REVIEW

Buruli ulcer disease: prospects for a vaccine

Kris Huygen · Ohene Adjei · Dissou Affolabi · Gisela Bretzel · Caroline Demangel · Bernhard Fleischer · Roch Christian Johnson · Jorge Pedrosa · Delphin M. Phanzu · Richard O. Phillips · Gerd Pluschke · Vera Siegmund · Mahavir Singh · Tjip S. van der Werf · Mark Wansbrough-Jones · Françoise Portaels

Abstract Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, is a neglected bacterial infection of the poor in remote rural areas, mostly affecting children. BUD is a mutilating disease leading to severe disability; it is the third most common mycobacterial infection in immunocompetent people after tuberculosis and leprosy. It is most endemic in West Africa, but cases have been reported from more than 30 countries. Treatment with antibiotics is possible, long-lasting and requires injections; there are cases of treatment failures, and the disease is prone to resistance. A vaccine against *M. ulcerans* would protect persons at risk in highly endemic areas, and could be used as a therapeutic vaccine to shorten the duration of treatment and prevent relapses. There is considerable evidence supporting the

K. Huygen (✉)
Scientific Institute of Public Health,
Rue Engelandstraat 642, 1180 Brussels, Belgium
e-mail: kris.huygen@iph.fgov.be

O. Adjei
Kumasi Centre for Collaborative Research, Kumasi, Ghana

D. Affolabi
Laboratoire de Référence des Mycobactéries, Cotonou, Benin

G. Bretzel
Department of Infectious Diseases and Tropical Medicine,
Ludwig-Maximilians-University, Munich, Germany

C. Demangel
Institut Pasteur, Paris, France

B. Fleischer
Bernhard Nocht Institute for Tropical Medicine,
Hamburg, Germany
e-mail: fleischer@bni-hamburg.de

R. C. Johnson
Programme National de Lutte contre l'Ulcère de Buruli,
Cotonou, Benin

J. Pedrosa
Life and Health Sciences Research Institute (ICVS),
School of Health Sciences, University de Minho, Braga, Portugal

D. M. Phanzu
Institut Medical Evangelique, Kimpese, DR Congo

R. O. Phillips
Kwame Nkrumah University of Science
and Technology, Kumasi, Ghana

G. Pluschke
Swiss Tropical Institute, Basel, Switzerland

V. Siegmund
European Research and Project Office GmbH,
Saarbrücken, Germany

M. Singh
LIONEX Diagnostics and Therapeutics GmbH,
Brunswick, Germany

T. S. van der Werf
University of Groningen Medical Centre,
Groningen, The Netherlands

M. Wansbrough-Jones
St George's University, London, UK

F. Portaels
Prince Leopold Institute of Tropical Medicine,
Antwerp, Belgium

notion that generation of a vaccine is feasible. This article reviews the present state of the art with special emphasis on the immunology of the infection and the prospects for development of a vaccine.

Keywords Buruli ulcer · *Mycobacterium ulcerans* · Mycolactone toxin · Vaccine · Neglected diseases · Review

Introduction

Buruli ulcer disease (BUD) has emerged since 1980 as an important cause of human suffering. It is a poverty-related mutilating disease caused by *Mycobacterium ulcerans*. BUD is the third most common mycobacterial disease in humans after tuberculosis and leprosy and the most poorly understood of these three diseases. The disease presents as an indolent necrotizing disease of the skin, subcutaneous tissue and bone and can afflict all age groups but children under 15 years represent the largest part of the BUD disease burden. The disease remained largely ignored by many national public health programs for decades [2]. In 1998, the World Health Organization recognized BUD as an emerging health problem, primarily because of its frequent disabling and stigmatizing complications [68].

Geographic distribution, incidence and prevalence

The disease is endemic in rural wetlands of tropical countries of Africa, America, Asia and Australia. Cases have also been reported in non-tropical areas of Australia, Japan and China. Known incidence rates are highest in West Africa, particularly in Benin, Côte d'Ivoire and Ghana where between 1,000 and 2,000 cases are reported annually [70]. In some West African countries, the number of BUD cases may even exceed those of tuberculosis and leprosy [11]. There is evidence of enormous under-reporting of the disease.

Little is known about the focal epidemiology of BUD. Incidence, prevalence, and other data are usually reported at the national or district level. These data show the importance of the disease but do not reveal the wide variations that often exist at the village level within a given district [26].

Reservoirs and transmission

BUD is an infectious disease but rarely, if ever, contagious. There is now sufficient evidence from microbiological and epidemiological data, including studies of risk factors, to consider BUD a water-related disease [13]. However, the exact mode(s) of transmission from the environment and the ultimate natural reservoir(s) of infection remain obscure. Humans probably become infected by traumatic introduction of *M. ulcerans* into skin from the overlying *M. ulcerans*-contaminated surface. Contamination of the skin could result from direct exposure to stagnant water, aerosols arising from ponds and swamp surfaces or fomites [10].

Since the initial discovery of *M. ulcerans* DNA in water bugs (Hemiptera) in Benin [39], aquatic insects have been suspected to play a role in transmission [30, 40]. However, the ultimate importance of *M. ulcerans*-colonized aquatic insect bites in the transmission of BUD remains unproven, justifying continued investigation of other forms of transmission of *M. ulcerans* to humans [44, 51]. In an outbreak in Australia, mosquitoes have been implicated in transmission [48].

Prevention

In tropical rural settings where BUD is endemic and scantily dressed people play and work, avoiding contact with the *M. ulcerans* contaminated environment is virtually impossible. Wearing protective clothing when farming [47] and immediate cleansing of any skin injury [33] may reduce rates of infection, but achieving these measures is seldom feasible.

