

Can Crohn's Disease Really be Caused by Mycobacterium avium Subspecies Paratuberculosis?-With My Alternative Theory that Reduction in Commensal Gut Bacteria and Resultant Impaired Inactivation of Digestive Proteases as the Primary Cause

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Abstract

The role of Mycobacterium avium subspecies paratuberculosis (MAP) in Crohn's disease (CD) has been debated for more than a century. Up to date, it remains a highly controversy issue as there are large amounts of "solid" scientific evidence on both sides. However, I feel many of these conflicts are superficial and the core issue is just the extreme scarcity of MAP in CD and thus the still unresolved conflict between sensitivity and specificity. Along with in-depth analyses of the likely intimate nature of CD, MAP, and the so-called cell-wall deficient spheroplasts, as well as weighted assessment of findings from treatment and epidemiology, here I suggested that the evidences against a critical role of MAP in CD greatly overweigh those support it. Here I also shared a unified hypothesis I developed during the last 15 years regarding the etiology of inflammatory bowel disease (IBD), including the cause and mechanism of IBD as well as the relationship between CD and ulcerative colitis (UC). I proposed that reduction in commensal microbiota in modern society and the resultant impairment in inactivation of pancreatic digestive protease in the lower gut rather than any specific pathogens may have played the primary causative role in both CD and UC.

Introduction

It has been more than a century since Dr. Dalziel proposed a possible same etiology of Johne's disease in cattle, now known as being caused by Mycobacterium avium subspecies paratuberculosis (MAP), with the chronic interstitial enteritis in human, now known as Crohn's disease (CD) [1]. Up to date, the critical role of MAP in CD remains a highly controversial issue, with large amounts of "solid" scientific evidences on both sides as reflected by the many review articles during the last several decades [2-48]. It is believed by some people that MAP has played a critical causative role in CD. It is claimed that MAP as the cause for CD had fulfilled the Koch's postulates to identify the causative agent of a disease [8,11,12], or even the modernized revised versions of Koch's postulates such as those being suggested by Fredricks and Relman [8,11], or Falkow [49], or Hill's criteria [49]. However, in my opinion, many of these claimed evidences are rather superficial or even contradictory. The core issue of conflicts and controversy is the extreme scarcity of MAP in the tissue. To my perception, the evidences against the critical role of MAP in CD greatly over-weigh those supporting it. Here I would like to share my thoughts on this issue, along with a hypothesis I first proposed 15 years ago regarding the etiology of inflammatory bowel disease (IBD) including the cause and mechanism of IBD as well as the relationship between CD and ulcerative colitis (UC). I propose that reduction in commensal microbiota along with improved hygiene condition and inhibition by antibiotics and dietary chemicals such as saccharin and sucralose in modern society and the resultant impairment in inactivation of pancreatic digestive protease in the lower gut rather than any specific pathogens may have played the primary causative role for both CD and UC.

Patients with CD Not Only have Increase in MAP but Also Many Other Microbes

The first isolation of MAP from CD patients in 1984 by Chiodini's group has been usually taken as a grand millstone that inspired the new round of interest [50,51]. However, Dr. Chiodini, who remains actively engaged in research in this area during the last thirty

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years, has just published the most recent study in 2015 aiming at getting insights into the bacterial populations more representative of the causes and agents of CD, with the finding of significant change of many kinds of gut bacteria in CD [52]. This study performed deep 16s microbiota sequencing on isolated ilea mucosal and submucosal tissues from 20 patients with CD and 15 non-IBD controls with a depth of coverage averaging 81,500 sequences in each of the 70 DNA samples yielding an overall resolution down to 0.0001% of the bacterial population. Among the 4,802,328 total sequences generated, 98.9% or 4,749,183 sequences aligned with the Kingdom Bacteria that clustered into 8545 unique sequences with <3% divergence or operational taxonomic units enabling the identification of 401 genera and 698 tentative bacterial species. It was found that there were significant differences in all taxonomic levels between the submucosal microbiota in CD compared to controls, including organisms of the Order Desulfobivibrionales that were present within the submucosal tissues of most CD patients but absent in the control group. This most recent study strongly declined a unique critical role of MAP in CD.

