Pathogenetic mechanisms of the intracellular parasite *Mycobacterium ulcerans* leading to Buruli ulcer

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The necrotising skin infection Buruli ulcer is at present the third most common human mycobacteriosis worldwide, after tuberculosis and leprosy. Buruli ulcer is an emergent disease that is predominantly found in humid tropical regions. There is no vaccine against Buruli ulcer and its treatment is difficult. In addition to the huge social effect, Buruli ulcer is of great scientific interest because of the unique characteristics of its causative organism, *Mycobacterium ulcerans*. This pathogen is genetically very close to the typical intracellular parasites *Mycobacterium marinum* and *Mycobacterium tuberculosis*. We review data supporting the interpretation that *M ulcerans* has the essential hallmarks of an intracellular parasite, producing infections associated with immunologically relevant inflammatory responses, cell-mediated immunity, and delayed-type hypersensitivity. This interpretation judges that whereas *M ulcerans* behaves like the other pathogenic mycobacteria, it represents an extreme in the biodiversity of this family of pathogens because of its higher cytotoxicity due to the secretion of the exotoxin mycolactone. The acceptance of the interpretation that Buruli ulcer is caused by an intracellular parasite has relevant prophylactic and therapeutic implications, rather than representing the mere attribution of a label with academic interest, because it prompts the development of vaccines that boost cell-mediated immunity and the use of chemotherapeutic protocols that include intracellularly active antibiotics.

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For more on ***M ulcerans* infection** see http://www.afip.org/hot-topics/bu/

Introduction

Buruli ulcer, caused by *Mycobacterium ulcerans*, is an emerging disease that at present is the third most common human mycobacteriosis worldwide after tuberculosis and leprosy.[1] Buruli ulcer is found mostly in humid tropical areas of Asia, Latin America, and Africa where its incidence has been increasing, surpassing tuberculosis and leprosy in some regions.[1] Buruli ulcer is a necrotising disease of the skin (mainly the subcutaneous tissue), that mostly affects children, producing massive, disfiguring ulcers and permanent disabling scars.[2,3] There is no vaccine against this disease, and treatment is difficult and generally requires surgery, usually accompanied by skin grafting and prolonged courses of antibiotics. Despite its huge social effect Buruli ulcer remains a largely neglected disease.[3,4]

Buruli ulcer is also of great scientific interest because of the unique characteristics of its pathogenicity that have been the source of contradictory and controversial interpretations, and remain largely enigmatic.

An extreme among mycobacteria

M ulcerans is genetically very close to the typical intracellular parasites *Mycobacterium tuberculosis* and *Mycobacterium marinum*,[5] and the greater than 98% nucleotide sequence identity between *M ulcerans* and *M marinum* provides evidence that *M ulcerans* evolved from *M marinum*.[5–7] However, over the course of its evolution *M ulcerans* acquired a giant virulence plasmid, pMUM001, responsible for the synthesis of the exotoxin mycolactone.[8] Acquisition of this plasmid has been deemed to be the main driver for Buruli ulcer emergence in people.[9] Additionally, by comparison with *M marinum*, *M ulcerans* has undergone extensive gene loss,[10,11] as is the case in the obligate intracellular parasite *Mycobacterium leprae*, although to a lesser degree.[12]

M ulcerans grows very slowly in vivo[13] and, like most *M marinum* strains, has an optimum growth temperature of about 32°C,[14] explaining the predilection of *M marinum* for the skin and its restricted systemic dissemination.[15–19] However, bones can be infected by *M ulcerans* because of contiguous spreading or by lymphatic or haematogenous dissemination.[2,20] Since *M ulcerans* strains isolated from human bone infections do not grow at 37°C,[14] osteomyelitis is one of the still enigmatic features of Buruli ulcer. Progression of the infection leads to coagulative necrosis of the dermis and subcutis, resulting in varied non-ulcerative clinical forms that can progress to ulceration following necrosis of the epidermis.[21]

Extensive necrosis is a hallmark of the histopathology of Buruli ulcer and has been associated with the high toxigenicity of *M ulcerans*. This characteristic of *M ulcerans*,[18,19,22] is unique among human pathogenic mycobacteria, and is due to the production of a family of cytotoxic exotoxins, the mycolactones.[23,24]

Therefore, three features of *M ulcerans* emerge as relevant for its pathogenicity: the first is the low optimum growth temperature that makes the skin its almost exclusive territory, the second is the slow growth rate that translates into slowly progressing lesions, and the third is the mycolactone-associated high cytotoxicity that adds to its mycobacterial nature with consequences for pathology and immunity.

The importance of mycolactone for pathogenesis

Mycolactones are unusual among bacterial exotoxins because they are poorly immunogenic polyketide-derived macrolides.[23,25] Mycolactone A/B is the most active and widespread, and it is characteristic of *M ulcerans* strains from Africa.[26] Experiments with externally added mycolactone A/B show that it has intense cytotoxic activity in vitro, affecting monocytes, macrophages, neutrophils,