Use of protected sources of water for domestic purposes reduces exposure to *M. ulcerans* contaminated water and consequently may reduce prevalence rates of BUD [26]. The problem of reducing risk factors for basic agricultural workers, fishermen and others who must put themselves at risk, remains, however, a serious concern.

Clinical manifestations

Mycobacterium ulcerans disease presents a spectrum of forms related partly to patient delay in admission to hospital [11]. Mean incubation periods are estimated to be 2–3 months.

The most common form of disease is a nodule which enlarges and ulcerates giving rise to a painless ulcer with undermined edges and edema of the surrounding skin. Ulcers develop by perforation of the underlying necrosis through the epidermis. Ulcers may remain small (minor ulcers), with a diameter up to 1–2 cm, and self-heal but usually enlarge (major ulcers) and destroy wide areas of skin. Major ulcers may self-heal eventually, resulting in atrophic stellate or symmetric scars with contractures and disabilities when located over joints which may ankylose

and become totally immobile. Major ulcers may progress to involve the subjacent bone and cause osteomyelitis [45].

Laboratory diagnosis

Although the experienced health care worker in endemic areas usually can make an accurate clinical diagnosis of BUD, microbiological confirmation is essential for the following reasons:

1. To confirm the precise prevalence and incidence of BUD in a given area;
2. To confirm new foci, especially where health care workers lack experience with BUD;
3. To help manage the disease by surgical and/or antimycobacterial treatment;
4. To confirm and differentiate relapse and reinfection after treatment.

Four laboratory tests are currently available to confirm the diagnosis of BUD [41, 50]: direct smear examination for acid-fast bacilli by Ziehl–Neelsen or auramine stain, in vitro culture, IS*2404* PCR and histopathological examination. PCR represents the gold standard for diagnosis in research studies because of its high sensitivity and specificity.

Treatment

For decades, excision surgery with primary closure or skin grafting was the recommended therapy of BUD. Recurrence rates after surgery vary between 6 and 28% [5, 12]. In the last few years, the use of oral rifampicin plus intramuscular streptomycin with or without surgery has been recommended by WHO [69]. Several centers in Africa have initiated therapy with antibiotics according to the WHO guidelines [69], and some studies suggest that following drug therapy for 8 weeks, most ulcers may heal without surgery. Recurrence rates within the year following the end of chemotherapy were less than 2% [6].

However, access to health services in endemic areas is usually restricted. Due to the painless nature of the lesions, patients often seek treatment late and tend to turn first to traditional healers. Thus, treatment is usually delayed, causing frequent and severe complications, leading to prolonged and expensive hospitalization.

Moreover, it has been determined for Central Cameroon that despite free-of-charge medical treatment, the cost burden of BUD accounts for 25% of households' yearly earnings, surpassing the threshold of 10%, which is generally considered catastrophic for the household economy,

and calling into question the sustainability of current BUD programs [21].

Pathogenesis and immunity

Mycobacterium ulcerans is genetically very close to *M. marinum* [55], an intracellular pathogen that triggers inflammatory responses and cell-mediated immunity (CMI) [32] but it is unique among pathogenic mycobacteria since it produces a family of toxic macrolides, the mycolactones, which are required for virulence [14, 17]. Mycolactones are secreted and diffuse into the infected tissues and surrounding areas, but the amount and precise distribution of the toxin in the lesions is not known. Mycolactones have a potent cytotoxic activity that induces apoptosis and necrosis of several cell types including adipocytes, fibroblasts and leukocytes, and participate in the tissue necrosis typical of the disease [1, 23, 36, 65]. Recently, studies using animal models have shown that mycolactone distributes beyond the sphere of its cytocidal action and gains access to the blood and lymphoid organs, where it concentrates in mononuclear cell subsets [24].

Cellular immune responses

Resistance to *M. ulcerans* has been associated with the development of Th1 type responses [19, 20, 47, 66] and as BUD disease progresses to healing, granuloma formation has been reported [22, 27, 54, 63], and the DTH burulin skin test [53] tends to change from negative to positive [16, 31]. In contrast, disseminated BUD disease and bone involvement have been reported to be associated with defects in granuloma formation [28, 34].

As in tuberculosis and leprosy, the macrophage activating cytokine IFN-γ seems to play a pivotal role in the control of *M. ulcerans* infection, and PBMC from BUD patients display a reduced capacity to produce this cytokine upon in vitro stimulation with whole *M. ulcerans* bacilli [18, 47]. Using RT-PCR analysis, Gooding et al. [19] have described the production of Th2 type cytokines, i.e., IL-4, IL-5, IL-6 and IL-10 by PBMC from Australian BUD patients. These findings could only be partly confirmed by Prévot et al. [47] in French Guyana, who were unable to detect any IL-4 or IL-13 activity in BUD patients, but who confirmed that the production of IL-10 following stimulation with whole, killed *M. ulcerans* was higher in BUD patients than in healthy controls. Westenbrink et al. [66] finally failed to detect an association of IL-4 or IL-10 with changes in IFN-γ in BUD patients . An extensive real-time PCR analysis on skin biopsies of 16 patients with early nodules and 28 patients with late-stage ulcers, showed a

significantly higher expression of IL-8 and other pro-inflammatory cytokines in 32 biopsies with neutrophilia than in those of 12 biopsies without acute inflammation [38]. As in the Prévot study, expression levels of IL-4 and IL-5 were below detection level, whereas some IL-10 message could be detected.

