansasii*[8], *Bacterioides fragilis*[9], and *Chlamydia pneumonia*[10], and viral agents including the measles virus[11,12] and *Cytomegalovirus*[13] have been proposed as the cause of CD, but none have been accepted due to a lack of firm evidence.

Wakefield *et al*[12] proposed that CD is due to chronic infection of submucosal endothelium in the gut by the measles virus, inducing a granulomatous reaction and a microinfarction pathological process. This theory was supported by epidemiological studies which associated perinatal exposure to the measles virus[14] and the attenuated measles vaccine[15] with an increased risk of the development of CD. Because of the absence of an increased risk for CD in vaccinated patients[16], the difficulty of detecting the virus in intestinal specimens[17] and low antibody titers to measles in CD patients[18], this hypothesis was rejected and measles has now been proposed as a possible cofactor rather than the primary causative agent of CD.

Table 1 The infective theory of inflammatory bowel disease etiology

Infectious agent	Ref.	Current position
Bacterial		
Listeria monocytogenes	[6]	Dismissed
Pseudomonas maltophila	[7]	Dismissed
Mycobacterium kansasii	[8]	Dismissed
Bacterioides fragilis	[9]	Dismissed
Chlamydia pneumoniae	[10]	Dismissed
Adherent-invasive *Escherichia coli*	[19-22]	Active
Proteobacteria	[23]	Active
Mycobacterium avium subspecies *paratuberculosis*	[24-26]	Active
Viral		
Measles virus	[11,12,14-18]	Dismissed
Cytomegalovirus	[13]	Dismissed

Supporters of the infective theory have considered the possible role of adherent-invasive *Escherichia coli* (AIEC) in IBD etiology. The link between IBD and AIEC was first suggested in 1978 by Tabaqchali *et al*[19], who noted high titers of antibodies against AIEC O-antigens in IBD patients. Later, this organism was often isolated from the mucosa of CD patients compared to healthy controls, due to its ability to adhere to gastrointestinal epithelial cells and to demolish the intestinal barrier by producing an α-hemolysin[20]. Further investigations revealed that AIEC stimulates the release of the pro-inflammatory cytokine, interleukin (IL)-8, which extensively replicates and induces tumor necrosis factor (TNF)α expression in macrophages, leading to the formation of granulomas. In an immunohistochemical study, 57% of tissue samples from CD patients contained *E. coli*, particularly near ulcers, along fissures, around abscesses, within the lamina propria and in the germinal centers of mesenteric lymph nodes[21]. In another study using polymerase chain reaction (PCR), *E. coli* DNA was found in 80% of intestinal granulomas in CD patients[22].

A recent review by Mukhopadhya *et al*[23] extensively described and eventually supported the role of Proteobacteria in the pathogenesis of IBD. They examined the importance of pattern recognition in the extracellular innate immune response of the host and postulated that Proteobacteria with adherent and invasive properties could exploit host defenses, driving proinflammatory changes, altering the intestinal microbiota, favoring dysbiosis and finally leading to the development of IBD.

Several clinical and histopathological similarities between IBD, particularly CD and JD, caused by MAP, indicated a potential role for this pathogen in CD etiology. Similar to *Mycobacterium tuberculosis* or *Mycobacterium leprae*, MAP is able to deregulate immune signaling as one of its survival strategies, resulting in a spectrum of disease manifestations, ranging from simple colonization with a healthy phenotype to severe inflammatory bowel disease[24]. This association has aroused great scientific interest since the 1980s, when Chiodini *et al*[25] isolated MAP and its DNA from biological tissues in CD patients.

Based on these observations, the link between MAP and CD has been widely investigated through direct and indirect methods for MAP detection: PCR for MAP-DNA in blood, stool and biopsies, MAP-specific serological and cell-mediated response, cultures of MAP from intestinal tissues, milk and blood samples[26].