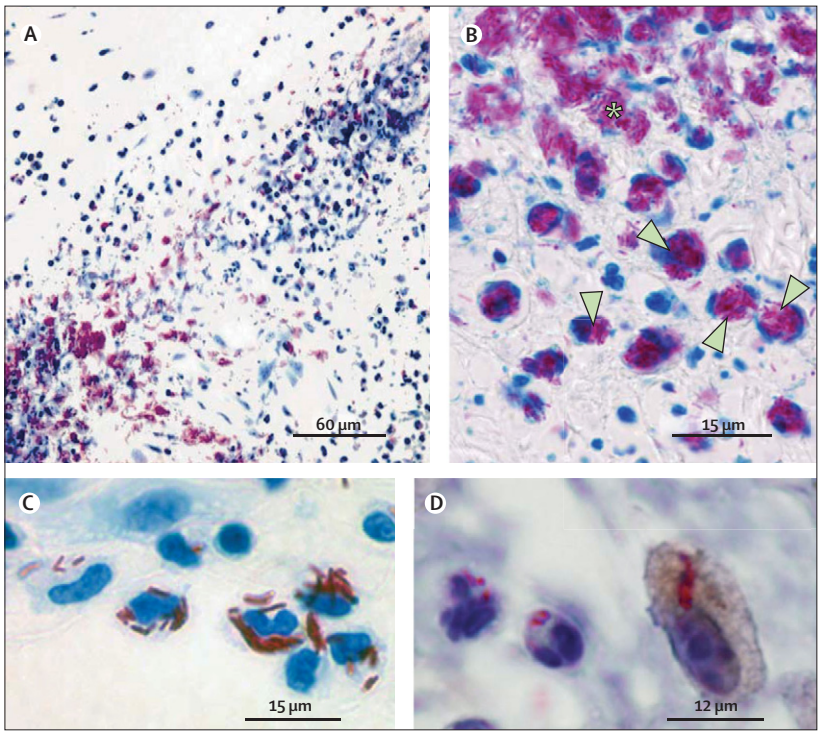

Figure 1: **Subcutaneous injection of toxigenic ITM 98-912 *Mycobacterium ulcerans* in mouse foot pads**
Sample collected 16 days after the injection of 5·9 log_{10} bacilli (A) when swelling was already evident but before ulceration. An inflammatory cellular infiltrate is present near the abundant extracellular bacilli. Reproduced with permission from the American Society for Microbiology.[29] Sample collected at 28 days after inoculation of 6 log_{10} bacilli (B), when the lesion was advanced but not yet ulcerated. This sample shows the transition from the peripheral inflammatory infiltrate with macrophages packed with acid-fast bacilli (arrows) to the acellular necrotic area with abundant extracellular bacilli (asterisk). Reproduced with permission from the American Society for Microbiology.[30] Intramacrophage bacilli (C) in the cellular infiltrate of the foot pad sample in part A. Sample collected 15 days after injection of 6 log_{10} bacilli (D). High magnification of the peripheral area of the inflammatory infiltrate after macrophage specific immunohistochemical staining with the F4/80 antibody. A macrophage contains *Mycobacterium ulcerans* bacilli. Reproduced with permission from the American Society for Microbiology.[30]

lymphocytes, fibroblasts, and dendritic, epithelial, and adipose cells.[24,25,27,28] With L929 cells 0·01 ng/mL of mycolactone A/B is enough to induce cell death.[26]

The cytotoxicity of mycolactone has been linked to its apoptogenic activity. Apoptosis was seen when several cell types were exposed in vitro to purified mycolactone,[24] when cultured primary mouse macrophages were infected with mycolactone-producing *M ulcerans* strains,[29] and in guineapigs[24] and mice[29,30] infected with toxigenic *M ulcerans*. Moreover, massive apoptosis has been reported in Buruli ulcer lesions.[31]

Tissue necrosis and immunosuppression have been associated with mycolactone cytotoxicity.[25,32] The apoptogenicity of mycolactone has been assumed to be the basis of the tissue destruction typical of infections with *M ulcerans*. However, it is not clear how this activity leads to extensive tissue necrosis. Indeed, the necrotic alterations in *M ulcerans* lesions exceed the cell destruction attributable to mycolactone cytotoxicity and extend to non-cellular components of the connective tissue.[33,34] The actual contribution of cytotoxicity due to *M ulcerans* to the various types of damage seen in Buruli ulcer necrotic epidermal, dermal, and subcutaneous lesions is not clear,[35] and the involvement of other mechanisms, including ischaemia associated with vascular pathology, deserve consideration.[2,36,37] Ischaemia-inducing vascular pathology, namely vasculitis and thrombosis leading to blockage of small-sized and medium-sized vessels in the subcutaneous tissue and dermis, has been described in human beings and animals infected with *M ulcerans*.[18,24,34,36,38]

Although usually less extensive, necrosis also happens in skin infections with *M marinum*, *Mycobacterium haemophilum*, and *M tuberculosis*,[35,39] and also in pulmonary *M tuberculosis* infections.[40] The mechanisms that form these necroses might contribute to the necrotic lesions in *M ulcerans* infections.

Immunosuppression in *M ulcerans* infections has been thought to be a consequence of the cytotoxicity of mycolactone towards immune cells, ultimately affecting pathogenicity. However, it is not clear what mycolactone cytotoxicity contributes to the immunosuppression reported in Buruli ulcer.[35,41]

Regarding both the immunosuppressive and necrotising activities of mycolactone, the local concentration of the toxin in tissues infected with *M ulcerans* is not known because it is poorly immunogenic, making it difficult to detect with immunocytochemistry. In mice subcutaneously infected with a mycolactone-producing *M ulcerans* strain, the intact mycolactone molecule was detected by mass spectrometry in mononuclear cells in the blood, lymph nodes, and spleen.[42] The technical approach used in that study did not allow the assessment of the distribution, concentration, and biological activity of mycolactone at the subcutaneous sites infected with *M ulcerans*. Therefore, in the in-vitro experiments addressing the effects of mycolactone on immune cells the toxin was added at arbitrary concentrations, so extrapolation to the real disease in vivo should be treated with caution. The lack of knowledge about the amount of cytotoxic factors released from *M ulcerans* during the early, active, or late stages of infection has already been stated.[35]

The in-vitro cytolytic activity of mycolactone affects many types of cells[25,27] and some studies show that it inhibits phagocytosis.[22,43–45] However, understanding how such extensive activity is expressed in vivo is difficult. Indeed, the toxin is highly diffusible[18,42] and yet cutaneous Buruli ulcer is associated with minimum systemic effects,[46] abundant phagocytes with intracellular *M ulcerans* are frequently present in areas close to accumulations of high numbers of extracellular bacilli (figures 1, 2, and 3)[29,30] that have been described as producers of high concentrations of mycolactone,[44,47,48] and mycolactone was not detected in the sera of mice with progressive subcutaneous infection by toxigenic *M ulcerans*.[42]