The *M. ulcerans* infection-associated reduction of IFN-γ responses is not restricted to mycobacterial antigens and resolves after surgical excision of the lesion [71], suggesting that bacterial factors such as mycolactone may diffuse from bacillar colonies and exert immunosuppressive effects at the systemic level. This hypothesis is supported by observations that non-cytotoxic doses of mycolactone efficiently suppress the functions of several types of mononuclear cells in vitro. At nanomolar concentrations, mycolactone inhibits the activation-induced production of IL-2 by human lymphocytes, and of TNF by monocytes and macrophages [7, 37, 61]. Mycolactone also blocks the capacity of dendritic cells (DCs) to prime cellular responses and produce chemotactic signals of inflammation [9]. Lymphocytes, monocytes, DCs and macrophages compose the mononuclear cell fraction of blood and lymphoid organs. The fact that mycolactone targets mononuclear cells in mice infected with *M. ulcerans* thus strongly suggests that these cell subsets are immunosuppressed in infected hosts, and that mycolactone impairs the development of cellular immunity. In this model, neutralizing the immunosuppression imposed by mycolactone using inhibitors of its biosynthesis, or ablating its biological activity in vivo, would considerably enhance the efficacy of therapeutic vaccines and antibiotic treatments.

So far, nearly all studies of the cellular immune response against *M. ulcerans* have used whole bacteria or burulin, which is a crude, heat-killed bacterial sonicate [38]. IFN-γ responses against culture filtrate antigens 423 and 425 were similar in pattern but lower than those against *M. ulcerans* sonicate [38]. A cross-reactive antigen that has been studied in some more detail in *M. ulcerans* infection is the mycolyl transferase antigen 85 (Ag85), which forms a major fraction of the secreted proteins in mycobacterial culture filtrates. This Ag85 is actually a 30–32 kDa family of three proteins (Ag85A, Ag85B and Ag85C) [67], which all possess a mycolyl transferase enzymatic activity required for the integrity of the cell wall [4]. Purified Ag85 from BCG induces strong T cell proliferation and IFN-γ production in most healthy individuals infected with *M. tuberculosis* or *M. leprae* and in BCG vaccinated mice, whereas in tuberculosis and leprosy patients the Ag85 specific IFN-γ production is much lower [29, 64]. Similarly, PBMC from BUD patients demonstrate lower IFN-γ responses against Ag85 purified from BCG than PBMC from healthy BCG vaccinated subjects [47].

Antibody responses

In studies with *M. ulcerans* culture filtrates, IgG antibody responses against the secreted *M. ulcerans* proteins were frequently found in BUD patients, but also in TB patients from BUD non-endemic regions [16, 35]. The IgM responses of BUD patients against the filtrate proteins were more distinct than those of healthy family members living in the same village [35] indicating B cell stimulation. Diaz et al. used the highly immunogenic *M. ulcerans* 18 kD small heat shock protein, which has no homologs in *M. bovis* and *M. tuberculosis* to monitor *M. ulcerans* specific IgG responses in BUD patients and household contacts from Ghana. Under stringent assay conditions 75% of patients, independent of disease stage, but also 38% of household contacts showed reactivity, whereas samples from Europeans and non-exposed Africans remained negative [15]. This indicates that specific humoral responses against *M. ulcerans* develop in exposed, but otherwise healthy individuals. Immune responses in healthy household contacts have also been described by immunoblot analysis in Australian samples [19], where a lower background staining than with African sera facilitated analysis with *M. ulcerans* cell extracts. The control of *M. ulcerans* infection may be primarily dependent on cell-mediated immunity involving activated macrophages, T cells, and Th1 type cytokines, as is thought to be the case for *M. tuberculosis* and *M. leprae* infection. However, antibodies could provide additional protective mechanisms against the largely extracellular *M. ulcerans*. Opsonisation might improve phagocytosis and killing by neutrophils, increase intracellular killing by macrophages or improve antigen presentation and induction of protective T cell responses.

Vaccination against *M. ulcerans*

Although significant progress has been made in management of this disease in endemic countries during the last decade, BUD remains a major economic and social burden for developing countries [21]. Therefore, vaccination programs remain the only viable prevention alternative. Evidence that a protective immune response can develop in humans is incomplete but, as described above, two serological studies have indicated that some household contacts of BUD patients have been exposed to *M. ulcerans* without developing disease. This may be because they developed a protective immune response or because of unrelated issues such as in inadequate infective dose of the organism or the wrong conditions for bacterial proliferation in exposed skin. In addition, there is much anecdotal evidence that some people develop pathological lesions after infection with *M. ulcerans* which heal in a short time without any

treatment. This was, for example, documented in the control group of a placebo-controlled trial of clofazimine for treatment of early BUD lesions from which 30% healed without going on to ulcerate [49]. This strongly suggests that the disease can be controlled by an appropriate host response, although there is no evidence that such individuals were protected against infection on future exposure.

The fact that a bimodal age distribution of *M. ulcerans* disease has been observed with peaks at 5–15 years and 75–79 years raises the possibility that latent infection can reactivate as immunity declines with age [11]. There is, however, no hard evidence to support this notion of reactivation at present.

Molecular and immunological tools have started to give us more insight into the mechanism of *M. ulcerans* infection, the progression to BUD and immune responses involved. However, a key question with respect to the development of an effective vaccine remains to be answered: is *M. ulcerans* essentially an intracellular or extracellular pathogen? In the former case, antibodies are likely to play little role in protection and vaccination should focus on the stimulation of cellular immune responses, particularly those conferred by the Th1 type helper T cell population. If extracellular, a vaccine aimed at stimulating antibody responses would be a more obvious and perhaps easier option. The third, and perhaps most probable, possibility is that both arms of the immune response are required for optimal protection, as is the case for a number of viral and parasite pathogens.