MAP CHARACTERISTICS AND CLINICAL IMPLICATIONS IN ANIMALS

MAP is a Gram-positive, facultative obligate intracellular bacterial pathogen, acid-fast and dependent on mycobactin for its replication (a liposoluble substance that permits iron utilization). It grows very slowly *in vitro*, taking 8-16 wk or more for duplication, and it appears in human tissue in a cell-wall-deficient state (CWD), also known as "spheroplast". This is the reason why, different from *Mycobacterium tuberculosis*, it cannot be highlighted by Ziehl-Neelsen staining[27]. The discrimination between MAP and other organisms belonging to the *Mycobacterium avium* complex (MAC) is known to be problematic. The identification of a species-specific insertion sequence, a fragment of DNA of 1451 base pairs known as IS900, allowed easier distinction using the PCR technique[28,29]. Through the use of IS900 restriction fragment length polymorphism, two variants of MAP have been identified: type C associated with cattle and S-type, less virulent, associated with sheep. Although these subtypes show a tendency to preferentially infect some animal species, cross-infection is possible and documented[30].

It is thought that the contagion among animals primarily occurs *via* the fecal-oral route, through the ingestion of milk, colostrum or animal feed contaminated by MAP[31]. The possibility of intrauterine transmission of this microorganism is also documented, especially in symptomatic animals (20%-40% infected born from a mother with clinically-manifested disease and 10%-20% infected born from a mother with subclinical infection). Once in an organism, MAP is able to invade macrophages in ileal lymphoid tissue and increase in number inside phagosomes, inhibiting their maturation and promoting the recruitment of inflammatory cells. This process, associated with T-lymphocyte activation and interferon (IFN)-γ production, results in granulomatous lesions that characterize JD, or paratuberculosis, a chronic widespread enteritis of cattle, sheep and other ruminants. The progression of disease is classically categorized into four stages: silent infection, subclinical stage, clinical stage and clinically evolved disease. Animals are generally infected at an early age. Clinical signs, dominated by diarrhea and weight loss, generally occur 3-5 years after infection. This long-lasting incubation period depends on the level of environmental contamination (MAP exposure dose), virulence of the MAP strain, the capacity of a single animal of hinder the infection (immune response is more efficient with age), and genetic susceptibility *vs* MAP (inter- and intra-species differences)[32]. MAP has been isolated from the feces and milk of ruminants with clinical and subclinical infection: this fact explains the wide diffusion of MAP infection and the vast environmental contamination[33]. MAP can survive for a long period in different ecosystems, due to its high environmental resistance, but replication outside infected animals is not possible due to its dependency on mycobactin. Several studies have been carried out to identify environmental sources of human exposure to MAP, including its presence in water supplies (domestic water and rivers)[34-36], milk, dairy products, and even in meat[37]. MAP's ability to spread *via* the milk of infected animals has been proven and it has been shown that this microorganism is able to survive routine pasteurization methods, with persistence of vital mycobacteria and antigenic components in commercial milk[38]. MAP is widely present in the human food chain, and even in dairy products representing a potential exposure source. Environmental diffusion of MAP could represent a favorable condition for IBD onset when associated with intestinal immunodeficiency in susceptible subjects[39].

"MAP HYPOTHESIS": SUPPORTING AND CONTRASTING EVIDENCE

The MAP hypothesis in CD is considered to have originated in the 1980s, when Chiodini *et al*[25] demonstrated that MAP isolated from human tissues can cause a granulomatous disease of the distal small intestine in experimental animals. The authors isolated from the intestinal tissues of two CD patients a mycobacterium which shared the acid-fast and mycobactin-dependent characteristics of MAP. When injected intravenously or intraperitoneally in experimental animals, this organism induced a disease characterized by hepatic and splenic granulomas in mice, but not in rats, guinea pigs, rabbits or chickens. It also resulted in granulomatous disease in the distal small intestine in young goats, when administered orally. Acid-fast bacilli were not found in autopsy sections of the intestine, however, a single organism with characteristics similar to the one administered was seen in microgranulomas of the mesenteric lymph nodes. The identification of MAP in human tissues is seldom reported, because of its CWD form, as Chiodini *et al*[25] stated. Two recent meta-analyses showed that the association between MAP and CD seems to be specific compared with individuals free of IBD. The pooled Odds Ratio from studies using PCR for tissue samples was 7.01 (95%CI: 3.95-12.4) and was 1.72 (95%CI: 1.02-2.90) in studies using enzyme-linked immunosorbent assay (ELISA) for serum[40,41].