Except for the presence of pMUM001, it is not clear what effect the genomic differences between *M ulcerans*

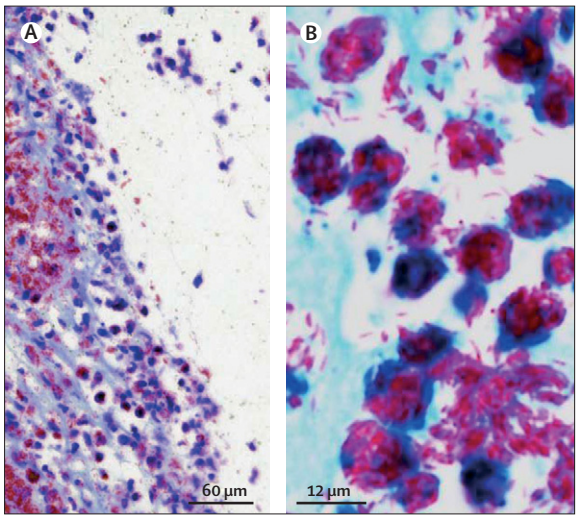

Figure 2: **Low dose inoculation of the mycolactone producer *M ulcerans* ITM 98-912 strain**
Histopathological pattern of the infection in the mouse foot pad 41 days after inoculation of 300 bacilli. This lesion was advanced but not yet ulcerated. The pattern, similar to that in figure 1A, shows abundant extracellular bacilli and a cellular infiltrate with intracellular bacilli (shown at higher magnification in part B).

and its ancestor *M marinum* have on pathogenicity. The virulence of *M ulcerans*, as for any other pathogen, must be multifactorial,[49] but since mycolactone has monopolised research on Buruli ulcer pathogenesis other virulence factors have so far had little attention. *M ulcerans* mycolactone-negative mutants are able to multiply within macrophages[30,44] and induce in mice an intracellular infection with inflammatory responses and granulomatous lesions.[44] Analysis of the *M ulcerans* proteome has started and is likely to reveal the virulence-related expression profile of this pathogen.[50]

Some factors besides mycolactone that are likely to be involved in the pathogenicity of *M ulcerans* deserve urgent research. First, all *M ulcerans* strains tested have 11 chromosomal protein-coding DNA sequences that seem to be specific to this bacterium and might contribute to pathology associated with Buruli ulcer.[6,9] Second, *M ulcerans* has been shown to form an extracellular matrix that is involved in virulence. Besides mycolactone, this coat contains many proteins and lipids or lipoglycans that are likely to play a part in the pathogenicity of *M ulcerans*.[51] Besides these extracellular-matrix lipids, like in other pathogenic mycobacteria,[52] several *M ulcerans* cell-wall lipids are likely to be involved in virulence.[53] Third, bacterial phospholipases are known virulence factors,[54] and phospholipase C and D activities and DNA sequences homologous to the genes encoding phospholipase C in *M tuberculosis* and *Pseudomonas aeruginosa* were detected in *M ulcerans*.[55]

In conclusion, mycolactone is a key factor in the virulence of *M ulcerans*,[23] but the actual size of its effect on pathogenesis is not clear. As discussed elsewhere,[56,57] this toxin provides a target for vaccination. However, the lipid nature of mycolactone complicates the development of immunity against this toxin. An alternative would be to target enzymes involved in the biosynthesis of mycolactone.

M ulcerans-associated cellular responses

Revisiting the past

The published work on *M ulcerans* infections has been dominated by the interpretation that, by contrast with other pathogenic mycobacteria, *M ulcerans* is an extracellular parasite that induces infections associated with minimum or absent inflammation, as has been reviewed elsewhere.[1,30,58,59] However, the early publications about Buruli ulcer and experimental infections with virulent *M ulcerans* strains of Australian and African origin[15–19] reported the presence of areas of tissue necrosis with abundant extracellular bacilli and inflammatory cellular infiltrates with phagocytes extensively colonised with bacilli, two features recognised as typical of mycobacterial infections. The first definitive description of Buruli ulcer[15] reported the presence of large numbers of bacilli within phagocytes, similar to infections with *M leprae*. In mouse infections, Frank Fenner[16] reported the presence of macrophages packed with bacilli, and William H Feldman and colleagues[17] reported a severe inflammatory reaction with inflammatory exudates and clusters of phagocytes containing clumps of bacilli. Two other early reports[18,19] on experimental *M ulcerans* infections in guineapigs also reported the presence of inflammatory infiltrates with bacilli within neutrophils and macrophages.

Descriptions of *M ulcerans* infections in human beings and animals on the basis of the interpretation that the cause of Buruli ulcer was an extracellular parasite were frequently accompanied by explicit or implicit recognition of features that do not fit with such an interpretation.[29,30] Namely, that those infections are associated with cell-mediated immunity (CMI) and delayed-type hypersensitivity responses (DTH), and that CMI-boosting BCG-type vaccines are worth testing as a prophylactic measure.

