The Beginning of the End?

Summary.
Mycobacterium avium ssp paratuberculosis (MAP) causes a chronic and often fatal inflammatory disease of cattle. Since 1913 MAP has been linked with Crohn's disease (CD) This link has not been conclusively proven, but since 1978 various international research groups have tried to connect CD with MAP. The missing piece of the jigsaw has been the failure to see and grow the organism, and thus be able to detect it in the diagnostic medical laboratories.
Researchers have used transmission electron microscopy (TEM) with some success but TEM is not routinely available in laboratories. Several researchers detected cell-wall-deficient mycobacteria (CWDM) in cultures but were unable to grow them in ways that could be used in diagnostic laboratories. This barrier was acknowledged by researchers, and there were extensive efforts to bypass the need to culture CWDM. These attempts were important for an understanding of the nature of CD, and the response of patients to antibiotics, but could not replace the lack of naturally generated CWDM from patients diagnosed as having CD.
At Otakaro Pathways Ltd we have been exploring methods to culture CWDM from blood and tissue samples.

It has been a long time since I first saw cell-wall-deficient mycobacteria (CWDM) down the microscope. That happened on a Saturday afternoon in 2005. From 2005 until 2011, I carried out occasional experiments to induce the CWDM to grow, in time away from my main job, which was supervising diagnostic microbiology laboratories.

When we established Otakaro Pathways, I had time to try and develop methods to be able to cultivate the elusive CWDM.

By 2015 this became the main goal of the company. We could see them, stain them, count them, but we could not make them replicate enough to be able to study them. We were not alone, but being able to see the CWDM in human blood samples gave us an advantage. You cannot grow them if you cannot see them.

The progress towards designing culture media for the CWDM was slow, with 10 failed experiments for every new ingredient in the culture medium. Every set of experiments took 3 months or longer. This meant we knew a lot about what did not work, and slowly learnt about the response of the CWDM in the various experimental media.

This process of discovery went on and on, until eventually, over the space of several years, we were able to understand the underlying properties of the organisms, and how to grow enough living bacteria for DNA isolation.

After that realisation, we were able to ask the big question –

What causes Crohn's disease?

I have been totally occupied with trying to understand CD for longer than I care to admit. I am not alone, as a recent paper in Nature reminded me[1]. The global rate of patients diagnosed with inflammatory bowel disease (IBD) is climbing alarmingly.

There have been several theories put forward for the cause of CD. These theories often attribute the cause to genetic, hereditary, and/or environmental factors. This covers most human diseases.

The increasing numbers of people diagnosed with IBD suggests we are not doing very well in controlling the global spread. Of the three options above, I strongly suspect that the cause is environmental. The accelerated global spread is not suggestive of genetic or hereditary changes. The environment is a popular suspect among scientists. If the cause was genetic, then the spread would not be of the magnitude seen in 2025.

Environmental suspects are numerous, everything from microplastics to colonisation have been called out as potential causes.

There is a saying that "to a man with a hammer, everything is a nail."
Research on Crohn's disease follows patterns, or phases of interest. Right now, the microbiome, diet and an abnormal immune response are high on the list.
Don't get me wrong; the microbiome, diet, and immune responses are important factors.

Microbiome

Some patients maintain remission with faecal microbiome transplant (FMT) which alters the gut microbiome of the patient.

The gut microbiome is the collection of bacteria, viruses, and fungi that populate the human intestine. MAP is incorrectly excluded as a suspect by researchers in this area. One reason is because it is almost never isolated, so is not considered. Another reason is that it is thought to have been ruled out as a cause of CD by because previous research on MAP was not conclusive. In fact, because MAP is present in the environment, there should be evidence of presence of MAP in the gut microbiome[2]. Logically, if researchers report that MAP DNA is present in the blood of healthy control subjects, then it must have entered the blood via the digestive tract.

Interestingly, the same general changes in the gut microbiome that are seen when a patient has intestinal tuberculosis, are also seen in Crohn's disease. It used to be common in laboratories to see the ratios of various gut bacteria change if a patient had an intestinal bacterial infection with Salmonella species.

