Cell Wall-deficient Bacteria as a Cause of Infections: a Review of the Clinical Significance

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Cell wall-deficient bacteria (CWDB) are pleomorphic bacterial forms. These atypical organisms may occur naturally or they can be induced in the laboratory. Their presence has been known about for over a century, but a definite link to clinical disease outcomes has not been demonstrated. A number of case reports and laboratory studies suggest some disease associations, however. Considerable controversy surrounds the true relevance of CWDB to disease; there is a widespread belief that they may represent a response by the walled organism to adverse extracellular conditions like antibiotic pressure. This review looks at studies published between 1934 and 2003, which were identified by Dialog DataStar using the key words ‘cell wall deficient bacteria and clinical significance and infections’ and by further scanning the reference list at the end of the papers retrieved. We conclude that the evidence for the clinical significance of CWDB in disease is not compelling.

KEY WORDS: ATYPICAL BACTERIA; CELL WALL-DEFICIENT BACTERIA; L-FORM; WILD-TYPE BACTERIA; CLINICAL SIGNIFICANCE

Introduction

The true relevance of cell wall-deficient bacteria (CWDB) to disease is unknown. CWDB are not well studied because they are difficult to characterize by light microscopy. They may persist in cells because they have no means of survival outside the cell, or because the host immunological response is thought to diminish when the bacterium loses its cell wall. For this reason, CWDB may accumulate in the body over time. While there is no direct evidence that their presence results in disease, laboratory data suggest that CWDB are intracellular organisms that can revert to wild-type bacteria (WTB) outside the cell. CWDB are suggested to up-regulate to a more aggressive pathological form when an adverse environment threatens their survival inside cells, and they may in that theoretical situation cause symptoms of disease. Difficulties in proving this theory limit the acceptance of CWDB as disease-causing organisms. CWDB do not fulfil the first and third postulates of Koch, and many microbiologists do not believe they cause any harm to the host. There is some correlation between the use of antibiotics and their appearance.
Cell wall-deficient bacteria may be defined as bacteria with altered morphology and cultural characteristics consistent with damaged or absent cell wall structures. The term L-form refers to bacteria without a cell wall in vitro that grow on solid media treated with penicillin and propagated in a characteristic manner. L-forms do not revert in penicillin-free media in which the absence of the cell wall has been demonstrated either by immunology or by electron microscopy (EM). Klieneberger originally used the term L-form in honour of the Lister institute while working there. In this review, we will take the L-form to mean a pleomorphic variant of bacteria with deficient cell wall characteristics that may require hypertonic media, and may revert to a walled organism. We will use the terms CWDB and L-form interchangeably.

Difficulties in identifying cell wall-deficient bacteria

The difficulties in accurately identifying CWDB in the laboratory are legion. Pfeiffer described in 1895 a non-rigid wall bacterial form of Vibrio cholerae that was difficult to see with the light microscope. Other workers from various laboratories confirmed the presence of bacteria that lacked a rigid cell wall and were difficult to grow by standard laboratory techniques. Ingredients in standard laboratory media suppress atypical bacterial forms by encouraging the growth of bacteria with cell walls only. Ingredients that appeared to suppress the growth of non-walled bacteria in vitro included riboflavin, yeast extract, peptone, un-purified agar and concentrated serum.

Cell wall-deficient bacteria, in vitro, are resistant to antibiotics that act on cell wall biosynthesis. They may remain more sensitive to bacteriostatic antibiotics than WTB. The cell wall of the Gram-negative bacterium consists of an outer membrane of lipopolysaccharide plus an inner peptidoglycan layer, while the cell wall of the Gram-positive bacterium is mainly made from a layer of peptidoglycan 50 – 100 molecules thick. Glycopeptides and β-lactams act by inhibiting the biosynthesis of peptidoglycan. Thus, they would preferentially induce the development of CWDB in Gram-positive organisms compared with Gram-negative bacteria.

Bisset and Bartlett demonstrated that CWDB persist within the red blood cells (RBC) of normal people. They identified atypical variants of Bacillus licheniformis within RBCs and proposed a life cycle. They hypothesized that the organisms they saw as part of the life cycle had previously been wrongly classified in other genera. It was their opinion that CWDB may pass through stages in the proposed life cycle, resembling organisms from the genera Mycobacterium, Corynebacterium, Listeria, Mycoplasma, Micrococcus and Bacillus at various points. Domingue et al., in similar experiments, could not replicate the results, however. They could not isolate more than one type of organism from RBC lysates and have argued that some of the life-cycle stages described by Bisset and Bartlett may represent elementary reproductive units of CWDB. The revertant may also vary serologically from the WTB (see rheumatic fever below). Theodore et al. showed by polyacrylamide gel electrophoresis that CWDB do not retain all of the cellular proteins of the WTB parent. Diagnostic difficulties may often arise in the identification and classification of CWDB because of the discovery of newer non-culturable organisms like Rochalimaea species or Bartonella species. CWDB may also be difficult to culture if the growth medium lacks ingredients essential for their survival. Markesich et al. showed that CWDB variants of Mycobacterium avium...
subspecies *paratuberculosis* (MAP) might be difficult to grow because the culture medium or host lacks the iron-chelating agent mycobactin, which is essential to MAP growth. The culture method required might involve an inappropriate length of incubation.\(^2\) The optimum pH for CWDB may differ greatly from that required to grow its WTB parent.\(^2\) The morphology of CWDB can also be demonstrated by a variety of newer techniques such as DNA detection, RNA detection, *in situ* hybridization, and the presence of specific proteins.\(^2\) - \(^5\) The methods involved in these newer techniques are often difficult to reproduce and many centres do not accept their validity.