Indirect support for the "MAP hypothesis" comes from Shanahan *et al*[42] who found a similarity with *Helicobacter pylori*, an infectious agent widely dispersed, but able to cause peptic disease and gastric cancer only in a minority of exposed subjects. Similarly, *Mycobacterium tuberculosis* affects about one third of the world's population, but only 5%-10% develop clinically manifested disease. Analogies exist for MAP, in that it is widely dispersed and affects animals and humans, possibly playing a role in CD

Table 2 Direct methods for dectection of *Mycobacterium avium* subspecies *paratuberculosis*

Method	Tissue analyzed	Ref.	+/-	Advantage	Defect
MAP culture				Effective isolation of microorganism	Organism's fastidious nature; slow-growth
	Intestinal biopsy	[55]	+		
	Peripheral blood	[57,59,60]	-		
PCR for MAP-specific IS900 DNA				Fast and easy isolation of MAP fragment	Not able to distinguish vital MAP *vs* fragment of killed MAP; confounded by frequent MAP opportunistic infection (oro-fecal route)
	Intestinal biopsy	[53-55]	+		
	Peripheral blood	[58]	+		
	Peripheral blood	[59,60]	-		
	Stools	[63]	-		
PCR for MAP-specific IS900 DNA				Demonstrate the presence of active/ vital metabolic processes	Difficult to reproduce (RNA half-life measured in min)
	Intestinal biopsy	[62]	+		

+: Supporting *Mycobacterium avium* subspecies *paratuberculosis* (MAP) hypothesis; -: Contrasting MAP hypothesis; PCR: Polymerase chain reaction; IS900: Insertion sequence 900; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid.

onset only in few subjects.

A recent study by Xia *et al*[43] showed significantly higher levels of antibodies against a specific mycobacterial protein, tyrosine phosphatase (PtpA), in CD patients when compared to healthy controls. PtpA is secreted during MAP infection in a time-dependent manner from the human-derived monocyte cell line THP-1. The same evidence was not observed in UC patients, suggesting the exclusive involvement of MAP in CD[43].

Although analogies exist between CD and JD, some discrepancies should be mentioned. JD does not present some of the basic patterns of CD: the segmental localization and features that can complicate the evolution of CD, such as fistula, fissures, bleeding, stenosis, adhesions, perforation and the formation of abscesses are not described in JD.

Against the "MAP hypothesis", immunosuppressive drugs, widely employed in IBD, should not be used in latent or clinically manifested mycobacterial infections, as they increase the risk of onset and/or worsening of the disease. Screening analyses are necessary to exclude any type of mycobacterial infections before administering anti-TNFα drugs. *Mycobacterium tuberculosis* massively proliferates with anti-TNFα drugs or steroid treatment, and the same for *Mycobacterium avium intracellulare* in the intestine of HIV-infected patients[5]. It is possible that intracellular cell wall deficient MAP may not be able to replicate despite ongoing immunosuppressive therapy. Corticosteroid therapy was associated with lower MAP detection rates in the study by Autschbach *et al*[44].

In patients treated with infliximab, a decreased level of antibodies against PtpA was found and no statistical differences were observed in comparison to the controls. This observation suggests that inhibition of TNFα may reduce MAP growth probably with a negative effect on the secretion of PtpA[43,45,46]. Alternatively, infliximab interferes with the antigen presentation system in macrophages dampening the immune B response and the antibody titer. In this respect, a statistical difference in antibody titer was observed between CD patients treated with azathioprine (AZA) and untreated patients; no significant difference was observed when patients were treated with either 5-ASA or steroids[43]. "*In vitro*" studies showed the ability of AZA[47] and other immunosuppressants, such as cyclosporine[48] and methotrexate[49], to inhibit MAP growth in the culture system.

In addition, epidemiological evidence supporting the association between MAP and CD is still lacking. The prevalence of IBD is more common in urban areas, while no increased incidence of disease was detected among people in rural areas, especially in farmers and their families, despite major exposure to MAP[50]. Furthermore, environmental conditions which should promote MAP transmission, such as poor hygienic conditions and overcrowding, seem to protect against CD[51,52]. The lack of evidence for horizontal or vertical transmission of CD leads to MAP being a zoonotic agent or an opportunistic pathogen in humans.