Experimental infections in mice have shown that *M. ulcerans* is efficiently internalized by mouse phagocytes in vitro [7, 61] and that it proliferates inside macrophages [61]. This intracellular phase is transient [7], except in the edges of the lesion [61]. Following injection in the dermis of the ears, bacilli are indeed internalized by professional cells and transported to the draining lymph nodes within host cells [7]. The inoculation site eventually becomes ulcerated with tissue necrosis and extracellular bacteria appear at later stages of the infection.

As of now, there is no specific vaccine against *M. ulcerans*, but some evidence in the literature has suggested a cross-reactive protective role of the *M. bovis* BCG vaccine used against tuberculosis. Two large randomized controlled trials of BCG vaccination for the prevention of BUD were conducted in Uganda during the late 1960s and early 1970s.

The first study was performed in a population of approximately 2,500 Rwandan refugees in the Kinyara refugee camp in the Bunyoro district of Uganda [62]. At the commencement of the study the entire population was surveyed and divided into groups according to whether they had previously had BUD, prior BCG or no prior BCG. Those without prior BCG were randomized to receive or not to receive a single dose of BCG, provided their tuberculin skin test result (TST) was <6 mm. The population was followed for 16 months. Overall efficacy of BCG was 47%. Protection rate was 72% in the first 6 months, but almost 0% in the second 6 months. In non-randomized subjects, prior BUD disease (healed lesion) and a positive TST were protective. The protective effect of a positive TST also declined during the study period. Protection afforded by BCG was not influenced by age at vaccination or TST conversion. In those who developed BUD, BCG and positive TST were associated with "reactive" histology and a higher proportion of "pre-ulcerative" lesions.

The second study was performed in a rural area along the south-east bank of the Nile in central Uganda [52]. The study population consisted of 9,396 people who lived in five administrative parishes. At the commencement of the study, the population was surveyed and offered TSTs. BCG was given at random to 50% of all these persons, irrespective of previous BUD lesions, BCG scar or tuberculin-status. The main findings were: overall protection rate of 47% during the first year of study. Protection appeared to decline beyond this time. BCG only provided protection in those with initial TST <4 mm and BCG reduced the size of BUD lesions at diagnosis. Healed BUD lesion or BCG scar offered some protection, but subsequent BCG vaccination did not show additional protection.

Taken together, both studies were consistent with BCG producing a significant but only short-lasting protection against BUD. Moreover, the results in the tuberculin-positive subjects suggested that a cross-protective immune response could also be induced by previous exposure to *M. tuberculosis*. Conversely, in 1987 Bahr et al. [3] described a cross-reactive skin test response to *M. ulcerans* in BCG vaccinated school children from Kuwait, a region where *M. ulcerans* has not been reported so far. All this is not really surprising in the light of the close phylogenetic relationship of mycobacteria from the *M. tuberculosis* complex (*M. tuberculosis–bovis–africanum*) on the one hand and *M. ulcerans-marinum* on the other [60].

One manifestation of BUD, against which BCG vaccination seems to exert a sustained, immunoprophylactic effect, is the disseminated osteomyelitic form. In 2002, Portaels et al. [42] reported a protective effect of BCG vaccination against osteomyelitis in children suffering from BUD in Benin. In her study, only 7.7% of the children younger than 15 years of age with BCG scars had this severe, disseminated form of BUD, whereas in the group of unvaccinated children, 33% suffered from osteomyelitis. Also in adults, BCG vaccination confers a certain degree of protection against osteomyelitis [43].

In experimental foot pad infection of C57BL/6 mice with *M. ulcerans*, *M. bovis* BCG vaccine offers only a short-term protection and the duration of this protection cannot be prolonged by a booster vaccination [58]. These

findings seem to indicate that the limited protection conferred by BCG against *M. ulcerans* is not so much caused by a waning of the immune protection, but rather that the *quality* of immune response induced by the vaccine is not optimal. It is possible that the cross-reactive immune response induced by the *M. bovis* BCG vaccine is not sufficient and that species-specific immune responses are required to effectively control the infection. A comparative study of the protective efficacy of two DNA vaccines encoding Ag85A from BCG and from *M. ulcerans*, respectively, indicates that this might indeed be the case [59].

Mycolactone is an obvious candidate for a vaccine, but by virtue of its chemical structure, the polyketide is not in itself immunogenic, and so far antibodies against mycolactone have not been detected either in healthy contacts or in BUD patients. The enzymes involved in the mycolactone biosynthesis are alternative vaccine candidates. In addition to the giant *mlsA1*, *mlsA2* and *mlsB* genes encoding the polyketide synthases (PKS), other genes have been identified that are involved in the synthesis of the toxin: three putative transcriptional regulatory genes: *fur*, *fis* and *tetR*, and two genes encoding enzymes involved in the synthesis of mycolactone precursor molecules: acetate kinase (*ack*) and 3-ketoacyl acyl carrier protein synthase III (*fabH*).

Although *M. ulcerans* and *M. tuberculosis/bovis* BCG share a large number of highly conserved antigens, even small sequence changes can result in the loss of immunodominant epitopes. Therefore, it has been argued that an attenuated, live vaccine based on *M. ulcerans* could offer a better and more specific protection than BCG [25]. Mycolactone negative isolates of *M. ulcerans* have been identified, both as spontaneous mutants that lack the yellow pigmentation of the mycolactone, and more recently by screening of kanamycin resistance in isolates generated by transposon mutagenesis [56].