Diet

Diet can control CD to a greater or lesser extent in patients. There is no general agreement on how particular diets work, but patients are referred to dieticians by gastroenterologists. There are also apparent racial and cultural differences[3], [4], dietary triggers of inflammation[5], and an underlying understanding of environmental factors[6], [7].

New probiotic therapies also offer promise as a means of controlling the gut microbiome[8] in both animals and humans[8].

Immune response

The use of biologics such as Humira and Remicade can control inflammation in Crohn's disease for varying periods.

The Role of MAP in Crohn's Disease.

This has been a subject of intense debate for nearly 40 years.
There are several theories about bacteria causing Crohn's disease.

E.coli is a suspect. The gut microbiome, a poorly understood host of bacteria, is also a suspect.

The media love stories about bacteria. We are told by the media that microbes are a barely-controlled threat to our existence. Certain species, usually recent crossovers from the environment to the human species, may be dangerous, but for a microbiologist, the entire bacterial universe is essential to life, and we are not the smartest species on the Planet, just the newest one.
We only know what we know.

In 1970, when I started in the laboratory, human infections with *Chlamydia trachomatis* were poorly understood. By the time the association and mode of transmission were known, it was far too late to halt the spread. The organism could not be easily detected or cultured in the laboratory, so diagnosis and therapies were "hit and miss", until molecular methods of detection were available to clinicians, and the true extent of infectious spread became obvious.

More recent examples of the emergence of new organisms and infectious diseases include *Helicobacter pylori*, HIV, COVID19, *Mycobacterium riyadhense*, *Campylobacter species*, *Clostridium difficile*, *Legionella pneumophilia*, and *Tropheryma whipplei* .
Emergence of new bacterial species is a common occurrence in Nature. We humans are unable to close the door to new evolving species; and there are so many doors.

<u>More Questions</u>

One of the questions I have been asked is-
"Where do the CWDM come from?"
The truthful answer right now, is that I am not certain, but there is a high [probability that I am going to find out. (with international help)

Another question often asked by clinicians and microbiologists has been-
"Is Crohn's disease caused by a *Mycobacterium species?"*

There is a high probability of this, so let's construct a case to try and answer that question. Dr Ellen Pierce put the question very well in a paper[9]
"Where are all the Mycobacterium avium spp paratuberculosis in patients with Crohn's disease?"
In her paper, Dr Pierce provides some very good guesses, that I can easily agree with –
They are within the body, hiding in plain sight. The ability to detect something is often based on the particular detection method, as well as where you look to find it. Dr Pierce's paper is intelligent and thoughtful and deserves more attention. I say this because there are now commercial staining methods widely available that will detect CWDM in tissue samples. Globally, right now, an anatomical pathologist will be looking at a tissue section that contains CWDM. Anatomical pathologists are relied upon to make the definitive diagnosis of Crohn's Disease.

Increasing Global numbers of patients with inflammatory bowel disease point to a growing health problem[1].
Spread of CD may be helped by a lack of laboratory methods for detection of CWDM.
In the history of Microbiology, this is not unusual. Bacteria evolve far more quickly than humans.
Mycobacterium tuberculosis complex, (MTBC) the old enemy, is the cause of tuberculosis.
MTBC is evolving faster than human attempts to control the disease.

It is not difficult to lay out a similar case for CD. This disease was known as “regional ileitis” before 1932. Researchers had made an earlier association between a disease called “chronic regional enteritis” in humans, and a disease called “Johne’s disease” in cattle in 1913[10].

In 1932 regional ileitis was renamed Crohn’s disease by US researchers, although the authors did not make the link with Johne’s disease[11].

In 1978 several British researchers[12] found what looked like cell wall deficient mycobacteria (CWDM) in human tissue culture samples from patients with CD. CWDM are mycobacteria without their outer cell wall. They had to use transmission electron microscopy (TEM) to see them, and fortunately they published photos[12]. Looking back down the years, the organisms they saw are strikingly like the CWDM we are now able to culture in our lab.
In the 1980s, Dr Rod Chiodini had similar success when using TEM on his cultures. Dr Chiodini later cultured bacillary forms of Mycobacterium paratuberculosis (MAP) from the tissue samples[13], [14]. MAP is the causative organism of Johne’s disease.