It is hard to explain the paradox that an infection, although able to stimulate such an intense inflammatory reaction within the gut in CD patients, is not associated with a strong cellular or serologic reactivity against MAP. Detection of MAP-DNA in the granulomas of intestinal CD is neither disease-specific nor bacterial-specific, because other forms of bacterial DNA are also present[22]. IS900 was scientifically proven to be species-specific in animals affected by JD, however, its specificity in human tissues or in environmental ecosystems is unknown. Past studies supporting the MAP hypothesis probably included a percentage of false positive findings, ascribable to other mycobacterial species. IS900 PCR should be used as a confirmation test, in addition to other methods able to detect MAP presence in tissues.

DIRECT METHODS FOR MAP DETECTION

Because of its presence in human tissues in an intracellular cell-wall-deficient shape, MAP cannot usually be identified by Ziehl-Neelsen staining. The direct detection of MAP in humans requires culture, PCR for MAP-DNA/RNA (Table 2).

Isolation of the microorganism by culture methods is considered the gold standard for MAP detection. This method remains problematic and time-consuming even in the best circumstances, due to the organism's fastidious nature and slow growth. Although the use of MGIT

(Mycobacterial Growth Indicator Tube - Becton Dickinson) reduces growth time to 10-12 wk, there are still difficulties in culture isolation. DNA amplification with PCR and *in-situ* hybridization are the two main methods for MAP detection, using the IS900 sequence as a target.

Several studies, based on cultural methods and/or molecular techniques, demonstrated a higher prevalence of both the microorganism and its DNA in CD patients compared to controls. The presence of MAP has been investigated in different tissues, such as intestinal biopsies and surgical specimens, blood and recently fecal samples.

Biopsy specimens have always been considered by researchers as irreplaceable biological samples and have been widely utilized for MAP detection. Sanderson *et al*[53] identified MAP IS900 DNA in 26 of 40 (65%) CD patients, 1 of 23 (4.3%) UC patients and 5 of 40 (12.5%) control subjects. Later, Bull *et al*[54] described a MAP-DNA prevalence of 92% in CD patients (34 of 37) *vs* 26% non-IBD controls (9 of 34) (P = 0.0002). In 2005, Sechi *et al*[55] found a highly significant difference between CD patients (83.3%) and the control group (10.3%) (P = 0.000001) for IS900 PCR presence in DNA extracts of fresh intestinal mucosal biopsies, as well as in culture using supplemented MGIT media (63.3% and 10.3%, respectively, P = 0.00001). In the same year, Autschbach *et al*[44] and Romero *et al*[56] published data on the prevalence of MAP in surgical tissues.

Several authors have investigated the presence of viable MAP through culture or the detection of MAP-DNA in peripheral blood using PCR. Naser *et al*[57] found a statistically significant positivity in the CD group compared to the control. This result was confirmed in the study by Bentley *et al*[58], who found a significant over-representation of MAP-DNA in patients with CD *vs* controls (33.8% *vs* 21.5%, P = 0.002) in a larger cohort. Two recent meta-analyses have been published showing a generally higher MAP prevalence in CD *vs* controls in different samples[40,41].

However, a number of investigations have failed to demonstrate any evidence of an association between CD and detection of MAP-DNA. Parrish *et al*[59] reported that all CD patients were negative for MAP using PCR and only one healthy control patient was positive; however, no viable MAP was cultured from this individual and all blood cultures were negative for MAP, despite the large study size (130 CD, 130 healthy controls). Other authors found a high prevalence of MAP even in non-IBD controls[60].

Molecular techniques for assaying MAP-DNA are not able to discern the presence of vital microorganisms from fragments of killed MAP. RT-PCR for IS900-RNA detection overcomes this problem by directly demonstrating the presence of active metabolic processes at the time of isolation[61]. The most significant study in this field, although limited to a small number of patients (CD n = 8, UC n = 2, and controls affected by colon-rectal cancer n = 2), identified the presence of MAP-RNA in all ileal mucosal samples from CD and UC patients[62]. RNA has a half-life of minutes, therefore, detection of this molecule cannot only be related to the wide environmental spread of MAP, as would be legitimate to suppose in the case of DNA detection.