Adjuvanted subunit based protein vaccines are an alternative to live attenuated vaccines. Subunit vaccines have the advantage of being well characterized and of posing no danger for application in HIV-positive populations. On the other hand, their antigenic repertoire is obviously limited, and the induced immune responses and memory are generally weak, requiring repeated boosting. So far only two subunit-based vaccines have been tested in experimental models. Tanghe et al. [57] were the first to report that vaccination with plasmid DNA encoding the mycolyl-transferase 85A from BCG could significantly reduce the bacterial load in the foot pads of *M. ulcerans* infected mice. Vaccination with recombinant Ag85A protein from *M. ulcerans* conferred also some protection and a DNA prime-protein boost immunization protocol resulted in a protective efficacy comparable to the one induced by the BCG vaccine [59]. The genes encoding the GroEL-2 protein Hsp65 are highly conserved among mycobacterial species, with 96 and 95%

identity at the amino-acid level between the *M. ulcerans* antigen and the homologous proteins in *M. tuberculosis* and *M. leprae*, respectively. Hsp65 is very immunogenic and vaccination of mice with a plasmid encoding hsp65-encoding DNA vaccine was also significantly protective against *M. ulcerans* infection, as demonstrated by the reduced bacterial loads in tails of infected BALB/c mice. In this mouse model, the protection levels conferred by DNA vaccines expressing hsp65, Ag85B, or both antigens were comparable, but nevertheless inferior to the one afforded by vaccination with BCG [8]. Although hsp65 is not currently considered as a vaccine candidate in humans because of its strong homology with human hsp60 and the risk of promoting auto-immune diseases, this experiment further demonstrates the possible role of subunit antigens in vaccination against BUD.

Outlook

In recent years, impressive progress has been made in research activities with respect to epidemiology, transmission, pathogenesis, immunology, diagnosis and treatment of BUD infection. Priority has been given to increased surveillance, early detection and treatment of the disease and there is still much to be done: antimycobacterial treatment has to be shortened and adapted to avoid injections, and the role, extent and timing of debridement surgery and skin grafting subsequent to antibiotic treatment have to be defined. However, good surveillance, detection and treatment tools will not be sufficient to decrease the incidence and prevalence of BUD. Prevention tools are mandatory and identification and development of vaccine candidates have now become a priority for better control of BUD. Generally, a vaccine against any neglected bacterial infection will only be used in highly endemic regions within endemic countries. A vaccine against *M. ulcerans* will be useful to protect children in hyperendemic foci but it may also have a role in therapy by shortening the duration of antibiotic treatment and preventing recurrences and severe forms of disease.

References

1. Adusumilli S, Mve-Obiang A, Sparer T, Meyers W, Hayman J, Small PLC (2005) *Mycobacterium ulcerans* toxic macrolide, mycolactone modulates the host immune response and cellular location of *M. ulcerans* in vitro and in vivo. Cell Microbiol 7:1295–1304. doi:10.1111/j.1462-5822.2005.00557.x
2. Aujoulat I, Huguet-Ribas MP, Koita Y (1996) Buruli ulcer: an ignored public health problem. Dev Sante 125:22–30 (in French)
3. Bahr GM, Chugh TD, Behbehani K, Shaaban MA, Abdul-Aty M, Al Shimali B, Siddiqui Z, Gabriel M, Rook GA, Stanford JL (1987) Unexpected findings amongst the skin test responses to

mycobacteria of BCG vaccinated Kuwaiti school children. Tubercle 68:105. doi:10.1016/0041-3879(87)90025-0

4. Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan BJ, Besra GS (1997) Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. Science 276:1420–14222. doi:10.1126/science.276.5317.1420

5. Buntine J, Crofts K (eds) (2001) Buruli ulcer: management of *Mycobacterium ulcerans* disease. World Health Organization, Geneva

6. Chauty A, Ardant MF, Adeye A, Euverte H, Guédénon A, Johnson C, Aubry J, Nuermberger E, Grosset J (2007) Promising clinical efficacy of streptomycin–rifampin combination for treatment of Buruli ulcer (*Mycobacterium ulcerans* disease). Antimicrob Agents Chemother 51:4029–4035. doi:10.1128/AAC.00175-07

7. Coutanceau E, Marsollier L, Brosch R, Perret E, Goossens P et al (2005) Modulation of the host immune response by a transient intracellular stage of *Mycobacterium ulcerans*: the contribution of endogenous mycolactone toxin. Cell Microbiol 7:1187–1196. doi:10.1111/j.1462-5822.2005.00546.x

8. Coutanceau E, Legras P, Marsollier L, Reysset G, Cole ST, Demangel C (2006) Immunogenicity of *Mycobacterium ulcerans* Hsp65 and protective efficacy of a *Mycobacterium leprae* Hsp65-based DNA vaccine against Buruli ulcer. Microbes Infect 8:2075–2081. doi:10.1016/j.micinf.2006.03.009

9. Coutanceau E, Decalf J, Martino A, Babon A, Winter N et al (2007) Selective suppression of dendritic cell functions by *Mycobacterium ulcerans* toxin mycolactone. J Exp Med 204:1395–1403. doi:10.1084/jem.20070234

10. Debacker M, Zinsou C, Aguiar J, Meyers WM, Portaels F (2003) First case of *Mycobacterium ulcerans* disease (Buruli Ulcer) that followed a human bite. Clin Infect Dis 36:e67–e68. doi:10.1086/367660

11. Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, Scott JT, Dramaix M, Portaels F (2004) *Mycobacterium ulcerans* disease: role of age and gender in incidence and morbidity. Trop Med Int Health 9(12) 1297–1304

12. Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, Portaels F (2005) Buruli ulcer recurrence, Benin. Emerg Infect Dis 11:584–589

13. Debacker M, Portaels F, Aguiar J, Steunou C, Zinsou C, Meyers W, Dramaix M (2006) Risk factors for Buruli ulcer, Benin. Emerg Infect Dis 12:1325–1331

14. Demangel C, Stinear TP, Cole ST (2009) Buruli ulcer: reductive evolution enhances pathogenicity of *Mycobacterium ulcerans*. Nat Rev Microbiol 7:50–60. doi:10.1038/nrmicro2016

15. Diaz D, Dobeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A et al (2006) Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to *Mycobacterium ulcerans*. Clin Vaccine Immunol 13:1314–1321. doi:10.1128/CVI.00254-06

16. Dobos KM, Spotts EA, Marston BJ, Horsburgh CR Jr, King CH (2000) Serologic response to culture filtrate antigens of *Mycobacterium ulcerans* during Buruli ulcer disease. Emerg Infect Dis 6:158–164

17. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R, Small PL (1999) Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283:854–857. doi:10.1126/science.283.5403.854

18. Gooding TM, Johnson PDR, Campbell DE, Hayman JA, Hartland E, Kemp AS, Robins-Browne RM (2001) Immune response to infection with *Mycobacterium ulcerans*. Infect Immun 69:1704–1707. doi:10.1128/IAI.69.3.1704-1707.2001

19. Gooding TM, Johnson PD, Smith M, Kemp AS, Robins-Browne RM (2002) Cytokine profiles of patients infected with *Mycobacterium ulcerans* and unaffected household contacts. Infect Immun 70:5562–5567. doi:10.1128/IAI.70.10.5562-5567.2002

20. Gooding TM, Kemp AS, Robins-Browne RM, Smith M, Johnson PD (2003) Acquired T-helper 1 lymphocyte anergy following infection with *Mycobacterium ulcerans*. Clin Infect Dis 36:1076–1077. doi:10.1086/368315

21. Grietens KP, Um Boock A, Peeters H, Hausmann-Muela S, Toomer E, Ribera JM (2008) It is me who endures but my family that suffers: social isolation as a consequence of the household cost burden of Buruli ulcer free of charge hospital treatment. PLoS Negl Trop Dis 2:e321. doi:10.1371/journal.pntd.0000321

22. Hayman J (1993) Out of Africa: observations on the histopathology of *Mycobacterium ulcerans* infection. J Clin Pathol 46:5–9. doi:10.1136/jcp.46.1.5

23. Hong H, Demangel C, Pidot SJ, Leadlay PF, Stinear T (2008) Mycolactones: immunosuppressive and cytotoxic polyketides produced by aquatic mycobacteria. Nat Prod Rep 25:447–454. doi:10.1039/b803101k

24. Hong H, Coutanceau E, Leclerc M, Caleechurn L, Leadlay PF, Demangel C (2008) Mycolactone diffuses from *Mycobacterium ulcerans*-infected tissues and targets mononuclear cells in peripheral blood and lymphoid organs. PLoS Negl Trop Dis 2:e325. doi:10.1371/journal.pntd.0000325

25. Huygen K (2003) Prospects for vaccine development against Buruli disease. Expert Rev Vaccines 2:561–569. doi:10.1586/14760584.2.4.561

26. Johnson RC, Sopoh GE, Boko M, Zinsou C, Gbovi J, Makoutode M, Portaels F (2005) Distribution de l'infection à *Mycobacterium ulcerans* (ulcère de Buruli) dans la commune de Lalo au Bénin. Trop Med Int Health 10:863–871. doi:10.1111/j.1365-3156.2005.01465.x

27. Kiszewski AE, Becerril E, Aguilar LD, Kader IT, Meyers WM, Portaels F, Hernandez PR (2006) The local immune response in ulcerative lesions of Buruli disease. Clin Exp Immunol 143:445–451. doi:10.1111/j.1365-2249.2006.03020.x

28. Lagarrigue V, Portaels F, Meyers WM, Aguiar J (2000) Buruli ulcer: risk of bone involvement! A propos of 33 cases observed in Benin. Med Trop 60:262–266

29. Launois P, DeLeys R, Niang MN, Drowart A, Andrien M, Dierckx P, Cartel JL, Sarthou JL, Van Vooren JP, Huygen K (1994) T-cell-epitope mapping of the major secreted mycobacterial antigen Ag85A in tuberculosis and leprosy. Infect Immun 62:3679–3687

30. Marsollier L, Robert R, Aubry J et al (2002) Aquatic insects as a vector for *Mycobacterium ulcerans*. Appl Environ Microbiol 68:4623–4628. doi:10.1128/AEM.68.9.4623-4628.2002

31. Marston BJ, Diallo MO, Horsburgh CR Jr, Diomande I, Saki MZ, Kanga JM, Patrice G, Lipman HB, Ostroff SM, Good RC (1995) Emergence of Buruli ulcer disease in the Daloa region of Cote d'Ivoire. Am J Trop Med Hyg 52:219–224

32. Mor N, Lutsky I, Levy L (1981) Response in the hindfoot pad and popliteal lymph node of C57BL mice to infection with *Mycobacterium marinum*. Isr J Med Sci 17:236–244