The Noncontagious Nature of CD

The MAP believers usually take the paper by Van Kruiningen et al [53] published in 1986 regarding the development of enteritis in a kid goat after inoculation of MAP isolated from CD patients as a hallmark evidence of fulfillment of the Koch's postulates [8]. However, Dr. Van Kruiningen, who has been the second author in Chiodini's

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paper regarding the first reported successful isolation of MAP from CD patients [50, 51], had later become a firm disbeliever of the MAP/CD association [47, 54]. In the recent paper [54], Dr. Van Kruiningen discussed his suspicion that the isolation of MAP from CD as possible contamination in regarding the persistent failure of the great efforts to infect the many kinds of animals with gut tissue from CD patients. This constant failure of inoculation with gut tissue from CD is in strong contrast with the finding that mucosal homogenate of animals with John's disease caused more severe pathological changes than the isolated cultured MAP [55]. Although study by Naser et al reported isolation of viable MAP from the milk of mothers with CD [56], there is still no evidence on the transmission of CD from mother to babes through milk. Again, this is in strong contrast with the fact that John's disease can be easily transmitted through the milk, and even the fetus can be get infected in the womb through the circulation. All these results indicated the noncontagious nature of CD.

Has Really All Animals Infected With MAP Developed Pathological Changes Similar as John's disease In Ruminants?

It is claimed by MAP believers that John's disease can be reproduced in virtually all kinds of animals. This seems not the case. Although MAP infection has been tried in a variety of animal models such as chickens, guinea pigs, hamsters, mice, and rabbits, many of them failed to consistently reproduce disease symptoms or the typical predominant gut damage as seen in John's disease [57]. For example, oral inoculation of rabbits with approximately 10^8 of live MAP culminated in clinical and histopathological lesions in only 62 to 75% of the animals, in contrast to the development of the disease in almost all calves orally inoculated with only 10^6 MAP [57]. In fact, large amounts of acid-fast MAP and sustained damage are only mainly seen in people with AIDS or animals with immunodeficiency such as in the nude (nu/nu), beige or SCID beige, or T-cell receptor (TCR) knockout mice [58-60], or in animals (mice, chicken, etc) treated with immunosuppression agents like cyclophosphamide or prednisolone [61-63]. Although the typical MAP and histological changes can be found in the mesenteric lymph node, liver, and spleen of some susceptible species of mice inoculated with large amounts of the bacteria, the intestine (ileum, cecum, and colon) usually does not contain lesions or acid-fast bacilli [57]. Even treated with immunedepression agents, MAP still became undetectable in the feces and organs of the infected chicks after a few months [63].

Where are the MAP for the Mass Damage Seen in CD?

Disease by mycobacteria such as *M. tuberculosis* and *M. leprae* is usually accompanied by the existence of large amounts of the acid fast bacteria that commonly correlates with the extent of tissue damage, along with the fact that paucibacillary leprosy is accompanied with much milder symptom, low contagiousness, easier treatment and better prognosis than multibacillary [64]. However, despite gut damage seen in CD patients are much severe than those seen in cattle with John's disease, large amounts of MAP can be found in the feces and the gut tissue in John's disease but seldom in CD, which has been one of the main reasons made many people to believe CD and John's disease are different diseases [10]. The extreme scarcity of MAP in CD seems to be the core issue of the many conflict findings in the MAP/CD research and resultant controversy.

Naser et al had been the first to report in 2004 the existence of viable MAP in the blood of 14/28 CD, 2/9 of UC and 0/11 non-IBD (NIBD), with the optimized culture of MAP through the well-developed

mycobacterial growth indicator tube (MGIT) [65]. It served as strong evidence for the likely positive link between MAP and CD. However, using this standardized method and blood samples from the same subjects, a follow-up blind multi-center study published in 2009 revealed that Naser's group detected MAP in 4/20 of CD, 9/20 of UC and 3/20 of NIBD, while the group at University of Wisconsin detected MAP in 6/20 of CD, 9/20 of UC and 1/20 of NIBD, and the group at the Center for Disease Control and Prevention detected MAP in 4/20 of CD, 1/20 of UC and 0/20 of NIBD, with no sample was positive by all three centers [66]. If there was indeed no problem with the method, the scarcity of MAP in the blood would be an important factor that led to the big discrepancies among the different centers. Two of the three centers, including the one led by Naser, found a higher rate of existence of MAP in the blood of patients with UC rather than CD, which negated the claimed unique critical role of MAP in CD.