In support of the interpretation that *M ulcerans* is an extracellular parasite that induces infections with minimum or absent inflammation the following arguments have been advanced. Buruli ulcer biopsies show predominantly extensive necrotic acellular areas with extracellular bacilli,[33,34,59,60] mycolactone has immunosuppressive activities that inhibit inflammatory and immune responses,[44–46,61] in some in-vitro models *M ulcerans* and mycolactone hampered the phagocytosis of the pathogen by macrophages,[22,43–45] and attempts to grow *M ulcerans* within cultured macrophages were unsuccessful.[43,44]

However, the following reassessment of these and other results shows that an alternative interpretation of *M ulcerans* pathogenicity is justified, one that is more in accordance with the mycobacterial nature of this

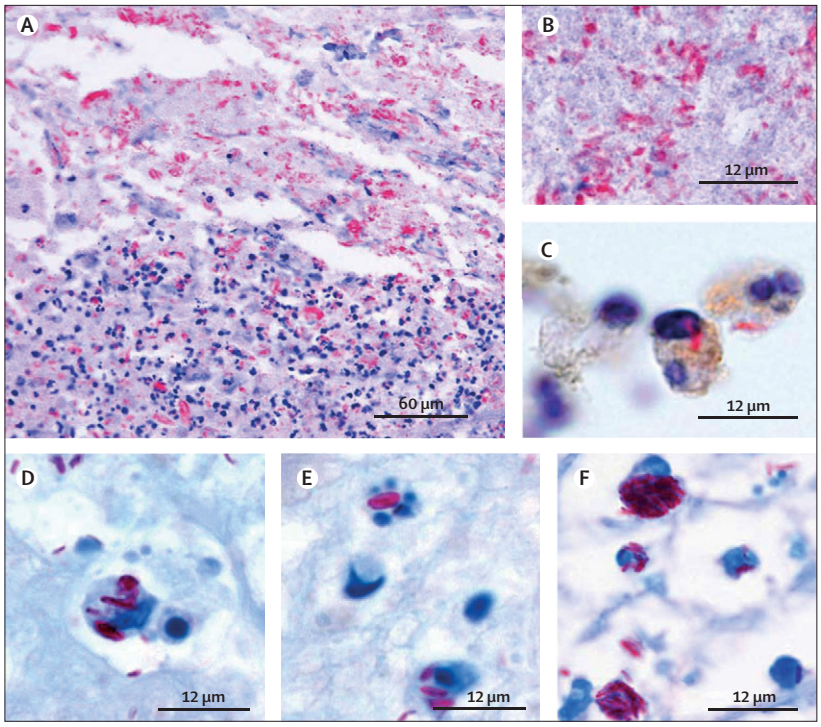

Figure 3: **Inflammatory infiltrates with intramacrophage *M ulcerans* bacilli in specific areas of biopsy specimens from non-treated African patients with active Buruli ulcer**
Extracellular bacilli in the necrotic acellular area of the lesion (upper region) and bacilli colocalising with phagocytes are present in the inflammatory infiltrate (A). Detail of the abundant extracellular bacilli in a necrotic acellular area (B) and bacilli within macrophages specifically stained with NCL-LN5 antibody (C), in the inflammatory infiltrate in the sample in panel A. High magnifications of the areas in the sample in part A containing inflammatory infiltrates colocalising with bacilli (D–F), showing intracellular bacilli associated with phagocytes with nuclear alterations characteristic of apoptosis (E) and intracellular globus-like clumps of bacilli (F). Reproduced with permission from the American Society for Microbiology.[30]

organism and with several reported aspects of Buruli ulcer immunity.

Association with inflammatory infiltrates

Biopsies of initial Buruli ulcer lesions are not available. In advanced Buruli ulcer it is unquestionable that a diagnostic hallmark is the presence of extensive necrotic acellular areas with a few inflammatory cells and frequently abundant extracellular *M ulcerans*, because it represents the predominant histopathological pattern. The mycobacterial nature of the cause of Buruli ulcer and the published early results on *M ulcerans* infections reviewed above, however, show that there are reasons to admit that such a histological pattern might only represent part of the picture of host–parasite interactions in spatial, temporal, and immunological terms.[29,30,62] This is more so since the proportion of necrotic areas increases with the progress of the infection. Moreover, it should be noted that necrotic areas with extracellular mycobacteria are also present at specific places and phases of development in lesions due to *M tuberculosis*,[40] *M haemophilum*, or *M marinum*.[35]

The dynamics of the cellular response of the host from the very beginning of the infection can only be assessed with animal models. Most animal studies on the phagocyte response to invasion by mycobacteria used high inocula[63–67] and revealed an early inflammatory infiltrate enriched with neutrophils, quickly followed by recruitment of inflammatory monocytes or macrophages that, in time, became predominant. This dual influx of inflammatory phagocytes has also been found in studies on rodents infected with *M ulcerans* using high inocula.[15,19,29,30,68] A time-lapse assessment of the subcutaneous cellular response to infection with virulent *M ulcerans* strains in mice showed that extensive inflammatory infiltrates with neutrophils and macrophages were present in specific areas of the lesion in early and advanced active disease (figures 1 and 2).[29] Since experimental infections after inoculation of low bacterial doses more closely resemble the natural infection, it is relevant that a similar histopathological scenario, although with a delayed onset, was seen when mice were infected with 300 virulent *M ulcerans* bacilli (figure 2).[29] In this and other studies of human,[15] guineapig,[19] and mouse[30] infections, *M ulcerans* bacilli were found not only extracellularly in the necrotic areas but also within neutrophils and, mostly, macrophages (figures 1, 2, and 3) during the entire infectious process, as in other experimental or natural mycobacterioses. However, one report[45] described transient intraphagocyte bacilli present only in the early phases of the infection and predominantly within neutrophils.