Many bacteria are known to become cell wall-deficient when subject to the relevant stresses. Those that have attracted clinical interest are listed in Table 1.

**LIFE CYCLE**

The life cycle of CWDB is not well known. CWDB are thought to replicate by various mechanisms including budding, filamentous growth and binary fission. The walled parent may do this serially, resulting in a non-rigid cell that is spherical and highly pleomorphic.\(^3\) Orskov\(^2\) demonstrated that the yield of CWDB from *Proteus* is improved by incorporating 2% caffeine in the culture; large vacuolated bodies characteristic of CWDB colonies in *Streptobacillus moniliformis* have been noted.\(^2\) Schellenberg\(^2\) noted large bodies in *Proteus vulgaris* L-forms propagated by division, whilst refractile granules budded from other bodies and gave rise to small L-form colonies. Atypical growth originating in the smallest granules of *P. mirabilis* and *Salmonella* isolates that expanded and formed either mycelia or large bodies were also observed.\(^2\) Bacilli may develop bulges that may further extend to become mycelia.\(^3\) Induced bacteria do not always develop into a spherical L-form. The Morax-Axenfeld bacillus is known to transform into CWDB by lengthening, then twisting over and over again. Areas of the strand separate by twisting, then swell to become spheres.\(^3\) CWDB induced by natural conditions are more likely to remain viable.\(^3\) Gonococci are unique because they may transform spontaneously into large bodies during continuous incubation. L-form colonies can be propagated indefinitely if grown in suitable media. Madoff\(^3\) argues that when the inducing agent is removed, the L-form may revert, or become stable and no longer capable of assuming the wild-type morphology. Fig. 1 captures the essence of this proposed life cycle in light of the evidence discussed in this review.

**EVOLUTIONARY ADVANTAGE**

An infectious origin has been postulated for a number of clinical conditions that do not fulfil Koch’s postulates. Bacteria with deficient cell walls may be isolated from atherosclerotic plaques of patients with acute coronary syndromes (ACS) and coronary artery disease (CAD).\(^3\) Atypical mycobacteria are implicated in the pathogenesis of sarcoidosis and Crohn’s disease.\(^2\) CWDB have also been isolated along with WTB in patients with overt infections such as urinary tract infections, infective endocarditis, osteomyelitis, Whipple’s disease and meningitis.\(^2\) Schmitt-Slomska *et al.*\(^2\) suggest that CWDB persist within cells because they can inhibit phagocytosis. Others argue that CWDB persist in cells as an evolutionary response to antibiotic pressure. The revertant emerges either when the inducing antibiotic is withdrawn or in the presence of altered host immune responses.
### TABLE 1: Clinically important bacteria that may become cell wall-deficient when subjected to the appropriate stresses

1. *Staphylococcus aureus*  
2. *Streptococcus pyogenes*  
3. *Streptococcus viridans*  
4. *Mycobacterium* species  
5. *Escherichia coli*  
6. *Shigella flexneri*  
7. *Enterococcus faecalis*  
8. *Clostridium welchii*  
9. *Clostridium tetani*  
10. *Neisseriae* species  
11. *Haemophilus influenzae*  
12. *Micrococcus* species  
13. *Bacillus* species  
14. *Lactobacillus* species  
15. *Brucella abortus*  
16. *Bordetella pertussis*  
17. *Serratia marcescens*  
18. *Pasteurella multocida*  
19. *Stenotrophomonas maltophilia*  
20. *Pseudomonas aeruginosa*  
21. *Corynebacterium* species  
22. *Vibrio* species  
23. *Listeria monocytogenes*  
24. *Legionella pneumophila*  
25. *Bacteroides funduliformis*  
26. *Rhizobium lupinus*  
27. *Proteus mirabilis*  
28. *Bifidobacterium bifidum*  
29. *Salmonella typhimurium*  
30. *Streptobacillus moniliformis*  
31. *Nocardia* species  
**Spirochaetes:**  
32. *Treponema pallidum*  
33. *Borrelia burgdorferi*  
34. *Leptospira interrogans*

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**FIGURE 1:** The proposed life cycle of cell wall-deficient bacteria (CWDB)

- **In standard laboratory media:** Wild-type parent  
- **In special media with osmotic support/antibiotics:** CWDB (subtle intracellular accumulation during the life of man)  
- **Laboratory filter:** Reversion to walled organism  
- **In special media without osmotic support/antibiotics:** (occurs at sites of cellular injury)  
- **Revertant:**
THE CELL WALL AND CELL WALL-DEFICIENT BACTERIA