In a recent paper, Naser et al presented a review with a listing of the more than twenty studies that support or against the association of MAP with CD by PCR [4]. Most of the negative findings failed to detect any MAP in CD and Naser et al stressed the necessity to use the nested PCR consisting of two rounds of amplification for a sufficient detection of MAP DNA, which further reflected the extreme scarcity of MAP in CD. Nevertheless, multiple studies showed that the so-called MAP specific sequence of IS900 can also be falsely detected positive in many other species such as *Mycobacterium chelonae*, *Mycobacterium terrae* and *Mycobacterium xenopi* strains [38, 67], *Mycobacterium scrofulaceum* [68], *Mycobacterium cosmeticum* [69], and *M. avium* subsp. *Avium* [70]. Another study found IS900 based MAP detection being false positive in one of five single-round PCR and two in four nested PCR [71]. Dr. Chiodini et al also raised big concern regarding the real specificity of IS900 assay for MAP and pointed out the potential big problem by identification of MAP based exclusively on IS900 as being used in many studies [10]. Thus, the increase in sensitivity is accompanied by the loss of specificity, which may be the still unresolved core issue responsible for the great discrepancies among the different studies and the MAP controversy.

Infection of MAP in all animals seems to have milder damage of the gut than CD but more acid fast MAP that correlates the clinical and pathological manifestations of the disease [10,57]. If CD really has a similar pathological mechanism as John's disease in cattle, it seems quite unlikely CD had caused by MAP, as a large amount of MAP can be found in the feces or gut tissue in cattle with John's disease even at the subclinical stage [72].

Spheroplasts: A Superbug or Defeated Remnants of MAP?

The virtually non-infectious nature of CD and the lack of typical acid-fast MAP as seen in the gut tissue of John's disease has been explained by the MAP believers as the existence of only cell wall-deficient spheroplastic forms of MAP in the gut tissue of CD patients, which had been also first reported by Chiodini and Van Kruiningen in 1986 [73]. However, Van Kruiningen published a paper in 1999 with a perception that there is no support for a common etiology in John's disease of animals and CD in humans and the significance of spheroplasts that appear more frequently on cultures is seriously in question [47].

Gut tissues of CD patients not only contain cell wall deficient MAP, but also spheroplasts of other mycobacteria [74]. People have used to compare MAP with *M. tuberculosis* or *M. leprae*. Studies had revealed that spheroplasts can also be formed in *M. tuberculosis* by lysozyme and certain enzymes of monocytes [75], while these spheroplasts

can only become pathogenic after being reverted to bacterial form [76], which has been also true for many other bacteria [77]. Similarly, prominent lysosomes, phagosomes or phagolysosomes containing one or more organisms of MAP can be found in macrophages of MAP infected mice, lambs, calves and adult cattle, and it is believed that the lipid-rich cell wall of MAP has played an important role in resistance to intracellular killing [78]. It is also found that animals susceptible to MAP have a predominance of small phagolysosomes, little enzyme activity, and numerous intact bacteria. In contrast, animals resistant to MAP usually had macrophages containing large phagolysosomes, high enzyme activity and bacillary debris, or organisms that had lysed, indistinct or degenerate cell walls [78]. Thus, the occasionally seen cell wall-deficient spheroplastic form of MAP would be more likely the constrained remnant of the invaded MAP by a robust reaction of the immune system, rather than a superbug with excessive virulence. Much of positive signals by the multiple rounds of PCR as recommended might be just debris of MAP or adjacent species of bacteria, which may explain the low rate of positive findings by culture.

Conflict Claims Regarding the Effectiveness of Treatment of CD

The MAP believers attribute the absence of sustained effect in the two year anti-MAP study in CD patients conducted in Australia [79] as the high resistance of MAP to the drug [6,8,9,80]. On the other hand, they also attribute the efficacy of immunosuppression agents to the killing of MAP [6,8,9,80]. Although immunosuppression agents may have some inhibitory effect on MAP, an in vitro study by Greenstein et al showed that 0.5 µg/ml clarithromycin exerted an even stronger inhibition on MAP than 4 µg/ml methotrexate or 6-MP [80]. Study had also showed that MAP is much more sensitive to drugs such as clarithromycin than many other mycobacterium such as *M. tuberculosis*, with the minimal inhibition concentration (MIC) being as low as 0.15 µg/ml for MAP but 5 µg/ml for *M. tuberculosis* [81]. The cell wall-deficient spheroplastic form of MAP as seen in CD would make them more susceptible to intracellularly acting antibiotics such as clarithromycin [77]. The study by Selby et al in Australian had treated CD patients with 750 mg/day clarithromycin, along with 450 mg/day rifabutin and 50 mg/day clofazimine for up to two years. In contrast, immunosuppression agents are usually used at a much lower dose and shorter period. Can these immunosuppression agents really exert an even strong inhibition on MAP than antibiotics like clarithromycin? Use of immunosuppression agents increased the risk of tuberculosis and many other pathogens [82], including MAP infection in animals [61,63]. Thus, the effective treatment with the multiple immunosuppression agents made CD unlikely to be caused by uncontrolled infection [83,84].