Importantly, and confirming the initial description of Buruli ulcer histopathology,[15] the presence of inflammatory infiltrates with intramacrophage *M ulcerans* in specific areas of the lesion has been reported in Buruli ulcer biopsies of untreated African patients with active disease (figure 3).[30,69] This observation shows that the cellular response and presence of intramacrophage *M ulcerans* are not restricted to experimental mouse infections that, by contrast with human disease,[1] do not heal spontaneously.[19]

In agreement with the observation of inflammatory infiltrates in infections with *M ulcerans*, high expression of interleukin 8 or macrophage inflammatory protein 2 and other proinflammatory cytokines was found in Buruli ulcer lesions of human beings[70] and in mice infected with *M ulcerans*.[68] This finding accords with others that cultured mouse macrophages infected with *M ulcerans* secrete macrophage inflammatory protein 2 and monocyte chemotactic protein 1,[45,71] chemokines that are known to recruit neutrophils and monocytes.[72,73]

Association with CMI and DTH

In response to stimulation, peripheral blood mononuclear cells of patients with active Buruli ulcer disease show reduced secretion of interferon γ[46,74–76] and interleukin 2.[42] These observations have been advanced in support of the view that infections with *M ulcerans* are associated with mycolactone-induced systemic immunosuppression[42,46,77] and are related to the in-vitro observed damage to immune cells induced by that toxin or by toxigenic bacilli.[22,44,45,50,61,71,77,78] However, repressed interferon γ and interleukin 2 immune responses also happen in

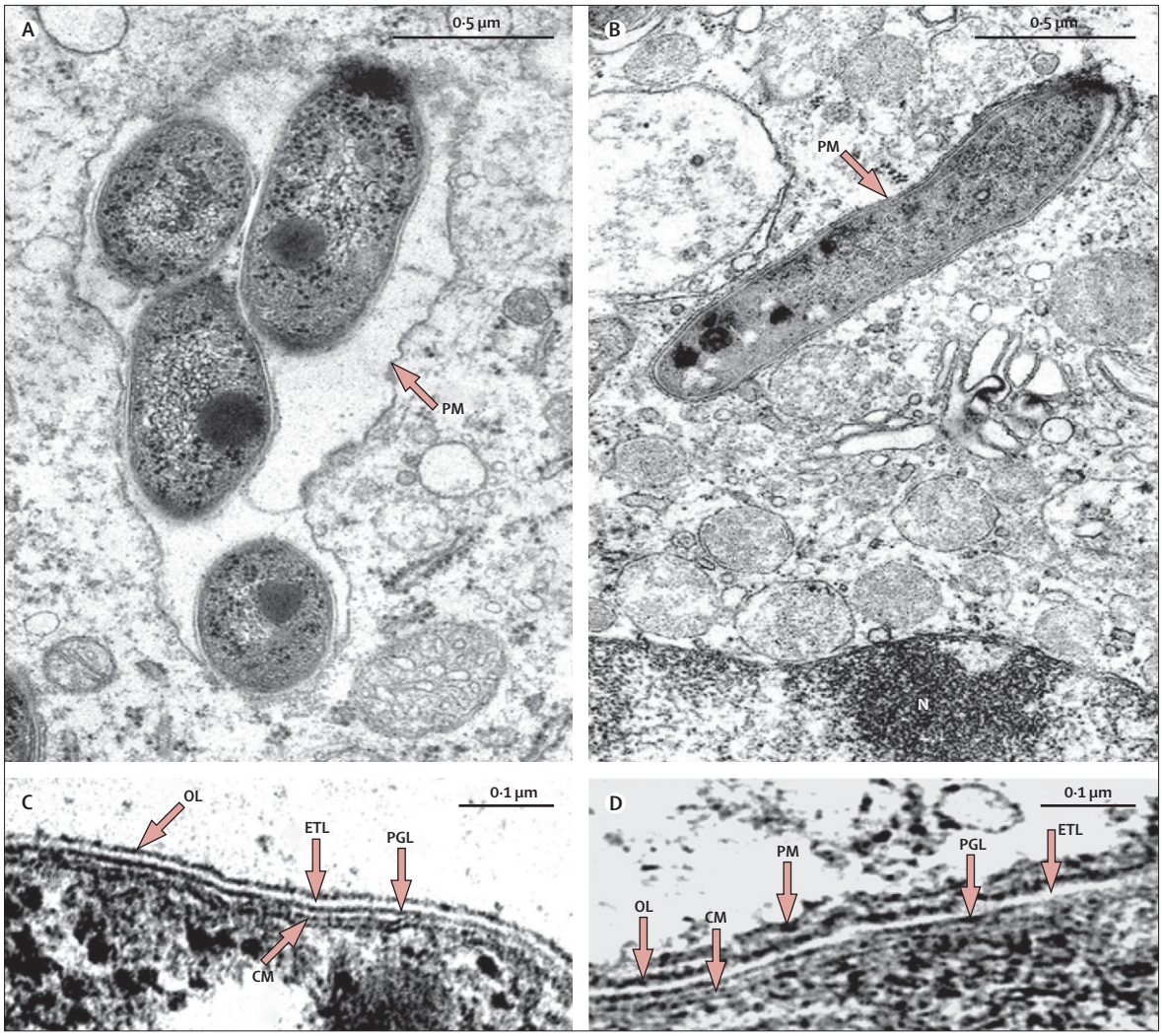

Figure 4: **Infection of cultured mouse bone marrow-derived macrophages**
Ultrastructure of intramacrophage *M ulcerans* 4 days after infection (multiplicity of infection=1) with mycolactone producer *M ulcerans* strain ITM 98-912 (A, B, and D). Under these conditions the bacilli multiply intracellularly.[30] Detailed image of the cell envelope of extracellular bacilli (C). The typical layers of mycobacterial envelopes are visible—namely, the cytoplasmic membrane (CM) covered by the cell wall with an innermost electron-dense peptidoglycan layer (PGL), an intermediate electron transparent layer (ETL), and an outer electron-dense layer (OL). High magnification of the envelope of an intramacrophage bacillus (D) at the zone labelled with an arrow in part B, showing the same envelope layers shown in part C plus a tightly apposed phagosomal membrane (PM). Reproduced with permission from the American Society for Microbiology.[30]

advancing tuberculosis[79–81] and the reduced production of interferon γ recovers upon antibiotic treatment in tuberculosis[81] and in Buruli ulcer.[82] Moreover, it is not clear how immune dysfunction measured in the peripheral blood relates to immunity at the location of infection.[80] Therefore, although there is depression of cytokine production in infections with *M ulcerans*, the contribution of mycolactone to this effect is not known.

There are several results that show that *M ulcerans* elicits CMI and DTH in animals and in Buruli ulcer despite the cytotoxicity of mycolactone towards immune cells.