The hallmark of CWDB is the lack of a cell wall and lack of sensitivity to penicillin. Lack of a complete cell wall allows the CWDB to pass through filters and distinguishes it from the revertant and WTB. Panos et al. showed that CWDB induced from Group A β-haemolytic streptococcus lacked muramic acid, glucosamine and thiamine in the murein layer. Strominger et al. showed that penicillin prevents the incorporation of uridine diphosphoacetylmuramic acid peptide into the WTB cell wall during cell wall synthesis, resulting in the development of atypical bacterial forms. Rabitol, polysaccharide A and muramic acid were also completely absent in both cyclerin-induced and penicillin-induced CWDB from Staphylococcus aureus. In addition, low galactose concentrations caused a strain of Bifidobacterium to lose the ability to synthesize a rigid murein, and a stress protein in CWDB cultures from Escherichia coli was not present in cultures of WTB.

A residual cell wall persists in most CWDB and revertants. Fodor showed that CWDB from S. aureus induced by penicillin could result in three distinct variants with varying N-acetyl amino sugar content. He concluded that antibiotic-induced changes do not follow a set pattern and that the three variants were inhibited at different stages of the muropeptide synthesis required for the bacterial cell wall. Fleck found lipoprotein, lipopolysaccharide and glycopeptide in the residual wall of CWDB induced from Proteus. He reported that diaminopimelic acid (DAP), glutamic acid and hexosamines were missing in the glycopeptide fraction, resulting in the formation of a plastic wall. Other groups have also reported structural defects in the mucopolysaccharide layer of CWDB, indicating that DAP is important in the reversion of CWDB. CWDB that do not synthesize DAP are known to degrade their own murein when grown in media not containing DAP. Classical growth could be restored when placed in DAP-enriched media.

Slabyj et al. found that the length of the teichoic acid chain in CWDB was about half that present in the Streptococcus pyogenes parent. The lipid composition of CWDB is increased compared with the WTB parent, and this may be because the cytoplasmic membrane of CWDB is known to undergo hypertrophy. Changes in lipid composition have been hypothesized to be the reason that CWDB derived from S. pyogenes are unable to synthesize a rigid cell wall. Comparative amino acid analyses of S. pyogenes and its CWDB variant have shown a 7-fold increase in the glucosamine content associated with membrane protein, although no qualitative differences occur. Variation in the ribosome loci may also occur, and this can be related to changes in the metabolic pattern of CWDB.

Cell wall-deficient bacteria from S. faecalis have the precursor particles of their ribosomes firmly attached to the membrane of the cytoplasm, and have sedimentation constants of 103S, 130S, 153S and more. There is, therefore, considerable variation in the known factors that affect bacterial cell wall components.

Disease associations

Many disease associations have been described. Patients infected with human immunodeficiency virus (HIV) and transplant recipients are confounders. These patients are immunosuppressed and they tend not to fit typical CWDB profiles. The key features of CWDB in various diseases are described overleaf.
INFECTIVE ENDOCARDITIS

The incidence of infective endocarditis in the UK has declined in recent years to 2:100,000 per year.\(^{50}\) Various organisms have been cultured from the blood of patients with infective endocarditis, although culture-negative endocarditis may account for up to 40% of reported cases.\(^{51}\) Blood cultures are commonly negative because of the use of antibiotics before the blood is taken.\(^{52}\) CWDB may be found in the peripheral blood of patients with infective endocarditis,\(^{53,54}\) and may also be demonstrated in vegetations on the valves of patients with infective endocarditis.\(^{55}\) Negative blood cultures may co-exist with the presence of CWDB in valvular vegetations. In one series, three of the four patients reported had negative blood cultures. The investigators interpreted this occurrence as indirect evidence of the pathogenic role of CWDB,\(^ {55}\) although the blood culture methods employed would have been unlikely to grow CWDB. In another series, 321 patients with infective endocarditis were recruited into a prospective cohort and treated them with standard antibiotics.\(^ {56}\) A total of 246 patients had a presumptive diagnosis of infective endocarditis. The yield of 34% positive blood cultures in Todd-Hewitt broth increased to 51% in osmotically normal semi-liquid agar with 0.2% thioglycollate, and further increased to 81.2% in the yolk sacs of non-embryonated hen’s eggs. The investigators concluded that the requirement for osmotic support improved the yield of culture positive results. The study was, however, not designed to show that atypical organisms were a cause of infective endocarditis. We know, therefore, that CWDB may be present in infective endocarditis,\(^ {57,58}\) but we do not know whether they are directly pathogenic.