I believe that Dr Chiodini’s work was brilliantly original and focused other researchers on CD and MAP. The story gets complicated from then until now, and is not mine to tell. Suffice to say that there had been a lot a lot of research on the link between CD and MAP, but by 2015 the question of whether MAP caused CD remained unfinished business.

On the pharmaceutical side, AMAT therapy[15], and variations on that antimicrobial formula produced exciting (sometimes unpublished) results. The use of AMAT in Crohn’s disease is reminiscent of the chlamydia story in which tetracyclines were used to treat chlamydial infections before the causative organisms were well-known and could be detected in the laboratory.

There is nothing new under the sun. The same microbiology story repeated many times over the last 150 years. Few people were able to isolate the MAP organism in either the cell-walled or the CWDM form from human patients with CD.

This is not to diminish the many attempts by researchers to culture the CWDM. There were numerous experiments, but by 2022, most researchers accepted that the culture of CWDM was very difficult to impossible[16] in the time required to make the test timely and therefore clinically useful[17].
This left the door open to arguments and unfounded criticisms of the MAP theory. In some ways that period showed how important it is to be able to see and culture the organism from patients diagnosed with CD.

What are the advantages to seeing the organism?

Seeing is definitely believing, and being able to see the organism under the microscope means that researchers can discover how to grow it. Once the organism can be cultivated, it can be tested against antibiotics, and the structure of the organisms can be described.
CWDM may be of interest to other pathology researchers. Anatomical pathology already has methods available to detect the CWDM in tissue from patients, and if the link with CD can be strengthened, then biochemists can look for the metabolites produced by CWDM that may cause inflammation (and they are doing that). Immunologists may also benefit from the availability of CWDM for research purposes. Animal testing can be used to replicate the disease in animal species. Finally, having a target to treat means that precisely engineered vaccines and new antibiotics could be available.

Structure of CWDM.

So far, we know that biofilm is important to survival of CWDM cultures, and that the cell wall of the CWDM is absent. (Biofilm appears to replace the cell wall) The CWDM replicate by several different methods, and there are often high numbers present in cultures, when using newly developed media for growth.
The lack of the cell wall means that only the inner membrane is present.
The inner membrane is made of protein, and has a tough but flexible structure.
The inner membrane protects the cytoplasm, where the chromosomal material is held. The cytoplasm is the engine room of the mycobacterial cell. Without the cytoplasm, cell death is inevitable.
The cell-walled version of MAP seen in animals is similar to a walnut, with a hard outer shell (the cell wall). If the shell of the walnut is removed, then only the soft kernel is left.

In the case of CWDM, the loss of the cell wall means that the strong protective outer armour is missing. I think this is one reason why the CWDM are mostly seen inside white cells (intracellular), where they are usually in the dormant state.
On release from the white cell, they become "activated." The CWDM rapidly produce biofilm, perhaps to attract another white cell, as well as providing some shelter from the host's immune response.
Biofilm is a slime-like material that preserves the living CWDM. Replication inside the biofilm is often seen by us.

Importantly, the CWDM replicate -this is proof of viability (that they are living organisms)
They cannot be seen in the stain routinely used in microbiology laboratories to see mycobacteria.
They will not grow in the commercially manufactured media made to culture mycobacteria, because the media contain inhibitory substances and lack the key nutrients and balance that help the CWDM to grow.
These are great ways to hide from humans trying to find them. When they enter white cells inside the body, it gives them an extra layer of camouflage.
They are called "stealth pathogens"[18] for the reasons stated above.

In summary, our work has shown us that CWDM exist, can be grown, counted, studied, and are associated with Crohn's disease. They produce virulence factors and are invasive.
Is this the Beginning of the End? We will know for certain when numbers of patients with active CD reduce dramatically. That should be our collective goal.

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