Recent studies have investigated the presence of IS900 DNA fragment in stools from CD patients, UC patients and from healthy controls. The use of human feces represents a new approach to investigate the "MAP hypothesis"; as it has the advantage that stools can be collected more easily than blood samples and biopsy specimens. In a previous study, the results revealed that detection of MAP DNA in human feces is very common: 21 of 31 CD (68%), 13 of 20 UC (65%) and 11 of 23 healthy controls were MAP-positive [CD *vs* controls: P = no significance (NS), UC *vs* controls: P = NS][63]. The IS900-positive percentage in IBD samples was higher than in healthy controls, but did not reach statistical significance, partially due to the small sample size. No significant correlation was observed when analyzing IS900 positivity in relation to disease activity in the IBD group[63].

INDIRECT METHODS FOR MAP DETECTION

Besides direct methods for MAP identification, indirect techniques are also available, with the aim of testing humoral and cellular-mediated immune response using the blood of IBD patients and healthy controls (Table 3).

ELISA represents the main diagnostic method for identifying cattle affected with JD, and is the most frequently used method to identify subjects immune to MAP. However, an ELISA validated for humans is not presently available.

Many studies have assayed the presence of MAP antibodies in CD patients and controls, obtaining contrasting results that should be related to the use of different methodologies and heterogeneous antigens (nuclear extracts, soluble antigens from cultured filtrates, recombinant antigens). For example, 57% of CD patients were positive for the detection of antibodies against a 38-kDa antigen, while 53% manifested specific antibodies for 24-kDa and 18-kDa bacterioferritin, but only 18% showed simultaneous seropositivity for all the three antigens[64]. Other studies demonstrated that the detection of anti-p36 antibodies is usually positive in CD patients (about 89%), while it seems to be less common in UC patients (about 15%). A specific reactivity against p35 and p36 is frequent in CD patients, but both these antigens are common to *Mycobacterium avium* subspecies *avium*, suggesting that cross-reactivity may influence results[65]. Other mycobacterial components used for testing humoral response against MAP are 14-kDa AhpC antigen and the heat shock proteins[66]. Of 13 studies included in a recent meta-analysis, 10 found that the prevalence of antibodies against MAP was higher in patients with CD than in controls, with a pooled odd ratio of 1.72 (1.02-2.90); similar pooled odd ratios were obtained when CD and UC pa-

Table 3 Indirect methods for detection of *Mycobacterium avium* subspecies *paratuberculosis*: humoral and cellular mediator of immune response to *Mycobacterium avium* subspecies *paratuberculosis*

Method	Tissue analyzed	Antigen/cellular mediator	Ref.
ELISA for antibody detection			
	Peripheral blood	38-kDa, 24-kDa, 18-kDa	[64]
		p35 *vs* p36	[65]
		14-kDa, AhpC, HSP	[66]
		MAP protoplasmic antigen	[67]
		18-kDa	[68]
		Anti-PPA	[69]
		Ptp-A	[43]
		Ptp-A, protein kinase G	[46]
ELISA for cytokine detection (proliferative assay)			
	Peripheral blood lymphocytes	INFγ; IL-17	[71,72]
	Gut biopsies lymphocytes T $CD4^+$	INFγ; IL-17	[73]
	Peripheral blood monocyte THP-1	TNFα	[74]

ELISA: Enzyme-linked immunosorbent assay; AhpC: Alkyl hydroperoxide reductase C; HSP: Heat shock proteins; PPA: Ptp-A protein tyrosine phosphatase; INF-γ: Interferon-gamma; IL-7: Interleukin 7; TNFα: Tumor necrosis factor alpha.

tients were compared (1.88; 1.26-2.81)[41].

Nevertheless, other studies on humoral response against MAP antigens did not find an association between CD and MAP: Brunello *et al*[67] reported no significant differences in the titers of IgG antibodies against MAP protoplasmic antigen between CD (3.7%) and UC patients (5%). Walmsley *et al*[68] determined anti-18 kDa mycobacterial bacterioferritin IgG or IgA titers in CD patients and controls without finding any differences, while the results from Bernstein *et al*[69] showed a seropositivity rate for anti-PPA (anti-purified protoplasmatic antigen) in 37.8% of CD patients, 34.7% of UC patients and 33.6% of healthy controls, with non-significant statistical differences among the groups.

Patients with IBD have an impaired barrier function of intestinal mucosa with increased bacterial translocation[70]. In relation to these data, increased antibody titer against *E. coli*, aerobes, anaerobes and enteric bacterial pathogens has been reported both at the mucosal and systemic level[21]. Thus, a pronounced humoral and cell-mediated immune response against bacteria is unavoidable. The same could be true for MAP, however, this is not enough to support its etiopathological role in CD onset.