Buruli ulcer lesions can heal spontaneously.[1] The development of T-helper-1 type of responses has been associated with resistance to *M ulcerans* and found in active infection and healing Buruli ulcer lesions.[46,74–76,83,84] Positive DTH burulin skin tests[85] were seen in 28 (71·8%) of 39 patients with Buruli ulcer,[86] a response similar to that reported for tuberculosis skin tests.[87] The positivity of the burulin test increased from early to advanced phases:[86,88] positive responses were seen in most of the patients with Buruli ulcer with healed (15 [93·8%] of 16) and ulcerative disease (11 [64·7%] of 17), but in few (two [33·4%] of six) patients with early ulcerative disease. As with tuberculosis,[89] granuloma formation happens when Buruli ulcer disease heals.[36,83,90,91]

The described histopathology of Buruli ulcer lesions healing in response to antibiotic treatment is similar to that of other mycobacterioses and involves CMI.[48,69]

BCG vaccination protects human beings against Buruli ulcer osteomyelitis,[92,93] and BCG and a DNA vaccine encoding antigen 85A from BCG protect against experimental infections with *M ulcerans*.[94–96]

Infection with HIV is associated with substantial CMI deficiency, allowing opportunistic infections with intracellular parasites including *M tuberculosis* and *Mycobacterium avium*.[97] Because of the geographical location of most Buruli ulcer disease foci in rural areas and children, HIV infections are infrequent in at-risk individuals in areas endemic for Buruli ulcer.[98] However, HIV infection has been associated with increased incidence of Buruli ulcer[99] and with aggressive disease with rapidly spreading osteomyelitis.[100]

The pattern of cytokine expression reported in Buruli ulcer lesions[62,70,75,83,101] corresponds with the development of CMI and DTH. These data suggest that the unique cytotoxicity of *M ulcerans* can coexist with the immunological expression of its mycobacterial nature.[29,30,62,70]

M ulcerans lifestyle in the host

M ulcerans as an intracellular parasite

That a microorganism is intracellular in a given moment only means that it is found inside a cell. Any intracellular or extracellular parasites can be seen within cells, mainly phagocytes. Labelling a microorganism as an intracellular parasite is a different issue that must include a set of characteristics regarding its life cycle in the host and the type of immune responses elicited.[102,103] Intracellular parasites produce diseases whose pathogenesis and immune responses require a phase of intracellular residence and multiplication.[102–104]

M ulcerans-related *M tuberculosis* and *M marinum* are labelled as intracellular parasites based on the following characteristics: first, they grow in vitro[105,106] and in vivo[58,107] within macrophages and have genes to promote their entry, survival, and multiplication within this host cell.[108,109] Second, these mycobacteria elicit CMI, DTH responses, and a granulomatous tissue reaction.[110,111]

M ulcerans and mycolactone were reported to inhibit phagocytosis by macrophages in vitro and this inhibition has been assumed to interfere with the in-vivo phagocytosis of *M ulcerans*, promoting the extracellular location of the bacilli.[22,43–45] However, substantial inhibition of phagocytosis in vitro was only seen with high concentrations of the toxin,[44] and the actual concentration of the toxin in infectious sites is unknown. Recent results show that mycolactone-producing *M ulcerans* strains are phagocytosed in vitro by macrophages (figure 4) at similar rates as *M tuberculosis* and *M bovis* BCG when a low multiplicity of infection was used.[30] Moreover, the presence of intraphagocytic bacilli in active untreated human and experimental *M ulcerans* infections suggest that even if some inhibition of phagocytosis is induced, substantial in-vivo uptake of the pathogen by macrophages does happen.

M ulcerans was found unable to grow within macrophages in vitro.[43,44] However, these results were from experiments using very high multiplicities of infection that induce quick killing of macrophages.[22,30,44,45] When this point was re-examined it was shown that virulent *M ulcerans* grows within cultured macrophages provided low multiplicities of infection are used,[30] as described for *M tuberculosis* and *M marinum*.[105,112,113]

The ability of *M ulcerans* to grow within macrophages in vitro is not direct evidence that this growth happens in vivo. This in-vitro characteristic has been considered as typical of intracellular parasites like *M tuberculosis* and *M marinum*, and has been thought to be a correlate of an intracellular lifestyle[114,115] and of disease promoting pathogenicity.[116,117] Moreover, in mouse foot pads injected with virulent *M ulcerans* (figures 1 and 2)[30] and in biopsies of Buruli ulcer (figure 3),[15,30] distended macrophages containing huge numbers of bacilli were seen, which were reminiscent of intramacrophage globi resulting from the intracellular multiplication of *Mycobacterium lepraemurium* and *M leprae*.[118]

The interpretation that the cause of Buruli ulcer has the essential hallmarks of intracellular parasites like the other pathogenic mycobacteria[30] is supported by the observations that *M ulcerans* is phagocytosed in vitro by macrophages and has the ability to grow within these phagocytes, is seen in large numbers within macrophages in active untreated infections, and elicits CMI and DTH responses. In favour of this interpretation is the observation (Torrado E, Trudeau Institute, Saranac Lake, NY, USA, personal communication) that several genes (including MUL_0769, MUL_4008, MUL_0823, MUL_0463, and MUL_2626) needed for growth of *M tuberculosis* and *M avium* within macrophages[108] are in the genome of *M ulcerans* strain Agy99,[6] although studies to clarify the function of these genes have not been done.

Cytotoxicity versus intracellular parasitism

Intracellular multiplication in the *M ulcerans* life cycle explains the reported features of immunity associated with infections and accords with the mycobacterial nature of *M ulcerans*, but faces a potential conflict of how to reconcile intracellular parasitism with cytotoxicity.