ACUTE CORONARY SYNDROMES

Myocardial infarction (MI) is often preceded by malaise and fever, suggesting that infections may play a role in coronary artery disease.\(^ {59,60}\) Chronic infection with \textit{Chlamydia pneumoniae} has been implicated as a risk factor for CAD and is thought to predispose to atherosclerosis through the action of chlamydial lipopolysaccharide.\(^ {61}\) \textit{C. pneumoniae} is an obligate intracellular parasite that survives as inclusion bodies within membrane-bound intra-cytoplasmic vacuoles. The chlamydial cell wall has both an inner and an outer tri-laminar membrane resembling those of Gram-negative bacteria. It is apparently cell wall-deficient during part of its life cycle. There is evidence that \textit{C. pneumoniae} persists in atherosclerotic plaques and several animal models show the potential for atherosclerotic development after inoculation with \textit{C. pneumoniae}.\(^ {62,63}\) Some studies have shown an association between \textit{Chlamydia} and cardiovascular disease.\(^ {64}\) A number of small antibiotic intervention trials have, however, produced conflicting results as to the clinical significance of this association.\(^ {65–67}\)

Table 2 presents the key features of all of the randomized clinical trials (RCTs) that have evaluated the efficacy of antibiotics in ACS. The WIZARD trial, which is the largest antibiotic intervention study to have been reported to date, showed that the macrolide azithromycin did not significantly reduce the risk of death or recurrent ischaemic events.\(^ {68}\) The WIZARD trial confirmed the findings of the AZACS trial,\(^ {69}\) which recruited 1439 patients with acute MI or ACS and randomized them to an active treatment arm of 500 mg of azithromycin on the first day followed by 250 mg daily for a further 4 days. The AZACS investigators found that short-term treatment with azithromycin did not reduce the development of recurrent
events in patients with ACS. This was in contrast to the smaller Finnish study CLARIFY, which recruited 148 patients randomized to receive clarithromycin or placebo. The CLARIFY study found that there was a reduction in the risk of ischaemic cardiovascular events in patients presenting with ACS.70 The STAMINA study recruited 325 patients randomized to two active treatment arms and one placebo arm,71 and it reported a reduction in cardiac deaths. The CLARIFY and STAMINA studies did not have adequate patient numbers to convincingly close the debate. Other large ongoing clinical trials (ACES and PROVE IT) may eventually contradict the WIZARD trial. It does seem that the overwhelming evidence today is that antibiotic treatment of atypical bacterial forms does not confer a survival advantage in ACS.

We note that all of the atypical bacteria RCTs reported to date involved the use of macrolides in the intervention arm. These macrolides are bactericidal, which as discussed above, may have no effect on CWDB populations. The choice of therapeutic intervention in these trials may therefore have biased the reported outcome. A weakness of the RCTs included in Table 2 is that none of them addressed the issue that their bactericidal intervention may be inducing L-forms of the organism they were trying to eradicate. The STAMINA study was the only clinical trial that avoided this criticism by employing an amoxicillin-based regimen versus azithromycin, but the two antibiotics were used in two different intervention arms. Combining both antibiotics in one intervention arm would have ensured the eradication of both CWDB and WTB. The early phase benefit reported by Gupta et al.,66 ROXIS,65 CLARIFY70 and STAMINA71 could be explained by initial eradication of WTB by the macrolide antibiotic employed. If the hypothesis that bacteria become cell wall-deficient in vivo is true, then it is easy to see that macrolides may induce CWDB, which may revert when the antibiotic pressure is removed, causing treatment failure. It is no surprise, therefore, that the AZACS and WIZARD trials did not show any long-term benefit with the use of macrolides. Another strength of the STAMINA study was that it was not based on serology. All serological testing described for atypical bacteria in the other clinical trials included in Table 2 were only valid for walled organisms. The investigators should have applied the lessons learnt from other diseases like typhoid and rheumatic fever, where the immune response produced by the WTB is different from that due to the CWDB variant, and used specific serological tests to detect them.72,73 If CWDB really play a role in cardiovascular disease, as hypothesized, a test to detect their presence should have been performed in these trials in addition to the serology of WTB. Interestingly, the STAMINA investigators comment that neither C. pneumoniae nor Helicobacter pylori serology was significantly related to treatment response.71

RHEUMATIC FEVER

Group A streptococci cause rheumatic fever in humans. Kaplan74,75 showed that antibodies produced in response to streptococcal infection cross-react with the endocardium and myocardium to cause cardiac damage. This usually manifests clinically as pancarditis with a rise in streptococcal antibody at more than 14 days following a streptococcal sore throat.76,77 Timakov and Kagan78 isolated CWDB from 12 patients in a series of 19 patients with rheumatic fever. Another Russian group reported finding high titres of antibody to streptococcal CWDB in 76% of patients
## TABLE 2: The key features of all of the antibiotic intervention randomized controlled trials that have been performed in acute coronary syndromes