33. Nackers F, Johnson RC, Glynn J, Zinsou C, Tonglet R, Portaels F (2007) Environmental and health-related risk factors for *Mycobacterium ulcerans* disease (Buruli ulcer) in Benin. Am J Trop Med Hyg 77:834–836

34. Noeske J, Kuaban C, Rondini S, Sorlin P, Ciaffi L, Mbuagbaw J, Portaels F, Pluschke G (2004) Buruli ulcer disease in Cameroon rediscovered. Am J Trop Med Hyg 70:520–526

35. Okenu DM, Ofielu LO, Easley KA, Guarner J, Spotts Whitney EA et al (2004) Immunoglobulin M antibody responses to *Mycobacterium ulcerans* allow discrimination between cases of active Buruli ulcer disease and matched family controls in areas where the disease is endemic. Clin Diagn Lab Immunol 11:387–391. doi:10.1128/CDLI.11.2.387-391.2004

36. Oliveira MS, Fraga AG, Torrado E, Castro AG, Pereira JP, Filho AL, Milanezi F, Schmitt FC, Meyers WM, Portaels F, Silva MT, Pedrosa J (2005) Infection with *Mycobacterium ulcerans* induces

persistent inflammatory responses in mice. Infect Immun 73:6299–6310. doi:10.1128/IAI.73.10.6299-6310.2005

37. Pahlevan AA, Wright DJ, Andrews G, George KM, Small PL et al (1999) The inhibitory action of *Mycobacterium ulcerans* soluble factor on monocyte/T cell cytokine production and NF-kappa B function. J Immunol 163:3928–3935

38. Phillips R, Horsfield C, Mangan JA, Laing K, Etuaful S, Awuah P, Nyarko K, Osei-Sarpong F, Biutcehr P, Lucas S, Wansbrough-Jones M (2006) Cytokine mRNA expression in *Mycobacterium ulcerans*-infected human skin and correlation with local inflammatory response. Infect Immun 74:2917–2924. doi:10.1128/IAI.74.5.2917-2924.2006

39. Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P-A, Meyers WM (1999) Insects in the transmission of *Mycobacterium ulcerans* infection (Buruli ulcer). Lancet 353:986. doi:10.1016/S0140-6736(98)05177-0

40. Portaels F, Chemlal K, Elsen P, Johnson PDR, Hayman JA, Kirkwood R, Meyers WM (2001) *Mycobacterium ulcerans* in wild animals. In: Collins MT, Manning B (eds) Mycobacterial infections in domestic and wild animals. Office International de Epizooties, vol 20. Scientific and Technical Review, Paris, pp 252–264

41. Portaels F, Johnson P, Meyers WM (eds) (2001) Buruli ulcer: diagnosis of *Mycobacterium ulcerans* disease. World Health Organization, Geneva

42. Portaels F, Aguiar J, Debacker M, Steunou C, Zinsou C, Guédénon A, Meyers WM (2002) Prophylactic effect of M*ycobacterium bovis* BCG vaccination against osteomyelitis in children with *Mycobacterium ulcerans* disease (Buruli Ulcer). Clin Diagn Lab Immunol 9:1389–1391. doi:10.1128/CDLI.9.6.1389-1391.2002

43. Portaels F, Aguiar J, Debacker M, Guédénon A, Steunou C, Zinsou C, Meyers WM (2004) BCG vaccination as prophylaxis against *Mycobacterium ulcerans* osteomyelitis in Buruli Ulcer disease. Infect Immun 72:62–65. doi:10.1128/IAI.72.1.62-65.2004

44. Portaels F, Meyers WM, Ablordey A, Castro A, Chemlal K, De Rijk P, Elsen P, Fissette K, Fraga A, Lee R, Mahrous E, Small P, Stragier P, Torrado E, Van Aerde A, Texeira da Silva M, Pedrosa J (2008) First cultivation and characterization of *Mycobacterium ulcerans* from the environment. PLoS Negl Trop Dis 2:e178. doi:10.1371/journal.pntd.0000178

45. Portaels F, Silva MT, Meyers WM (2009) Buruli ulcer. Clin Dermatol (in press)

46. Pouillot R, Matias G, Mbondji Wondje C, Portaels F, Valin N, Ngoss F, Njikap A, Marsollier L, Fontanet A, Eyangoh S (2007) Risk factors for Buruli ulcer disease in Cameroon. PLoS Negl Trop Dis 1:e101. doi:10.1371/journal.pntd.0000101

47. Prévot G, Bourreau E, Pascalis H, Pradinaud R, Tanghe A, Huygen K, Launois P (2004) Differential production of systemic and intralesional gamma interferon and interleukin-10 in nodular and ulcerative forms of Buruli disease. Infect Immun 72:958–965. doi:10.1128/IAI.72.2.958-965.2004

48. Quek TY, Athan E, Henry MJ, Pasco JA, Redden-Hoare J, Hughes A, Johnson PD (2007) Risk factors for *Mycobacterium ulcerans* infection, southeastern Australia. Emerg Infect Dis Nov 13(11):1661–1666

49. Revill WD, Morrow RH, Pike MC, Ateng J (1973) A controlled trial of the treatment of *Mycobacterium ulcerans* infection with clofazimine. Lancet 2: 873–877, 95

50. Siegmund V, Adjei O, Nitschke J, Thompson W, Klutse E, Herbinger KH, Thompson R, van Vloten F, Racz P, Fleischer B, Loescher T, Bretzel G (2007) Dry reagent-based polymerase chain reaction compared with other laboratory methods available for the diagnosis of Buruli ulcer disease. Clin Infect Dis 45:68–75. doi:10.1086/518604