Since intracellular parasites are dependent on living host cells and many of their functions,[119] the classic view was that they must have low or no cytotoxicity.[102] However, several intracellular parasites including *M tuberculosis*, *M haemophilum*, and *M marinum* are cytotoxic to macrophages.[112,113,120,121] The direct cytotoxic activity of those mycobacteria towards host cells has been linked to specific molecules unrelated to mycolactone.[112,113,120,122] Direct cytolytic activity by intramacrophage pathogens after intracellular multiplication has also been reported for other intracellular parasites,[123–125] and association of that activity to tissue invasiveness and virulence has been established for *M tuberculosis* and *M marinum*.[112,113,126] Therefore,

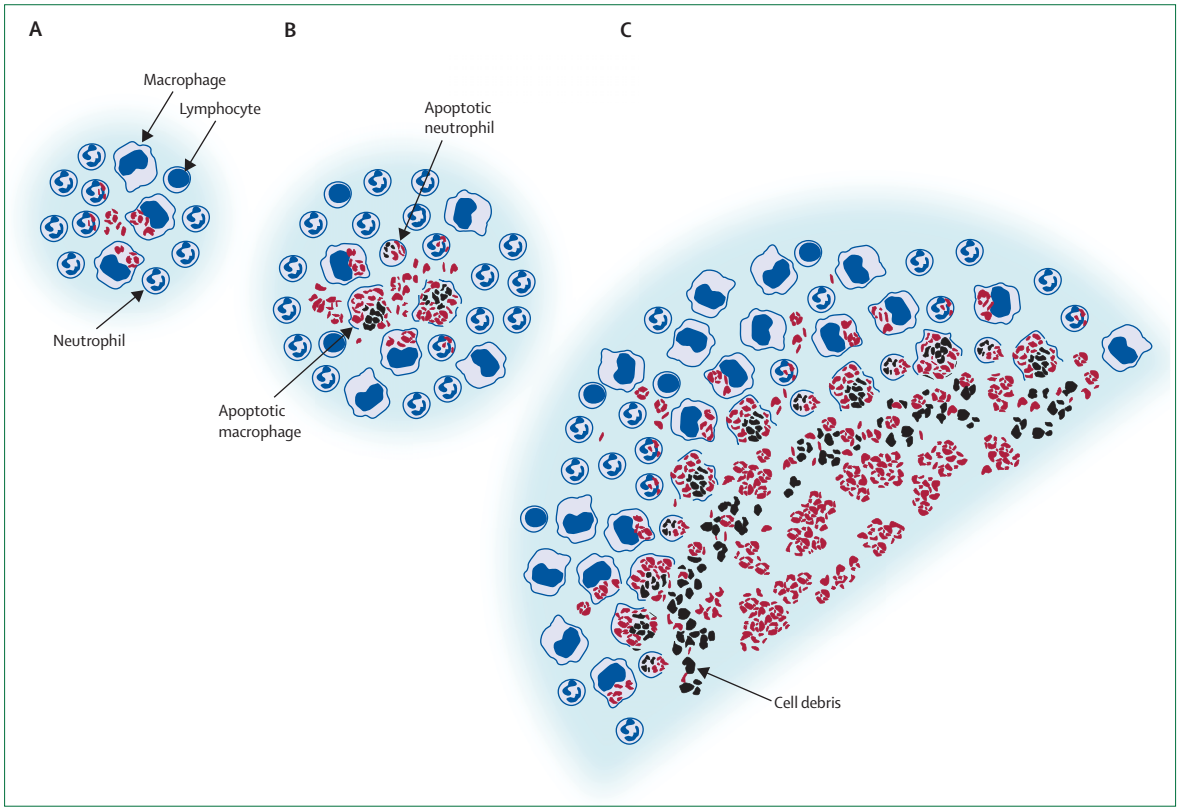

Figure 5: **Schematic representation of the initiation and establishment of a Buruli ulcer lesion**
Early stage of infection (A) with *M ulcerans* (red bacilli) phagocytosed by neutrophils and macrophages in the acute inflammatory infiltrate. A more advanced stage (B) characterised by the presence of an area with inflammatory cellular infiltrate with intraneutrophil and intramacrophage bacilli, and apoptotic neutrophils and macrophages. The advanced stage of the lesion with extensive necrotic, acellular areas (C) containing abundant clumps of extracellular bacilli, cellular debris, and neutrophils and macrophages with intracellular bacilli at the edge of the necrotic areas.

M ulcerans is not alone in the group of intracellular parasites and in the *Mycobacterium* genus as a cytotoxic microorganism, although the intense activity of its main cytotoxic molecule, mycolactone, makes it more difficult to reconcile cytotoxicity with intracellular parasitism. However, the negative consequences of cytotoxicity on intracellular growth of *M ulcerans* could be controlled by regulating the expression of mycolactone genes. Mechanisms for the temporal regulation of the cytolytic activity of intracellular parasites have been reported,[127,128] including quorum-sensing systems in *M tuberculosis* and *M marinum*.[129]

M ulcerans within neutrophils

Phagocytosis of *M ulcerans* by neutrophils in vivo was reported in early[15,19] and recent[29,30,45] publications. In more detailed studies, bacilli within neutrophils were seen in the initial phases of the infection and transiently[45] or throughout the infectious process.[29,30] Although the pathogen was inoculated in high numbers in these studies, this does not reproduce the early phase of the natural infection where the invading mycobacteria are most likely phagocytosed by the resident tissue macrophages—highly phagocytic cells located in all body territories[130] that are the first phagocytes the invading pathogens encounter.[131] This phagocytosis is the case, for example, at the beginning of pulmonary tuberculosis.[40] Phagocytosis by neutrophils is most typical in infections by extracellular parasites[132] that are eliminated after a brief period within the neutrophils.[133] Therefore, the presence of *M ulcerans* within neutrophils in vivo is irrelevant for the characterisation of *M ulcerans* as an intracellular parasite.