| Trial name   | Year | Patient characteristics                                      | Intervention                                                                 | Results                                                                                                                                                       |
|--------------|------|-------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ROXIS$^{65}$ | 1997 | 202 patients with ACS                                      | Roxithromycin 150 mg twice daily for 30 days versus placebo                                                                          | A 90% reduction in combined endpoint of death due to cardiac ischaemia, MI and severe recurrent ischaemia ($P = 0.018$)                                      |
| Gupta et al.$^{66}$ | 1997 | 60 male survivors of acute MI with elevated anti-\textit{C. pneumoniae} antibody titres of 1:64 | Azithromycin 500 mg/day for 3 days ($n = 280$) or 500 mg/day for 6 days ($n = 12$) versus placebo ($n = 12$) | Groups with seropositivity had a four-fold increased risk for adverse cardiovascular events compared with seronegative groups (OR, 4.2; 95% CI, 1.2 – 15.5; $P = 0.03$). Treatment with azithromycin reduced cardiovascular events in seropositive groups (OR, 0.9; 95% CI, 0.2 – 4.6; $P = NS$) |
| ACADEMIC$^{67}$ | 1999 | 302 CAD patients with \textit{C. pneumoniae} IgG titres $\geq$ 1:16 | Azithromycin 500 mg/day for 3 days, then 500 mg/week for 3 months versus placebo | No differences in antibody titres and clinical events at 6 months                                                                                           |
| CLARIFY$^{70}$ | 2002 | 148 patients with ACS                                      | Clarithromycin 500 mg daily versus placebo for 85 days                                                                         | A 42% reduction in the composite risk of death, MI and ACS compared with placebo (RR, 0.54; 95% CI, 0.25 – 1.14; $P = 0.10$)                                   |
| STAMINA$^{71}$ | 2002 | 325 patients with ACS or acute MI                           | Amoxicillin 500 mg twice daily plus metronidazole 400 mg twice daily plus omeprazole 20 mg twice daily; or azithromycin 500 mg once daily plus metronidazole 400 mg twice daily plus omeprazole 20 mg twice daily; or placebo | A 36% reduction in cardiac deaths and readmission with ACS in patients receiving antibiotics compared with placebo ($P = 0.02$)                                      |
| AZACS$^{69}$ | 2003 | 1439 patients with acute MI or ACS                         | 500 mg of azithromycin on the first day followed by 250 mg daily for 4 days                                                        | No reduction in death, recurrent MI or recurrent ischaemia ($P = 0.664$; 95% CI, 0.72 – 1.24)                                                                  |
TABLE 2 (continued):
The key features of all of the antibiotic intervention randomized controlled trials that have been performed in acute coronary syndromes

<table>
<thead>
<tr>
<th>Trial name</th>
<th>Year</th>
<th>Patient characteristics</th>
<th>Intervention</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIZARD68</td>
<td>2003</td>
<td>7747 patients with previous MI and \textit{C. pneumoniae} titres of 1:16</td>
<td>Azithromycin 600 mg/day for 3 days then 600 mg/week for 11 weeks versus placebo</td>
<td>A 7% reduction in death, re-infarction, coronary revascularization, or hospitalization for angina at 14 months (non-significant)</td>
</tr>
<tr>
<td>ACES</td>
<td>In progress</td>
<td>4000 adults with stable CAD</td>
<td>Azithromycin orally once a week for 1-year and 4-year follow-up for the composite primary endpoint of CAD death, nonfatal MI hospitalization for ACS, and coronary revascularization.</td>
<td>Results awaited</td>
</tr>
<tr>
<td>PROVE IT</td>
<td>In progress</td>
<td>4000 patients with ACS in the last 10 days and dyslipidaemia</td>
<td>Pravastatin 40 mg daily or atorvastatin 80 mg daily or gatifloxacin versus placebo</td>
<td>Results awaited</td>
</tr>
</tbody>
</table>

ACS, acute coronary syndromes; MI, myocardial infarction; \textit{C. pneumoniae}, \textit{Chlamydia pneumoniae}; OR, odds ratio; CI, confidence interval; NS, not significant; RR, relative risk, CAD, coronary artery disease.
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during the acute phase of rheumatic fever in a series that recruited 1343 patients. It has been proposed that CWDB in rheumatic fever may be difficult to recognize because revertants may vary serologically from the WTB. This may be because a genetic change in the organism converts it from β-haemolytic streptococcus to the α-haemolytic organism. CWDB have been reported to be present in the blood of patients with rheumatic fever. As the incidence of this disease has declined sharply in the USA and western Europe since the advent of antibiotics, it is unlikely that sufficient patient numbers will ever be found to test for clinical significance of CWDB in rheumatic fever in a properly designed study relevant to western societies.