Moreover, the cell-mediated response against MAP, dosing IFN-γ and other inflammatory cytokines, have also been analyzed[71,72].

In-vitro IFN-γ lymphocyte production, derived from peripheral blood after stimulation with MAP PPD-antigen, was simultaneously evaluated with other MAP detection methods (PCR for MAP-DNA in tissues, direct culture isolation, ELISA for MAP-specific antibodies and cell-mediated response). The authors observed higher levels of MAP-specific IFN-γ in the control group compared to IBD patients, supporting the hypothesis of a wide environmental diffusion of MAP and that healthy subjects could have a stronger cell-mediated immune response, better than the IBD patients.

However, Olsen *et al*[73] found that isolated T $CD4^+$ clones from gut biopsies of patients with active CD significantly proliferate in response to MAP-specific antigens *vs* controls and were able to produce IFN-γ and IL-17.

Protein tyrosine phosphatase (PtpA) and Protein Kinase (PknG) are a very recent indirect method of detecting the presence of MAP. Both proteins are involved in the signal transduction system of MAP and seem to have a critical role for survival of the pathogen within human macrophages, modulating the host's immune response[45]. PtpA, is a specific mycobacterial protein tyrosine phosphatase secreted in a time-dependent manner during MAP infection from the human-derived monocyte cell line, THP-1, and can inhibit phagosome acidification and phagosome-lysosome fusion by dephosphorylating the host sorting protein VPS33B. When sera from CD and UC patients and controls were screened for these antibodies, there was a significantly higher titer in CD patients *vs* controls. In contrast, no significant difference was measured in UC patients[43].

Nakase *et al*[74] demonstrated that THP-1 cells infected with MAP produced a higher amount of TNFα when compared with either *Mycobacterium avium* or *Mycobacterium smegmatis*, suggesting that MAP is directly involved in the up-regulation of this cytokine.

ROLE OF COMBINED ANTIMYCOBACTERIAL THERAPY IN CD

In animals, JD is a chronic progressive fatal condition. Not even pre-emptive administration of antibiotics prevents the infection. *In vitro* analysis showed that MAP is not responsive to traditional anti-tuberculous drugs. In addition, *in vivo* the association of isoniazid, ethambutol and ripampicin lacks efficacy in patients with CD, as demonstrated by a two-year clinical trial, followed by a three-year follow-up period[75]. Because of its typical CWD form, antibiotics that interfere with bacterial wall synthesis do not show efficacy against MAP, which is similar to drugs that impede bacterial duplication at different levels, as MAP

has a low replication rate. Combined antibiotic therapies, including ripampicin and macrolide-derived drugs, for instance clarithromycin and azithromycin, demonstrated *in-vivo* and *in-vitro* efficacy both against MAP and other members of the *Mycobacterium avium* complex[76]. Similar to *Mycobacterium tuberculosis*, the importance of a therapeutic scheme consisting of at least three drugs in order to prevent drug-resistance was also confirmed for other mycobacterial species.

It has been suggested that the most irrefutable evidence of the involvement of a microbial agent in CD etiology would be the long-term remission of clinical manifestations following the clearance of infection[5]. With regard to this issue, Borody *et al*[77] treated a series of 12 patients suffering from severe, obstructive or penetrating CD with a triple antibiotic therapy consisting of rifabutin, clofazimine and clarithromycin, and obtained a clinical, endoscopic and histological remission rate of almost 50%. In an English clinical trial based on the use of rifabutin, clarithromycin and azithromycin from 6 to 35 mo, with a follow-up of 7 to 41 mo, therapy was tolerated by 46 of 52 (89%) enrolled patients; in particular, 17 of 19 patients (91%) who were steroid-dependent before treatment were steroid-independent. Furthermore, the authors observed an important improvement in CDAI, maintained over the following 24 mo[76]. In an American clinical trial, 36 patients positive for p35 and p36 MAP-specific antigens were followed for a period of 4-17 mo; 21 of 29 CD patients (58.3%) who seemed to tolerate antibiotic therapy with rifabutin and clarithromycin showed a clinical sustained improvement[78].