M ulcerans bacilli might be transferred within neutrophils to the draining lymph nodes.[45] However, the coexistence in *M ulcerans* within lymph nodes and of the bacilli within neutrophils is not evidence that bacilli have been transferred between the two. Mycobacteria might have been internalised in the lymph nodes by local phagocytes after having been transferred as free bacilli.[66]

Extracellular multiplication in vivo

Extracellular multiplication in vivo happens during specific phases of the infectious processes of several intracellular parasites.[134] The presence of extracellular bacilli in large clumps in necrotic areas (figures 1 and 3) suggests that extracellular multiplication of *M ulcerans* would take place in active, advanced disease.[15,16]

Accumulation of extracellular bacilli in necrotic areas has been reported in lesions due to *M tuberculosis*,[40,135] *M haemophilum*,[35] and *M marinum*.[35,113] Moreover, extracellular multiplication of *M tuberculosis* happens in tuberculosis.[40,135] However, the contribution of extracellular multiplication to the pathogenesis of infection with *M ulcerans* in necrotic areas, including through the secretion of mycolactone, has not been evaluated.

How long extracellular *M ulcerans* remain viable in the extensive areas of tissue necrosis is unknown. If in the ulcerated lesions viable extracellular *M ulcerans* are lost to the exterior, as with *M tuberculosis* in necrotic lung lesions,[40] they could contribute to the survival of the species in endemic areas.[11]

Extracellular *M ulcerans* might also be of relevance in the context of the antibody-mediated immunity in infections by some intracellular parasites,[136] including *M tuberculosis*.[137] Several reports describe the production of antibodies to *M ulcerans* in mice and patients with Buruli ulcer,[46,74,86,95,138,139] but a possible protective activity of these antibodies has not been assessed. However, the characteristic presence of abundant extracellular *M ulcerans* bacilli in advanced Buruli ulcer lesions has to be taken into consideration when developing new chemotherapeutic and prophylactic strategies.[57]

A model of the progression of infection

A model of the progression of *M ulcerans* infectious disease has been suggested (figure 5)[29,30] on the basis of the reviewed data, integrating aspects of Buruli ulcer microbiology and immunology. This model implies that *M ulcerans* behaves as an intracellular parasite that induces inflammatory cellular responses. During the active phase of the infection, *M ulcerans* parasitises macrophages and multiplies within them, continuously colonising incoming monocytes or macrophages and progressively invading healthy tissues. The persistent influx of leucocytes to the site of active infection provides immune cells that interact with *M ulcerans*, triggering leucocyte chemotaxis and CMI; the ability of this CMI to halt the progress of the infection, mediate immunopathology, or lead to self cure depends upon multiple factors including dose of infection, virulence of bacteria, and the immunocompetence of the host. The band of cellular infiltrate with *M ulcerans* multiplying within macrophages therefore represents the front where crucial events in the development of the disease occur. As the front advances with invasion of healthy tissues, leucocyte lysis at the trailing edge of the infiltrate creates a continuously enlarging acellular necrotic area with freed bacilli that probably multiply extracellularly. In advanced disease, the necrotic lesion further expands with extensive coagulation necrosis of the subcutaneous tissue, dermis, and epidermis, extensive vasculitis, and, in some cases, ulceration. In time, immunity becomes protective with production of a granulomatous tissue reaction and healing.

According to this view of Buruli ulcer pathogenesis, inflammatory infiltrates in advanced lesions occupy a smaller area compared with that of the necrotic zones because of the destruction of the continuously attracted infiltrates of immune cells by mycolactone rather than, as proposed by others,[22,23,44,77] because of the mycolactone-induced inhibition of cellular responses. That is, in advanced lesions of Buruli ulcer, the inflammatory cellular infiltrates are minor in spatial but not in immunological terms.

Search strategy and selection criteria

Data for this Review were identified by searching PubMed and the references in review papers for relevant articles in English and French from 1948 until May, 2009. All but one of the referenced papers are in English. Search terms included "*Mycobacterium ulcerans*", "Buruli ulcer", "intracellular parasites", "cell mediated immunity", "apoptosis", and "cytotoxicity". Preference was given to articles dealing with *M ulcerans* pathogenesis, mycolactone cytotoxicity, and bacterial growth within macrophages.

Conclusions

We propose that *M ulcerans* is an intracellular parasite that induces immunologically relevant inflammatory cellular responses. This interpretation clarifies some controversial points within the published work on Buruli ulcer and explains reported association with CMI and DTH, including development of T-helper-1 responses, some degree of protection by BCG-type vaccines, and higher incidence of the disease in CMI-deficient individuals. Rather than representing the mere attribution of a label with academic interest, this interpretation has relevant prophylactic and therapeutic implications because it prompts the development of vaccines that boost CMI and the use of chemotherapeutic protocols that include antibiotics active against intracellular mycobacteria.

Several features of *M ulcerans* biology represent an exaggeration of characteristics of the closely related species *M tuberculosis* and *M marinum*. Illustrated by the stronger ability of *M ulcerans* to induce cytolysis, produce necrotising lesions, and to depress immune responses. Mycolactone seems centrally responsible for the unique characteristics of the cause of Buruli ulcer, but the importance of the effects of this toxin on pathogenesis and whether it is the sole component in the induction of those exaggerated features awaits clarification. The clarification of the role of mycolactone will need to include the search for additional virulence factors, a much neglected area of study. Fresh research on *M ulcerans* infections in patients with Buruli ulcer and in animals is needed to better understand the relevance of data from experiments where mycolactone is added to cultured cells or is injected into animals,

the concentration, distribution, and stability of the exotoxin in infected sites, the regulation of mycolactone synthesis and secretion by intracellular and extracellular bacilli, and ability of extracellular bacilli to multiply. Research on these areas is essential for much needed advancement in the control of Buruli ulcer, an important and challenging disease that still remains largely neglected.

Contributors

All authors contributed equally to the preparation of this Review.

Conflicts of interest

We declare that we have no conflicts of interest.

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