SARCOIDOSIS
Sarcoidosis is a multi-system disease of unknown aetiology characterized by the presence of non-caseating granulomata. The method described by Ziehl and Nielsen in 1886 is the gold standard for identifying wild-type mycobacteria reviewed in Barksdale and Kim. CWDB variants of mycobacteria are Ziehl-Nielsen-negative, however, but may revert to WTB that can only be stained after prolonged culture. The diagnostic difficulties in proving the presence of CWDB in sarcoidosis has led to the development of a number of hypotheses for the role of mycobacteria in the pathogenesis of sarcoidosis. Mankiewicz proposed that people with Mycobacterium tuberculosis infection produce mycobacteriophage-neutralizing antibodies, and that those who cannot produce these antibodies develop non-caseating granulomata that manifest clinically as sarcoidosis. Hanngren et al. described the interaction of virus and mycobacteria and suggested that concurrent viral infection depressed T-cell function and altered the immune response to mycobacteria. Steffen et al. showed by polyacrylamide gel electrophoresis that the gel pattern of M. tuberculosis was similar to that of the CWDB of sarcoidosis. Mangiapan and Hance have suggested that sarcoidosis might be a clinical response to an infection with a small number of viable mycobacteria that persist in the tissues following a strong granulomatous response associated with good elimination of the organisms from the body. Other aetiological agents are implicated in the pathogenesis, including mycoplasma, Nocardia species, Corynebacterium species, clay soil, talc, oxalosis, beryllium, pine tree pollen and immune complexes. Many early reports favoured a link with CWDB, so it has become fashionable in recent years to explore the association of CWDB or atypical mycobacterial forms with sarcoidosis. The recent ACCESS study was designed to evaluate if CWDB were the cause of sarcoidosis. It recruited 1442 subjects into a prospective, case controlled multi-centre study. The ACCESS investigators used methods previously described, and they did not find any difference in the presence of CWDB isolates in the blood of patients recently diagnosed with sarcoidosis compared with control subjects. This finding contradicts previous reports that employed the same methods and looked at smaller populations of patients with sarcoidosis. CROHN’S DISEASE
This is another disease characterized by the presence of non-caseating granulomata. Crohn’s disease is remarkably similar to Johne’s disease, which is an intestinal disease seen in animals caused by MAP. Patients with Crohn’s disease are known to show a high degree of anergy to tuberculin, a diminished response of cultured circulating lymphocytes to phytohaemagglutinin, and...
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an altered response to dinitrochlorobenzene suggesting that cell-mediated immune responses are depressed in this disease. Two clinical types have been described – perforating and non-perforating.94 Non-perforating Crohn’s disease runs a less aggressive clinical course, and it is associated with features of intestinal obstruction rather than perforation, raised interleukin-1β levels, and has been described as being akin to tuberculoid leprosy.95 In contrast, the perforating type of Crohn’s disease is characterized by fistulae formation, abscesses, and has been likened to lepromatous leprosy. It is thought that the immune response of the individual determines which of the two disease types manifests clinically. CD4+ cells are also thought to play a major role. Infliximab, a monoclonal mouse antibody to tumour necrosis factor-α, reduced disease activity in a cohort of patients with the perforating type of Crohn’s disease.96 This has been likened to the use of granulocyte-macrophage colony stimulating factor, interleukin-2, interferon and interleukin-12 in modulating disease severity and prevention of antibiotic resistance in tuberculosis.97 One longitudinal cohort study demonstrated the presence of mycobacterial CWDB in 12% of patients with Crohn’s disease and 16% of patients with ulcerative colitis.92 M. avium subspecies paratuberculosis has been found in the milk of lactating women with Crohn’s disease.98 The MAP associated with Crohn’s disease may present in the immunocompromized patient with variable pathogenicity.99 Hermon-Taylor et al.100 published a case report of MAP associated with a Crohn’s-like illness. Some workers argue that the presence of MAP RNA from tissue samples of patients with Crohn’s disease may be proof of a causal link,101 but some antibiotic and anti-mycobacterial intervention trials have not shown any benefit due to anti-mycobacterial treatment.102 – 105 Greenstein106 argues that this failure of drug intervention has to do with the use of ineffective anti-microbial agents and inappropriate duration of treatment. Other antibiotic intervention trials showed variable benefit (Table 3).107,108 Moss et al.109 showed empirical improvement in 44 patients treated for 6 months with standard antimicrobials. Other groups showed medium-term improvement in markers of disease severity when combination therapy with a macrolide was employed, but these studies did not recruit patient populations that were large enough to draw strong conclusions.110 – 112 The results of these trials were also difficult to analyse because the investigators used different drug interventions and different outcome measures, and larger studies are needed before antibiotic treatment of Crohn’s disease can be routinely recommended.

OSTEOMYELITIS AND ARTHRITIS

A number of case reports in the literature implicate CWDB variants of S. aureus, Listeria monocytogenes, Salmonella enteritidis, Aeromonas hydrophila and Clostridium species as causes of osteomyelitis and arthritis. CWDB have been found in cultures of joint aspirates,113 and in three cases of pyoarthrosis that later reverted.114 S. aureus CWDB were also found in a case of recurrent osteomyelitis of the femur that was resistant to methicillin and oxacillin.115 Gordon et al.116 reported four cases of difficult-to-culture bacteria in chronic osteomyelitis that eventually grew S. aureus in hypertonic medium and CWDB in one case. Using cytochrome oxidase, other researchers characterized CWDB from the synovial fluid of patients with gonococcal arthritis.117 Palmer118 has identified S. enteritidis revertants in the synovial fluid of a patient with Salmonella arthritis and difficult-to-treat A. hydrophila in leukaemic patients with
### TABLE 3: The key features of intervention trials that have shown variable benefits of antibiotic treatment in Crohn’s disease