Epidemiological Findings

It is believed by some people that CD may be caused by MAP through the contaminated milk, beef, tap water, soil, or aerosols of river [4,9]. As studies showed no increase of CD in farmers, veterinarians, or animal workers with certainly increased exposure to MAP [85, 86], they attribute this discrepancy to increased immunity during the routine exposure [9]. However, all these epidemiological findings and explanations become frail in facing such fact that Sweden has hardly any MAP contamination in the environment but highest incidence of CD in the world over a long history [87], suggesting factors other than MAP would have played a predominant role. The finding of the increased milk consumption but decreased risk of CD in the recent large-scale, multiple countries study in Europe provided another critical piece of evidence that negated causative role of MAP in CD [88].

Therefore, to my perception, evidences against a critical role of MAP in CD greatly outweigh the evidences supporting it.

My Theory on the Cause of CD and UC

More than a decade ago at about the same time when the first risk gene, NOD2/CARD15/IBD1, of IBD was found [89,90], I found evidence suggesting impairment in the inactivation of digestive proteases, which is likely mediated by the deconjugated bilirubin, as the result of inhibition of gut bacteria by dietary chemicals such as saccharin may have played critical role in the pathogenesis of IBD [91]. It provided simple explanations for many puzzles in IBD such as the emerging of clustered cases of IBD around the beginning of last century, the dramatic increase of IBD in the western countries since 1950s, and the leveling off or decrease of IBD as observed in multiple studies during later 1970s and early 1980s at the time when saccharin was found capable of causing cancer in animals. Later, I further found evidence suggesting sucralose, a new generation of artificial sweetener that was first approved in Canada in 1991 followed by many other countries, may also linked to IBD through a similar mechanism as saccharin, which may have contributed to the recent worldwide increase of IBD [92,93]. This led me eventually coming up with a unified hypothesis on the etiology of IBD, including the cause and mechanism of IBD as well as the relationship between UC and CD [94]. It provided further explanations for the many puzzles in IBD such as the mysterious remarkable increase of IBD in Alberta of Canada since early 1990s, in Brisbane of Australia since middle 1990s, in north California of the United States since the end of 1990s, and in South-Eastern Norway since middle 2000s, shortly after the approval of sucralose in Canada in 1991, in Australia in 1993, in the United States in 1998, and by the European Union in 2004, as well as the especially remarkable recent increase of IBD in children, the shift in the occurrence from UC to CD over time, the increased appearance of CD in the colon, etc [94,95]. This possible link was further demonstrated by multiple more epidemiological studies published thereafter from countries across the world such as the United States [96], Canada [97], Sweden [98], Singapore [99], Saudi Arabia [100], China [101], Korea [102,103], as well as some peculiar changes in IBD such as the recent decrease in CD but increase in UC in the children in Sweden [98], the shared trend of change of pediatric IBD in Sweden with the general population IBD in Denmark but not pediatric IBD in Norway [104], etc [105,106].

To my perception, CD is more likely similar to IBD in dogs and cats rather than Johne's disease in cattle [107]. I proposed that the reduction in commensal microbiota in modern society and the resultant impairment in inactivation of pancreatic digestive protease in the lower gut rather than any specific pathogens may have played the primary causative role for both CD and UC, with the main difference being the relative sterility at the location of the damaged gut and the correspondent predominant reaction either by macrophage or neutrophils [91,94].

Concluding Remarks

For the evidences above, I feel the evidences against a critical role of MAP in CD greatly outweigh those supporting it. Here I advocate again checking out the possible link among reduction in the commensal gut microbiota, activity of digestive proteases, and IBD.

Competing Interests

The authors declare that they have no competing interests.

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