51. Silva MF, Portaels F, Pedrosa J (2007) Aquatic insects and *Mycobacterium ulcerans*: an association relevant to Buruli ulcer control? PLoS Med 4:e63. doi:10.1371/journal.pmed.0040063

52. Smith PG, Revill WDL, Lukwago E, Rykushin YP (1976) The protective effect of BCG against *Mycobacterium ulcerans* disease: a controlled trial in an endemic area of Uganda. Trans R Soc Trop Med Hyg 70:449–457. doi:10.1016/0035-9203(76)90128-0

53. Stanford JL, Revill WD, Gunthorpe WJ, Grange JM (1975) The production and preliminary investigation of Burulin, a new skin test reagent for *Mycobacterium ulcerans* infection. J Hyg (Lond) 74:7–16

54. Stienstra Y, Van Der Graaf WT, te Meerman GJ, The TH, de Leij LF, van der Werf TS (2001) Susceptibility to development of *Mycobacterium ulcerans* disease: review of possible risk factors. Trop Med Int Health 6:554–562. doi:10.1046/j.1365-3156.2001.00746.x

55. Stinear TP, Jenkin GA, Johnson PD, Davies JK (2000) Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. J Bacteriol 182:6322–6330. doi:10.1128/JB.182.22.6322-6330.2000

56. Stinear TP, Mve-Obiang A, Small PLC, Frigui W, Pryor MJ, Brosch R, Jenkin GA, Johnson PDR, Davies JK, Lee RE, Adusumilli S, Garnier T, Haydock SF, Leadlay PF, Cole ST (2004) Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*. Proc Natl Acad Sci USA 101:1345–1349. doi:10.1073/pnas.0305877101

57. Tanghe A, Content J, Van Vooren JP, Portaels F, Huygen K (2001) Protective efficacy of a DNA vaccine encoding antigen 85A from *Mycobacterium bovis* BCG against Buruli ulcer. Infect Immun 69:5403–5411. doi:10.1128/IAI.69.9.5403-5411.2001

58. Tanghe A, Adnet PY, Gartner T, Huygen K (2007) A booster vaccination with *Mycobacterium bovis* BCG does not increase the protective effect of the vaccine against experimental *Mycobacterium ulcerans* infection in mice. Infect Immun 75:2642–2644. doi:10.1128/IAI.01622-06

59. Tanghe A, Danzy J-P, Pluschke G, Huygen K (2008) Improved protective efficacy of a species-specific DNA vaccine encoding mycolyl-transferase Ag85A from *Mycobacterium ulcerans* by homologous protein boosting. PLoS Negl Trop Dis 2:e199. doi:10.1371/journal.pntd.0000199

60. Tonjum T, Welty DB, Jantzen E, Small PL (1998) Differentiation of *Mycobacterium ulcerans*, *M. marinum*, and *M. haemophilum*: mapping of their relationships to *M. tuberculosis* by fatty acid profile analysis, DNA–DNA hybridization, and 16S rRNA gene sequence analysis. J Clin Microbiol 36:918–925

61. Torrado E, Adusumilli S, Fraga AG, Small PL, Castro AG et al (2007) Mycolactone-mediated inhibition of tumor necrosis factor production by macrophages infected with *Mycobacterium ulcerans* has implications for the control of infection. Infect Immun 75:3979–3988. doi:10.1128/IAI.00290-07

62. Uganda Buruli Group (1969) Lancet 18th, 111

63. Van der Werf TS, Van der Graaf WT, Tappero JW, Asiedu K (1999) *Mycobacterium ulcerans* infection. Lancet 354:1013–1018. doi:10.1016/S0140-6736(99)01156-3

64. Van Vooren JP, Drowart A, De Bruyn J, Launois P, Millan J, Delaporte E, Develoux M, Yernault JC, Huygen K (1992) Humoral responses against the 85A and 85B antigens of *Mycobacterium bovis* BCG in patients with leprosy and tuberculosis. J Clin Microbiol 30:1608–1610

65. Walsh DS, Meyers WM, Portaels FP, Lane JE, Mongkolsirichaikul D, Hussem K, Gosi P, Myint KS (2005) High rates of apoptosis in human *Mycobacterium ulcerans* culture-positive buruli ulcer skin lesions. Am J Trop Med Hyg 73:410–415

66. Westenbrink BD, Stienstra Y, Huitema MG, Thompson WA, Klutse EO, Ampadu EO, Boezen HM, Limburg PC, Van der Werf TS (2005) Cytokine responses to stimulation of whole blood from patients with Buruli ulcer disease in Ghana. Clin Diagn Lab Immunol 12:125–129. doi:10.1128/CDLI.12.1.125-129.2005

67. Wiker HG, Harboe M (1992) The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. Microbiol Rev 56:648–661

68. World Health Organization (2000) Buruli ulcer. In: Asiedu K, Scherpbier R, Raviglione M (eds) *Mycobacterium ulcerans* infection. World Health Organization, Geneva

69. World Health Organization (2004) Provisional guidance on the role specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer). World Health Organization, Geneva

70. World Health Organization (2008) Buruli ulcer disease. *Mycobacterium ulcerans* infection: an overview of reported cases globally. Wkly Epidemiol Rec 83:145–156

71. Yeboah-Manu D, Peduzzi E, Mensah-Quainoo E, Asante-Poku A, Ofori-Adjei D et al (2006) Systematic suppression of interferon-gamma responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen *Mycobacterium ulcerans*. J Leukoc Biol 79:1150–1156. doi:10.1189/jlb.1005581