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Drug intervention</th>
<th>Findings</th>
</tr>
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<tbody>
<tr>
<td>Moss <em>et al.</em>[^109^] recruited 44 patients</td>
<td>1978</td>
<td>Treated for 6 months with standard anti-microbial agents</td>
<td>Improvement in symptoms due to Crohn’s disease</td>
</tr>
<tr>
<td>Gui <em>et al.</em>[^110^] recruited 52 patients, and 46 completed the study</td>
<td>1997</td>
<td>Rifabutin and clarithromycin or azithromycin. Patients treated for a mean period of 18.7 months (range, 6 – 35 months) and followed-up for 25.1 months (range, 7 – 41 months)</td>
<td>Inflammatory markers ESR ($P = 0.009$) and CRP ($P = 0.03$) were reduced at 18 months and serum albumin was increased at 12 months ($P = 0.04$). Harvey-Bradshaw Crohn’s disease activity index was reduced after 6 months ($P = 0.004$, paired Wilcoxon test). Reduction was maintained at 24 months ($P &lt; 0.001$). A total of 33% of patients required surgery to relieve intestinal obstruction</td>
</tr>
<tr>
<td>Douglass <em>et al.</em>[^108^] recruited 25 patients, and four patients were lost to follow-up. Nine patients withdrew from the study because of treatment failure</td>
<td>2000</td>
<td>Rifabutin 450 mg once daily, clarithromycin 250 mg three times daily and clofazamine 2 mg/kg once daily for 12 months. Mean follow-up of 5 months</td>
<td>Complete remission in nine out of 25 patients (36%). The majority of patients responded within 3 months. Patients with colonic disease had better prognosis than those with small bowel disease</td>
</tr>
<tr>
<td>Shafran <em>et al.</em>[^111^] recruited 36 patients, and 29 completed the study</td>
<td>2002</td>
<td>Clarithromycin 250 mg twice daily and rifabutin 150 mg twice daily, accompanied with a probiotic. Patients were monitored for 4 – 17 months</td>
<td>Twenty-one (58.3%) out of 29 patients improved on treatment. Improvement was defined as a decrease of 70 points between entrance and exit Crohn’s disease activity index scores. Three patients (8.3%) had significant improvements, but required other Crohn’s medications. Five patients (13.8%) showed no improvement</td>
</tr>
<tr>
<td>Borody <em>et al.</em>[^112^] recruited 12 patients</td>
<td>2002</td>
<td>Rifabutin 450 mg daily, clarithromycin 750 mg daily and clofazamine 2 mg/kg daily, and followed up for up to 54 months</td>
<td>Harvey-Bradshaw Crohn’s disease activity index in full or partial response to therapy fell from an initial $13.4 \pm 1.91$ to $0.5 \pm 0.47$ ($n = 8$, $P &lt; 0.001$) after 52 – 54 months. Six out of 12 patients had reversal of severe disease, and 8% required surgery to relieve intestinal obstruction</td>
</tr>
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ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.
arthritides. He argued that a mixture of organisms could cause septic arthritis, with one genus existing as CWDB and the other as WTB. There was no uniformity in the methods described in these studies and most of the studies that have been conducted in the setting of rheumatology and osteomyelitis are at least 20 years old. We have not found any RCTs in this setting that have attempted to link CWDB to clinical outcomes, so the conclusions from the case reports discussed above should be interpreted with caution.

**BONE MARROW TRANSPLANT**

Woo et al.\(^\text{119}\) found that about 25% of in-hospital allogenic bone marrow transplant (BMT) patients with a diagnosis of culture-negative febrile episodes were infected with CWDB. The clinical significance of this finding within the setting of bone marrow transplantation is that 75 – 80% of neutropenic fevers encountered in BMT patients are thought to be caused by infections.\(^\text{120}\) Of these, only 16% were previously reported from the same group in Hong Kong as being culture positive.\(^\text{121}\) A potential problem with this study was that the regimen used to increase the number of circulating stem cells may have had an impact on the increased percentage of CWDB harvested from the blood of these patients. In Europe, more than 72% of all autologous stem cell transplantation is by peripheral blood stem cells (PBSC).\(^\text{122}\) PBSC are associated with faster haemopoietic reconstitution, reduced antibiotic support treatment and less chemotherapy-related toxicity than BMT in both allogenic and autologous settings. A study that included both patient populations, comparing BMT versus PBSC, would have been more appropriate, and as a result the clinical relevance of the findings is questionable.

**URINARY TRACT INFECTIONS**

Cell wall-deficient bacteria have been reported in haematuria and pyelonephritis.\(^\text{123,124}\) A prospective cohort study of 57 patients with pyelonephritis or chronic bacteriuria found that 17% had CWDB in the urine, but the study lacked the statistical power to conclude that CWDB caused urinary tract infections.\(^\text{125}\) Domingue et al.\(^\text{126}\) isolated CWDB that subsequently reverted to *Staphylococcus haemolyticus* and *Streptococcus agalactiae* from a 22-year-old woman. The patient later presented with haematuria and culture-negative urine. The authors concluded that the woman had an infection due to CWDB persisting in the genitourinary tract that caused idiopathic haematuria after standard antibiotic treatment. The weakness in their conclusion lies in the fact that atypical organisms that do not grow well on standard media can be routinely cultured in supra-pubic samples of asymptomatic women.\(^\text{127,128}\) Also, their observations were not based on protein sequencing or nucleic acid analysis of the bacterial cellular characteristics and it is difficult to accept that the bacteria cultured emanated from the same colony. Case reports may not be enough to change clinical practice. Observations of this nature should ideally be made in a population big enough to make extrapolations to a larger patient group if possible. We have not found any RCTs that looked at CWDB in urinary tract infections.