These results seem promising, suggesting that at least a sub-group of CD patients benefit from antibiotic treatment. Nevertheless, these trials are not controlled, are based on a small number of patients and with variability concerning the antibiotics used. Selby *et al*[79] published the result of a large controlled trial in which 213 patients with active CD were randomized to receive triple intracellularly active antibiotic treatment (clarithromycin, rifabutin, clofazimine) or placebo for two years, followed by one year of follow-up, in addition to a 16-wk tapering course of steroids. They observed a relapse rate of 59% in the antibiotics arm *vs* 50% in the placebo arm ($P = 0.54$) among the 32 patients who completed the study at the end of the two-year period. No statistically significant differences between the two arms of the study were noted either during the two-year treatment, or during the follow-up period.

Incongruity of these results may be due to the heterogeneity of the patient selection criteria, the drug type and dosage, the use of mono-therapy or combined therapy and the duration of treatment. Primary end-points were different (drug-capacity of inducing and/or maintaining clinic and/or endoscopic disease remission, relapse percentages after treatment suspension) with consequently different interpretation of the results. Furthermore, none of the previous studies included evaluation of MAP presence in the enrolled population. The eradication of MAP after antibiotic therapy would strongly support the role of MAP in CD etiology.

FINAL CONSIDERATIONS AND PERSPECTIVES

The present data do not demonstrate that MAP is the causal agent in CD, however, a certain degree of involvement of this bacterium in the physiopathological steps of the disease is reasonable. The literature contains contrasting and contradictory evidence on this association with a lack of uniformity in the materials and methods adopted by different researchers for MAP detection and inappropriate patient and control selection parameters. The variability in quality and quantity of analyzed samples, the possible contamination during collection, transport and/or manufacturing of samples, produced further bias among studies[80]. In order to consider an infectious agent as the cause of disease, it is necessary to demonstrate that infection precedes the disease onset. Even studies on the causative role of MAP in CD did not sufficiently support this association because they did not provide enough evidence between the timing of infection and the onset of intestinal disease. The detection of MAP-DNA in the blood of CD patients might suggest that a viable form of the organism is present in CD; however, this could be a secondary phenomenon due to increased intestinal permeability and/or the inability of macrophages to kill MAP in CD patients, rather than an etiological explanation for the disease.

To further elucidate the role of MAP in CD and IBD it would be useful to simultaneously evaluate different biological samples (blood, biopsies and stools) and to clarify whether the detection of MAP-DNA in stools or in biopsies may be related to its viable presence in blood, representing a possible marker of active infection. To our knowledge only two small studies on this issue have been published to date[71,72].

There is no effective and readily accessible method available to diagnose MAP-infection in humans. CD histology suggests that the host's immune response is primarily cell-mediated. Therefore, studies which investigate cell-mediated reactivity against MAP, through the use of T-cell-based IFN-γ assays, could gain more relevance. The reliability of the quantiferon-TB Gold test in evaluating the contact with non-tubercular mycobacteria is still in doubt. The positive cut-off level for the Quantiferon test in patients with non-tuberculous mycobacterial disease has not been determined[81]. The development of "home-made" INF-γ release assays that specifically dose the quantity of this cytokine after stimulation with MAP-specific antigen, including proper negative and positive controls, is required.

As demonstrated, steroid therapies and combinations with immunosuppressive agents increase the risk of an indeterminate result in the Quantiferon test for the detection of latent tuberculosis infection[82]. We know that

MAP infected human macrophages disrupt the host's immune response for its own benefit and a high amount of the cytokine, TNF-alpha, was found in MAP associated CD patients[74].

The controversy regarding MAP and IBD has persisted far too long. Firstly, it is necessary to ratify criteria for sample collection, test performance and interpretation of results. Secondly, in order to establish a causal role of MAP in the etiology of CD, it is necessary to determine if clearance of MAP using drugs that specifically act against this organism, selectively change the natural history of the disease, guarantee a sustained clinical remission and an improvement in histological activity.

The interest in a possible link between MAP and CD would be of clinical relevance (development of diagnostic methods) and for the prevention of the disease (implementation of public health measures, modifications in food processing practices, develop screening MAP infection).

Heterogeneous clinical and histological features, disease course and response to therapies make CD a highly polymorphic entity[83] and is better identified as a "syndrome". In this respect, CD may not have a singular etiology, but rather result from the concomitant action of multiple causal agents and triggering factors, including MAP.

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