**MENINGITIS**

There have been a number of case reports and small prospective series in which CWDB were isolated from clinical samples in meningitis. Alexander-Jackson\(^\text{129}\) isolated mycobacteria revertants from the cerebrospinal fluid (CSF) of patients with tuberculous meningitis. CWDB have also been isolated from the CSF of patients with meningitis and
were commonly isolated from brain abscesses complicating the disease. Difficult-to-culture organisms were cultured in hypertonic media from 21/89 cases of meningitis, and Timokov found CWDB in a prospective study of 56 patients. Species of bacteria associated with CWDB in meningitis include Staphylococci, Streptococci, Pseudomonas, Mycobacteria, Proteus, Salmonella, Escherichia coli, Haemophilus influenza, Nocardia and Neisseriae. These CWDB variants may occur during or before antibiotic treatment, but there have not been any RCTs that linked CWDB to disease outcomes in meningitis.

WHIPPLE’S DISEASE
This is another disease in which the tuberculin response is depressed. First described by Whipple in 1907, this is a rare condition that typically presents with gastrointestinal symptoms, arthralgia, weakness, fever, lymphadenopathy, weight loss and cachexia. It is now known to be caused by another difficult-to-culture organism called Tropheryma whippellii, previously known as the Whipple bacillus. It is similar to the atypical intracellular forms seen in sarcoidosis. The organism has been shown by EM to possess a fragile tri-laminar cell wall.

EYE DISEASE
In the last 20 years, CWDB have been reported to be present in cases of uveitis, chronic orbital inflammatory disease and in retinal pigment disease, but the clinical significance is not yet clear.

OTHER DISEASE CONDITIONS
There are other case reports suggesting that CWDB may be involved in pneumonia, empyema, systemic lupus erythematosus and scleroderma. The data are based on case reports and are not robust.

Clinical relevance
The issue of atypical bacteria and whether or not they cause infections and disease is an important one. Since the last decade of the 19th century, many researchers have reported isolating CWDB from patients with different diseases. Many people believe CWDB cause disease, and have perpetuated this theory.

The techniques used to identify CWDB are not uniform and many are questionable. In addition the evidence used to argue for their clinical significance is mainly based on case reports or small prospective series. For these reasons many are skeptical about the clinical significance of CWBD and recent clinical trials have also questioned the relevance of these atypical organisms to disease.

What we need to do in every poorly understood clinical or laboratory infectious event is consider the most plausible explanation. Koch’s postulates are not an ‘all or none rule’ and Koch himself recognized this. He could not apply his postulates to cholera despite indirect evidence that it was microbiological in origin and Mycobacterium lepra is widely regarded as causing leprosy, despite the postulates of Koch not being met. Hepatitis also presents a discrepancy as it was universally regarded as being viral in origin years before the viruses were identified. We need to consider, therefore, the possibility that CWDB may cause disease by hitherto unknown mechanisms that do not fulfill Koch’s postulates.

In the future, we should aim to eradicate both populations of CWDB and WTB in human beings and see if this changes the disease outcome. Our clinical trials should involve the use of combinations of bactericidal and bacteriostatic drugs similar to
therapeutic interventions in tuberculosis. Without employing drugs that would eliminate both variants in bacterial populations, the true relevance of CWDB to pathogenesis and prognosis will remain elusive.

**Conclusion**

That CWDB may be involved in disease pathogenesis is attractive, but as yet, this hypothesis has not been borne out by RCTs. We still lack clarity about the fundamental pathogenic significance of these organisms. Increasingly, we are questioning the generic nature of many of the mechanisms proposed. We question the definition of Domingue et al.\(^1\) that CWDB should be defined as organisms shown by EM to be partially or completely cell wall-deficient that may revert to walled forms in vitro. CWDB can be characterized by other techniques that do not involve EM, and may occur in nature or can be induced in the laboratory.\(^2\) We accept Madoff’s argument\(^3\) that CWDB can be propagated indefinitely in the presence of an inducing medium, and that the CWDB may revert (the so-called unstable L-form) or may be stabilized when the inducing agent is removed. We recognize that there is neither universal agreement on the definition of CWDB nor universal acceptance of the methods for inducing them. We would like to see the development of more robust and reproducible methods for identifying CWDB. Many of the techniques in use today were described in the middle of the 20th century. We argue that techniques described over 40 years ago and fastidious laboratory culture methods of the same period may not compensate for the limits of our present knowledge. The CWDB life cycle within cells needs to be clearly defined. We also need to clarify whether the CWDB observed within cells in a non-disease state eventually cause disease, like the WTB. This is especially important in light of recent clinical trials that question the likely clinical relevance of these organisms. Bridging the gap from these RCTs to clinical relevance requires us to conduct robust studies, replicated in multiple settings, that provide irrefutable evidence to show that CWDB play a role in established infections.

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**Conflicts of interest**

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